SELECTIVE KILLING OF HUMAN T CELL LYMPHOTROPIC VIRUS TYPE I-TRANSFORMED CELL LINES BY A DAMAVARICIN Fc DERIVATIVE

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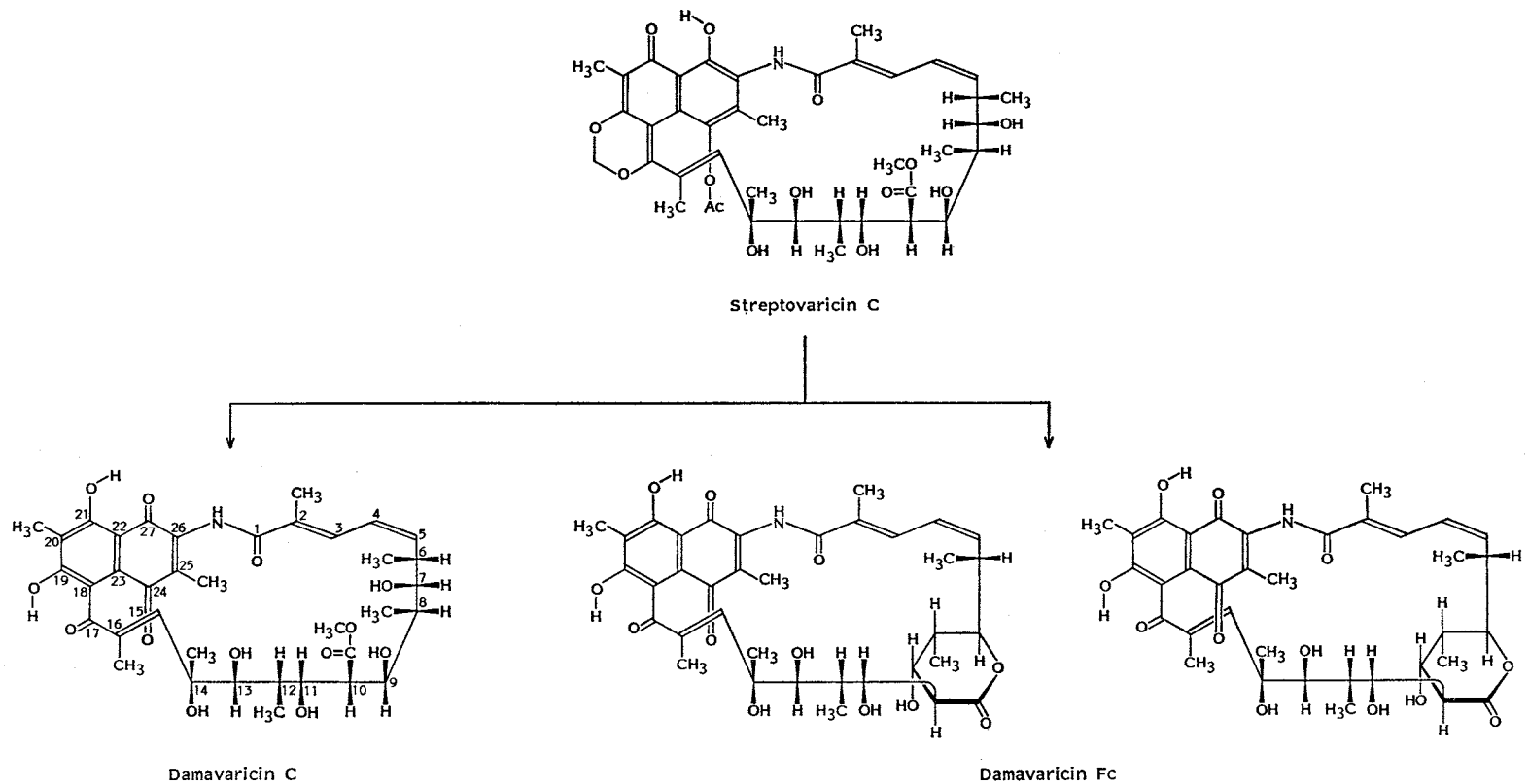
n-Pentyl ether of damavaricin Fc (*n*-pentyl DvFc) preferentially killed human T-cell lymphotropic virus type I (HTLV-I)-transformed cell lines. The mechanism of action of the drug was investigated using MT-4 cells. Cytotoxic action was diminished by the removal of *n*-pentyl DvFc from the culture or by the addition of sulfhydryl compounds such as 2-mercaptoethanol and dithiothreitol. The killing activity of *n*-pentyl DvFc was also diminished by membrane-acting agents including quinidine and diphenylhydantoin. Influx and subsequent efflux of Ca^{2+} were observed when either HTLV-I infected (MT-4 cells) or uninfected cells were treated with *n*-pentyl DvFc. An efflux of K^+ was observed in HTLV-I infected MT-4 cells immediately after the exposure of the cells to *n*-pentyl DvFc. The K^+ efflux, however, was not observed in the uninfected T cells. *n*-Pentyl DvFc seems to act primarily on the cell surface of MT-4 cells, leading to the perturbation of membrane function. The restoration of cell growth, however, is critically dependent on the presence of dithiothreitol and 2-mercaptoethanol, implying a role for a free sulfhydryl group in the killing activity.

Damavaricin Fc is an atropisomeric mixture of two compounds produced by treatment of streptovaricin C with oxygenated concentrated ammonia - methanol. Damavaricin Fc, in which the ansa bridge lies above the aromatic nucleus, has the P helicity as well as streptovaricin C, whereas atropisodamavaricin Fc, in which the ansa bridge lies below the aromatic nucleus, has the M helicity (Fig. 1)[1]. To avoid complicating nomenclature, hereafter we will use damavaricin Fc to refer to this atropisomeric mixture. Streptovaricins belong to the ansa-ring class of antibiotics and in addition to antibiotic activity, they also inhibit retrovirus reverse transcriptase activity *in vitro*. We have previously reported the biological activities of several derivatives of damavaricin Fc including those which have different alkyl ether linkage at the C-19 position of the naphthoquinone ring of the molecule. Some of these derivatives inhibited proliferation of a mouse retrovirus[1].

Recently, it has been discovered that several retroviruses can cause severe human diseases[2~4]. Human T-cell lymphotropic virus type I (HTLV-I) is the causative agent of adult T-cell leukemia[5], one of the most aggressive human leukemias, as well as HTLV-I associated myelopathy (HAM)[6],

Fig. 1. Structure of damavaricin Fc.



and tropical spastic paraparesis[7]. However, no effective therapy for HTLV-I-related disease is currently known.

We wished to determine whether damavaricin Fc derivatives would inhibit the growth of HTLV-I-transformed cells. In the present study, we describe the selective killing of HTLV-I-transformed cells by the *n*-pentyl ether of damavaricin Fc (*n*-pentyl DvFc), and its mode of action.

Materials and Methods

Cells

Peripheral blood lymphocytes (PBL) were obtained after Ficoll-Hypaque centrifugation of peripheral blood from normal healthy donors. The established cell lines used in this study were the HTLV-I-negative MOLT-4[8], SKW, and NALM16 cells[9]; and the HTLV-I-positive T-cell lines MT-1[10], MT-2, MT-4[11], 467[12], KAN[13] and TLOml cells[14]. FUK cells were established from PBL of an adult T-cell leukemia (ATL) patient by culturing with interleukin-2 (IL-2) (unpublished). They were cultured in RPMI 1640 medium (Gibco Co.) supplemented with 10% or 20% fetal calf serum (FCS) (M.A. Bioproduct, Walkersvile, MD), benzylpenicillin (100 U/ml) and streptomycin (100 μg/ml).

Chemicals

n-Pentyl DvFc was prepared as previously described[1]. 5,5-Diphenylhydantoin (5,5-DPH) and quinidine hydrochloride were purchased from Sigma Chemical Company. The radioactive compounds [*methyl*-^{3}H]thymidine (20 Ci/mmol), [5-^{3}H]uridine (30 Ci/mmol), L-[4,5-^{3}H]leucine (63 Ci/mmol), $^{45}Ca^{2+}$ (40 mCi/mg calcium) and $^{86}Rb^+$ (8 mCi/mg rubidium) were obtained from Amersham Co., Ltd. Dithiothreitol and 2-mercaptoethanol were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Sensitivity of the Cells to *n*-Pentyl DvFc

1 ml of cell suspension (6×10^5 cells/ml) was added to 1 ml of medium containing various concentration of *n*-pentyl DvFc, and the mixtures were incubated at 37°C in a CO_2 incubator. Growth and viability of the cells were monitored by cell counting and by Trypan blue dye exclusion.

Assay for Alkaline Phosphodiesterase

MT-4 cells were ruptured in ice-cold phosphate-buffered saline (PBS, 10 mM potasium phosphate, pH 7.4, 135 mM NaCl) with a Dounce homogenizer and the extract was used as a source of a typical sulfhydryl (SH) enzyme. The reaction mixture (1 ml) contained: Tris-HCl (pH 9.0) 50 mM, $MgCl_2$ 5 mM, thymidine-5-monophosphate-*p*-nitrophenyl ester 1 mM, extract from 5×10^6 MT-4 cells, and various concentrations of *n*-pentyl DvFc. After incubation at 37°C for 45 minutes, the enzyme reaction was terminated by the addition of 300 μl of TCA. The deproteinized supernatant was added to 400 μl of 2.5 N NaOH, and the optical density at 400 nm was compared with standard *p*-nitrophenol to determine the amount of substrate hydrolyzed.

Biosynthesis of DNA, RNA, and Proteins

MT-4 cells (3×10^5 cells/ml) were incubated with various concentration of *n*-pentyl DvFc in culture medium (total volume; 400 μl) containing labeled precursors ([^{3}H]thymidine, [^{3}H]uridine, or [^{3}H]leucine, all at 4 μCi/ml) for 40 minutes. Cells were washed with PBS, and treated with 400 μl of 10% cold TCA. Acid-insoluble fractions were collected on glass fiber filters (Advantec GC50) and washed with 5% cold TCA, and the radioactivity was counted.

Rubidium Efflux Measurements

HTLV-I infected (MT-4) or uninfected (MOLT-4) cells (2.4×10^6 cells/ml) were loaded overnight with 10 μCi/ml of $^{86}Rb^+$ in RPMI 1640 containing 10% FCS. Cells were diluted 10-fold into non-radioactive medium, spun down ($400\times g$, 5 minutes) and resuspended in the same medium at 6×10^6 cells/ml. *n*-Pentyl DvFc (5 μg to 3×10^5 cells) was added to the culture and incubated at 37°C in a CO_2 incubator. At the indicated times, 100 μl of cell suspension was removed and layered over 500 μl

of a corn oil and dibutylphthalate (3:10) mixture[15], and centrifuged in an Eppendorf microfuge (15,000 rpm, 40 seconds). The tip of tube containing the cell pellet was cut off and the radioactivity was determined.

Calcium Flux Measurement

MT-4 or MOLT-4 cells (6×10^6 cells/ml) were incubated in the presence of $^{45}CaCl$ (50 μCi/ml). After 10 minutes, *n*-pentyl DvFc (5 μg to 3×10^5 cells) was added to the culture. At the indicated times, 120 μl of cell suspension was transferred into 1 ml of simplified medium (NaCl 145 mM, KCl 5 mM, Na_2HPO_4 1 mM, $MgSO_4$ 0.5 mM, glucose 5 mM, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10 mM, ethylene glycol bis(2-aminoethylether)tetraacetic acid (EGTA) 1 mM, pH 7.4). Cells were spun down ($10,000\times g$, 30 seconds) and resuspended in simplified medium, transferred to a membrane filter (pore size, 0.45 μm), washed, and the radioactivity was determined.

Results

Sensitivity of HTLV-I-transformed Cells to *n*-Pentyl DvFc

All HTLV-I-positive cell lines tested were highly sensitive to *n*-pentyl DvFc as compared

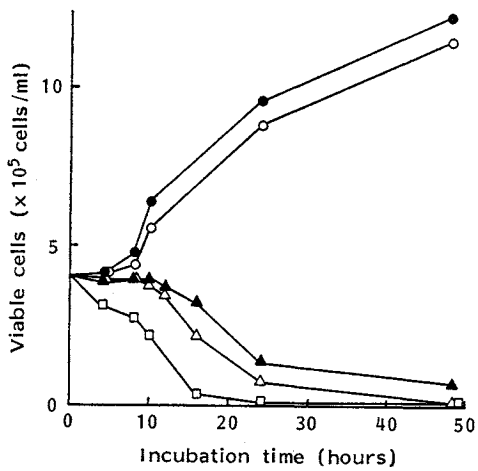
Table 1. Effect of *n*-pentyl DvFc on cell growth.

Cell lines	Minimum concentration of *n*-pentyl DvFc inhibiting cell growth by 50% (μg/ml)[a]
PBL (resting)	44
PBL (PHA-activated)	32
HTLV-I-negative cell lines	
MOLT-4	16
SKW	25
NALM16	27
HTLV-I-positive T-cell lines	
FUK	1
467	3
KAN	6
TLOml	1
MT-1	4
MT-2	7
MT-4	2

[a] The values present the concentration of *n*-pentyl DvFc showing 50% cell growth compared with DvFc-free control after incubation for 72 hours.

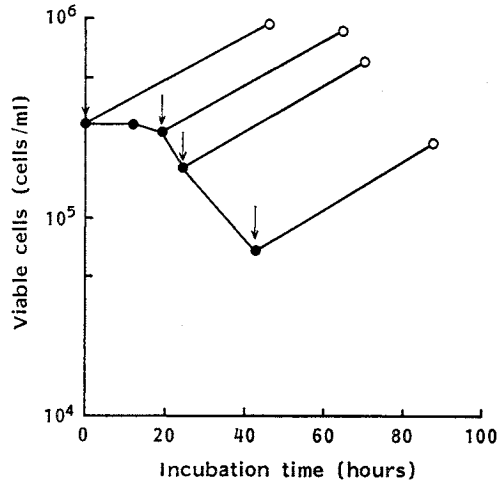
Fig. 2. Effect of *n*-pentyl DvFc on the growth of MT-4 cell.

The concentrations of DvFc were 0 (●), 1 (○), 5 (▲), 10 (△), and 25 (□) μg/ml.



MT-4 cells (4×10^5 cells/ml) were incubated in the presence of *n*-pentyl DvFc. Cell growth and viability were monitored by Trypan blue dye exclusion.

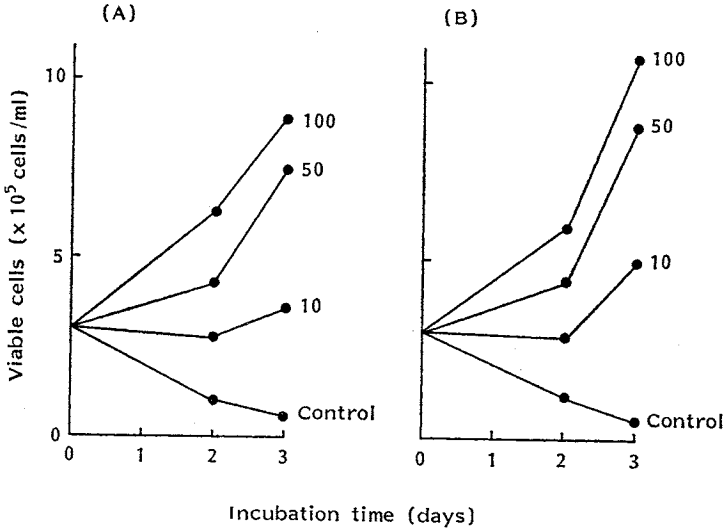
Fig. 3. Effect of the removal of *n*-pentyl DvFc on the growth of *n*-pentyl DvFc-treated MT-4 cells.



MT-4 cells (3×10^5 cells/ml) were incubated in the presence of *n*-pentyl DvFc (5 μg/ml). At indicated times (presented by arrow heads), viable cells were determined by Trypan blue dye exclusion (●), 1 ml of culture was taken and cells were washed twice with 1 ml of drug-free medium by centrifugation ($400\times g$, 3 minutes). Cells were resuspended in medium, incubated for 46 hours, and viable cells were determined (○).

Fig. 4. Recovery of cell killing by *n*-pentyl DvFc with SH-compound.

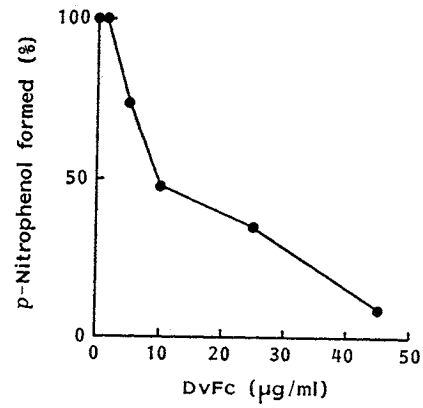
MT-4 cells (3×10^5 cells/ml) were incubated in the presence of *n*-pentyl DvFc (5 μg/ml) and various concentration of dithiothreitol (A) or 2-mercaptoethanol (B).



Cell growth and viability were monitored with the Trypan blue dye exclusion test. The values in the figure are the concentration (μM) of SH-compound.

with HTLV-I-negative cell lines (Table 1). Among the sensitive cell lines, MT-4 was chosen to study the mode of action of *n*-pentyl DvFc. To determine the time course of cytotoxic action of *n*-pentyl DvFc, logarithmically growing MT-4 cells were exposed to various concentrations of *n*-pentyl DvFc and the cell number was measured at various times. MT-4 cells showed no growth in the presence of *n*-pentyl DvFc at concentrations of more than 5 μg/ml under these conditions (Fig. 2). However, these MT-4 cells were viable for at least 12 hours after exposure to *n*-pentyl DvFc (5 μg/ml) as indicated by the following experiments. MT-4 cells were incubated in the presence of *n*-pentyl DvFc (5 μg/ml), and at various times, were washed with *n*-pentyl DvFc-free medium, and were incubated in the absence of the drug. The washed cells were able to resume growth in drug-free medium even after 42 hours (Fig. 3). Similar results were obtained in experiments using 10 μg/ml of *n*-pentyl DvFc. These results imply that *n*-pentyl DvFc binds to MT-4 cells reversibly.

Fig. 5. Inhibitory effect of *n*-pentyl DvFc on the membrane bound enzyme, alkaline phosphodiesterase of MT-4 cells.



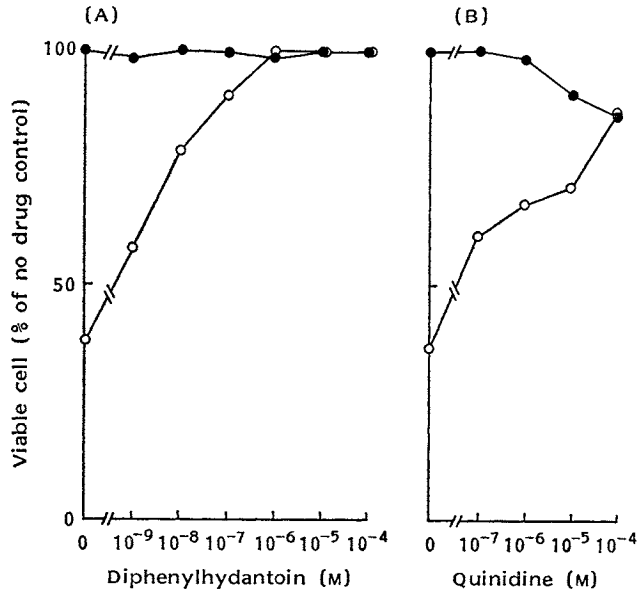
MT-4 cells were ruptured and the extract was used as a source of SH enzyme.

Effect of Sulfhydryl Compounds

It was reported that the activity of naphthomycin, an antibiotic of the ansamycin group, was prevented by SH compounds[16]. Therefore we examined whether the cytotoxic action of *n*-pentyl

DvFc might also be prevented by SH compounds. Both dithiothreitol and 2-mercaptoethanol diminished the action of *n*-pentyl DvFc on MT-4 in a dose-dependent manner (Fig. 4). These results indicate that *n*-pentyl DvFc may act on the SH groups of proteins. To confirm the action of *n*-pentyl DvFc on the SH groups of proteins, we measured the inhibitory effect of *n*-pentyl DvFc on alkaline phosphodiesterase, a known SH enzyme located in the cell membrane of lymphocytes[17]. *n*-Pentyl DvFc inhibited this enzymic activity *in vitro* (Fig. 5). 50% inhibition was seen at a *n*-pentyl DvFc

Fig. 6. Effect of membrane-acting agents on the cytotoxic activity of *n*-pentyl DvFc in MT-4 cells.

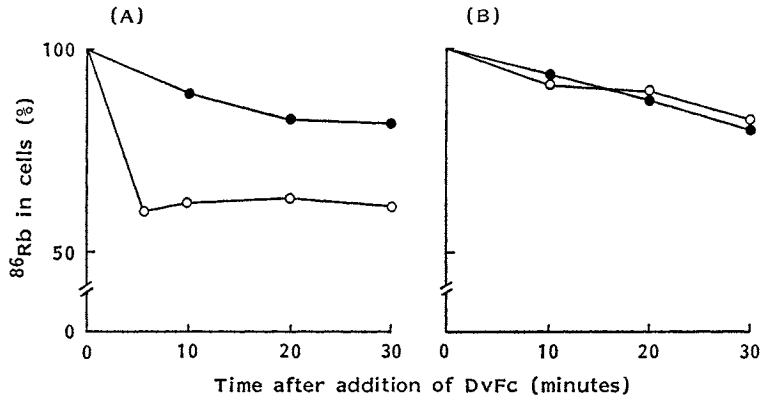


Logarithmically growing MT-4 cells were incubated for 24 hours in the presence of both *n*-pentyl DvFc (5 μg/ml) and membrane-acting agents (○), or of the later only (●).

(A) Effect of DPH. 100% corresponds to 7.2×10^5 cells/ml. (B) Effect of quinidine. 100% corresponds to 8.8×10^5 cells/ml.

Fig. 7. Effect of *n*-pentyl DvFc on the $^{86}Rb^+$ efflux from MT-4 cells.

● *n*-Pentyl DvFc-free control, ○ *n*-pentyl DvFc-treated.



Logarithmically growing MT-4 (HTLV-I infected) (A) and MOLT-4 (uninfected) (B) cells were loaded with $^{86}Rb^+$, and cellular $^{86}Rb^+$ was measured as described in the Materials and Methods.

concentration of 10 μg/ml. These results suggest that *n*-pentyl DvFc may react reversibly with the SH groups of the membrane proteins of MT-4 cells.

Effect of *n*-Pentyl DvFc on Macromolecular Synthesis

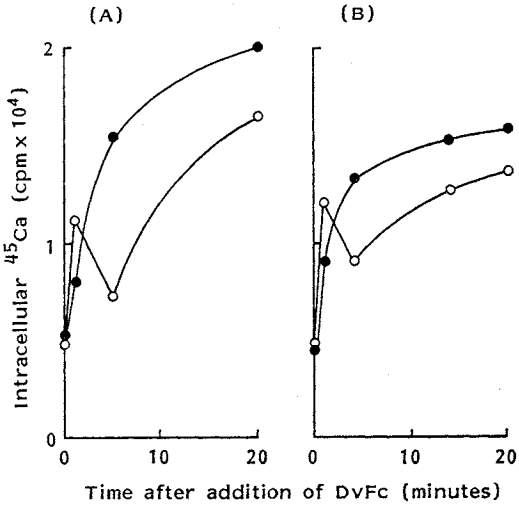
To determine the effect of *n*-pentyl DvFc on macromolecular synthesis, we measured the incorporation of radiolabeled precursors by MT-4 cells into DNA ([^{3}H]thymidine), RNA ([^{3}H]uridine), and protein ([^{3}H]leucine). DNA synthesis was inhibited by *n*-pentyl DvFc in a dose dependent manner (data not shown). Neither RNA nor protein synthesis was affected significantly.

Reversal of the Action of *n*-Pentyl DvFc by Membrane-acting Agents

The results described above indicate that *n*-pentyl DvFc might act directly on the cell membrane of MT-4 cells. We investigated the effect of membrane-active agents such as quinidine and DPH. These drugs are a K^+ channel blocker and an anticonvulsant, respectively. Both types of drugs overcame the inhibitory effects of *n*-pentyl DvFc (Fig. 6). However, another K^+ channel blocker, 4-aminopyridine (4-AP) did not show this effect. Verapamil, a Ca^{2+} channel blocker, was also effective at concentration of 10^{-7} to 10^{-4} M (data not shown).

Fig. 8. Effect of *n*-pentyl DvFc on the permeability of calcium ion.

● *n*-Pentyl DvFc-free control, ○ *n*-pentyl DvFc-treated.



Logarithmically growing MOLT-4 (A) and MT-4 (B) cells were incubated in the presence of ^{45}CaCl for 10 minutes, and *n*-pentyl DvFc was added to the culture. At indicated times, intracellular ^{45}Ca was measured as described in the Materials and Methods.

Effect of *n*-Pentyl DvFc on Ion Permeability

To confirm whether *n*-pentyl DvFc actually caused K^+ efflux in MT-4 cells, $^{86}Rb^+$-loaded MT-4 cells were exposed to *n*-pentyl DvFc and the cellular ^{86}Rb level was examined. Efflux of 40% of cellular ^{86}Rb was observed within 6 minutes after the exposure of cells to *n*-pentyl DvFc. Efflux of ^{86}Rb was not observed in the HTLV-I negative MOLT-4 cells under the same condition (Fig. 7). The two drugs which reversed the killing of HTLV-I infected cells by *n*-pentyl DvFc, DPH[18] and verapamil[19], are known to inhibit Ca^{2+} influx into cells. Therefore Ca^{2+} influx into MT-4 cells might be changed by the action of *n*-pentyl DvFc. MT-4 cells were loaded with ^{45}Ca and then exposed to *n*-pentyl DvFc in the presence of $^{45}Ca^{2+}$. There was a transient increase in intracellular ^{45}Ca ion followed by a decrease within 10 minutes (Fig. 8). Since this phenomenon was also observed in MOLT-4 cells, it cannot be responsible for the selective killing of MT-4 cells.

Discussion

We have been engaged in a series of studies to elucidate the relationship between the structure and the biological activity of streptovaricin C derivatives (damavaricin Fc and damavaricin C)[1,20~22].

The damavaricin Fc as well as damavaricin C were produced from streptovaricin C[23]. Whereas damavaricin C retained antibacterial activity, damavaricin Fc lost it[1]. It is suggested that the lactone-ring formed between C-7 and C-10 is closely related to the loss of antibacterial activity. The low toxicity of damavaricin Fc has been demonstrated on mammalian cells *in vitro*[1] and also in animals when orally administered (unpublished data). Therefore, damavaricin Fc may have a potential for the therapy of adult T cell leukemia or HTLV-I-related diseases. This drug may be applicable to the treatment of ATL by adoptive immunotherapy such as lymphokine-activated killer cells[24].

Since *n*-pentyl DvFc has shown the most effective inhibitory activity against focus formation by mouse sarcoma virus/mouse leukemia virus complex[1], we selected this derivative for study. We have shown the selective killing of the HTLV-I-transformed cells by *n*-pentyl DvFc, and have given circumstancial evidence for the mechanism of cell-killing. The results suggest that the drug acts on the cell membrane. It has been reported that HTLV-I-transformed cells have an altered cell surface: They express the IL-2 receptor[25] and Ia antigen[14]. In the present study, we demonstrated that K^+ efflux occured in HTLV-I-transformed cells immediately after exposure to *n*-pentyl DvFc. Since K^+ efflux was specific for HTLV-I infected cells, K^+ efflux seemed to be an essential event at an early phase in the course of the killing action. The killing action by *n*-pentyl DvFc, however, was not reversed by 4AP, which is a blocking agent of voltage-sensitive K^+ channel[26]. This suggests that the K^+ efflux caused by *n*-pentyl DvFc was not through the voltage-sensitive channel but through the Ca^{2+}-dependent one. In fact, Ca^{2+} influx seems to occur in MT-4 cells immediately after the addition of DvFc, but MOLT-4 cells also showed a similar pattern of Ca^{2+} influx, suggesting that drug induced Ca^{2+} influx was not specific to infected cells. We speculate that if the level of intracellular Ca^{2+} was similar between these two cell lines (MT-4 and MOLT-4) after exposure to *n*-pentyl DvFc, the Ca^{2+}-dependent K^+ channel of MT-4 cell is more sensitive to the level of Ca^{2+} than that of MOLT-4 cell.

What is the target site for the action of *n*-pentyl DvFc? From the results in which the killing is prevented by the removal of *n*-pentyl DvFc or by the addition of SH-compounds, we consider that *n*-pentyl DvFc interacts with membrane proteins containing SH groups in MT-4 cells. HTLV-I antigens expressed on the cell membrane are unlikely to be the target molecules, because TLOml cells which are also HTLV-I-infected but do not express HTLV-I antigen *in vitro*[14] were also killed by *n*-pentyl DvFc. DNA synthesis was inhibited after addition of DvFc to HTLV-I infected cells. The inhibitory activity of *n*-pentyl DvFc on SH enzymes may effect DNA synthesis, but this is probably a secondary effect.

From the results described above, we conclude that *n*-pentyl DvFc acts directly on the membrane and that the membranes of HTLV-I-transformed cells respond differently from the membranes of uninfected cell lines. The precise mechanism is not clear at this moment. The relationship between the Ca^{2+} channel and the K^+ channel in HTLV-I-transformed T cells as compared to untransformed cells appears to be different.

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