MODULATION OF THE CYTOTOXICITY OF THE ANTITUMOR ANTIBIOTIC PEPLOMYCIN BY MEMBRANE-INTERACTING DRUGS AND BY INCREASED LEVELS OF CALCIUM IONS

Satoshi Mizuno,*1 Akiko Ishida,*1 Yoshimasa Uehara*2 and Tomoko Nanjo*1

*1Department of Antibiotics, National Institute of Health, Kamiosaki 2-10-35, Shinagawa-ku, Tokyo 141 and *2Institute of Microbial Chemistry, Kamiosaki 3-14-23, Shinagawa-ku, Tokyo 141

The cytotoxic effect of peplomycin (PEP), a new derivative of bleomycin glycopeptide antibiotics, toward HeLa cells and mouse FM3A cells was markedly enhanced by combination of PEP with non-toxic doses of the membrane-interacting drugs verapamil (0.1–0.2mM) and dibucaine (0.15–0.25mM), or with CaCl₂ (10–16mM). Treatment with verapamil or CaCl₂ following PEP treatment also effectively enhanced the cytotoxic effect of PEP, suggesting an interaction with PEP-induced damage. Cellular uptake of PEP did not increase in dibucaine-treated cells, suggesting no correlation between membrane permeability to PEP and enhanced cytotoxicity. Increased Ca²⁺ did not enhance the cytotoxic effects of adriamycin, mitomycin C, cis-diamminedichloroplatinum(II), vinblastine or macromomycin, thus suggesting a unique interaction with PEP. The enhancing action of verapamil and Ca²⁺ was greatly promoted by 41°C hyperthermia, although 41°C alone scarcely enhanced PEP cytotoxicity. Cytochalasin B and α-tocopherol, but not cytochalasin D, reversed the enhanced cytotoxicity produced by PEP and verapamil or dibucaine, but did not reverse the enhanced cytotoxicity by PEP combined with increased Ca²⁺. The enhancing action of Ca²⁺ was, however, antagonized by ruthenium red and lanthanum chloride, which are potent inhibitors of Ca²⁺ uptake by cells, or by magnesium chloride. Verapamil and increased Ca²⁺ promoted the decomposition of the DNA-membrane complex induced by PEP in HeLa cells, and also impaired the regeneration of the decomposed DNA complex.

Key words: Peplomycin — Bleomycin — Membrane-directed drugs — Calcium ions — Enhanced cytotoxicity

Bleomycins are glycopeptide antibiotics that are used in the treatment of human cancers, particularly squamous cell carcinoma and malignant lymphomas.34) Bleomycin is unique in that it causes neither bone marrow toxicity nor immunosuppression, although pulmonary toxicity is a major side effect. PEP* is a clinically useful new derivative of bleomycin that shows reduced pulmonary toxicity.31) The primary action of bleomycin causing cessation of cell proliferation involves breakage of cellular DNA.7) The cytotoxic effect of bleomycin is enhanced by hyperthermia both in vitro6,8) and in vivo16,17) suggesting the potential clinical usefulness of thermochemotherapy based on this drug in cancer treatment.

We previously showed that the cytotoxic effect of bleomycin in cultured mammalian cells was markedly enhanced by ethanol and local anesthetics.19–21) The enhancing
action of ethanol and the anesthetics on bleomycin cytotoxicity was promoted by moderate hyperthermia (40–41°C). This enhancing effect seemed to be selective for bleomycin, since the drugs did not enhance the cytotoxicity of other antitumor drugs, including adriamycin, mitomycin C and cis-platin, although hyperthermia enhanced the activity of all of the antitumor drugs. In addition, we have recently shown that several other membrane-directed drugs, including verapamil, prenylamine, clomipramine, chlorpromazine and dipyridamole, and increased levels of calcium ions enhance PEP cytotoxicity. Both the drugs and Ca²⁺ are known to interact extensively with cell membranes, affecting the levels of membrane-bound Ca²⁺ and inducing structural and functional modification of membranes. To elucidate the mechanism of enhancement of PEP cytotoxicity by the drugs and Ca²⁺, we further studied the effects of verapamil, dibucaine and increased Ca²⁺ on PEP cytotoxicity, and the results are reported in this paper.

**Materials and Methods**

**Cells** HeLa S3 cells were grown on the surface of plastic flasks in Eagle's minimum essential medium (MEM) supplemented with 10% calf serum. Mouse FM3A cells originally established from a mammary carcinoma of C3H mice were maintained as a suspension culture in Eagle's MEM with 0.1% Bactopeptone (Difco) and 10% calf serum. The cells were cultured at 37°C in a CO₂ incubator in 95% air and 5% CO₂ at 100% humidity. HeLa cells were detached with 0.1% trypsin for subculture.

**Drugs** PEP, [³H]PEP (0.75 mCi/mg) and cis-platin were supplied by Nippon Kayaku Co., Tokyo. Adriamycin and mitomycin C were supplied by Kyowa Hakko Co., Tokyo. Macromycin was supplied by Kanegafuchi Chemical Ind., Takasago, and verapamil was supplied by Elsi Co., Tokyo. Dibucaine-hydrochloride, cytochalasins B and D, vinblastine sulfate and A23187 were purchased from Sigma Chemicals. Ruthenium red and δ,δ,α-tocopherol were purchased from Wako Pure Chemicals, Osaka. Cytochalasins and α-tocopherol were dissolved in dimethylsulfoxide and ethanol, respectively. A23187 was dissolved in dimethylformamide : ethanol (1:3). Other drugs were dissolved in 0.9% NaCl solution. [³H]PEP chelated with cobalt or cupric ions ([³H]PEP(Co) or [³H]PEP(Cu)) was prepared and purified as described elsewhere.

**Treatment with Drugs** The cells were used at the exponential phase. HeLa cells were grown in plastic tubes with a flat surface (Nunc) containing 2 ml of medium. After 2 days of culture, the medium was replaced with fresh medium (pH 7.4–7.5) and the cells (5 × 10⁶ cells) were treated with drugs for 1 hr at 37°C in a water bath. After incubation, the cells were rinsed with Hank's balanced salt solution and trypsinized for survival determination. FM3A cells (3 × 10⁶ cells) were suspended in 2 ml of fresh medium and treated with drugs at 37°C for 30 min. After incubation, the cells were washed with Hank's solution by centrifugation, suspended in fresh medium at 4–5 × 10⁶ cells/ml, and cultured in plastic tubes (16 × 125 mm) for the assay of cell growth. The numbers of cells were counted with a Coulter counter. The pH of the medium increased slightly to about 7.6 during the incubation for drug treatment of both cell lines. The experiments on recovery of HeLa cells from drug-induced damage were performed as follows; after drug treatment, the cells were rinsed once with Hank's solution, reincubated in fresh medium and then trypsinized for the colony formation assay.

**Determination of Cell Survival** The survival of HeLa cells was determined by following clonal growth as described previously.

**Determination of Uptake of PEP** FM3A cells (5 × 10⁶ cells) in 1 ml of fresh medium were treated with dibucaine and then incubated with [³H]PEP chelated with cobalt or cupric ions in the presence of dibucaine. Aliquots of the incubation mixture were taken and layered on a mixture of silicone oil and liquid paraffin (84:16 by volume; specific gravity 1.05 at 25°C) in microcentrifuge tubes and uptake was determined by centrifugation of the cells through the oil at 12,000 g for 1 min in an Eppendorf microcentrifuge. After removal of the reaction mixture and oil, the cells were solubilized in 0.5N KOH and counted with a liquid scintillation spectrometer. The radioactivities due to [³H]PEP in the extracellular water were corrected by using [¹⁴C]inulin as the standard.

**Sucrose Density Gradient Analysis of the DNA Complex** HeLa cells (5 × 10⁶ cells) were labeled with 0.2 μCi/ml of [³H]thymidine (200 Ci/mmol) at 37°C for 18 hr. The cells were treated with drugs in fresh medium at 37°C for 1 hr. After incubation, the cells were rinsed with Hank's...
solution, trypsinized and suspended in cold saline. In some experiments on the repair of damage in the DNA complex, the cells (after rinsing) were reincubated with or without drugs at 37° for 1 hr and then trypsinized. A gradient consisting of 4.5 ml of 5–20% sucrose (w/v) in 2M NaCl and 10mM sodium citrate (pH 9.0) over 0.4 ml of 60% sucrose saturated with CsCl was overlayed with 0.3 ml of lysis solution containing 0.2%, sarkosyl, 0.08% sodium deoxycholate, 2M NaCl, 20mM EDTA and 10mM sodium citrate (pH 9.0). The cell suspension (5 × 10^4 cells/50 μl saline) was layered on the gradient for 1 hr at 20° in the dark, and then centrifuged at 20° for 1 hr at 20,000 rpm using a Beckman SW50.1 rotor. After centrifugation, fractions were collected from the bottom into tubes containing 0.1 mg calf thymus DNA as a carrier. Five percent trichloroacetic acid-insoluble materials were collected on glass fiber papers. The papers were dried and counted with a liquid scintillation spectrometer.

### RESULTS

**Enhanced Cytotoxicity by PEP Combined with Verapamil, Dibucaine and Increased Ca^{2+}**

HeLa cells were treated with PEP combined with verapamil, dibucaine and increased CaCl₂ at 37° for 1 hr, and cell survival was determined by clonal growth assay. Fig. 1 shows that the cytotoxic effect of PEP increases with increasing dose of PEP from 10 to 30 μg/ml in combination with verapamil (0.1mM), dibucaine (0.15 mM) or CaCl₂ (14mM). Eagle’s MEM contained 2mM Ca^{2+}, and therefore the amount of added CaCl₂ was 12mM. These doses of verapamil, dibucaine and CaCl₂ showed no cytotoxicity. The cytotoxic effect of PEP (20 μg/ml) combined with graded

![Graph 1](image1.png)

**Fig. 1.** Enhancement of PEP cytotoxicity by verapamil, dibucaine and increased Ca^{2+}

HeLa cells were incubated with graded doses of PEP alone (○) or combined with verapamil (0.1mM) (■) dibucaine (0.15mM) (▲) or CaCl₂ (12mM) (●) at 37° for 1 hr; bars, SD (shown when larger than the symbol).

![Graph 2](image2.png)

**Fig. 2.** Enhancement of PEP cytotoxicity by increased CaCl₂

HeLa cells were incubated with various concentrations of CaCl₂ (2–16mM) in the medium with (●) or without (○) PEP (20 μg/ml) at 37° for 1 hr.
dose of verapamil (0.05–0.2 mM) increased with increasing dose of verapamil and time of PEP and verapamil treatment up to 1 hr, and treatment with PEP and 0.2 mM verapamil (non-toxic) for 1 hr decreased cell survival to about $1 \times 10^{-5}$. Fig. 2 shows that the cytotoxic effect of PEP combined with CaCl$_2$ in the range from 2 to 16 mM increases only slightly from 2 to 8 mM, but markedly with increasing CaCl$_2$ from 8 to 16 mM. The enhancing effect of increased Ca$^{2+}$ (14 mM) was greatly influenced by the pH of the medium within the narrow range from 7.4 to 7.7; the enhancement was only slight at pH 7.4 and 7.5, but sharply increased from 7.5 to 7.7 (Fig. 3). In contrast, the enhancing effect of verapamil (0.1 mM) was evident at pH 7.4 and 7.5, and increased as the pH was increased to 7.6 and 7.7.

Enhancement of PEP cytotoxicity was also clearly shown by the rapid assay of inhibition of cell growth. FM3A cells were treated with PEP combined with increasing doses of CaCl$_2$, verapamil or dibucaine.

HeLa cells were incubated with PEP (20 µg/ml) without (○) or with CaCl$_2$ (14 mM) (●) or verapamil (0.1mM) (■) at 37° for 1 hr in the growth medium supplemented with 30 mM 4-[(2-hydroxyethyl)-1-piperazinio]ethanesulfonic acid buffer of the indicated pH value instead of bicarbonate buffer.

The cells were incubated at 37° for 30 min with PEP (20 µg/ml) and CaCl$_2$ (A), verapamil (B) or dibucaine (C). After incubation, the cells were washed, suspended in fresh medium at $4-5 \times 10^4$ cells/ml and cultured. (A) Control (○), CaCl$_2$ (10 mM) (△), PEP (●), PEP + CaCl$_2$ (8 mM) (■), PEP + CaCl$_2$ (10 mM) (▲); (B) control (○), verapamil (0.2 mM) (△), PEP (●), PEP + verapamil (0.1 mM) (■), PEP + verapamil (0.2 mM) (▲); (C) control (○), dibucaine (0.25 mM) (△), PEP (●), PEP + dibucaine (0.15 mM) (▲), PEP + dibucaine (0.25 mM) (■).
PELOMYCIN AND MEMBRANE-DIRECTED DRUGS

It was previously shown that the enhancement of cytotoxicity by dibucaine and verapamil seemed to be selective for bleomycin antibiotics.\textsuperscript{20,22} Table I shows that increased Ca\textsuperscript{2+} enhances PEP cytotoxicity, but did not enhance the cytotoxic effects of several other antitumor drugs, including adriamycin, mitomycin C, cis-platin, macromomycin and vinblastine.

**Promotion by 41\textdegree C Hyperthermia of Drug- or Ca\textsuperscript{2+}-induced Enhanced Cytotoxicity of PEP** We previously showed that local anesthetics interacted with hyperthermia to enhance the cytotoxic effect of PEP synergistically.\textsuperscript{21} The enhancing effect of verapamil and Ca\textsuperscript{2+} on PEP cytotoxicity was also markedly promoted at 41\textdegree C, although 41\textdegree C hyperthermia alone barely enhanced PEP cytotoxicity (Table II).

**Effect of Dibucaine on Cellular Uptake of PEP** It has been shown that cobalt ions increase cellular uptake of PEP, and that there are two components (saturable and non-saturable) of the uptake of PEP.

**Table I. Effect of Increased CaCl\textsubscript{2} on the Cytotoxic Effects of Several Antitumor Drugs**

<table>
<thead>
<tr>
<th>Antitumor drugs (µg/ml)</th>
<th>Cell counts (×10\textsuperscript{-3} cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CaCl\textsubscript{2} (2mM)</td>
</tr>
<tr>
<td>None</td>
<td>11.8</td>
</tr>
<tr>
<td>PEP (20)</td>
<td>7.4</td>
</tr>
<tr>
<td>Adriamycin (0.5)</td>
<td>6.0</td>
</tr>
<tr>
<td>Mitomycin C (2.0)</td>
<td>4.2</td>
</tr>
<tr>
<td>cis-Platin (0.1)</td>
<td>5.5</td>
</tr>
<tr>
<td>Macromomycin (0.02)</td>
<td>5.2</td>
</tr>
<tr>
<td>Vinblastine (10)</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Table II. Enhanced PEP Cytotoxicity by Verapamil and CaCl\textsubscript{2} Combined with 41\textdegree C Hyperthermia

<table>
<thead>
<tr>
<th>Drugs (mM)</th>
<th>Cell counts (×10\textsuperscript{-3} cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37\textdegree C</td>
</tr>
<tr>
<td>None</td>
<td>11.5</td>
</tr>
<tr>
<td>Verapamil (0.1)</td>
<td>11.8</td>
</tr>
<tr>
<td>PEP</td>
<td>8.7</td>
</tr>
<tr>
<td>PEP+verapamil (0.05)</td>
<td>5.4</td>
</tr>
<tr>
<td>PEP+verapamil (0.1)</td>
<td>2.3</td>
</tr>
<tr>
<td>PEP+CaCl\textsubscript{2} (6.0)</td>
<td>5.6</td>
</tr>
<tr>
<td>PEP+CaCl\textsubscript{2} (7.5)</td>
<td>2.7</td>
</tr>
</tbody>
</table>

FM3A cells were incubated with drugs in the presence of 2 and 12mM CaCl\textsubscript{2} at 37\textdegree C for 30 min. After incubation, cells were washed, suspended in fresh medium at 4×10\textsuperscript{5} cells/ml, and cultured for the assay of growth. Cells were counted after 3 days of culture.

Fig. 5. Effect of dibucaine on the uptakes of PEP (Co) and PEP (Cu)

FM3A cells were treated with dibucaine (0.25mM) for 10 min (A), or 0.5mM for 25 min (B) and then treated with [\textsuperscript{3}H] PEP (Co) (0.6 (A) or 20 (B) µg/ml) or [\textsuperscript{3}H] PEP (Cu) (1.4 (A) or 20 (B) µg/ml). At the indicated times duplicate 0.1 (A) or 0.2 (B) ml aliquots were removed and the uptake was estimated. (○) control+PEP (Co), (□) control+PEP (Cu), (●) dibucaine+PEP (Co), (■) dibucaine+PEP (Cu)
To examine the effect of dibucaine on the uptake of PEP, FM3A cells were treated with dibucaine (0.25 mM for 10 min, or 0.5 mM for 25 min), and then incubated with [3H]PEP(Co) and [3H]PEP(Cu). As seen in Fig. 5, the rate of uptake of PEP(Co) was several times higher than that of PEP(Cu). The uptake of the two kinds of metal-chelated PEP did not increase but rather decreased in the dibucaine-treated cells at two different doses of PEP. The results suggest that dibucaine neither increases the membrane permeability to PEP nor decreases the efflux of PEP after it enters the cells.

Enhancement of Cytotoxicity by Verapamil or Increased Ca²⁺ Following PEP Treatment

HeLa cells were treated with PEP (20 μg/ml) for 1 hr. After incubation, the cells were washed and further incubated for 30 and 60 min in fresh medium without or with verapamil (0.15 mM) or with CaCl₂ (total 14 mM). HeLa cells were incubated with PEP (20 μg/ml (solid line) or 40 μg/ml (dotted line)) at 37°C for 1 hr. After incubation, the cells were washed and further incubated for 30 and 60 min in fresh medium without or with verapamil or with CaCl₂ (total 14 mM) (△). Table III. Reversal of Enhanced Cytotoxicity of PEP Combined with Verapamil or Dibucaine by Cytochalasin B and α-Tocopherol

Table III: Reversal of Enhanced Cytotoxicity of PEP Combined with Verapamil or Dibucaine by Cytochalasin B and α-Tocopherol

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Cell counts (× 10⁻⁵ cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td>Control</td>
<td>10.0</td>
</tr>
<tr>
<td>PEP</td>
<td>7.3</td>
</tr>
<tr>
<td>PEP + verapamil</td>
<td>0.7</td>
</tr>
<tr>
<td>PEP + dibucaine</td>
<td>1.0</td>
</tr>
</tbody>
</table>

FM3A cells were incubated with cytochalasin B (CB) (20 μM) or D (CD) (20 μM) or α-D,L-tocopherol (αTc) (1 mM) at 37°C for 1 min, and were then further incubated for 30 min with PEP (10 μg/ml) and verapamil (0.2 mM) or dibucaine (0.25 mM) in the presence of CB, CD, and αTc. Cells were counted after 3 days of culture. CB, CD, or αTc alone had no cytotoxicity, and addition of the solvents (0.25%) alone had no cytotoxic effect.
clonal growth assay or reincubated with or without verapamil (0.15mM) or CaCl₂ (14mM) for up to 1 hr. The survival of cells that were plated immediately after PEP treatment increased 2- or 3-fold within the recovery incubation of 30 min, and reached a plateau between 30 and 60 min (Fig. 6). On the other hand, the cells incubated with verapamil or increased Ca²⁺ following PEP treatment showed a decrease in survival which depended on the time of incubation for up to 1 hr. The decrease in survival leveled off between 1 and 2 hr (not shown). The reduction in survival caused by verapamil treatment after PEP treatment rapidly diminished as the time interval between the two treatments increased, and treatment 1 hr after PEP treatment failed to enhance the cytotoxicity.

Reversal of Enhanced Cytotoxicity by Cytochalasin B, α-Tocopherol, and by Ca²⁺-Transport Inhibitors Cytochalasin B (20μM), but not cytochalasin D, showed a reversal effect on the enhanced PEP cytotoxicity induced by verapamil and dibucaine (Table III). The antioxidant α-tocopherol could also partially reverse the effects of verapamil and dibucaine. Reversal by cytochalasin B and α-tocopherol was also observed when cells were incubated with the drugs together with verapamil or dibucaine following PEP treatment. Neither drug, however, could reverse the enhanced cytotoxicity induced by PEP combined with increased Ca²⁺. The effect of increased Ca²⁺ was, however, reversed almost completely by inhibitors of Ca²⁺ uptake, i. e., ruthenium red and lanthanum chloride, and also by increased MgCl₂ (8mM) (Table IV). However, the Ca²⁺ transport inhibitors were unable to reverse the enhanced cytotoxicity due to PEP and verapamil or dibucaine.

Effect of Verapamil and Increased Ca²⁺ on PEP-induced Damage in the DNA-membrane Complex When mammalian cells were lysed under mild conditions, the DNA sedimented very rapidly, and this rapidly sedimenting material consisted of DNA complexed with lipid components, probably derived from nuclear membrane.26)

![Fig. 7](image_url)
This DNA complex also seemed to contain a protein component. Miyaki et al. showed that bleomycin induced decomposition of the DNA complex at low doses which hardly induced double-strand scission of free DNA. The decomposition of the DNA complex in HeLa cells induced by PEP was promoted by verapamil and increased Ca\(^{2+}\) (Fig. 7A). Reincubation of the cells after PEP treatment regenerated the rapidly sedimenting DNA complex (Fig. 7B). However, the presence of verapamil or Ca\(^{2+}\) in the repair incubation inhibited the regeneration of the decomposed DNA complex.

**DISCUSSION**

The present data confirmed our previous findings that verapamil, dibucaine and increased Ca\(^{2+}\) enhance PEP cytotoxicity, and further characterized the drug- and Ca\(^{2+}\)-induced enhancement of the cytotoxicity. It is known that verapamil and dibucaine as well as Ca\(^{2+}\) interact extensively with proteins and phospholipids in cell membranes, inducing various structural and functional modifications of the membrane. Therefore, the drug- and Ca\(^{2+}\)-induced enhancement of PEP cytotoxicity must be a membrane-related phenomenon. Enhancement of PEP activity by membrane-interacting drugs was also demonstrated in the antibacterial effect on Bacillus subtilis (unpublished data), indicating that the phenomenon is not limited to eukaryotic cells. Our data indicate that cellular uptake of [\(^3\)H]PEP does not increase in dibucaine-treated cells. Moreover, treatment with verapamil and increased Ca\(^{2+}\) after treatment with PEP enhanced PEP cytotoxicity. These results suggest that membrane permeability to PEP is not directly related to the enhanced cytotoxicity, and that verapamil- or Ca\(^{2+}\)-induced membrane modification may interact with PEP-induced cellular damage. However, our results do not rule out the possibility of increased PEP availability to particular sensitive targets as a result of changes in its intracellular distribution.

Tsuruo et al. have shown that verapamil enhances the cytotoxic effect of vincristine and vinblastine in P388 leukemia and its vincristine-resistant cells, and causes accumulation of vincristine by inhibiting vincristine efflux from the cells. However, under our experimental conditions of a much shorter time of drug treatment (1 hr) with a higher dose of verapamil (0.15mM) and using HeLa cells and FM3A cells, we observed only a slight increase in vincristine and vinblastine cytotoxicities compared with a much greater increase in PEP cytotoxicity (unpublished data).

Cytochalasin B, an inhibitor of microfilament function by inhibiting actin polymerization and also of glucose carrier activity by binding to plasma membrane sites, caused a reversal of the enhanced PEP cytotoxicity induced by verapamil and dibucaine. Cytochalasin D, which rather specifically inhibits microfilament function, hardly reversed the enhanced cytotoxicity. In addition, phloretin, a specific inhibitor of glucose carrier activity and a competitor of cytochalasin B for the glucose carrier-related membrane site, showed no reversal of the enhanced cytotoxicity (unpublished data). These results suggest that neither microfilaments nor glucose carrier activity are related to the enhancement of the cytotoxic effect of PEP, and cytochalasin B may compete with verapamil or dibucaine for other membrane sites which are unrelated to glucose carrier activity. The antioxidant \(\alpha\)-tocopherol partially reversed the enhancement of PEP cytotoxicity by the drugs, suggesting an involvement of lipid peroxide formation by the action of PEP in the enhanced cytotoxicity. It was recently shown that bleomycin-Cu complex increased the lipid peroxide level in cultured lung fibroblasts, and \(\alpha\)-tocopherol reduced the increased level of lipid peroxides.\(^9\) Cytochalasin B and \(\alpha\)-tocopherol showed no
reversal or only a slight reversal of enhanced PEP cytotoxicity induced by increased Ca^{2+}, but the action of Ca^{2+} was specifically antagonized by ruthenium red and lanthanum chloride, potent inhibitors of Ca^{2+} uptake by cells.\textsuperscript{3,13,29) A high dose of MgCl\textsubscript{2} (8mM) also antagonized the action of Ca^{2+}. In addition, a Ca^{2+} ionophore (antibiotic A23187) induced no enhancement of PEP cytotoxicity (unpublished). These results suggest that an increase in membrane-bound Ca^{2+} levels mediates the enhancement of PEP cytotoxicity, and the membrane sites of Ca^{2+} binding may be different from those of verapamil binding. Increased Ca^{2+} has been shown to induce rapid and reversible phase separations in phospholipid bilayer membrane by interacting with phosphatidylserines.\textsuperscript{21) }

Studies in vitro and in vivo have shown that cells have the capacity to repair at least part of the damage induced by bleomycin.\textsuperscript{1) Recovery from PEP-induced potentially lethal damage is measured in terms of the increase in the survival as a function of time before trypsinization for colony formation and after PEP treatment. Treatment with verapamil or with increased Ca^{2+} after PEP treatment impaired the recovery of cells from PEP-induced damage, and induced enhancement of cell killing (Fig. 6). Furthermore, verapamil and Ca^{2+} promoted the decomposition of the DNA-membrane complex in HeLa cells, and also blocked the regeneration of the decomposed DNA complex (Fig. 7). The results imply that recovery from damage might be related to the repair of damage in the DNA complex, and the repair may be impaired in the cells where membranes are modified by verapamil or Ca^{2+}. Cell damage caused by bleomycin is more similar to X-ray damage than to UV damage.\textsuperscript{5,19) Cell exposure to ethanol enhances the cytotoxic effect of bleomycin and also that of X-ray irradiation.\textsuperscript{14,19) Verapamil and increased Ca^{2+}, however, showed no enhancement of the cytotoxic effect of \textsuperscript{60}Co irradiation at 37° in FM3A cells (unpublished data).}

Acknowledgments

The authors wish to thank Drs. H. Umezawa and S. Okamoto for helpful suggestions and encouragement during the course of these studies.

(Received July 5, 1983/Accepted Aug. 6, 1983)

References


22) Mizuno, S. and Ishida, A. Potentiation of bleomycin cytotoxicity by membrane-interacting drugs and increased calcium ions.