EFFECT OF BLOOD, ASCITES, AND TUMOR CELL DENSITY ON CYTOCIDAL ACTION OF NEOCARZINOSTATIN

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Cytocidal activity of Neocarzinostatin (NCS) was analyzed in vitro by using cultured and ascitic L1210 cells. NCS shows rapid and typical concentration-dependent cytotoxic action against L1210 cells. The concentration required for 90% cell-kill (MLD90) and for all 10^6 cell-kill (MIC/10^6) was 5×10^-2 and 4×10^-1 μg/ml, respectively, when L1210/C cells were exposed to NCS at the concentration of 2×10^5/ml in RPMI-1640 medium. When L1210/C cells at the concentration of 1.3×10^7/ml were exposed to NCS, cytotoxic activity of NCS decreased, and MLD90 and MIC/10^6 increased to 1.75×10^-1 (3.4×) and 2.83 μg/ml (6.5×), respectively. Also, when a small fraction of whole BDF1 mouse blood, red blood cells, or spleen cells was present in the reaction mixture, cytotoxic activity of NCS appeared to decrease. Furthermore, when washed hemorrhagic ascitic L1210 cell suspension was exposed to NCS, 100 times or more concentration of NCS was required for 90% cell-kill or all 10^6 cell-kill. The effect of plasma or serum on cytotoxic activity of NCS was minimum. These results indicate that cytotoxic activity of NCS is greatly inhibited through contact with tumor cells, blood cells, spleen cells, and/or hemorrhagic ascites. This may be one of the reasons why L1210 cells, which show high sensitivity against NCS in vitro, are less sensitive in vivo. In order to explain these findings, the possibility of inactivation of NCS by the cells or reduction of free active NCS molecules by the binding or adsorption with the cells is discussed. This characteristics in action of NCS should be taken into account in clinical use.

Neocarzinostation (NCS) is a proteinous antitumor antibiotic and has been shown to have a rapid and concentration-dependent cytotoxic action in vitro.5, 6, 7 It has also been reported that in vitro cultured tumor cells had a high sensitivity against NCS.5, 6, 8) In the case of in vitro cultured L1210 mouse leukemia cells, the concentrations required for 90% cell-kill (MLD90) and for all 10^6 cell-kill (MIC/10^6) by 30-min exposure have been reported to be 0.05 and 0.17 μg/ml, respectively.5) In spite of high sensitivity of L1210 cells against NCS in vitro, intraperitoneally transplanted L1210-bearing mice could not be cured by the intraperitoneal injection of NCS, even at 2 mg/kg which is almost equal to its LD50.9 It is unlikely that the in vivo dosage of NCS is too small to obtain cure of L1210-bearing mice, compared with the level of in vitro sensitivity of L1210 cells against NCS. Namely, there is a great discrepancy in the effect of NCS between in vitro and in vivo.

NCS has no time-dependent action in vitro. Namely, the effective concentration of NCS was almost the same regardless of duration of exposure time.5–8 It has been reported that several other antitumor antibiotics had not only concentration-dependent action, but also time-dependent action.5–8 Therefore,
NCS has very unique characteristics in cytotoxic action among antitumor antibiotics. These data suggested that one of the reasons for this unique action of NCS in vitro may be the rapid inactivation of NCS through its contact with tumor cells. Then, we have to think of many normal cells existing concomitantly with tumor cells in vivo. The cytotoxic action of NCS might be reduced by its contact not only with tumor cells but also with normal cells. This may be responsible for the discrepancy of effect of NCS between in vitro and in vivo. In the present investigation, we conducted the experiments in order to solve the discrepancy of effect of NCS between in vitro and in vivo.

**Materials and Methods**

**Tumor Cells** In vitro cultured L1210/C cells and ascitic L1210 mouse leukemia cells were used in the experiment. L1210/C cells were cultured in RPMI-1640 (Nissui Seiyaku Co., Tokyo) supplemented with 10% fetal bovine serum (Microbiological Associates, U.S.A.), grown in suspension state in humidified CO2 incubator (Forma Scientific, water jacket type) at 37.5°C. Ascitic L1210 cells were propagated intraperitoneally in BDF1 mice.

**In vitro Assay Method for Cytotoxic Action of Neocarzinostatin** Soft agar cloning assay was based on the determination of growth capacity of cells after exposure to antitumor agents, as described before. In this work, blood, ascites, or spleen cells were added to the reaction mixture to see the effect of these components on the cytotoxic activity of NCS.

Fresh whole blood, red blood cells, plasma, spleen cells from BDF1 mouse, or O-type human fresh red blood cells were added to the reaction mixture. The heparinized blood was taken and spun down at 1500 rpm for 10 min to separate plasma from red blood cells. The red blood cells were washed 3 times and suspended in RPMI-1640 medium. The cell suspension was made by pumping through 16 gauge needle and filtered through a stainless steel mesh. After the viable cell number was counted in hemocytometer, the cell suspension was adjusted to adequate cell density.

The reaction mixture consisted of 1.0 ml of L1210/C cell suspension in RPMI-1640 with adequate cell density, 0.3 ml of fetal bovine serum, 1.0 ml of normal cell suspension, 0.2 ml of RPMI-1640 medium, and 0.5 ml of Neocarzinostatin solution of suitable concentration in RPMI-1640 medium (total, 3 ml). The reaction mixture was mixed rapidly and incubated for 30 min at 37°C in humidified CO2 incubator. Then, they were spun down at 1200 rpm for 5 min and the precipitate was washed 1 to 3 times with RPMI-1640 medium and suspended in 3 ml of complete growth medium (RPMI-1640 supplemented with 10% fetal bovine serum). The surviving fraction of cells was measured by the soft agar cloning assay as described before.

**In vitro-in vivo Assay for Ascitic L1210 Cells**

It is inadequate and inconvenient to use a large amount of L1210 cells for the in vitro drug treatment assay, because cell viability cannot be maintained in the in vitro culture system of high density of tumor cells and, therefore, a few tumor cells remaining viable in about 108 L1210 cells treated with NCS cannot be detected by the in vitro soft agar cloning assay. In order to analyze the effect of high density of L1210 cells on cytotoxic activity of NCS, in vitro-in vivo assay system is applied.

Seven-day-old hemorrhagic ascites of BDF1 mice, after intraperitoneal implantation of 106 L1210 cells, was taken into heparinized RPMI-1640 medium. After spinning down at 1500 rpm for 5 min, the cells were washed 3 times with RPMI-1640 and suspended in RPMI-1640 medium of equal volume as the original ascites. L1210 cells were counted using a hemocytometer. Then, the cell suspension was diluted or concentrated appropriately. Original ascites which we used here contained 8.4 × 107/ml of L1210 cells and 20% packed red blood cells. The reaction mixture consisted of 8 ml of the cell suspension, 1 ml of fetal bovine serum, and 1 ml of NCS solution of a suitable concentration in RPMI-1640 medium, and the mixture was incubated at 37°C for 30 min in humidified CO2-incubator. After spinning down at 1500 rpm for 5 min, the cells were washed 3 times with RPMI-1640 and suspended in 5.5 ml of RPMI-1640 medium.

Five-tenths ml of the cell suspension was inoculated intraperitoneally into BDF1 mouse. Ten mice were used for each experimental group. Survival days of mice were observed and increase in life span (ILS, %) was calculated from the following equation:

\[
\text{ILS (\%)} = \left( \frac{\text{average survival days (ASD) of treated group} - \text{ASD of control group}}{\text{ASD of control group}} \right) \times 100
\]
The number of viable cells in the inoculated L1210 cells was estimated by extrapolation from ILS (%), using cell dose-survival curve of L1210-bearing mice, which was expressed by $Y = -24X + 144$, where $Y$ is ILS (%) and $X$ is the logarithmic value of L1210 cell dose inoculated intraperitoneally.

Hematocrit value of contaminated red blood cells in hemorrhagic ascites was measured by the microcentrifugal method. More than 95% of nucleated cells in hemorrhagic ascites were L1210 leukemia cells.

**Drug Sensitivity of L1210 Cells** The concentrations required for 90% cell-kill and all 10⁶ cell-kill at a given exposure time were expressed as MLD₉₀ and MIC/10⁶, respectively, which were calculated from the dose-response curves of cytocidal action of NCS. NCS was obtained from Kayaku Antibiotic Research Laboratory, Tokyo.

**RESULTS**

**Effect of L1210 Cell Density and Normal Cells on Cytocidal Action of Neocarzinostatin against Cultured L1210/C Cells**

The dose–response curves of cytocidal action of NCS are shown in Fig. 1. The surviving fraction of L1210 cells decreased exponentially as the concentration of NCS increased. However, the slope of dose–response curves became milder as the tumor cell concentration increased in the reaction mixture. This indicates that the sensitivity of L1210 cells to NCS appears to decrease as the tumor cell concentration increases.

The cytocidal action of NCS was much influenced by the concomitant presence of normal blood cell components of BDF₁ mice. The slope of dose–response curves of cytocidal action of NCS became milder by concomitant presence of final 10% whole blood, 1.3 × 10⁷ cells/ml of spleen cells, and 5% of packed red blood cells of BDF₁ mouse in the reaction mixture (Fig. 2). Ten percent of BDF₁ plasma does not have much effect on NCS activity.

Also, the human O-type washed fresh red blood cells inhibited the cytocidal activity of NCS (Fig. 3). The inhibition became stronger as the concentration of red blood cells increased.

**Effect of Hemorrhagic Ascites on Cytocidal Activity of Neocarzinostatin against Ascitic L1210 Cells** Average survival days of 10 mice inoculated with 10⁶ untreated ascitic L1210/C cells were 7.7 days (0% ILS). After a mixture of 6.7 × 10⁷ cells/ml of ascitic L1210 cell suspension with 20% packed red blood cells was incubated with a given concentration of NCS, 6 × 10⁷ L1210

![Fig. 1. Effect of tumor cell density on cytocidal activity of NCS](image-url)
cells or $3 \times 10^6$ L1210 cells were inoculated intraperitoneally into BDF$_1$ mice. The concentration–survival curves are shown in Fig. 4. Percentage ILS increased linearly as the concentration of NCS increased up to 20 $\mu$g/ml in the reaction mixture. However, the concentration–survival curves reached a plateau at the concentration over 30 $\mu$g/ml of NCS. Percentage ILS did not exceed 90% in the case of intraperitoneal transplantation of $6 \times 10^7$ L1210 cells and 120% in the case of $3 \times 10^6$ cells. This result indicates that a small fraction of L1210 cells was found to survive, even after $6.7 \times 10^7$ L1210 cells/ml with 20% packed red blood cells was incubated with higher concentration (30~
CYTOCIDAL ACTION OF NEOCARZINOSTATIN

60 μg/ml) of NCS for 30 min at 37°. Namely, ca. 2 × 10^2 out of 6 × 10^7 cells or ca. 10^3 cells out of 3 × 10^6 cells were estimated to remain viable after incubation with higher concentration of NCS. On the contrary, when the same L1210 cells of 3.2 × 10^6/ml with 1% packed red blood cells were incubated with lower concentration of NCS (namely, 20-fold dilution of the original ascitic cell suspension used), there were no viable cells found in the L1210 cell suspension. In addition, the slope of the concentration-survival curves becomes much steeper, and the lower concentration of NCS is enough to kill all of 3 × 10^6 L1210 cells. This indicates that the cytocidal activity of NCS was greatly inhibited by the presence of a higher concentration of tumor cells and the concomitant presence of normal cell components in hemorrhagic ascites.

Apparent Sensitivity of L1210 Cells to Neocarzinostatin Measured after Various Drug Treatment Conditions The values of MLD_{90} and MIC/10^6 of NCS against L1210 mouse leukemia are shown in Table I. MLD_{90} and MIC/10^6 values are 5.16 × 10^{-2} and 4.35 × 10^{-1} μg/ml, respectively, when the cytocidal action of NCS was measured under the condition in which 2 × 10^5/ml L1210 cell suspension in RPMI-1640 medium without serum is incubated with a given concentration of NCS. These levels of MLD_{90} and MIC/10^6 were normalized to 1.0 in order to compare with sensitivities measured under other conditions.

The level of the sensitivity appeared to decrease as the tumor cell concentration or the concentration of blood cell components in the reaction mixture increased. Ninety-fold increase in tumor cell concentration from 1.5 × 10^6/ml to 1.3 × 10^7/ml in the reaction mixture reflects 3.4-fold increase in MLD_{90} and 6.5-fold increase in MIC/10^6.

The effect of BDF_1 mouse plasma or fetal bovine serum on cytocidal activity of NCS is minimum. However, the concomitant presence of 5~25% fresh BDF_1 mouse whole blood, fresh red blood cell, and O-type human fresh red blood cells in the reaction mixture reflect 3.5- to 6.5-fold increase in MLD_{90} and 7.4- to 12.8-fold increase in MIC/10^6. MLD_{90} and MIC/10^6 values appeared to increase by 3.4-fold and 5.3-fold, respectively, when the spleen cells at the concentration of 1.3 × 10^7/ml are present in the reaction mixture.

![Graph](Fig. 4. Effect of hemorrhagic ascites on cytocidal activity of NCS)
When the ascitic L1210 cells are used at the concentration of $6.7 \times 10^7$/ml with 20% packed red blood cells in the reaction mixture containing 100% cell components of the original hemorrhagic ascites, MLD$_{50}$ is 5 µg/ml (97-fold increase) and MIC/10$^6$ is over 60 µg/ml (over 137-fold increase). However, when 20-fold diluted L1210 cell suspension was used in the reaction mixture, MLD$_{50}$ and MIC/10$^6$ were $7.5 \times 10^{-1}$ µg/ml (14.5-fold increase) and 4.05 µg/ml (9.45-fold increase), respectively. Thus, the sensitivity of L1210 cells appeared to change markedly according to the condition of drug treatment. In general, increase in cell density of tumor cells, blood cells, as well as hemorrhagic ascites, rather than concentration of serum or plasma, resulted in the inhibition of cytocidal activity of NCS.

### DISCUSSION

It has been reported that NCS showed a rapid and typical concentration-dependent action but not time-dependent action in vitro.$^{5,6,7,11}$ This suggests that the cytocidal action of NCS may be easily inhibited by its exposure to L1210 cells. It has been reported that more than 70% of NCS lost its bactericidal activity by incubation with the tissue homogenate, especially from the liver, testis, and kidneys, for 1 to 2 hr.$^{11}$ It has been found that intact NCS is quite resistant against proteolytic digestion by trypsin, chymotrypsin, papain, and pronase, but sensitive to serine-type, SH-type, and other proteolytic enzymes such as cathepsin and peptidase.$^{2-4}$

NCS is also easily degraded into small fragments in vitro with serum or mammalian cultured cells.$^{2,4}$ These findings suggest that the inhibition of cytocidal activity of NCS.

<table>
<thead>
<tr>
<th>Amount of L1210/C cells</th>
<th>Other components</th>
<th>MLD$_{50}$ (µg/ml)</th>
<th>Ratio$^b$</th>
<th>MIC/10$^6$ (µg/ml)</th>
<th>Ratio$^b$</th>
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<tbody>
<tr>
<td>BDF$_1$ mouse$^a$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$6.7 \times 10^7$/ml</td>
<td>100% hemorrhagic ascites</td>
<td>5.00</td>
<td>(97.0)</td>
<td>60</td>
<td>(&gt;130)</td>
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<tr>
<td>$3.2 \times 10^6$/ml</td>
<td>5% hemorrhagic ascites</td>
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<td>(14.5)</td>
<td>4.05</td>
<td>(9.54)</td>
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<td></td>
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<td>25%</td>
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<td>(0.93)</td>
<td>$4.20 \times 10^{-1}$</td>
<td>(0.96)</td>
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<td>Fetal bovine serum</td>
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<tr>
<td>$2.0 \times 10^5$/ml</td>
<td>80%</td>
<td>$6.25 \times 10^{-2}$</td>
<td>(1.2)</td>
<td>$7.50 \times 10^{-1}$</td>
<td>(1.7)</td>
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<tr>
<td></td>
<td>60%</td>
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<td>$7.50 \times 10^{-1}$</td>
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<tr>
<td></td>
<td>20%</td>
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<td>(1.1)</td>
<td>$5.71 \times 10^{-1}$</td>
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<td>(1.0)</td>
<td>$4.35 \times 10^{-1}$</td>
<td>(1.0)</td>
</tr>
<tr>
<td>$1.3 \times 10^7$/ml</td>
<td></td>
<td>$1.75 \times 10^{-1}$</td>
<td>(3.4)</td>
<td>2.83</td>
<td>(6.5)</td>
</tr>
<tr>
<td>$3.2 \times 10^6$/ml</td>
<td>(---)</td>
<td>$1.00 \times 10^{-1}$</td>
<td>(2.1)</td>
<td>1.34</td>
<td>(3.1)</td>
</tr>
<tr>
<td>$1.4 \times 10^6$/ml</td>
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<td>(1.4)</td>
<td>$6.66 \times 10^{-1}$</td>
<td>(1.5)</td>
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<tr>
<td>$1.5 \times 10^5$/ml</td>
<td></td>
<td>$5.50 \times 10^{-2}$</td>
<td>(1.0)</td>
<td>$4.70 \times 10^{-1}$</td>
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$a)$ +10% fetal bovine serum  
$b)$ MLD$_{50}$ or MIC/10$^6$ divided by each standard sensitivity  
$c)$ Standard sensitivity
by L1210 cells, blood cells, or cell components of hemorrhagic ascites might be due to inactivation of NCS by the proteolytic enzymes located on the cell membrane. Another possibility for the inhibition of cytocidal activity of NCS by the cells may be the binding or adsorption of NCS with cell membrane of tumor cells or blood cells.

Studies on the distribution of NCS in vivo have shown that the residual antibacterial activity of intact NCS or radioactive counts of 14C-succinyl-NCS, which was administered with protease inhibitors, was high in the kidneys, skin, stomach, pancreas, lung, urinary bladder, and bone marrow. These tissues have a low capacity for inactivation of NCS. It has been known that a certain type of proteinases play a main role in inactivation of NCS. The inhibitors of proteinase activity also inhibit degradation of NCS. These facts suggest that the combined use of proteinase inhibitors with NCS might potentiate the clinical effectiveness of NCS.

We demonstrated that a small fraction of L1210 cells is still viable, even after higher concentration of NCS was exposed to ascitic L1210 cells suspension. It might be suggested that 1 cell out of $3 \times 10^5$ L1210 cells might be resistant, based on the experiment. However, when 20-fold diluted ascitic L1210 cell suspension was incubated with NCS, NCS became more cytotoxic and no viable cells were found in all $3 \times 10^6$ L1210 cells. Moreover, in the diluted L1210 cell suspension, L1210 cells were killed more efficiently by lower concentration of NCS. These facts indicate that a small fraction of viable cells remaining after exposure to a higher concentration of NCS is not resistant cells, but small fraction of L1210 cells can survive because of rapid inhibition of action of NCS by cell components in the original ascites.

The level of sensitivity of L1210 cell appeared to change about 100 times according to the drug treatment condition. This characteristic in the action of NCS should be taken into account in its clinical use.

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10) Shimoyama, M., Kimura, K., Gann, 63, 773–783 (1972).