Effect of Neocarzinostatin on DNA Synthesis in L1210 Cells

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SUMMARY

The mechanism of action of neocarzinostatin has been investigated. The synthesis of DNA in L1210 cells in vivo was selectively inhibited by the drug. DNA synthesis in vitro catalyzed by crude DNA-dependent DNA polymerase of L1210 cells was sensitive to the drug in a concentration-dependent manner. This inhibition was reversed by increasing the concentration of DNA. Evidence for a DNA-drug interaction was obtained from the results of the preincubation of DNA with the component in the DNA polymerase reaction and from thermal denaturation studies. The degradation of cellular DNA in vivo by the drug was also suggested by the sedimentation profile in the alkaline sucrose gradient centrifugation.

INTRODUCTION

NCS, an acidic polypeptide, is an antitumor antibiotic with a molecular weight of 9,000 (9) or 10,700 daltons (13) isolated from Streptomyces carzinostaticus F-41. NCS has been shown to have a significant growth-inhibitory effect on mouse ascites tumor (3, 10). Recently, this antibiotic was reported to be effective against human leukemias (15). Although several papers (17, 18) showed that NCS inhibited DNA synthesis strongly in Sarcina lutea and in cultured mammalian cells such as HeLa cells (8), the exact mechanism of action of NCS is not entirely clear. This report demonstrates the activity of NCS toward L1210 cells and describes the experiments relevant to the mechanism of action of the antibiotic. The results presented indicate that NCS is an effective inhibitor of DNA-dependent DNA polymerase of L1210 cells.

MATERIALS AND METHODS

Chemicals. Lyophilized NCS was obtained from Kayaku Antibiotics Research Company Ltd., Tokyo, Japan, and stored in the dark at −20°C. NCS was dissolved in distilled water before use. Calf thymus DNA was purchased from Worthington Biochemicals Corp., Freehold, N. J. dTTP, dCTP, dGTP, and dATP were obtained from Sigma Chemical Co., St. Louis, Mo. dATP-3H, deoxythymidine-3H, uridine-3H, and adenine-14C were purchased from the Radiochemical Center, Amersham, England.

Cells. Cell suspensions were prepared from L1210 ascites tumors, grown in C57BL/6 × DBA/2 mice, which were kindly provided from Dr. M. Shimoyama and Dr. K. Kimura, National Cancer Center, Tokyo, Japan. Cells (2 × 10⁶) were inoculated i.p. 7 days prior to each experiment. The cells, obtained from the peritoneal cavity, were treated once with Tris-buffered ammonium chloride to remove red cell contamination (2) and then washed twice with 0.85% NaCl solution and once with Eagle’s minimum essential medium. All isolation procedures were performed at 0–4°C.

Incubation. The cells were resuspended in Eagle’s minimum essential medium supplemented with 30% calf serum to give a final concentration of 3 × 10⁶ cells/ml. The number of cells in the suspension was assessed by the standard procedure of the leukocyte counting, using a diluting pipet and a hemocytometer. Tubes containing 1 ml of the suspension were warmed for 15 min at 37°C and 10 μl of the stock NCS solution were added to the cell suspension to give the desired concentration. The pH of the incubation mixture was not altered by adding the NCS solution. The control tube contained distilled water in place of NCS. After the cells were preincubated with NCS, thymidine-3H (5 Ci/mmol) or uridine-3H (2.3 Ci/mmol) was added to each tube to a final concentration of 1 μCi, and then the incubation was continued for 30 min at 37°C. The reaction was stopped by chilling the tubes on ice, and the cells were layered by gentle suction onto glass filters (Whatman GF/C, 2.4 cm), prewetted with 0.85% NaCl solution. The filters were washed with cold 0.85% NaCl solution 3 times and further washed with cold 10% Cl₃-COOH containing 1% sodium pyrophosphate 4 times, dried, and counted in toluene base scintillation fluids.

When adenine-14C (8.5 mCi/mmol) was used as a radioactive precursor, the number of cells was increased (5 × 10⁷ cells/ml) and 1.5 ml of cell suspensions were placed in incubation tubes. After the cells were exposed to NCS for 60 min at 37°C, the cells were labeled with adenine-14C (1.3 μCi/tube) for 60 min at 37°C. DNA and RNA were isolated by the method of Hecht and Potter (7) after removal of the acid-soluble fraction. Absorbance of each fraction at 260 and 280 nm was measured, and aliquots of fractions were dissolved in the solvent system of Bruno and Christian (4). The radioactivity was determined in a Packard Tri-Carb scintillation counter. The counts were corrected for quenching by the internal standard method.

Under the conditions described above, the control experiments showed that survival of L1210 cells, judged by the ability of cells to exclude 0.025% trypan blue, was more than 90%.
90% and the incorporation of the label was linear for 3 hr.

Isolation of Crude DNA Polymerase from L1210 Cells. The extract containing DNA-dependent DNA polymerase activity was isolated from L1210 cells suspended in 5 volumes of buffered sucrose medium according to the method of Bollum (1). The suspension was homogenized in a Potter-Elvehjem homogenizer using a Teflon tight pestle followed by centrifugation at 100,000 × g. The resulting supernatant was used as DNA polymerase in this experiment.

Assay Procedure of DNA Polymerase Activity. Reaction mixture (total volume, 0.25 ml) contained: 20 mM Tris HCl buffer, pH 7.4; 8 mM MgCl₂; 1 mM 2-mercaptoethanol; 0.4 mM each of dGTP, dCTP, and dTTP; and 1 μCi of dATP-³H (14 Ci/mmol), calf thymus DNA (heat denatured), and the enzyme. The reaction was performed at 37° for 30 or 60 min. The reaction was stopped by adding 2 ml of cold 10% Cl₃CCOOH. Bovine serum albumin (100 μg) was added as a carrier. After 15 min at 4°, precipitates were collected on glass fiber filters (Whatman GF/F, 2.4 cm in diameter), washed 4 times with 5-ml portions of a solution of 10% Cl₃CCOOH and 1% sodium pyrophosphate, dried, and counted in toluene base scintillation fluid. In this condition used, the incorporation of dATP-³H into the acid-precipitable fraction was linearly increased for 180 min.

Thermal Profile of DNA. The thermal denaturation profile of calf thymus DNA was determined using a Gilford 240 recording spectrophotometer, equipped with an automatic sample changer. The temperature of the sample cuvettes was increased at a rate of 1°/min with the linear temperature programmer.

Analysis of the Size of Cellular DNA by Alkaline Sucrose Gradient. For this purpose, cultured L1210 cells were used, maintained in Rosell Park Memorial Institute Medium 1640 supplemented with 10% calf serum. Before treatment with the drug, the cultures in logarithmic growth were incubated for 24 to 48 hr with thymidine-³H (5 Ci/mmol, 1 μCi/ml). After the radioactive precursor was washed out, the resuspended cells (5 × 10⁴ cells/ml) were cultured at 37° for 60 min in the presence of an appropriate concentration of NCS. For analysis of DNA damage by the alkaline sucrose gradient centrifugation, the technique developed by Sawada and Okada (19) was used with slight modifications. In this method, 5 × 10⁴ cells in a volume of 0.1 ml were placed in a high alkaline-salt lysing solution layered on top of a linear 5 to 20% alkaline sucrose gradient, containing 0.3 N NaOH; 0.7 M NaCl; 0.001 M EDTA; and 0.01 M Tris, pH 12. Lysis was allowed to continue for 17 ± 5 hr at 5° in the dark. After the cell lysis was completed, the gradients were centrifuged for 120 min at 38,000 rpm on a Hitachi RPS 40 swing rotor, and about thirty 10-drop fractions were collected directly on disc filters (Whatman No. 3MM, 2.4 cm in diameter). The filters were washed successively in a cold solution of 10% Cl₃CCOOH and 2% sodium pyrophosphate, in ethanol, and in acetone; then they were dried and counted in toluene base scintillation fluid. Results are expressed as the percentage of the total radioactivity recovered in each fraction.

RESULTS

Effects of NCS on the Synthesis of DNA and RNA. L1210 cells were incubated with thymidine-³H or uridine-³H after the cells were preincubated for 60 min in the presence of NCS.

As shown in Chart 1a, the incorporation of thymidine-³H into the acid-precipitable fraction was markedly inhibited by NCS. Chart 1b shows that the decrease of the incorporation was linear, when counts of acid-insoluble fractions were plotted against the concentration of NCS added on a semilogarithmic paper. On the other hand, the incorporation of uridine-³H into the acid-precipitable fraction was less inhibited. The effect of NCS for the biosynthesis of nucleic acids in L1210 cells was also investigated using adenine-¹⁴C as a radioactive precursor. As shown in Chart 2, analysis of the labeled macromolecules by the method of Hecht and Potter (7) revealed that DNA synthesis was inhibited markedly, while little inhibition of RNA synthesis was observed at a concentration as high as 10 μg/ml, suggesting that NCS inhibited DNA synthesis selectively. The effect of NCS on DNA synthesis started rapidly. As shown in Chart 3, 15-min exposure of cells to NCS caused a deep inhibition of the incorporation of thymidine-³H into the acid-precipitable fraction and the inhibition was almost completed by 60 min.

In order to determine whether NCS affects the uptake of the precursor of DNA, the uptake and the metabolism of thymidine-³H in L1210 cells were measured. As shown in Table 1, the incorporation of thymidine-³H into the acid-soluble fraction was not inhibited by NCS at concentrations of 0.5 to 5 μg/ml, which gave about 50% inhibition on DNA synthesis. Moreover, the radioactivity in the acid-soluble

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Effect of NCS on DNA Synthesis

PERCENT INCORPORATION OF ADENINE-14C INTO NUCLEIC ACID

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>DNA</th>
<th>RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>70%</td>
<td>30%</td>
</tr>
<tr>
<td>0.5</td>
<td>60%</td>
<td>40%</td>
</tr>
<tr>
<td>1.0</td>
<td>50%</td>
<td>50%</td>
</tr>
<tr>
<td>5.0</td>
<td>40%</td>
<td>60%</td>
</tr>
<tr>
<td>10.0</td>
<td>30%</td>
<td>70%</td>
</tr>
</tbody>
</table>

Chart 2 Effects of NCS on nucleic acid synthesis in L1210 cells. L1210 cells (5 x 10^7 cells/ml Eagle's medium with 30% calf serum, 1.5 ml/tube) were preincubated for 60 min at 37°C in the presence of NCS; then the cells were labeled with adenine-14C for 60 min at 37°C. DNA and RNA fractions were extracted as described in "Materials and Methods." DNA and RNA contents in each fraction were measured by the absorbance at 260 nm, as 1 µg/ml amounts of DNA or RNA has the absorbance of 0.022 at 260 nm. Results were expressed as the cpm incorporated per µg DNA or RNA; 3930 cpm/µg DNA and 3600 cpm/µg RNA were incorporated without NCS.

fraction existed almost completely as nucleotides. The results suggested that NCS did not affect the precursor level.

Effect of NCS on DNA-dependent DNA Polymerase. The effect of NCS on cell-free DNA synthesis catalyzed by L1210 cell extract was tested. As shown in Table 2, the DNA synthesis was DNA dependent, and addition of all 4 deoxyribonucleotides to the reaction mixture was required. The capacity of NCS to inhibit DNA polymerase seemed to be less than expected, but inhibition was nevertheless relatively efficient and dependent on the drug concentration. It was found that the DNA concentration used in Table 2 was below saturation. Conversely, the degree of inhibition depended on the amounts of template DNA in a reaction mixture. As shown in Table 3, inhibition decreased with increasing amounts of DNA used as templates. Chart 4 clearly shows that 41% inhibition of DNA polymerase activity by NCS could be reversed completely by increasing amounts of DNA templates at the infinite level.

The possibility of the direct interference of NCS with DNA polymerase was considered. On the addition of NCS, 80 µg/ml, inhibition of DNA synthesis catalyzed at various concentrations of the enzyme occurred; these results, shown in Table 4, indicate that there was no appreciable correlation between the concentration of the enzyme and inhibition rates by NCS of DNA synthesis.

In order to rule out the further possibility of NCS-enzyme complex or NCS-deoxyribonucleotide complex formations as the mechanism of inhibition, the enzyme or each of the other components contained in a reaction mixture was separately preincubated with NCS at 37°C for 30 min, and then the reaction was started by adding the missing components. As shown in Table 5, inhibition of DNA synthesis of L1210 cells by NCS. L1210 cells (3 x 10^6 cells/ml) were incubated for the desired time at 37°C with NCS; then the cells were labeled for 30 min with thymidine-3H. We obtained 7.9 x 10^6 cpm without the preincubation with NCS.

Table 1

<table>
<thead>
<tr>
<th>NCS (µg/ml)</th>
<th>Acid-insoluble (x 10^3 cpm)</th>
<th>% inhibition</th>
<th>Acid-soluble (x 10^3 cpm)</th>
<th>% recovery</th>
<th>% of nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>68.6</td>
<td></td>
<td>20.5</td>
<td>100</td>
<td>99.6</td>
</tr>
<tr>
<td>0.5</td>
<td>34.1</td>
<td>50.3</td>
<td>19.3</td>
<td>90.5</td>
<td>98.4</td>
</tr>
<tr>
<td>1</td>
<td>30.2</td>
<td>51.0</td>
<td>19.5</td>
<td>91.1</td>
<td>99.9</td>
</tr>
<tr>
<td>5</td>
<td>27.8</td>
<td>59.6</td>
<td>18.6</td>
<td>87.0</td>
<td>99.8</td>
</tr>
</tbody>
</table>

- % inhibition of acid-insoluble fraction.
- % recovery of acid-soluble fraction.
- % of nucleotides in acid-soluble fraction.
Table 2

Inhibition of DNA polymerase by NCS

Incubation mixture (0.25 ml) contained denatured calf thymus DNA, 40 µg/ml, and L1210 cell extract, 392 µg/ml, as DNA polymerase. Incubation was carried out for 60 min at 37°. Each value is the average of triplicate samples.

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>dATP-3H incorporated (cpm)</th>
<th>Inhibition by NCS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>10,038</td>
<td>0</td>
</tr>
<tr>
<td>- DNA</td>
<td>395</td>
<td></td>
</tr>
<tr>
<td>- dXTP (cold)</td>
<td>286</td>
<td></td>
</tr>
<tr>
<td>+ NCS, - DNA</td>
<td>376</td>
<td></td>
</tr>
<tr>
<td>+ NCS 20 µg/ml</td>
<td>9,406</td>
<td>6</td>
</tr>
<tr>
<td>+ NCS 40 µg/ml</td>
<td>8,985</td>
<td>11</td>
</tr>
<tr>
<td>+ NCS 80 µg/ml</td>
<td>6,660</td>
<td>34</td>
</tr>
</tbody>
</table>

Table 3

Inhibition of DNA polymerase by NCS

Incubation mixture (0.25 ml) contained the designated concentration of denatured calf thymus DNA and L1210 cell extract, 392 µg/ml, as DNA polymerase. Incubation was carried out for 60 min at 37°. Each value is the average of triplicate samples.

<table>
<thead>
<tr>
<th>Primer DNA (µg/ml)</th>
<th>dATP-3H incorporated (cpm)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without NCS</td>
<td>With NCS, 60 µg/ml</td>
</tr>
<tr>
<td>10</td>
<td>7,698</td>
<td>4,471</td>
</tr>
<tr>
<td>18</td>
<td>10,341</td>
<td>7,001</td>
</tr>
<tr>
<td>35</td>
<td>16,403</td>
<td>12,601</td>
</tr>
<tr>
<td>140</td>
<td>25,555</td>
<td>22,064</td>
</tr>
</tbody>
</table>

Table 4

Effect of NCS on DNA synthesis at various concentrations of the enzyme

Incubation mixture (0.25 ml) contained the designated concentration of crude DNA polymerase, denatured calf thymus DNA, 20 µg/ml, and NCS, 80 µg/ml. Incubation was carried out for 30 min at 37°. Each value is the average of triplicate samples.

<table>
<thead>
<tr>
<th>DNA polymerase (µg/ml)</th>
<th>dATP-3H incorporated (cpm)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without NCS</td>
<td>With NCS, 80 µg/ml</td>
</tr>
<tr>
<td>0</td>
<td>256</td>
<td>349</td>
</tr>
<tr>
<td>98</td>
<td>2,287</td>
<td>1,255</td>
</tr>
<tr>
<td>196</td>
<td>6,071</td>
<td>3,236</td>
</tr>
<tr>
<td>588</td>
<td>8,906</td>
<td>5,997</td>
</tr>
</tbody>
</table>

Table 5

Influence of the preincubation of NCS with the component in the DNA polymerase reaction

Incubation mixture contained denatured calf thymus DNA, 20 µg/ml, and crude DNA polymerase, 196 µg/ml, in the standard assay mixture. NCS, 40 µg/ml, was separately preincubated with crude DNA polymerase, DNA, or 4 common deoxyribonucleotide triphosphates at 37° for 30 min, and then the reaction was started by adding the missing components for 30 min at 37°. Each value is the average of triplicate samples.

<table>
<thead>
<tr>
<th>Preincubation</th>
<th>dATP-3H incorporated (cpm)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>6,684</td>
<td>4,334</td>
</tr>
<tr>
<td>DNA polymerase</td>
<td>3,917</td>
<td>3,485</td>
</tr>
<tr>
<td>dXTP (cold)</td>
<td>4,650</td>
<td>4,119</td>
</tr>
</tbody>
</table>

Chart 4. Dependency on DNA concentration, shown by double reciprocal plots of the data in Table 3.

shown in Table 5, the preincubation of NCS with the template DNA showed the most prominent inhibition of DNA synthesis (35.2%). On the other hand, the preincubation of NCS with the enzyme or with deoxyribonucleotides caused less inhibition (approximately 11%) than the preincubation of NCS with DNA, suggesting that NCS interacts with DNA but not with the enzyme.

Effect of NCS on Thermal Denaturation Profile of Calf Thymus DNA. In order to show the interaction of NCS with DNA, the thermal denaturation profile of calf thymus DNA was investigated. As shown in Chart 5, the Tm of DNA decreased slightly (1°), after the incubation of DNA with NCS, 60 µg/ml, for 15 min at room temperature (23°). The reduction of Tm caused by NCS was reproducible in several experiments and was also observed when the solvent containing Mg2+ ion was used (the result is not shown). Chart 5 also shows the effect of adriamycin on Tm of calf thymus DNA. Adriamycin, the derivative of daunomycin (5), interfered with DNA strand (21) and caused the decrease of Tm.

Effect of NCS on the Size of Cellular DNA. It has been reported that NCS induced the degradation of DNA in S. lutea (18). Attempts were made to investigate the effect of NCS on the size of cellular DNA by alkaline sucrose gradient centrifugation. The size of the DNA strand in control cells observed by this method has been estimated to be 170 S with the reference of T4 phage DNA. The sedimentation profiles

Chart 5. Thermal profile of calf thymus DNA. The experiments were performed in 1 mM Na2HPO4 and 0.1 mM EDTA, pH 7.5. Concentration of DNA was 20 µg/ml.

a dXTP, deoxyribonucleoside triphosphate.
for DNA from NCS-treated L1210 cells showed a remarkable shift of the sedimentation peak toward a smaller molecular size as shown in Chart 6, suggesting that NCS caused the degradation of DNA.

**DISCUSSION**

Our results show that NCS inhibits DNA synthesis selectively in L1210 cells; it inhibits L1210 DNA polymerase activity in vitro, presumably as the result of a DNA-NCS interaction; and it causes the degradation of cellular DNA as shown by the sedimentation profiles of cellular DNA in alkaline sucrose gradient centrifugation.

The mode of action of NCS seems to be complicated. It has been reported that NCS caused the immediate inhibition of DNA synthesis in S. lutea without any interaction with DNA (18). On the other hand, Tsuruo et al. (20) reported that NCS interacted with DNA and inhibited DNA synthesis in vitro when catalyzed by *Escherichia coli* DNA polymerase.

The results presented here suggest that the drug can interact with DNA, causing a change of DNA structure such as strand scission and inhibiting the synthesis of DNA catalyzed by the crude DNA polymerase. Quite a few drugs are known to interact with nucleic acids, producing biological effects. Several antibiotics such as phleomycin (6) and daunomycin (5) interact with DNA resulting in an increase of $T_m$, while NCS, like bleomycin (14), induces a decrease of $T_m$, probably by strand scission.

Another polypeptide antibiotic, macromomycin, was shown to inhibit DNA synthesis by binding to cell membranes (11). However, in contrast to NCS, it was reported that macromomycin neither induced the degradation of cellular DNA nor inhibited DNA synthesis in vitro in the nuclei of Yoshida sarcoma cells.

Although there are no distinct data that demonstrate the penetration of whole NCS molecules into target cells, our results suggest that the mechanism of inhibition of DNA synthesis by NCS may be directly related to the interaction of NCS or particular parts of NCS molecules with DNA template. It has been shown that NCS retains its biological activity after the treatment with a proteolytic enzyme such as trypsin or chymotrypsin (12).

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