IN VITRO RELEASE OF THYMINE FROM DNA BY NEOCARZINOSTATIN

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SUMMARY: In vitro degradation of DNA to acid soluble products was induced by the combined action of neocarzinostatin and sulfhydryl agent as 2-mercaptoethanol, dithiothreitol, or reduced glutathione, but not other reducting agent as ascorbic acid or $NaBH_4$. From the analysis by Sephadex G-10 gel filtration, acid soluble products were found to be thymine and oligonucleotide, but not thymidylic acid and thymidine. Release of adenine or guanine from DNA was not detected.

From these results, it is suggested that DNA chain breakage by the combined action of neocarzinostatin and 2-mercaptoethanol may be due to an indirect phosphodiester bond breakage with release of thymine.

INTRODUCTION

NCS, an antitumor antibiotic isolated from Streptomyces carzinostatics (1), is an acidic single-chained polypeptide with a molecular weight of approximately 10,700. Its amino acid sequence has been recently determined (2). The antitumor activity was demonstrated against various tumor cells such as Sarcoma 180 (3), Leukemia SN-36 (4), Leukemia L-1210 (5), and Hela cells (6). The effects of NCS on cells include: inhibition of mitosis (7), inhibition of DNA synthesis (7-10), and DNA chain breakage (10-12). It was recently reported that DNA chain breakage was produced in vitro by NCS, both in the presence (13) and in the absence (12) of 2-mercaptoethanol. DNA degradation into acid soluble products was not observed in vitro in both reports.

Abbreviations: NCS, neocarzinostatin; TMP, thymidylic acid.

In this communication, we report that extensive degradation of DNA into acid soluble products was induced by the combined action of NCS and 2-mercaptoethanol, and that thymine was specifically released from DNA.

MATERIALS AND METHODS

NCS was received from Kayaku Antibiotics Research Company Ltd. Tokyo.

Labeled DNA. [methyl-^{3}H]Thymine-labeled E. coli DNA was prepared as previously reported (14), except that [methyl-^{3}H]thymidine (20 Ci/mmol) from the Radiochemical Center, Amersham, England, was used, and E. coli-T(W3110) was used instead of E. coli B3. [^{14}C]Adenine, guanine-labeled B. subtilus DNA, prepared by using B. subtilus HLL3g possessing purB6 and [8-^{14}C]adenine, was received from Dr. T. Kada, National Institute of Genetics, Mishima, Japan.

Estimation of the rate of DNA chain breakage. The standard reaction mixture (0.3 ml), containing 0.05 M Tris-HCl buffer (pH 8.0), NCS, 3 mM 2-mercaptoethanol and 23 µg [methyl-^{3}H]thymine-labeled E. coli DNA (3000 cpm/µg), was incubated at 37°C for 20 min. After the addition of 60 µl of carrier albumin and 60 µl of cold 35 % CCl_3COOH, the mixture was centrifuged at 3000 rpm for 10 min, and 0.2 ml of the supernatant was counted in 10 ml of scintillator (40 g naphthalene, 4 g PPO, 0.2 g POPOP, 100 ml methanol, 20 ml ethyleneglycol in 1000 ml dioxane).

RESULTS AND DISCUSSION

NCS has been thought not to produce the scission of DNA strand in vitro (11). However, recently in vitro scission of DNA strand by NCS has been reported (12,13). Beeman et al. (13) showed that the incubation of SV-40 DNA with the antibiotic in the presence of 2-mercaptoethanol resulted in the conversion of superhelical DNA to nicked circular duplex DNA. The conversion of DNA was not shown in the absence of 2-mercaptoethanol, while Tatsumi et al. (12) showed that DNA chain breakage was produced by NCS alone. We also observed that DNA strand scission was produced by 1 µg/ml NCS alone, and it was greatly enhanced by 3 mM 2-mercaptoethanol (Fig. 1). Little acid soluble material was produced in the presence of both 1 µg/ml NCS and 3 mM 2-mercaptoethanol (Fig. 2B). These results

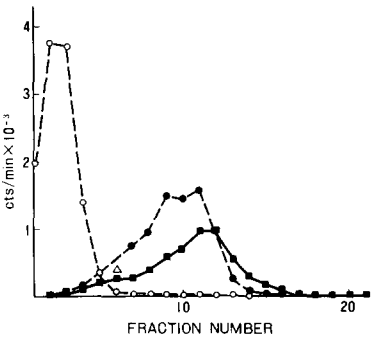

Fig. 1. Alkaline sucrose gradient centrifugation. The standard reaction mixture (0.3 ml), containing 23 µg [methyl-^{3}H]thymine-labeled E. coli DNA and 0.05 M Tris-HCl buffer (pH 8.0), was incubated for 10 min at 37°C, and was made alkaline (finally 0.3 M NaOH and 50 mM ethylenediamine tetraacetate). An aliquot (0.15 ml) was centrifuged on alkaline sucrose gradient as described previously (14). Fractions are numbered from top to bottom. Additions: ■—■ , none; ●--● , 1 µg/ml NCS; O--O , 1 µg/ml NCS and 3 mM 2-mercaptoethanol.

showed that DNA chain breakage was endonucleolytic. DNA chain breakage was also observed with neutral sucrose gradient centrifugation, suggesting that DNA strand scission was not only due to the formation of alkali-labile bond. The rate of DNA degradation increased linearly with increasing concentrations of 2-mercaptoethanol, and reached a plateau at 5-6 mM (Fig. 2A). With high concentration of NCS, extensive degradation of DNA into acid soluble materials was observed in the presence of 2-mercaptoethanol (Fig. 2B). Twenty per cent of the total radioactivity was rendered acid soluble in 20 minutes by the combined action of 300 µg/ml NCS and 3 mM 2-mercaptoethanol.

To clarify the mode of breakage of the DNA strands, we analyzed the acid soluble products by Sephadex G-10 gel filtration (Fig. 3). Significant release of thymine from DNA, accompanied by DNA chain breakage, was detected. TMP and thymidine were not detected. Release of adenine or guanine from [^{14}C]adenine, guanine-labeled B. subtilus DNA was not observed (data not

shown). These results show that the DNA chain breakage by NCS may be due to an indirect phosphodiester bond breakage with release

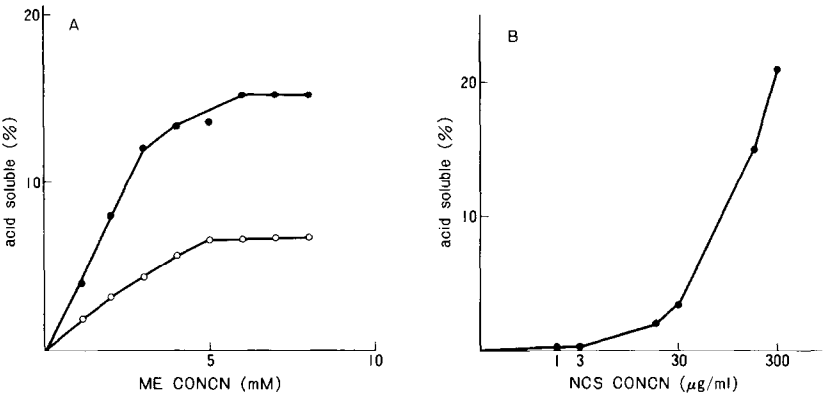

Fig. 2. Effect of increasing concentrations of 2-mercaptoethanol (ME) and NCS on DNA degradation by NCS. The reaction conditions are described in Materials and Methods. A. ●—● , 150 μg/ml NCS; O—O , 30 μg/ml of NCS. B. ●—● , 3 mM 2-mercaptoethanol.

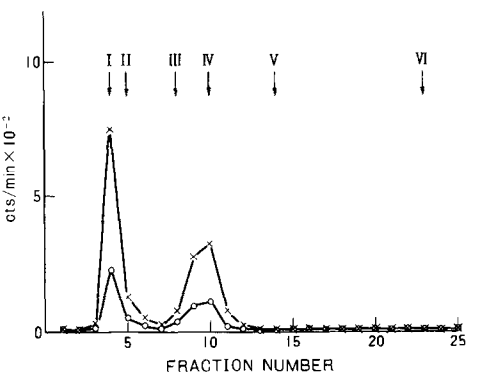

Fig. 3. Analysis of acid soluble products by Sephadex G-10 gel filtration. Reaction mixtures (0.6 ml) contained 0.05 M Tris-HCl buffer (pH 8.0), 3 mM 2-mercaptoethanol, 46 μg [methyl-^{3}H] thymine-labeled E. coli DNA (3000 cpm/μg), and 30 μg/ml NCS (O—O) or 300 μg/ml NCS (x—x). Reactions were run at 37°C for 30 min, and 0.5 ml of CCl_3COOH soluble fraction (see Materials and Methods) was adjusted to pH 10.5 by addition of 0.1 ml of 2N NaOH and applied on to Sephadex G-10 column (0.9 X 19 cm) equilibrated with 0.05 M NaCl-0.05 M glycine-NaOH (pH 10.5) and eluted with the same solution (15). Fractions (1.6 ml) were collected and 0.5 ml was counted in 10 ml of scintillator and 0.1 ml of 35 % CCl_3COOH. Arrows show elution positions of oligonucleotide(I), TMP(II), thymidine(III), cytosine(III), thymine(IV), guanine(V), and adenine(VI).

of thymine. Release of thymine has been also observed *in vitro* by X-irradiation (16-18) and bleomycin (19-21), and *in vivo* by X-irradiation (22,23).

The rate of DNA degradation by the combined action of NCS and 2-mercaptoethanol reached a plateau after 30-40 min., suggesting the possibility that NCS might be inactivated under the experimental condition. This conclusion was supported by the finding that the activity of NCS was lost about 50 % after a 5 min-preincubation with 3 mM or 10 mM 2-mercaptoethanol, and over 90 % after a 20 min-preincubation with 10 mM 2-mercaptoethanol.

The enhancement of degradation of DNA by 2-mercaptoethanol was also shown in the case of bleomycin (24). The degradation of DNA by bleomycin was enhanced not only by sulfhydryl compounds but also by other reducing or oxidizing agents (19,25) or O_2^- (21). We found that the degradation of DNA by NCS was also stimulated by other sulfhydryl compound as dithiothreitol or reduced glutathione, but not by ascorbic acid, $NaBH_4$, H_2O_2, or O_2^-. It is suggested that degradation of DNA by NCS was enhanced specifically by sulfhydryl compounds. The rate of DNA degradation by combined action of NCS and 2-mercaptoethanol displayed a broad pH optimum at 7.6-8.8, and was only 1 % of maximum at pH 5.

At present, the mechanism of NCS action on DNA and its activation by sulfhydryl compounds is not understood. The molecule of the antibiotic has four half cystine residues and is cross-linked by two disulfide bridges (2). Therefore, the possibility exists that NCS is converted into an active form by reduction of disulfide bridges, and later into an inactive form. This problem is now under investigation.

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