PRE-NEOCARZINOSTATIN, A SPECIFIC ANTAGONIST OF NEOCARZINOSTATIN

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Streptomyces carzinostaticus produces the antitumor protein, NCS, and at the same time a related protein which antagonizes the antimicrobial effect of NCS. This has been designated pre-neocarzinostatin (pre-NCS). The physicochemical properties of pre-NCS are very close to those of NCS, except for a difference in isoelectric point. Production of pre-NCS in the culture filtrate precedes production of NCS. Pre-NCS has no antimicrobial and antitumor effect but antagonizes NCS activity.

Pre-NCS, when given 2 hours prior to NCS, markedly diminishes the NCS-induced inhibition of *Sarcina lutea* growth and DNA synthesis in HeLa cells. However, the antagonistic action of pre-NCS is lost when pre-NCS is washed out from HeLa cell culture after 2 hours incubation. When pre-NCS was given intraperitoneally to mice bearing Sarcoma 180 ascites tumor, subsequent treatment with NCS failed to inhibit the tumor growth. On the other hand, the acute toxicity of NCS given intravenously was not abolished by preceding intravenous injection of pre-NCS.

The acute toxicity of pre-NCS by intravenous injection was greater than 100 mg/kg in mice.

The antitumor protein, neocarzinostatin (NCS) was isolated from culture filtrates of *Streptomyces carzinostaticus* by ISHIDA *et al.*[1,2] and its therapeutic effect on various experimental tumors, including mouse leukemia L-1210 and SN-36 has been reported.[3,4] NCS was found to inhibit specifically the synthesis of DNA in *Sarcina lutea*[5] and HeLa cells[6], and to induce DNA degradation when a relatively high concentration was used.[7,8]

Recently, the presence of another protein chemically similar to NCS was noticed in the culture filtrate of *S. carzinostaticus*. This second protein, designated as pre-neocarzinostatin (pre-NCS), antagonistically suppresses the inhibitory activity of NCS against *S. lutea* and HeLa cells. The present communication deals with the production, purification and NCS-antagonism of pre-NCS.

Materials and Methods

Determination of antagonistic activity of pre-NCS *vs* NCS:

(1) Routine assay of NCS and pre-NCS activity: The assay methods used during the process of production and purification of NCS and pre-NCS were as follows:

NCS; the conventional paper-disc diffusion method on a peptone agar plate (0.5% peptone and 0.25% NaCl, pH 6.8) with *S. lutea* PCI 1001 as a test organism.[1] Standard specimen of NCS used in this experiment was a gift from Kayaku Antibiotic Research Co., Ltd., Tokyo, Japan.

Pre-NCS; paper-discs containing appropriate amounts of pre-NCS were placed on the same peptone agar plate inoculated with *S. lutea* PCI 1001, and incubated for 3 hours at 37°C for the diffusion of pre-NCS. After removal of these discs, a sheet of filter paper (Whatman, No. 1) containing 0.05 ml/cm^2 of 2 mcg/ml solution of NCS was applied to the entire surface

of the assay plate for 20 minutes at room temperature, and then removed. After incubation for 20 hours at 37°C, a distinct growth zone of the test organism is found only in the presence of pre-NCS. A linear relationship was found between the diameter of the growth zone and the logarithmic concentration of pre-NCS over the range of 3 mcg/ml to 500 mcg/ml.

(2) Precise turbidimetric assay for the antagonistic action of pre-NCS: For more precise assay of pre-NCS, a turbidimetric assay was adopted. *S. lutea* PCI 1001 grown on trypticase soy broth (TSB: BBL) and *Bacillus subtilis* PCI 219 grown on nutrient broth were used as the test organisms. Another test microorganism used was *Mycoplasma gallisepticum* PG 31, which was grown in a liquid medium composed of 7 parts of PPLO medium (Difco), 2 parts of unheated horse serum and 1 part of 25% yeast extract[9] for both propagation and assay. This medium contained 500 units/ml of penicillin G, 500 mcg/ml of thallium acetate, and 1% glucose with 0.002% phenol-red as an indicator for acid production. The minimum growth-inhibitory concentration (MIC) of NCS and the minimum angatonistic concentration of pre-NCS which supresses the inhibitory action of NCS were determined by turbidimetric measurements of the growth after incubation for 10 hours in TSB for the bacteria and for 37 hours for the mycoplasma.

(3) *In vivo* effect of pre-NCS using mouse ascites tumor: The antitumor activity of NCS and antagonistic activity of pre-NCS effect were determined using sarcoma 180 in ascites form in *dd* mice weighing 18~22 g. The tumor cells used for the inoculation were obtained 7 days after intraperitoneal transplantation of the tumor cells. The cells were suspended in saline at a concentration of 2×10^7 cells/ml, and 0.1 ml of the suspension was inoculated into each mouse.

Treatment of tumor-bearing mice with NCS, pre-NCS, or a combination of these two was started 24 hours after tumor inoculation and the drug treatment continued once daily for 6 consecutive days. Evaluation of the effect of the treatment was made by measuring the volume of ascites 13 days after inoculation. Detailed schedules of pretreatment with pre-NCS or NCS followed by challenge with NCS or pre-NCS, respectively, are described in the text.

Protein content

Protein content was measured by the BIURET reaction as modified by GORNALL *et al.*,[10] using egg albumin (Armour Pharmaceutical Co., Kankakee, Illinois) as the standard.

Analytical disc gel electrophoresis

Disc electrophoresis was carried out according to the method of NAGAI[11] at pH 8.3 in 0.1 M sodium borate buffer or at pH 4.7 in 0.1 M sodium citrate buffer. A current of 2.5 mA per tube (0.5×12 cm) was applied for 3 hours. After electrophoresis the gel was stained for protein with Amidoblack 10 B and the excess dye was rinsed away with 7% acetic acid.

Production of pre-NCS and NCS

The procedures used were similar to those described earlier.[1] A slant culture of *Streptomyces carzinostaticus* var. F-41 was made on KRAINSKY's agar medium by incubation at 27°C for 5 days and this was used as a stock culture. The seed culture was prepared by inoculating one loopful of the stock culture into 100 ml of seed culture medium consisting of 2% starch, 2% soy bean meal, 0.5% yeast extract, 0.25% NaCl, 0.0005% $MnCl_2$, 0.0005% $CuSO_4$, 0.0005% $ZnSO_4$ and 0.2% $CaCO_3$ in distilled water, pH 7.2, in 500-ml SAKAGUCHI flasks. The flasks were incubated at 27°C for 20 hours on a reciprocating shaker at 120 strokes/minute. Five liters of seed culture was transferred to a 600-liter fermentation tank containing 200 liters of medium of the following composition: 4.0% glucose, 0.5% Casaminoacid, 0.5% NaCl, 0.2% $CaCO_3$, 0.1% K_2HPO_4, and 0.25% $MgSO_4$ (pH 7.2). Incubation was allowed to proceed for 50 hours at 27°C under standard conditions of agitation and aeration. For determination of the amounts of pre-NCS and NCS produced during the course of fermentation, aliquot filtrates were take at 12, 24, 27, 37, 40, 43 and 48 hours. Both NCS and pre-NCS were precipitated with 60% ammonium sulfate, filtered through a column of

Sephadex G-50, and approximately 30 mg (dry weight) of the crude preparation obtained from protein peak was subjected to analytical diethyl-aminoethyl (DEAE)-cellulose column chromatography (1.5×30 cm). Elution was effected by a stepwise NaCl gradient in 0.02 M Tris buffer, pH 7.2. The amount of protein in the fractions corresponding to pre-NCS and NCS was determined by planimetric measurement of the area under the curve for the extinction at OD_{280}.

Results

Production of NCS and Pre-NCS

Early production of pre-NCS was followed by NCS production. A typical fermentation course is shown in Fig. 1. The maximum titer (280 mcg/ml) of per-NCS was attained after 27 hours, and then the titer gradually decreased. In cotrast, the titer of NCS gradually increased from 20 to 48 hours. When the harvest was made at 27 hours, the ratio of protein obtained in the NCS- and pre-NCS-peaks in analytical DEAE cellulose chromatography was 1 : 3, while a late harvest at 48 hours gave the ratio of 7 : 1.

Purification of Pre-NCS

In order to obtain purified pre-NCS in large quantity, the culture broth (200 liters) harvested at 27 hours was adjusted to pH 3.5 with a saturated oxalic acid solution, and 1% w/v of a 1 : 1 mixture of Celite 545 and kaolin was added to remove impurities. The mixture was stirred for 15 minutes, and filtered in a filter press. To 170 liters of the filtrate, 110.5 kg (65% w/v) of solid ammonium sulfate was added to precipitate both NCS and pre-NCS. The precipitate was collected by filtration followed by dissolution in water (if necessary, adjusted to pH 5.0 with 1% NaOH) and the solution was dialyzed against distilled water for 24 hours at 4°C with frequent changes of water. The residual solution was freeze-dried. From 200 liters of culture broth, approxi-

Fig. 1. Production of pre-NCS and NCS in the culture filtrate of *Streptomyces carzinostaticus*.

Values are given as the amount of protein (mcg) per milliliter. The amount of protein in the per-NCS and NCS fractions after DEAE cellulose chromatography was determined by planimetric measurement of the area under the extinction curve at OD_{280}.

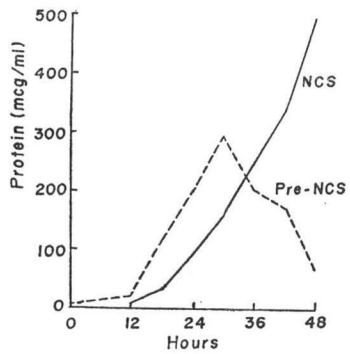
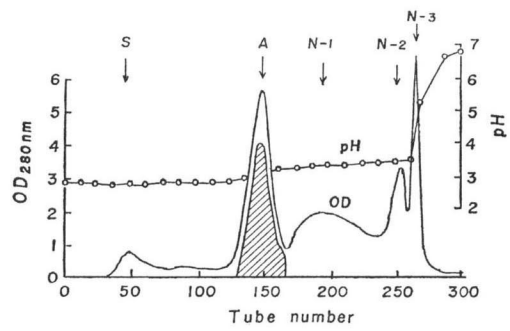


Fig. 2. Chromatography of crude pre-NCS on a CM-cellulose column.

Crude pre-NCS (2.5 g) was applied to a CM-cellulose column (3.0×40.5 cm), and eluted successively with 0.1 N acetic acid, 0.1 N acetate buffer of pH 3.2, 0.1 N acetate buffer of pH 3.4 and finally with 0.1 N sodium acetate. The optical density at 280 nm indicated five peaks. Respective yields were S, 488 mg; A, 756 mg; N-1, 276 mg; N-2, 180 mg; and N-3, 59 mg by dry weight. Antagonistic activity of pre-NCS against antibacterial activity of NCS was found in the cross-hatched area.

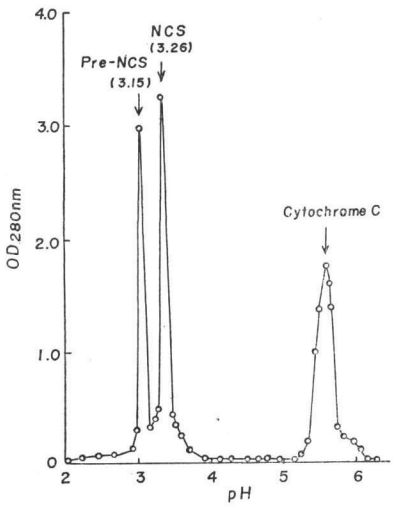


mately 40 g of a mixture of pre-NCS and protein impurities was obtained. The dried material was dissolved in 300 ml of water, and passed through a column of Sephadex G-25 (7 liters). Eluates in a void volume of the column were combined and concentrated by freeze-drying, yielding 32 g of dry material.

Further purification was effected by column chromatography on carboxymethyl (CM)-cellulose as shown in Fig. 2. In this experiment, 2.5 g of crude material was applied to a CM-cellulose column (3.0×40.5 cm), and eluted successively with 550 ml each of 0.1 N acetic acid, 0.1 N acetate buffer of pH 3.2, 0.1 N acetate buffer of pH 3.4 and finally with 0.1 N sodium acetate. As shown in Fig. 2, five fractions, *i.e.*, S, A, N-1, N-2 and N-3 were obtained. Fractions N-1, N-2 and N-3 contained NCS activity while fraction A (A stands for antagonistic) had no NCS activity but contained pre-NCS activity. Fraction S had neither NCS nor pre-NCS activity. The peak portion of fraction A was freeze-dried and rechromatographed on a CM-cellulose column under the same conditions. Fraction A obtained from this chromatography was dialyzed against water, desalted by Sephadex G-25, and freeze-dried, giving 9.75 g of amorphous powder. Fractions N-1 and N-2 purified by the same procedure gave a white fluffy powder after freeze-drying, yielding 3.51 g and 2.34 g, respectively.

Fig. 3. Isoelectric fractionation of pre-NCS and NCS.

An electrofocusing column of 110 ml capacity and Ampholine carrier ampholites capable of producing a gradient of pH from 3 to 6 (1% in final concentration) were used. The electrofocusing was carried out at 700 volts and 2~4°C for 37 hours. The content of the column was then fractionated into 1.5-ml fractions, and each fraction was examined for pH value, protein content, and pre-NCS or NCS activity. The pH values were measured at 10°C. Cytochrome C (*Rhodospirillum rubrum* heme protein) was used as a reference, the isoelectric point of which is 5.6.



Physicochemical Properties of pre-NCS

When the purity of pre-NCS preparation was examined by polyacrylamide gel electrophoresis at both alkaline and acidic pH, a single protein band was detected. As shown in Fig. 3, when the same pre-NCS preparation was subjected to isoelectric fractionation with Ampholine carrier ampholytes, pre-NCS was electrofocused in a single band at pH 3.15, while NCS banded sharply at pH 3.26 under the same experimental conditions. We take these values as the isoelectric points of these proteins.

Antagonistic Action of Pre-NCS against NCS

(1) Antibacterial Activity

In contrast to NCS, pre-NCS was found to have no bacteriostatic nor bactericidal activities (Table 1). However, pre-NCS was found to antagonize the inhibitory action of NCS, when microorganisms were pre-treated with pre-NCS prior to the addition of NCS (Table 1). As illustrated in Fig. 4, NCS completely inhibited the growth of *S. lutea* PCI 1001 at concentrations of more than 1.0 mcg/ml during the observation period of 10

Table 1. Biological activities of pre-NCS and NCS

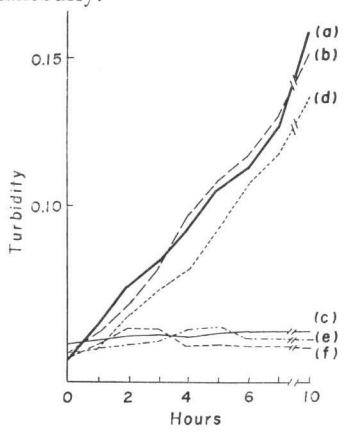
Activities		pre-NCS	NCS (N-1)
Antibacterial activity (mcg/ml)*	*Sarcina lutea*	>1,000	0.3
	Bacillus subtilis	>1,000	2.0
	Mycoplasma gallisepticum	>1,000	1.0
Cytotoxicity (mcg/ml)**	HeLa	> 200	0.1
LD_{50} (iv) (mg/kg)	in mice	> 100	1.85
Antitumor activity (mg/kg)***	s-180	> 10	0.03

* MIC : Minimum inhibitory concentration
** MCC: Minimum cytotoxic concentration
*** When given once daily for 6 consecutive days starting 24 hours after tumor transplantation.

Fig. 4. Antagonistic action of pre-NCS to the antibacterial activity of NCS.

An overnight culture of *Sarcina lutea* PCI 1001 was diluted in TSB medium, incubated for 3 hours and then diluted in the same medium to obtain 5×10^3 cells/ml. Pre-NCS and/or NCS at indicated concentrations were added (at time 0) and the bacterial growth was followed by turbidimetry at 620 nm.

(a), control; (b), pre-NCS (100 mcg/ml); (c), NCS (1~10 mcg/ml); (d), pretreated with pre-NCS (0.1 mcg/ml) 10 minutes before the addition of NCS (1.0 mcg/ml); (e), pre-treated with NCS (1.0 mcg/ml) 10 minutes before the addition of pre-NCS (10 mcg/ml); (f), treated with both pre-NCS (10 mcg/ml) and NCS (1 mcg/ml), simultaneously.

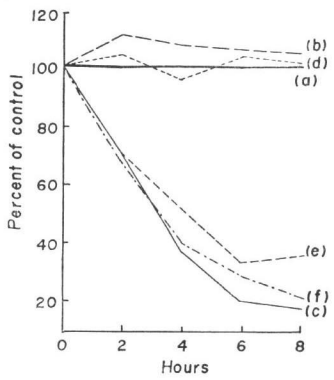


hours. However, when the culture was pre-treated with 0.1 mcg/ml of pre-NCS 10 minutes before the addition of 10 mcg/ml of NCS, bacterial growth continued unabated. On the other hand, addition of a higher con-

Fig. 5. Antagonistic action of pre-NCS against the inhibitory effect of DNA synthesis in HeLa S-3 cells with NCS.

A clonal line of HeLa S-3 cells grown in YLE medium consisting of 0.1% yeast extract and 0.5% lactalbumin hydrolysate in EARLE's solution supplemented with 0.45% glucose and 10% bovine serum was used. Two-day-old tube cultures of exponentially growing state received pre-NCS and/or NCS of indicted concentration at time O. For the determination of the synthesis of DNA, cells were exposed to 0.5 μCi/ml of ^{3}H-thymidine (TdR, 5.0 mCi/mole). After appropriate period of incubation, the incorporation was stopped by the addition of 10% perchloric acid (PCA), and the cell sheets were successively washed with 5% and 1% cold PCA. The PCA-insoluble fraction was taken up in 1 ml of NH_4OH, an aliquot of 0.5 ml was mixed with 10 ml of BRAY's solution, and radioactivity was determined in a Packard Tri-Carb scintillation spectrometer.

(a), Control; (b), pre-NCS (100 mcg/ml); (c), NCS (10 mcg/ml); (d), pre-treated with pre-NCS (100 mcg/ml) two hours before the addition of NCS (10 mcg/ml); (e), pre-treated with NCS (10 mcg/ml) two hours before or (f), simultaneously to the addition of pre-NCS (100 mcg/ml).



centration of pre-NCS (10 mcg/ml) either 10 minutes after or at the time of addition of NCS (1 mcg/ml) did not interfere with the antibacterial activity of NCS.

In order to rule out the possible direct interaction of pre-NCS with NCS, 5 ml each of 2,000 mcg/ml solution of pre-NCS and NCS were mixed and the mixture was incubated at 37°C for 4 hours in TSB or saline. The mixture was then filtered through Sephadex G-50 column and chromatographed on a CM-cellulose column to separate pre-NCS from NCS. The isolated NCS free of pre-NCS exhibited the same potency as was found before the incubation, indicating that pre-NCS does not impair directly the NCS activity.

(2) Inhibition of DNA Synthesis in HeLa S-3 Cells

NCS inhibits specifically the synthesis of DNA in HeLa S-3 cells.[6] In order to determine whether pre-NCS antagonizes the inhibitory action of NCS on DNA synthesis in HeLa S-3 cells, the effect of pre-NCS on the rate of incorporation of ^{3}H-thymidine into DNA of HeLa cells was examined in the presence or absence of NCS. Experimental procedures and results obtained are illustrated in Fig. 5. The incorporation of ^{3}H-thymidine into DNA wrs strongly inhibited 6 hours after the addition of NCS at a concentration of 10 mcg/ml and was reduced >80% at 8 hours. In contrast, pre-NCS alone did not inhibit DNA synthesis, even at a concentration

Table 2. Antagonistic action of pre-NCS to NCS in mice bearing Sarcoma 180

Intraperitoneal administration	Dose (mg/kg)		Results on 13th day			
	Pre-NCS	NCS	Ascites volume*	Tumor index	Antitumor effect*	Surv./Treat.
NCS alone		1.0	0.0	0.0	+	5/5
		0.5	0.0	0.0	+	5/5
		0.25	0.0	0.0	+	5/5
		0.125	0.4	0.04	+	4/4
		0.06	0.7	0.07	+	5/5
		0.03	1.9	0.19	+	4/4
Pre-treatment with Pre-NCS	10	1.0	2.7	0.21	+	4/5
	10	0.5	9.7	0.97	−	4/5
	10	0.25	10.0	1.00	−	2/5
	1.0	0.125	1.8	0.18	+	3/5
	1.0	0.06	7.4	0.74	−	3/5
	1.0	0.03	9.1	0.91	−	4/5
Post-treatment with Pre-NCS	10.0	1.0	0.0	0.0	+	5/5
	10.0	0.1	0.1	0.01	+	5/5
	10.0	0.03	0.9	0.09	+	5/5
Simultaneous treatment	10.0	1.0	0.0	0.0	+	5/5
	10.0	0.1	0.2	0.02	+	4/4
Pre-NCS alone	10.0		10.9	1.09	−	0/5
	1.0		9.2	0.92	−	2/5
Saline			9.9	1.00		21/25

* Ascites volume on 13th day was the main index to evaluate the antitumor effect of NCS. + indicates a positive antitumor effect and −, a negative antitumor effect *i.e.* the antagonistic effect of pre-NCS in combination test.

of 100 mcg/mg. When 100 mcg/ml of pre-NCS was added to a HeLa cell culture 2 hours before the addition of 10 mcg/ml of NCS, no inhibition of the incorporation of ^{3}H-thymidine was observed. When pre-NCS was added simultaneously or 2 hours after the addition of NCS, pre-NCS did not diminish the effect of NCS. These results clearly indicate that, for pre-NCS to antagonize the NCS effect, cells must be treated with pre-NCS prior to adding NCS.

The antagonistic action of pre-NCS in HeLa cells, however, was readily reversible after washing. Although the data are not shown, when HeLa cells were exposed to 100 mcg/ml of pre-NCS for 2 hours at 37°C, then washed three times with HANKS' balanced salt solution, the cells were found to be completely susceptible to 10 mcg/ml of NCS. In contrast, as has been pointed out in a previous publication,[6] the inhibitory action of NCS on DNA synthesis in HeLa S-3 cells was irreversible, after treatment for 2 hours.

(3) Anti-tumor Activity of NCS and Pre-NCS

As shown in Table 2, NCS markedly inhibits the growth of sarcoma 180 cells in ascitic form over the dose range of 1.0~0.03 mg/kg/day, when given intraperitoneally once daily for 6 consecutive days starting 24 hours after tumor implantation. Pre-NCS alone, however, did not inhibit the growth of the same tumor cells even at a dose as high as 10 mg/kg/day, when given by the same schedule.

In the combination tests, the tumor-bearing mice received pre-NCS 2 hours prior to or at the same time or 2 hours after the administration of NCS at the doses indicated in Table 2. The dose of 10 mg/kg/day of pre-NCS given prior to the administration of 0.5 mg/kg/day of NCS (20 : 1) abolished the chemotherapeutic effect of NCS. However, at the doses of 10 mg/kg/day of pre-NCS *vs* 1.0 mg/kg/day of NCS (10 : 1) or 1.0 mg/kg/day *vs* 0.125 mg/kg/day (8 : 1), no significant antagonistic effect of pre-NCS was observed. When the tumor-bearing mice were first treated with NCS and then with pre-NCS, or treated with pre-NCS and NCS simultaneously, no antagonistic action of pre-NCS was observed as shown in Table 2.

Failure to Diminish Acute Toxicity of NCS by Pre-NCS in Mice

The possibility was examined that pre-NCS might reduce the acute toxicity of NCS in mice. The acute LD_{50} in mice by intravenous injection was determined to be 1.85 mg/kg for NCS and greater than 100 mg/kg for pre-NCS. The acute toxicity caused by 18 mg/kg of NCS in mice, however, was not diminished by the injection of pre-NCS 2 hours previously even at a dose of 100 mg/kg.

Discussion

A protein possessing a selective antagonistic activity to the inhibitory action of NCS on various cells was found in a crude preparation of NCS. This protein was separated from NCS by chromatography on CM-cellulose column, being eluted from the column before NCS and, therefore, designated as "pre-NCS".

Although pre-NCS shares many physico-chemical properties with NCS (MAEDA, H. *et al.*, to be reported), clear-cut distinction could be made between these two in terms of (a) biological activities, (b) elution pH from the CM-cellulose or DEAE-cellulose column, concordant to the difference in isoelectric point, and (c) reversibility of pre-NCS action and irreversibility of NCS action on HeLa cells. One can conclude on the basis of these experimental results that both NCS and pre-NCS share a reversible binding site in *S. lutea*, HeLa cells and sarcoma

180 tumor cells but do not share the irreversible binding site which is found only with NCS and may lead to the inhibition of DNA synthesis in these cells.[8] Further chemical studies to be reported may explain such discrepancy on the basis of a similar primary structure but different secondary or tertiary structures. One exception to the antagonistic actions of pre-NCS to NCS was that the toxicity of NCS was not reversed by preceding pre-NCS treatment. This may be explained by the different distribution of the two compounds in mice after intravenous injection, the result to be reported elsewhere.

How does pre-NCS antagonize the action of NCS? Pre-NCS molecule having a similar primary structure to NCS might be able to adsorb to the NCS receptor site in NCS-sensitive cells. Lack of antagonism found when pre-NCS and NCS are added simultaneously to either bacterial or mammalian cells might be due to a difference in binding to the receptor site between NCS and pre-NCS. This may be possible when the number of receptor sites is limited. However, no detailed analysis to see whether the relation is competitive or not was made. Moreover, the fact that 1 mcg of pre-NCS was sufficient to antagonize 100 mcg of NCS in *S. lutea* (1 : 100), whereas 100 mcg of pre-NCS was necessary to negate the effect of 10 mcg of NCS in HeLa cells (10 : 1) must be explained, although the presence of a pre-NCS inactivating principle in HeLa cell culture medium (unpublished observation) might partly explain such discrepancy.

The production of NCS in the culture filtrate was preceded by that of pre-NCS (Fig. 1), suggesting a possible chemical conversion of pre-NCS to NCS. Studies on the biogenesis of NCS[12] revealed the presence of a precursor protein of NCS in the mycelium at an early growth stage and this precursor was found to be converted to active NCS at a late stage of biogenesis.[12] Although the chemical characterization of this precursor protein is not yet accomplished, this precursor protein can be released in the culture filtrate and may be pre-NCS.

The detoxin complex, obtained from *Streptomyces* culture filtrate, negated the inhibitory action of blasticidin S selectively against *B. cereus*, but not against *Piricularia oryzae*.[13] As a result, when curative and preventive effects of the combined preparation of blasticidin S and detoxin complex against rice blast disease were examined, the effectiveness of the blasticidin S was not decreased by the addition of detoxin, but the phytotoxicity was depressed markedly. Although such a favorable effect in chemotherapeutic experiment could not be obtained with a pre-NCS and NCS combination, the fact that an antagonistic principle to an antibiotic can be found in the same culture filtrate should be emphasized.

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