Inhibition of Ligand-independent Cap Formation of Mouse Lymphocytes and Raji Cells by Neocarzinostatin

Ichiro Yahara, Shintaro Iwashita, Takusaburo Ebina, Masanobu Satake, and Nakao Ishida

The Tokyo Metropolitan Institute of Medical Science, Honkomagome 3-18, Bunkyo-ku, Tokyo 113, Japan [I. Y., S. I.], and Department of Bacteriology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Sendai 980, Japan [T. E., M. S., N. I.]

ABSTRACT

Neocarzinostatin (NCS), an antitumor antibiotic, was found to inhibit cap formation of mouse lymphocytes and Raji cells. A treatment of lymphocytes with NCS in concentrations as low as 0.25 μg/ml is sufficient to cause a strong inhibition of ligand-independent cap formation. Compared to lymphocytes, Raji cells are less sensitive to the effect of NCS. The effect of NCS on cap formation could not be attributed to growth inhibition induced by the drug, for the inhibition of cap formation is induced readily and was detected even with resting cells such as lymphocytes. Inhibitors of DNA synthesis such as bleomycin, mitomycin C, and 1-β-D-arabinofuranosylcytosine showed no inhibitory effect on cap formation. Moreover, neither a RNA synthesis inhibitor nor a protein synthesis inhibitor affected the inhibitory effect of NCS, suggesting that the principal target of NCS that caused the inhibition of cap formation is not DNA. Inasmuch as the systems of cap formation used in the present study do not require the function of microtubules, the inhibitory effect of NCS on cap formation could not be accounted for by the effect on microtubules. An active transport, uptake of 2-deoxy-glucose, is not affected by NCS but is completely inhibited by azide. This fact suggests that cellular metabolisms required for cap formation are not depressed by NCS. Although NCS, unlike cytochalasin B, does not induce rounding up of fibroblasts spread over a substrate, both drugs induced disappearance of membrane ruffles of these cells. From these results and those previously reported, the mode of action of NCS on the membrane-cytoskeletal (microtubules and microfilaments) system is discussed.

INTRODUCTION

NCS, a polypeptide antibiotic with a molecular weight of 10,700 isolated from Streptomyces carzinostaticus (9), reveals antitumor activities in various systems including murine ascites tumors and human myelogenous leukemia (1, 7, 10). Ebina et al. (4) have demonstrated that NCS prevents cultured mammalian cells from progressing through the cell cycle, resulting in arrest of cell growth either at the G2 or the G1-early S phases. Ebina et al. (3, 5) have further shown that NCS inhibits cap formation of Daudi cells, cell spreading of HeLa cells, and the vinblastine-induced paracrystal formation of tubulin, the subunit protein of the microtubules. There are similar functions to colchicine which is known to prevent the above 3 phenomena and to dissociate microtubules. Both mitosis (8) and DNA synthesis (15) of cultured cells are known to be dependent upon the cytoplasmic microtubule organization. Therefore, Ebina et al. (5) have suggested that the dual effect of NCS on the cell cycle might be interpreted as a result of the binding of this antibiotic to specific cell surface receptors, which consequently modulates the cytoplasmic microtubules via the hypothetical transmembrane mechanism originally proposed by Edelman and Yahara (6, 17).

However, from the following facts previously obtained, the possibility that NCS affects the membrane-microfilament system cannot be excluded: (a) cytochalasin B, a microfilament-acting agent, has the same effect on cell spreading as did NCS and colchicine (5); (b) calcium ionophores and local anesthetics which inhibit cap and paracrystal formation (5) also modify microfilament function as well as microtubule function (11, 13); and (c) recent work showed that NCS, unlike colchicine, has no effect on microtubule assembly in vitro or disrupts the network of microtubules in vivo, although it was observed by immunofluorescence microscopy using antitubulin antibody that a microtubular organization appeared to be coarsely and rigidly changed in NCS-treated 3T3 and HeLa cells (12). Therefore, it seems that NCS might affect other systems than microtubules in some assay systems.

On the other hand, Yahara and Kakimoto-Sameshima (18) have demonstrated that most of the mobile receptors on mouse lymphocytes were redistributed into caps when the cells were incubated in hypertonic medium in the absence of ligands. The process of Li-cap formation cannot be inhibited by cytochalasin B and Con A but not by colchicine. Therefore, it can be deduced that Li-cap formation is a biological phenomenon that depends on the state of the cell membrane-microfilament system.

The purpose of this paper is to show that NCS also affects the membrane-microfilament system in addition to the membrane-microtubule system as reported previously (3, 5). That is, the present paper shows that NCS does not simply mimic the effect of a single drug such as colchicine, cytochalasin B, or Con A, but that it has a very complicated mode of action on the membrane-cytoskeletal system.

MATERIALS AND METHODS

Cells. Spleen cells were prepared from ICR mice which were randomly bred in a closed colony in the Shizuoka Laboratory Animal Center (Shizuoka, Japan). Unfractionated spleen cells were usually used for capping experiments as the experimental results obtained with these cells were found to be essentially the same as those obtained from lymphocytes purified by
centrifugation on a Ficoll-Hypaque solution (Pharmacia Fine Chemicals, Uppsala, Sweden). The cells were washed with PBS and suspended in the indicated medium.

A Burkitt's lymphoma cell line, Raji, was cultured in Roswell Park Memorial Institute Tissue Culture Medium 1640 supplemented with 10% fetal calf serum. Cultured cells with a density of 3 to 5 x 10^5 cells/ml were harvested by centrifugation, washed with Roswell Park Memorial Institute Tissue Culture Medium 1640, and suspended in the indicated medium.

The viability of the cells was determined by trypan blue exclusion and was around 90% for lymphocytes and 95% for cultured Raji cells even after exposure to 2x PBS at 37°C for 30 min. Secondary cultures of mouse embryonic fibroblasts prepared from BALB/c mice were provided by A. Hiragun.

**Reagents.** The materials used in this study were: NCS, Kayaku Antibiotics Research Co., Ltd., Tokyo, Japan; bleomycin, Nippon Kayaku Co., Ltd., Tokyo, Japan; mitomycin C, Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan; 1-β-D-arabinofuranosylcytosine, Kohjin Co., Ltd., Tokyo, Japan; Fl-anti-lg, prepared as described previously (16); Fl-Con A, Miles-Yeda Ltd., Rehovot, Israel; fluorescein isothiocyanate conjugated goat IgG directed against rabbit IgG, Miles-Yeda Ltd.; 2-deoxy-D-[1-3H]glucose, Radiochemical Centre, Amersham, England; cytochalasin B, Aldrich Chemical Co., Inc., Milwaukee, Wis. Each component of PBS was doubled in the 2x PBS solution (18). Monospecific antitubulin antibody was purified from rabbit antiserum directed against porcine brain tubulin as previously described (19).

**LI-Cap Formation.** LI-cap formation was carried out as described previously (18). Briefly, the cells (0.5 to 2 x 10^7/ml) were incubated in 2x PBS at 37°C for 15 min and fixed with 1% formaldehyde in the same medium. The fixed cells were washed and incubated with an appropriate fluorescent reagent to determine the distribution of surface receptors to be examined. For lymphocytes, Fl-anti-lg (100 μg/ml) and Fl-Con A (100 μg/ml) were used to determine the distribution of surface immunoglobulins and Con A receptors, respectively. Fl-Con A was used for Raji cells. At least 400 labeled cells were observed to determine the percentage of labeled cells showing caps.

**LD-Cap Formation.** Lymphocytes were preincubated with 10^-4 m colchicine in PBS at 37°C for 30 min if indicated. The cells were incubated with the indicated fluorescent ligand, 100 μg Fl-anti-lg per ml or 50 μg Fl-Con A per ml, in PBS at 37°C for 10 min. The cells were washed, suspended in PBS, and examined under a fluorescence microscope (18).

**Visualization of Microtubules.** The microtubule organization of the lymphocytes was visualized according to the method described previously (19).

**Uptake of 2-Deoxy-Glucose.** Cells (1 x 10^6) suspended in 1 ml of Hank's balanced salt solution (minus glucose) buffered with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.0) were incubated with 2 μCi 2-deoxy-[1-3H]glucose (specific activity; 17.5 Ci/mmol) at 37°C for 10 min. The cells were collected on Millipore filters HAWPO25 (22-mm diameter) and washed with cold PBS. The radioactivity retained on each filter was determined in Aquasol (New England Nuclear, Boston, Mass.) with a liquid scintillation counter.

**Scanning Electron Microscopy.** The details of the procedures for the preparation of the specimens and the conditions for electron microscopic observations were described previously (18).

**Rounding Up of Spread Fibroblasts.** A confluent culture of MEF was trypsinized, and the rounded cells were replated on glass coverslips (22 x 22 sq mm) in plastic dishes containing Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The cells were incubated at 37°C, and the inhibitors were added to the indicated cultures 2 hr after plating. The morphological changes of these cells after the addition of inhibitors were monitored by photomicrography.

**RESULTS**

**Effect of NCS on LI-Cap Formation.** Both LI-cap formation of mouse splenic lymphocytes and Raji cells were tested. The cells were preincubated with various concentrations of NCS (0.1 to 100 μg/ml) in PBS at 37°C for 10 min before incubation in 2x PBS containing the same concentrations of NCS. The results were not greatly influenced by the presence of NCS in the hypertonic medium probably because of irreversible binding of the drug to the cells during preincubation. The cell suspensions were incubated in 2x PBS with NCS at 37°C for 15 min, and then fixed and labeled with appropriate fluorescent reagents. As can be seen in Table 1, NCS in concentrations as low as 1 μg/ml strongly inhibited the LI-cap formation of the lymphocytes. Even with 0.25 μg NCS per ml, about 40% of the LI-cap formation was inhibited. The viability of the cells after treatment with NCS remained stable (Table 1), suggesting that nonspecific cell damage is not the cause of the inhibitory effect on cap formation. In contrast to the characteristic morphology of the cells showing cap formation in 2x PBS (Fig. 1a), noncap forming cells in the presence of NCS appear to be hairy (Fig. 1b). Scanning electron micrographs of these cells are shown in Fig. 2. As reported previously, most of the microvilli on the surface of LI-cap forming cells accumulate in the region of the cap (Fig. 2a). In contrast, the microvilli of NCS-treated cells are elongated in the hypertonic medium compared to those in PBS and are distributed randomly over the cell surface (Fig. 2b). Three other inhibitors of DNA synthesis, 1-β-D-arabinofuranosylcytosine, bleomycin, and mitomycin C, were also tested to show whether they affected LI-cap formation. None of these drugs was found to inhibit LI-cap formation, however (Table 2). Cytochalasin B, a microfilament-acting agent, had the same effect on LI capping as did NCS, but colchicine, a microtubule-acting agent, did not.

LI-cap formation of Raji cells was found to be less sensitive to NCS than that of mouse lymphocytes. More than 50% of the LI-cap formation of these cells was inhibited by 2.5 μg NCS.

**Table 1**

<table>
<thead>
<tr>
<th>NCS (μg/ml)</th>
<th>Mouse lymphocytes</th>
<th>Raji cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>inhibition (%)</td>
<td>viability</td>
</tr>
<tr>
<td>0</td>
<td>35.2</td>
<td>86</td>
</tr>
<tr>
<td>0.1</td>
<td>29.9</td>
<td>85</td>
</tr>
<tr>
<td>0.25</td>
<td>21.8</td>
<td>90</td>
</tr>
<tr>
<td>1</td>
<td>3.5</td>
<td>90</td>
</tr>
<tr>
<td>2.5</td>
<td>2.1</td>
<td>89</td>
</tr>
<tr>
<td>10</td>
<td>0.7</td>
<td>85</td>
</tr>
<tr>
<td>25</td>
<td>NT</td>
<td>74</td>
</tr>
<tr>
<td>50</td>
<td>NT</td>
<td>85</td>
</tr>
</tbody>
</table>

*Cap formation is expressed as the percentage of cells showing cap among the total labeled cells.

NT, not tested.
per ml. The differences in dose response between lymphocytes and Raji cells remain to be elucidated. Numbers or affinities of membrane receptors for NCS might be different on these 2 kinds of cells. The results shown in Table 2 indicate that none of the synthesis of DNA, RNA, or protein is necessary for Ll-cap formation and for its inhibition by NCS.

**Effect of NCS on Membrane Transport.** The possible effect of NCS on membrane transport was examined and compared with those of azide or cytochalasin B, both of which are known to inhibit Ll-cap formation (Ref. 19; Table 4). It was observed that NCS does not inhibit uptake of 2-deoxy-glucose by Raji cells (Table 5). In contrast, both sodium azide, a metabolic inhibitor, and cytochalasin B, an antimicrofilament drug, strongly inhibit the uptake of the sugar.

**DISCUSSION**

The present report showed that the pretreatment with NCS...
in concentrations as low as 1 \( \mu g/ml \) inhibited the LI-cap formation of lymphocytes within a short period (Table 1; Figs. 1 and 2) and that other antitumor drugs which are supposed to interact with DNA did not inhibit this LI-cap formation (Table 2). These observations, as well as the fact that lymphocytes are resting cells, suggest that NCS does not inhibit cap formation as a result of the inhibition of DNA synthesis.

Although the mechanism of LI-cap formation in hypertonic medium is complicated as was shown in a previous paper (18), the fact that LI-cap formation is strongly inhibited by cytochalasin B, NaN3, or Con A suggests that the membrane-microfilament system and energy metabolism are involved. Therefore, it is quite possible that NCS also affects the membrane-microfilament system. On the other hand, based on the observations that NCS mimics the effects of colchicine, Ebina et al. (5) proposed a hypothesis that NCS affects cytoplasm microtubules transmembranously so that central cap formation, cell spreading, and vinblastine-induced microtubular paracrystal formation are inhibited. However, previous and present immunofluorescent studies using antitubulin antibodies showed that NCS, unlike colchicine, does not disrupt the microtubular network of the fibroblasts (12) and lymphocytes (Fig. 3), and it seems that NCS conversely induces rigidity to the microtubules so that it might immobilize the surface receptors and induce the inhibition of cap formation. These observations suggest that NCS mimics the effect of Con A, which is also a polypeptide, with a molecular weight of about 26,000 (2) and a strong inhibitor of cap formation in which it alters the anchoring structures of the surface receptor (17). However, unlike Con A (16), NCS does not inhibit patch formation.

When compared with cytochalasin B, a known inhibitor of microfilament (14), NCS inhibits central cap formation of Daudi cells but cytochalasin B does not (5), although both inhibit LI-cap formation (Table 2). Cytochalasin B inhibits uptake of sugar but NCS does not (Table 5). Moreover, NCS does not induce the rounding up of spreading fibroblasts while cytochalasin B does (Fig. 4). Therefore, the effects of NCS seem to be different from those of cytochalasin B.

When compared with NaN3, a known inhibitor of energy metabolism, both NCS and azide inhibit cap formation, but unlike NCS, azide inhibits uptake of sugar (Table 5).

These biological activities of NCS in comparison with those of colchicine, cycochalasin B, NaN3, and Con A are summarized in Table 6. It appears that NCS mimics some of the effects of each drug. Therefore, it is strongly suggested that NCS has a complicated mode of action on the membrane-cytoskeletal system, but the detailed mechanism of its effect is still unknown and will be the main subject of future research. As NCS inhibits membrane ruffling and cap formation (Fig. 4), one possibility is that the system which regulates the movement of the cytoskeletal system might be modulated by this antibiotic although the network structures of the microfilaments and microtubules are preserved.

**ACKNOWLEDGMENTS**

We thank Drs. H. Mitsui and A. Hiragun for Raji and MEF cells.

**REFERENCES**

11. Poste, G., Papahadjopoulos, D., and Nicolson, G. L. Local anesthetics affect transmembrane cytoskeletal control of mobility and distribution of cell surface

---

Table 6

<table>
<thead>
<tr>
<th>Biological activities</th>
<th>Cells</th>
<th>NCS</th>
<th>Colchicine</th>
<th>Cytochalasin B</th>
<th>Metabolic inhibitor</th>
<th>Con A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of PC formation</td>
<td>HeLa (3)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Disruption of MT network</td>
<td>3T3 (12)</td>
<td>LyHeLa (12)</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Inhibition of IgM capping</td>
<td>Lympocyteb (16)</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>LD capping</td>
<td>Lympocyteb (16)</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Induction of round up</td>
<td>MEfb</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Inhibition of sugar uptake</td>
<td>Rajb</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

*PC*, vinblastine-induced paracrystal formation of tubulin; *MT*, microtubule.

---

This table compares the biological activities of various drugs affecting membrane-cytoskeletal system. The table indicates that NCS mimics the effects of Con A and colchicine, which are known to inhibit cap formation. However, NCS does not inhibit patch formation, in contrast to colchicine. The table also shows that NCS affects cell spreading and membrane ruffling, suggesting a complex mode of action on the membrane-cytoskeletal system.
Fig. 1. Inhibition of LI-cap formation by NCS. a, lymphocytes showing polar distribution (cap) of surface immunoglobulin after incubation in 2 × PBS; b, 1 μg NCS per ml was added to cells before incubation. × 2200.

Fig. 2. Surface morphology of lymphocytes. a, a lymphocyte showing accumulated microvilli after incubation in 2 × PBS; b, a cell with prolonged microvilli after treatment with NCS (1 μg/ml) in 2 × PBS. × 11,500.

Fig. 3. Microtubule organization of lymphocytes. a, control; b, treated with NCS (10 μg/ml). × 1800.
Fig. 4. Spreading MEF cells. a to c, control; d to f, 10 μg NCS per ml; g to i, 10 μg cytochalasin B per ml. Numbers on the upper left corner of each photograph, time (min) after the addition of inhibitors. Arrows, membrane ruffles. × 560.
Inhibition of Ligand-independent Cap Formation of Mouse Lymphocytes and Raji Cells by Neocarzinostatin

Ichiro Yahara, Shintaro Iwashita, Takusaburo Ebina, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/39/11/4687

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.