PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 145, 1197–1200 (1974)

Streptonigrin Inhibition of 3-Methylcholanthrene Transformation *in Vitro*[1] (37980)

P. J. PRICE, W. A. SUK, G. J. SPAHN,[2] M. A. CHIRIGOS,[3] J. A. LANE, AND R. J. HUEBNER[3]

Microbiological Associates, Bethesda, Maryland 20014

We have previously described a Fischer rat embryo transformation system which, at a low passage level (<60), requires both the addition of exogenous type "C" RNA virus (MuLV) and chemical carcinogen for transformation (1). At higher passage levels (>60), certain chemicals transform the cells in the absence of added virus, although gs-1 antigen of the endogenous type C RNA rat virus is often expressed in the transformed cultures (2). The relationship between cell transformation and activation of some form of viral expression has been demonstrated (3–5), and it has been postulated, but not proved, that the viral genome may be responsible for the actual transformation event (5).

The antibiotic streptonigrin (Sn) (6) has been shown to inhibit both murine sarcoma and leukemia virus expression *in vivo* (7–11) and *in vitro* (7, 8) as well as inhibit the RNA-dependent DNA polymerase of the avian myeloblastosis virus (7). In this paper, we present evidence that Sn also inhibits *in vitro* transformation of high-passage rat embryo cells by 3-methylcholanthrene (3MC).

Materials and Methods. Reduction in plating efficiency was used to determine the maximum nontoxic dose of Sn (Table I). The sequence of treatment with 3MC and Sn in the high-passage Fischer rat embryo cells (F1706 P_{98}) is depicted in Table II. The growth and transfer medium consisted of Eagle's minimal essential medium in Earle's salts (EMEM) supplemented with

[1] This work was supported by Contract NIH-NCI-70-E-2068 B within The Virus Cancer Program of the National Cancer Institute.

[2] Frederick Cancer Center, Frederick, MD 21701.

[3] National Cancer Institute, Bethesda, MD 20014.

TABLE I. Toxicity of Sn as Determined by Reduction in Plating Efficiency.[a]

Sn/ml	Absolute (%)	Relative (%)	Average No. of cells/colony
Control	30.0	100	60
0.02 ng	20.7	102	61
0.04 ng	28.0	93	63
0.08 ng	28.3	94	61
0.16 ng	29.6	99	64
0.33 ng	18.3	61	42
0.66 ng	14.7	49	16

[a] 100 cells were added to each 60-mm plastic dish (Lux), and 1 hr later, the medium was replaced with the Sn media. Five days later, the cells were fixed and stained and macroscopic colonies counted. Absolute plating efficiency is the actual percentage of cells which give rise to colonies. Relative plating efficiency is the percentage of cells which give rise to colonies, relative to the control, in which the absolute plating efficiency is arbitrarily set at 100%.

TABLE II. Sequence of Treatment.[a]

Day 1—Transfer 1:2.
Day 2—Feed with streptonigrin (Sn).
Day 3—Refeed with Sn and 3-methylcholanthrene (3MC).
Day 5—Transfer 1:2 in medium containing both Sn and 3MC.
Day 7—Refeed with Sn and 3MC.
Day 8—Refeed with Sn. (The 3MC is no longer incorporated in the medium.)
Day 9—Transfer 1:2 in the presence of Sn and incorporate Sn into the medium for every feed and transfer.

[a] Positive controls are treated only with 3MC, and negative controls with acetone (1:1000) in Eagle medium. 3MC or acetone is removed on Day 8 and the controls are then passaged serially along with the Sn-treated cultures.

10% fetal bovine serum, 2 m*M* L-glutamine, 100 μg gentamycin, and either 0, 0.16, 0.33, or 0.66 ng Sn per ml. The 3MC was diluted in acetone to 1,000 μg/ml, and further diluted in growth medium to 0.5 and 0.1 μg 3MC/ml. Acetone similarly diluted was used in one set of controls. Where indicated, Sn was incorporated in the medium throughout the entire course of the experiment. (The Sn was supplied by the Division of Cancer Control, National Cancer Institute, National Institutes of Health.) The stock solution was made up at 10 μg/ml and stored frozen.

At each subculture, one set of flasks was set aside to be held indefinitely without subdivision (holding series) and the other was subdivided 1:2 weekly to provide two new

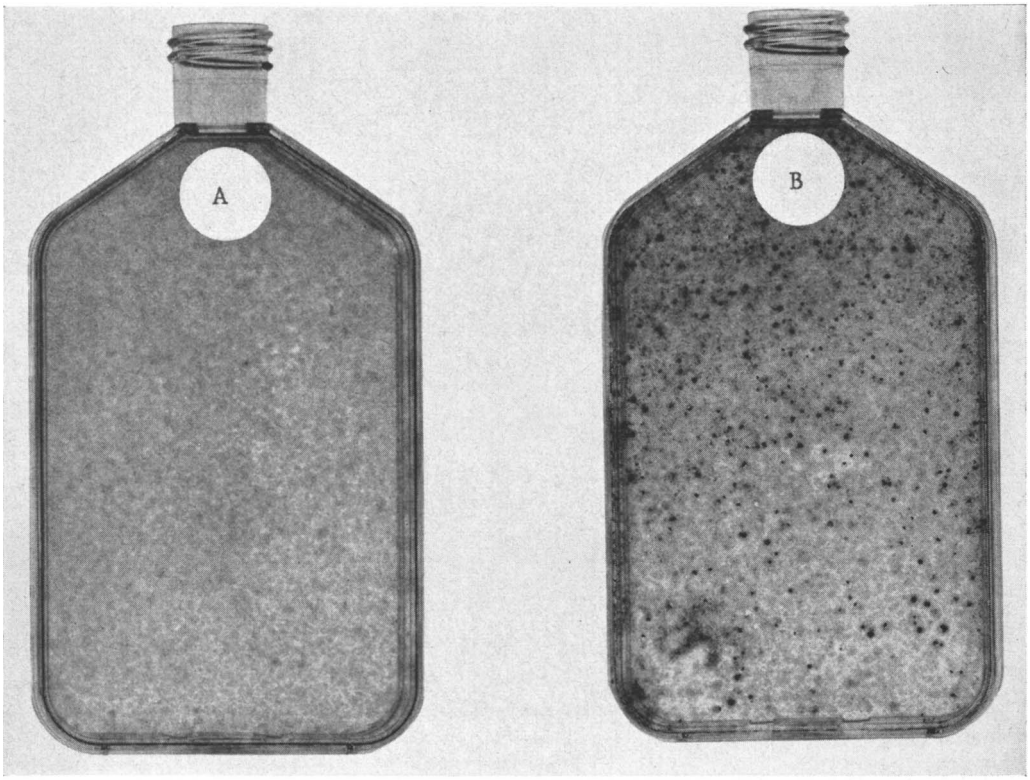

FIG. 1. Inhibition of 3MC-induced transformation by Sn. (A) 0.16 ng Sn + 0.1 μg 3MC (P_9). (B) 0.1 μg 3MC (P_9).

TABLE III. Sn Protection from Transformation.

Treatment	Transformed foci	Tumors
Experiment No. 1		
0.1 μg 3MC/ml	+ (P_1)	13/13 (56) [a]
0.5 μg 3MC/ml	+ (P_3)	ND[b]
0.1 μg 3MC + 0.16 ng Sn/ml	− (P_{27})	0/6 (76)
0.5 μg 3MC + 0.16 ng Sn/ml	− (P_{27})	ND
0.1 μg 3MC + 0.33 ng Sn/ml	− (P_{27})	0/6 (76)
0.5 μg 3MC + 0.33 ng Sn/ml	− (P_{27})	ND
0.1 μg 3MC + 0.66 ng Sn/ml	− (P_{27})	ND
0.5 μg 3MC + 0.66 ng Sn/ml	− (P_{27})	ND
Acetone Control	− (P_{27})	0/11 (76)
Experiment No. 2		
0.1 μg 3MC/ml	+ (P_4)	ND
0.5 μg 3MC/ml	+ (P_6)	ND
0.1 μg 3MC + 0.16 ng Sn/ml	− (P_{27})	ND
0.5 μg 3MC + 0.16 ng Sn/ml	− (P_{27})	ND
0.1 μg 3MC + 0.33 ng Sn/ml	− (P_{27})	ND
0.5 μg 3MC + 0.33 ng Sn/ml	− (P_{27})	ND
0.1 μg 3MC + 0.66 ng Sn/ml	− (P_{27})	ND
0.5 μg 3MC + 0.66 ng Sn/ml	− (P_{27})	ND
0.16 ng Sn/ml	− (P_{27})	ND
0.33 ng Sn/ml	− (P_{27})	ND
0.66 ng Sn/ml	− (P_{27})	ND
Acetone control	− (P_{27})	ND

[a] No. with tumors/No. inoculated (No. of days).
[b] ND = not done.

sets of cultures, one for the holding series and one for subdivision (vertical series). Transformed cultures are characterized by the appearance of progressively growing foci of cells lacking contact inhibition and orientation.

Tumorigenicity was determined by subcutaneous inoculation of 1×10^6 cells from the 11th vertical subculture of the first experiment into newborn Fischer rats (F344/f Mai).

Results. The maximum nontoxic dose of Sn was found to be 0.16 ng/ml (Table I). Incorporation in the growth medium of 0.33 or 0.66 ng of Sn reduced the relative plating efficiency by 39 and 51%, respectively. At both levels, however, resistant cells were selected, and when tested after 5 subcultures in the presence of Sn, the relative plating efficiency rose to 100% with 0.33 ng and 68% with 0.66 ng per ml.

Experiment No. 1. Duplicate cultures treated with 0.1 and 0.5 μg 3MC, in the absence of Sn, showed transformed foci one and three vertical subcultures, respectively, after 3MC treatment. After 27 population doublings (at which point the experiment was terminated), all three levels of Sn (0.16, 0.33, and 0.66 ng/ml) protected the cells from transformation by 3MC (Fig. 1) (Table III). The cultures which were transformed by 0.1 μg 3MC produced tumors at the site of inoculation in 13 out of 13 untreated newborn Fischer rats within 56 days postinoculation. Cultures protected from transformation by Sn failed to produce tumors (Table III).

Experiment No. 2. Duplicate cultures treated with 0.1 and 0.5 μg 3MC, in the absence of Sn, showed transformed foci four and six vertical subcultures, respectively, after 3MC treatment. When Sn was removed, 27 population doublings after 3MC treatment, all Sn-protected cultures were still normal (Table III). This experiment was not transplanted to animals.

Discussion. Streptonigrin, an antibiotic produced from broth filtrates of *Streptomyces flocculus,* has been shown to be an inhibitor of type C RNA virus expression. *In vivo,* Sn produced marked increases in survival time in mice inoculated with Rauscher (11) or Friend (10) leukemia viruses, and retardation and eventual regression of MSV(M)-induced tumor growth (7). *In vitro,* Sn has been shown to inhibit XC syncytia formation (12) by Moloney, Rauscher, and AKR leukemia viruses, and to inhibit focus formation by MSV(M) (7, 8). Streptonigrin has also been shown to inhibit the production of RNA-dependent DNA polymerase by the avian myeloblastosis virus (7).

In the present study, Sn was found to be very effective in nanogram amounts in protecting cells from chemically induced transformation. The lowest dose studied (0.16 ng/ml) showed no toxicity as determined by a reduction in plating efficiency and colony size. The mechanisms of protection are unknown. However, we do know from the controls in these tests, that at the high-passage level used in this study, the chemical carcinogen alone can transform the cells. In addition, rat leukemia virus (RaLV) gs-1 expression is detectable 8–10 subcultures following transformation and is not detectable prior to transformation (2). It is thought, as yet without direct proof, that the high-passage cells can be transformed by the chemical carcinogen in the absence of added virus because of a reduction in the cellular control of the endogenous virus.

Shif *et al.* (13) have recently shown that 5′-iododeoxyuridine can induce endogenous type "C" RNA virus in both the normal and chemically transformed high-passage F1706 cells and that 0.16 ng/ml Sn inhibits this induction. Thus, the inhibition of transformation by the chemical carcinogen in the presence of Sn suggests that chemical transformation may be associated with the activation of endogenous type "C" viral information.

Summary. At a nontoxic dose, the antiviral antibiotic streptonigrin protected Fischer rat embryo cells grown *in vitro* from transformation by 3-methylcholanthrene.

The authors are deeply grateful to Dr. Aaron E. Freeman for his advice in the preparation of this manuscript.

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Received June 28, 1973. P.S.E.B.M., 1974, Vol. 145.