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ORIGINAL ARTICLE

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Antitumor resistance induced by zinostatin stimalamer (ZSS), a polymer-conjugated neocarzinostatin (NCS) derivative

I. Meth A tumor eradication and tumor-neutralizing activity in mice pretreated with ZSS or NCS

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Abstract Zinostatin stimalamer (ZSS) is a new anticancer agent derived from neocarzinostatin (NCS), which is synthesized by conjugation of one molecule of NCS and two molecules of poly(styrene-co-maleic acid). ZSS exhibited potent in vitro and in vivo antitumor activity in preclinical experiments, and a clinical trial of the intra-arterial administration of ZSS with iodized oil on hepatocellular carcinoma showed potent antitumor activity. We investigated the effect of ZSS and NCS on antitumor resistance and found that pretreatment with either drug suppressed the growth of MethA tumors in Balb/c mice and induced tumor eradication when given separately by single administration at therapeutic doses between 1 day and 4 weeks before tumor transplantation. The findings that the cytocidal activity of these drugs was not detected in vivo at the time of tumor transplantation and that tumor regression was preceded by a period of transient growth suggested that tumor regression was due to host-mediated antitumor activity induced by these drugs. Pretreatment with ZSS or NCS also suppressed the growth of Colon 26 carcinoma and Sarcoma 180. The finding that NCS showed the same effect as ZSS suggests that poly(styrene-co-maleic acid) is not essential for the induction of host-mediated antitumor activity. Furthermore, apo-ZSS, which lacks cytocidal activity, did not induce antitumor activity. From this, it is suggested that the cytocidal effect of ZSS involves the induction of host-mediated antitumor resistance. In athymic Balb/c *nu/nu* mice, pretreatment with ZSS or NCS did not induce tumor eradication, suggesting that mature T lymphocytes play an important role in tumor eradication. Challenging MethA was rejected without transient growth in mice that had been cured of MethA, but challenging Colon 26 was not, showing that anti-MethA resistance was augmented selectively in the MethA-eradicated mice. Splenocytes from MethA-bearing mice pretreated with the drug showed tumor-neutralizing activity beginning 14 days after tumor transplantation. Tumor-neutralizing activity was only induced after MethA transplantation. The effector cells of this tumor-neutralizing activity were Thy1.2$^+$ T lymphocytes that had been passed through a nylon-wool column, but no significant augmentation of cell-mediated cytotoxic activity of splenocytes from MethA-eradicated mice was observed in vitro.

Key words Zinostatin stimalamer · Neocarzinostatin Antitumor resistance · Tumor-neutralizing activity MethA

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Introduction

Zinostatin stimalamer (ZSS) is a polymer-conjugated drug, recently marketed as a chemotherapeutic agent for the teatment of liver cancer in Japan. ZSS (average molecular mass 15 kDa) was synthesized by conjugation with one molecule of neocarzinostatin (NCS), a proteinaceous antitumor agent (molecular mass 11 753 Da) [10, 15], and two molecules of poly(styrene-co-maleic acid) (average molecular mass 2500 Da). ZSS has superior stability in plasma and tissues and shows more efficient accumulation in tumor tissue compared with NCS [16, 18]. ZSS exhibited potent in vitro and in vivo antitumor activity against various human and murine tumor cells by inhibiting DNA synthesis [26, 33]. ZSS has a narrower molecular mass distribution than SMANCS, a similar agent in early stages of development [17, 27].

Most anticancer chemotherapeutic agents induce immunosuppression by antiproliferative action. For the purpose of examining the effects of ZSS and NCS on antitumor defense, pretreatment with these drugs before tumor transplantation was conducted in order to exclude any direct cytocidal effect. If these drugs did not augment antitumor resistance and depressed the host defense systems, tumor growth would have been accelerated in the pretreated mice compared with controls. However, we found that pretreatment with ZSS or NCS induced tumor growth inhibition and tumor eradication. This study was conducted to clarify the mechanism of the antitumor action of ZSS and NCS.

Materials and methods

Animals and tumor cells

Female Balb/c, male athymic Balb/c *nu/nu*, male ICR, male and female C57BL/6, and male and female DBA mice, all aged 6–8 weeks, were purchased from Japan SLC (Shizuoka, Japan). MethA, a methylcholanthrene-induced fibrosarcoma, and Colon 26, an *N*-nitroso-*N*-methyl-urethane-induced undifferentiated adenocarcinoma, were maintained by intraperitoneal and subcutaneous passage, respectively, in Balb/c mice. Sarcoma 180, a mouse sarcoma, EL4, a 9,10-dimethyl-1,2-benzanthracene-induced lymphoma, and P815, a mastocytoma, were maintained by intraperitoneal passage in ICR, C57BL/6N and DBA/2 mice respectively. YAC-1, a Moloney-leukemia virus-induced lymphoma, was maintained in culture in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Commonwealth Serum Lab., Melbourne). For the measurement of antitumor activity, MethA or Colon 26 cells were inoculated subcutaneously into Balb/c or Balb/c *nu/nu* mice, and Sarcoma 180 cells were inoculated subcutaneously into ICR mice in the flank. EL4, P815, YAC-1 and MethA were used as target cells in the cell-mediated cytotoxicity assay.

Drugs

Zinostatin stimalamer (ZSS, Yamanouchi Pharmaceutical, Tokyo) and neocarzinostatin (NCS, Kayaku, Tokyo) were dissolved in sterile phosphate-buffered saline (PBS) just before use. Preparation and intravenous administration of these drugs were performed under subdued lighting to prevent light inactivation. Apo-ZSS, which has no active prosthetic groups and no cytocidal activity, was synthesized from one molecule of the apoprotein of NCS and two molecules of poly(styrene-co-maleic acid).

Antitumor activity

Mice were treated with or without the anticancer drug once between 1 day and 4 weeks before tumor transplantation (day 0). Tumor diameters were measured twice a week with calipers. Tumor weights were estimated from their volume using the following formula [9]:

$$\text{tumor volume (mm}^3\text{)} = [\text{short diameter (mm)}]^2 \times \text{long diameter (mm)}/2$$

At the end of some experiments, tumors were removed and weighed.

Antitumor resistance after tumor eradication

Balb/c mice were treated with 2.5 mg/kg ZSS or 1 mg/kg NCS 3 days before transplantation of MethA (7.5×10^5 cells, day 0). On day 29, cured mice were divided into two groups, and transplanted subcutaneously with MethA (7.5×10^5 cells) or Colon 26 (2×2 mm block) as tumor challenge studies. Tumor weights were determined as described above. Balb/c mice that had not been pretreated were transplanted with MethA or Colon 26 at the time of tumor challenge were used as a control.

Preparation and fractionation of spleen cells

Spleens were aseptically removed from normal mice, MethA-bearing mice or drug-pretreated MethA-bearing mice, and splenocytes were prepared as described previously [19]. The cells were treated with TRIS-buffered ammonium chloride (0.83%) to deplete erythrocytes and were washed three times with RPMI-1640 medium.

To remove T lymphocytes, splenocytes were treated with anti-Thy1.2 monoclonal antibody (Cedarlane Labs, Ontario) and Low-Tox-M rabbit complement (Cedarlane Labs) for 1 h at 37° C. Cells recovered in experiment 1 were examined by flow cytometry. This fraction contained 12.8% L3T4 dull-positive cells and 0.8% Lyt2.2-positive cells (Thy1.2-negative fraction).

For enrichment of T lymphocytes, splenocytes were treated with a nylon-wool column. Nylon wool (Wako Pure Chemical Industries, Osaka) was packed into a syringe (0.3 g) and autoclaved. Splenocytes (10^8 cells/ml in minimal essential medium, MEM, with 10% heat-inactivated FBS) were loaded on the nylon-wool column and incubated for 1 h at 37° C. Cells that passed through the column after addition of MEM medium with 10% heat-inactivated FBS were collected. L3T4-positive cells, Lyt2.2-positive cells and double-positive cells ($L3T4^+Lyt2.2^+$) in this fraction represented 61.5%, 26.2% and 0.7% of cells in experiment 1, and 59.5%, 27.5% and 0.8% of cells in experiment 2 respectively.

For flow-cytometric analysis, cells were stained with fluorescein-isothiocyanate-conjugated anti-Lyt2.2 monoclonal antibody and phycoerythrin-conjugated anti-L3T4 monoclonal antibody (Becton Dickinson, Mountain View, Calif.) for 15 min at 4° C. After washing, more than 5×10^4 cells were analyzed using an Epics Profile (Coulter Electronics, Hialeah, Fla.). The mean percentages of $L3T4^+$, $Lyt2.2^+$ and double-positive cells of unfractionated splenocytes from ZSS-pretreated MethA-bearing mice in each experiment were 31.2%, 14.4% and 0.7%, respectively.

In vivo tumor neutralization assay (Winn assay)

A modified Winn assay [34] was used. Effector cells (splenocytes) were mixed with the target cells (5×10^5 MethA cells) at the effector:target ratios of 50:1 and 25:1, and inoculated into Balb/c or Balb/c *nu/nu* mice. Tumor diameters were measured and tumor weights were calculated as described above.

Cell-mediated cytotoxicity assay

Natural killer (NK) cell activity, lymphokine-activated-killer (LAK)-like activity and tumor-specific T cell activity were determined by a 4-h ^{51}Cr-release assay using YAC-1, EL4 or P815, and MethA cells as the target respectively. Non-specific monocyte cytotoxicity was measured by an 18-h ^{51}Cr-release assay using MethA or P815 cells as the target. Target cells for the cytotoxicity assay (1×10^6 cells) were labelled with 37 kBq or 74 kBq sodium [^{51}Cr]chromate (Amersham Japan, Tokyo) for 1 h at 37° C and washed three times with RPMI-1640 medium with 10% heat-inactivated FBS. The cells

were then incubated again (1 h) in RPMI-1640 medium with 10% heat-inactivated FBS and washed (three times). Effector cells (splenocytes) and target cells (1×10^4 cells) were incubated in 96-well microplates at effector : target ratios of 100 : 1, 50 : 1, 25 : 1 and 12.5 : 1 for 4 h or 18 h at 37° C in a humidified atmosphere containing 5% CO_2. Supernatants were harvested using a supernatant collection system (Skatron, Lier, Norway) and radioactivity release was determined using an Auto Well gamma system (ARC-300; Aloka, Mitaka, Japan). Spontaneous release was determined in the absence of effector cells and maximum release was determined after treating the target cells with 2% Triton-X. Each assay was performed in triplicate and the percentage of specific ^{51}Cr release was calculated by the following formula:

specific release (%) =

[(experimental release − spontaneous release)/

(maximal release − spontaneous release)] × 100

CTL assay

Cytotoxic T lymphocytes (CTL) were induced by a mixed lymphocyte/tumor culture (MLTC) of splenocytes and MethA cells. MethA cells were treated with 50 μg/ml mitomycin C (Kyowa Hakko Kogyo, Tokyo) for 1.5 h at 37° C in a humidified atmosphere of 5% CO_2, and washed three times. Splenocytes (responders; 1×10^7 cells) were mixed with or without mitomycin-C-treated MethA cells (stimulators; 2×10^5 cells) in 24-well microplates, and cultured for 5 or 6 days at 37° C in a humidified atmosphere of 5% CO_2 in RPMI-1640 medium containing 1 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 5 mM HEPES and 10% heat-inactivated FBS. Cells were suspended in RPMI-1640 with 10% heat-inactivated FBS and used as effector cells in the CTL assay. Freshly harvested MethA cells were labelled with ^{51}Cr as target cells, and CTL activity was determined by 4-h or 5-h ^{51}Cr release.

Statistical analysis

Statistical analysis of the data was performed by Student's *t*-test.

Results

Antitumor activity induced by pretreatment with ZSS or NCS

The administration of ZSS 1 day before MethA transplantation strongly suppressed tumor growth at doses of more than 1 mg/kg, and the tumor was judged to be completely eradicated in 14 of 40 mice on day 24. NCS also suppressed tumor growth at doses of more than 0.5 mg/kg; however, the lethal dose of NCS in mice was lower than that of ZSS (estimated LD_{50} values were 1.7 mg/kg and more than 4 mg/kg respectively) (Table 1). The optimal doses of ZSS and NCS, when injected 1 day before tumor transplantation, were 2 mg/kg and 1 mg/kg, respectively, in the first series of experiments. Since additional experiments showed that 2.5 mg/kg ZSS was more effective and not toxic, subsequent experiments were done at 2.5 mg/kg ZSS, with some exceptions.

Optimum conditions for pretreatment with ZSS or NCS

To determine the relationship between the antitumor effect and the duration of drug pretreatment, mice were administered 2.5 mg/kg ZSS or 1 mg/kg NCS on various days before MethA transplantation. Single administration of these drugs between 1 and 7 days before transplantation strongly inhibited tumor growth and induced tumor eradication at a high incidence (Fig. 1A, B). Although unexpected, pretreatment with ZSS or NCS at −4 weeks was also effective (Fig. 1C). NCS exhibited almost the same effect following pretreatment between weeks −4 and −1, whereas the effects of ZSS tended to decrease with time. The optimal dose of ZSS was then determined by pretreatment at −1 or −3 weeks. Tumor eradication was achieved effectively at doses of 1 mg/kg or more and of 0.625 mg/kg or more by pretreatment at −1 week and −3 weeks respectively. At the latter time, however, 1.25 mg/kg ZSS was more effective than 2.5 mg/kg for tumor eradication (Table 2). These results showed that, if the interval between the administration of ZSS and tumor transplantation was longer than 7 days, the optimal dose of ZSS for tumor eradication was about the same as that of NCS.

Kinetics of MethA eradication induced by pretreatment with ZSS or NCS

Tumor regression was always observed after a period of transient tumor growth. As shown in Fig. 2, the tumor volumes of ZSS- and NCS-pretreated mice increased similarly to those of tumor-bearing untreated mice (tumor control mice) until about day 10, and then decreased gradually. In 7 of the 8 ZSS-pretreated mice and 5 of the 7 NCS-pretreated mice, tumors completely disappeared between days 21 and 42. In the remaining mice, tumors enlarged again after transient remission (Fig. 2A).

Effect of pretreatment with apo-ZSS

To determine whether a direct cytocidal effect of ZSS is involved in the induction of antitumor immunity by pretreatment with ZSS, treatment with ZSS without cytocidal activity was examined. NCS is a single-chain polypeptide with 113 amino acids (apoprotein) containing an endiyene prosthetic chromophore, which is essential for cytocidal activity. The noncytocidal ZSS derivative, apo-ZSS, was synthesized from the apoprotein of NCS and poly(styrene-co-maleic acid). In contrast to ZSS, apo-ZSS showed no effect on MethA growth under the same treatment protocol (Table 3). These results suggest that the cytocidal activity of ZSS appears to be involved in the induction of antitumor immunity.

Table 1 Antitumor effect induced by pretreatment with zinostatin stimalamer (*ZSS*) or neocarzinostatin (*NCS*) on MethA tumor. Mice were or were not administered a drug (−1 day) and transplanted with 8×10^5 MethA cells (0 day). Tumor was removed and weighed on day 24

Pretreatment drug (mg/kg)	Tumor weight (g) mean ± SE	Inhibition (%)	Tumor-free[a]	Survival[b]/total
ZSS				
0.5	1.79 ± 1.51	45.1	1	10/10
1.0	0.15 ± 0.20**	95.4	4	10/10
2.0	0.01 ± 0.02**	99.7	8	10/10
4.0	0.06 ± 0.05**	98.2	1	9/10
NCS				
0.5	0.58 ± 0.61*	82.2	2	10/10
1.0	0.17 ± 0.53**	94.8	8	10/10
2.0	0.01 ± 0.02**	99.7	2	3/10
None (MethA only)	3.26 ± 2.48		0	9/10

* $P < 0.05$, ** $P < 0.01$

[a] Number of tumor-free mice (day 24)

[b] Number of surviving mice (day 24). One mouse of the control group died of tumor on day 22, and 1 mouse treated with 4 mg/kg ZSS and 7 mice treated with 2 mg/kg NCS died from drug toxicity within 2 weeks after injection

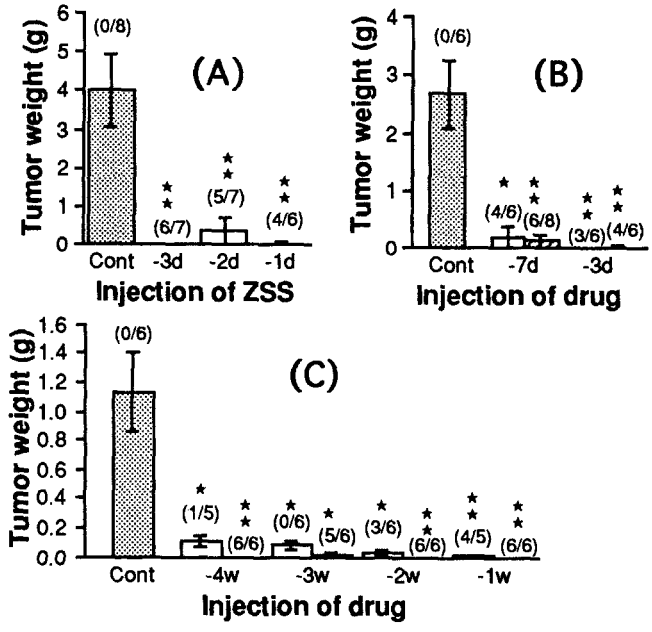

Fig. 1A–C Effect of zinostatin stimalamer (*ZSS*) or neocarzinostatin (*NCS*) pretreatment on MethA tumor growth. ZSS (2.5 mg/kg, □) or NCS (1 mg/kg, ▨) was injected i.v. into Balb/c mice at the indicated times (*d* days, *w* weeks) prior to MethA transplantation (**A**, **B** 8×10^5 cells, **C** 5×10^5 cells; s.c., day 0). Tumors were removed from mice and weighed on day 25 (**A**), day 21 (**B**) or day 24 (**C**). Of 9 control mice, 1 died of tumor on day 25 (**A**). Each value represents the mean tumor weight ± SE. Ratios above the bars represent the number of tumor-free mice/total. *, **, *** Significant differences between the control (▤) and each experimental group (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). *Cont* control.

Effect of pretreatment with ZSS or NCS on the growth of Colon 26 and Sarcoma 180

Pretreatment with ZSS and NCS significantly inhibited the growth of both Colon 26, derived like MethA from Balb/c mice, and Sarcoma 180, derived from another strain. However, tumor eradication was not observed (Fig. 3).

Participation of T lymphocytes in MethA eradication

To determine the role of T lymphocytes in the induction of antitumor activity, the effect of pretreatment of these drugs was examined in Balb/c *nu/nu* mice, which are athymic and lack mature T lymphocytes. As shown in Fig. 4, ZSS and NCS suppressed the growth of MethA, but no mice were cured. These results indicated that mature T cells were essential for MethA eradication.

Antitumor resistance after tumor eradication

To examine the augmentation of antitumor resistance after tumor eradication induced by ZSS or NCS pretreatment, cured mice were challenged with MethA or Colon 26. As shown in Table 4, rechallenged MethA were rejected in all mice without the transient growth observed in the first tumor inoculation. In contrast, the growth of Colon 26 was inhibited only partially. These results suggest that after MethA eradication by pretreatment with ZSS or NCS, antitumor resistance had been augmented tumor-selectively in the mice.

Tumor-neutralizing activity of spleen cells from ZSS- or NCS -pretreated MethA-bearing mice.

On day 7, tumor weights of MethA-bearing mice pretreated with ZSS or NCS were almost the same as those of nontreated MethA-bearing mice (tumor control). Weights were obviously decreased on day 14, however, and all mice were cured by day 32. Splenocytes from the ZSS- or NCS-pretreated mice were harvested on days

Table 2 Dose response of MethA growth to ZSS pretreatment. Mice were or were not administered ZSS on the indicated day and transplanted with 5×10^5 MethA cells on day 0. Tumor was removed and weighted on day 28 (experiment 1) or day 35 (experiment 2)

Expt.	Pretreatment with ZSS (mg/kg)	Tumor weight (g) mean ± SE	Inhibition (%)	Tumor-free[a]/total
1	− 7 days 0.5	0.48 ± 0.24	55.1	2/7
	− 7 days 1.0	0.01 ± 0.01***	99.1	6/7
	− 7 days 2.5	0.00 ± 0.00***	100	7/7
	MethA only	1.07 ± 0.21		0/10
2	− 21 days 0.15625	2.22 ± 0.58	3.9	1/6
	− 21 days 0.315	2.17 ± 0.54	6.0	1/6
	− 21 days 0.625	0.18 ± 0.15***	92.2	4/6
	− 21 days 1.25	0.00 ± 0.00***	100	6/6
	− 21 days 2.5	0.03 ± 0.02***	98.7	4/6
	None (MethA only)	2.31 ± 0.64		0/10

*** $P < 0.001$

[a] Number of tumor-free mice at the end of the experiments

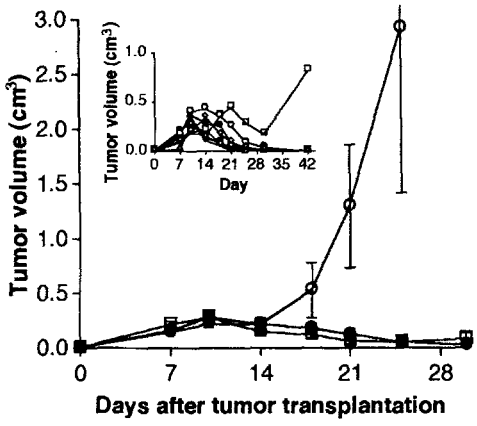

Fig. 2 Kinetics of tumor growth in mice pretreated with ZSS or NCS. Balb/c mice were administered ZSS (2.5 mg/kg, ●) or NCS (1 mg/kg, □) or neither (○), followed 3 days later by transplantation of 5×10^5 MethA cells s.c. Tumor diameters were measured on the indicated days and tumor volume was calculated as described in Materials and methods. Each group consisted of 7 or 8 mice. Each value represents the mean tumor volume ± SE. One mouse of the control group died of tumor on day 30. *Insert*: individual tumor growth in ZSS-pretreated mice

7, 14 and 32, and the anti-MethA activity was determined by in vivo tumor-neutralizing assay. Activities of splenocytes from the tumor control group and normal mice were also determined. As shown in Fig. 5, day-7 splenocytes from ZSS-pretreated mice showed slight inhibition of the growth of MethA, whereas splenocytes from NCS-pretreated mice and the tumor control group showed no significant effect when compared with those from normal mice. Day-14 splenocytes from ZSS- and NCS-pretreated mice inhibited MethA growth completely and all of the mice survived. Moreover, day-14 splenocytes from the tumor control group also inhibited tumor growth and 3 of 6 tested mice were cured. Day-32 splenocytes from ZSS- or NCS-pretreated mice showed anti-MethA activity similar to that of day-14 splenocytes, but those from the tumor control group no longer suppressed the growth of MethA.

Tumor-neutralizing activity was not observed in splenocytes from MethA- untransplanted mice pretreated with ZSS or NCS (data not shown).

Effector cells of tumor-neutralizing activity

To characterize the effector cells of the tumor-neutralizing activity, splenocytes were fractionated into T-cell-rich or -depleted splenocytes and determined by tumor-neutralizing assay. To exclude the effect of T lymphocytes of recipient mice, Balb/c *nu/nu* mice were used as recipients. The results are shown in Table 5. T-cell-rich splenocytes that had been passed through nylon wool absolutely suppressed MethA growth like unfractionated splenocytes (experiments 1, 2), whereas Thy1.2-negative splenocytes exhibited only weak or no growth inhibition (experiments 1 and 2 respectively) and the tumor grew to where it became lethal. These results indicate that T lymphocytes might be the main effector cells of tumor-neutralizing activity.

Cytotoxic activity of splenocytes from ZSS- or NCS- pretreated MethA-bearing mice

Two experiments were performed as follows. Splenocytes from ZSS- and NCS-pretreated mice were harvested during the period of tumor growth (experiment 1 on day 6, experiment 2 on day 7), during the period of tumor regression (experiment 1 on day 13, experiment 2 on day 15), and during the period of tumor eradication (experiment 1 on day 21, experiment 2 on day 29). The cytotoxic activity of splenocytes harvested in the various periods was evaluated using MethA, NK-sensitive YAC-1, NK-insensitive and/or monocyte-sensitive P815 or EL4 as target cells. In spite of different harvest times and E:T ratios, splenocytes from ZSS- or NCS-

Table 3 Effect of pretreatment with apo-ZSS, which is devoid of direct cytocidal activity, on MethA growth. Mice were treated with 2.5 mg/kg ZSS or apo-ZSS, or not treated, on the indicated day and transplanted with 7.5×10^5 MethA cells (0 day). Tumor diameters were measured on day 29

Treatment	Tumor volume (cm^3) mean ± SE	Inhibition (%)	Tumor free[a]/total
ZSS			
− 3 days	0.10 ± 0.10***	98.3	9/10
1 day	0.38 ± 0.13***	93.7	4/10
Apo-ZSS			
− 3 days	5.66 ± 0.85	6.1	0/10
1 day	4.84 ± 0.61	19.7	0/10
None (MethA only)	6.03 ± 1.07		0/11

*** $P < 0.001$

[a] Number of tumor-free mice (day 29)

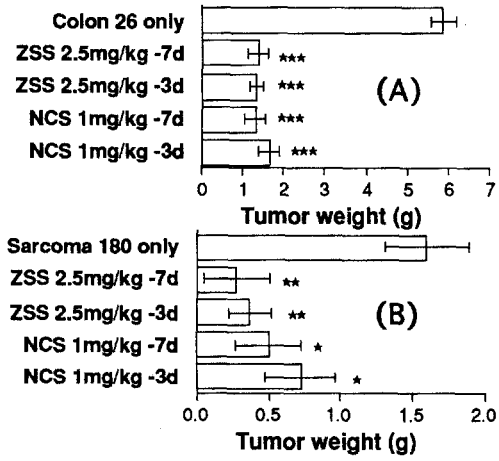

Fig. 3A, B Effect of pretreatment with ZSS or NCS on the growth of Colon 26 (**A**) and Sarcoma 180 (**B**). Mice were administered ZSS (2.5 mg/kg) or NCS (1 mg/kg) or neither 3 or 7 days before tumor transplantation (day 0). **A** Balb/c mice were inoculated with Colon 26 (2 × 2 mm block), and tumor weights were determined on day 21. The control group consisted of 11 mice and experimental groups of 7 mice each. Of 11 control mice, 2 died of tumor before the end of the experiment. **B** ICR mice were transplanted with Sarcoma 180 (1×10^6 cells), and tumor weights were determined on day 28. Control and experimental groups consisted of 9 and 7 mice respectively. Each value represents the mean tumor weight ± SE. *, **, *** Significant differences between the control and respective experimental groups (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

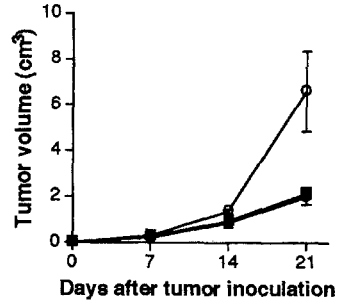

Fig. 4 Effect of pretreatment with ZSS and NCS on MethA growth in Balb/c *nu/nu* mice. Balb/c *nu/nu* mice were administered ZSS (2.5 mg/kg, ●) or NCS (1 mg/kg, □) or neither (○) i.v. 3 days before inoculation of MethA (1×10^6, s.c.). Control and experimental groups consisted of 8 and 6 mice respectively. Each value represents the mean tumor volume ± SE. Differences between control and experimental groups were statistically significant at $P < 0.05$, except results for the NCS-treated group on day 7

Table 4 Augmentation of tumor-selective antitumor resistance in mice cured of MethA by pretreatment with ZSS or NCS. Mice were administered 2.5 mg/kg ZSS or 1 mg/kg NCS 3 days before inoculation of 7.5×10^5 MethA cells (day 0). On day 29, cured mice were divided into two groups and reinoculated s.c. with MethA (7.5×10^5) or Colon 26 (2-mm block) respectively

Pretreatment drug (mg/kg)	Challenged tumor	Tumor-free[a]/total
ZSS 2.5	MethA	9/9
NCS 1	MethA	9/9
None	MethA	0/10
ZSS 2.5	Colon 26	0/9
NCS 1	Colon 26	0/10
None	Colon 26	0/9

[a] Number of tumor-free mice (day 21)

pretreated mice exhibited no significant cytotoxic activity against any target cells as compared with those from the tumor control group and normal mice. Representative results of these studies are shown in Table 6.

Besides the assays described above, CTL activity following MLTC of splenocytes and MethA cells was examined. Two experiments were performed using splenocytes from ZSS- or NCS-pretreated mice in various periods of tumor regression, as in the above experiments. Table 7 shows the results for splenocytes of the tumor eradication period. A significant difference was only seen between splenocytes without stimulator from normal mice and NCS-treated tumor-bearing mice at an E:T ratio of 50:1 ($P < 0.05$) in experiment 1. But at other E:T ratios (100:1, 25:1, 12.5:1) and in another experiment, no significant difference was observed between these spleen cells. In splenocytes harvested in other periods, no significant activity beyond that of the control splenocytes was detected in splenocytes from ZSS- or NCS-pretreated mice.

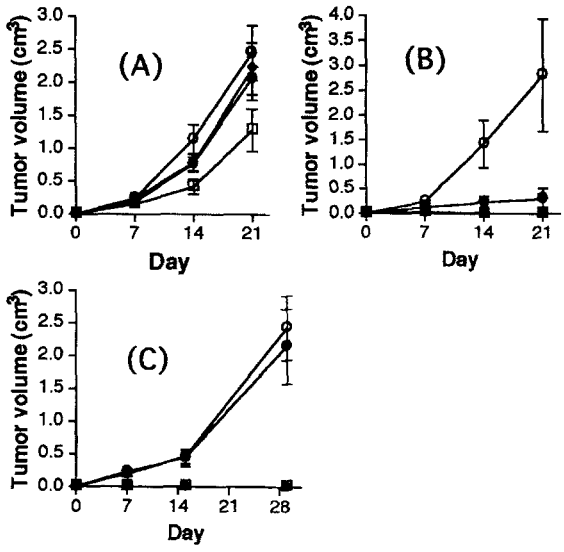

Fig. 5A–C. Tumor-neutralizing activity of splenocytes from MethA-bearing mice pretreated with ZSS or NCS. Mice were treated with ZSS (2.5 mg/kg, □) or NCS (1 mg/kg, ◆) or neither (●) 3 days before MethA transplantation (5×10^5 cells, s.c., day 0). Splenocytes were harvested on days 7 (**A**), 14 (**B**), and 32 (**C**) and inoculated into recipient Balb/c mice with MethA (5×10^5 cells) at an E:T ratio of 50:1 (**A, B**) or 25:1 (**C**). Splenocytes from normal mice (○) were also tested as control. Each value represents the mean tumor volume ± SE of 5–10 mice in each group. Differences between control and experimental groups were statistically significant at $P < 0.05$ (**A** ● 7 days, □ 14 days), $P < 0.01$ (**A** □ 7 days, **B** ● 7 days, **C** □ 7 days, ◆ 7 days), $P < 0.001$ (**B** □ 7, 14, 21 days; ◆ 14, 21 days, **C** □ 15, 29 days, ◆ 15, 29 days)

Discussion

The present study provides clear evidence that pretreatment with ZSS or NCS induces MethA tumor eradication. Although no significant cytocidal activity could be detected in vivo 24 h after intravenous injection of ZSS or NCS [6, 32], MethA was eradicated by a single injection of ZSS or NCS between 1 day and 4 weeks before tumor transplantation (Table 1, Fig. 1). The eradication of MethA always followed a preceding period of transient tumor growth (Fig. 2). These facts strongly suggest that MethA was eradicated by host-mediated antitumor activity induced by the drugs. Because NCS showed the same tumor eradication effect as ZSS, it was considered that the poly(styrene-co-maleic acid) component was not essential for the anti-tumor-resistance-stimulatory activity of ZSS. Further, apo-ZSS induced neither a therapeutic effect by post-treatment nor host-mediated antitumor activity by pretreatment (Table 3). These data therefore suggest that the cytocidal activity of ZSS or NCS is attributable to the induction of antitumor resistance.

The fact that MethA tumors were not eradicated in Balb/c *nu/nu* mice strongly suggests that T lymphocytes play an important role in the antitumor mechanism of this compound (Fig. 4). Furthermore, since tumor-neutralizing activity was seen only after MethA transplantation, it seems likely that antigen presentation was required for the induction of tumor-neutralizing activity. In fact, T lymphocytes were the main effector cells of tumor-neutralizing activity, and tumor-selective antitumor resistance was augmented in mice in which the tumor had been eradicated (Tables 4, 5).

The tumor-neutralizing activity appeared after day 14, when tumor regression had started, but not on day 7, at which time transient tumor growth was occurring. These results support the idea that the effector cells for tumor eradication and the effector cells in the tumor-neutralizing assay may be the same. On the other hand, splenocytes from tumor-bearing mice showed tumor-neutralizing activity on day 14, but no activity on day 32 (Fig. 5). This suggests that antitumor effector cells appeared once in the spleens of tumor-bearing mice, but that subsequent activity might be

Table 5 Tumor-neutralizing activity of splenic T cells from MethA-bearing mice pretreated with ZSS. Balb/c mice were administered 2.5 mg/kg ZSS 3 days before inoculation of 5×10^5 MethA cells (day 0). Splenocytes were harvested on day 14 (experiments 1, 2) or day 21 (experiment 3), and treated with anti-Thy 1.2 antibody and complement (*Thy1,2-depleted*) or a nylon wool column (*Nylon-column-passed*). To determine the tumor-neutralizing activity, splenocytes and MethA (5×10^5 cells) were inoculated into Balb/c *nu/nu* mice at an E:T ratio of 25:1 (day 0). Tumor diameters were measured on day 21 (experiments 1, 2) or day 18 (experiment 3)

Expt.	Splenocytes	Tumor volume (cm^3) mean ± SE	Inhibition (%)	Tumor free[a]/total
1	Unfractionated	0.00 ± 0.00***	100	5/5
	Thy 1.2-depleted	3.45 ± 0.51*	32.2	0/5
	Nylon-column-passed	0.00 ± 0.00***	100	5/5
	None	5.09 ± 0.40		0/5
2	Unfractionated	0.00 ± 0.00***	100	5/5
	Nylon-column-passed	0.00 ± 0.00***	100	5/5
	None	5.02 ± 0.61		0/7
3	Unfractionated	0.00 ± 0.00***	100	5/5
	Thy 1.2-depleted	2.29 ± 0.36	13.9	0/5
	None	2.66 ± 0.34		0/5

* $P < 0.05$, *** $P < 0.001$
[a] Number of tumor-free mice (day 21)

Table 6 Antitumor cytotoxicity of splenocytes from ZSS- and NCS-pretreated MethA-bearing mice. Mice were administered 2.5 mg/kg ZSS or 1 mg/kg NCS or neither (control) 7 days before inoculation of 5×10^5 MethA cells (day 0). Splenocytes were harvested on day 21 (experiment 1) or day 29 (experiment 2). Cytotoxicity was examined by 4-h ^{51}Cr-release assay at an E:T ratio of 100:1

Splenocytes from	Specific ^{51}Cr release (%)							
	Experiment 1				Experiment 2			
	MethA	YAC-1	EL-4	P815	MethA	YAC-1	EL-4	P815
Normal mice	14.4 ± 0.8	3.1 ± 1.3	4.1 ± 1.3	0 ± 0	14.8 ± 2.6	1.7 ± 0.3	1.2 ± 0.2	0 ± 0
Untreated tumor bearer	17.5 ± 1.8	4.5 ± 1.5	1.8 ± 0.3	0 ± 0	9.4 ± 1.5	3.9 ± 0.7	1.5 ± 0.5	0 ± 0
ZSS-treated tumor bearer	13.7 ± 1.2	2.0 ± 1.1	1.3 ± 0.5	0 ± 0	5.8 ± 0.3	2.8 ± 0.7	0.6 ± 0.3	0 ± 0
NCS-treated tumor bearer	11.3 ± 1.3	0.1 ± 1.0	3.6 ± 0.9	0 ± 0	7.0 ± 1.3	1.8 ± 0.8	1.7 ± 0.2	0 ± 0

Table 7 T cell-medited cytotoxicity against MethA after mixed lymphocyte/tumor-cell culture of splenocytes from ZSS- and NCS-pretreated MethA-bearing mice. Mice were administered 2.5 mg/kg ZSS or 1 mg/kg NCS or neither 7 days before inoculation of 5×10^5 MethA cells (day 0). Splenocytes were harvested on day 22 (experiment 1) or day 26 (experiment 2), and cultured with or without mitomycin-C-treated MethA for 5 days (experiment 1) or 6 days (experiment 2) at a responder:stimulator ratio of 50:1. Cytotoxicity was examined by a 4-h ^{51}Cr-release assay at the indicated E:T ratio

		Specific ^{51}Cr release (%)			
		Expt. 1		Expt. 2	
Responder splenocytes from	Stimulator	100:1	50:1	100:1	50:1
Normal mice	+	9.2 ± 2.9	6.8 ± 1.1	5.4 ± 0.6	3.4 ± 0.6
	−	7.4 ± 2.9	5.3 ± 2.7	6.6 ± 3.8	5.8 ± 2.5
Untreated tumor bearer	+	9.1 ± 1.4	9.7 ± 3.8	3.4 ± 1.2	2.2 ± 1.6
	−	11.6 ± 3.6	7.0 ± 3.7	4.8 ± 0.6	3.9 ± 2.2
ZSS-treated tumor bearer	+	10.7 ± 2.3	7.1 ± 2.8	4.1 ± 0.8	5.4 ± 1.4
	−	12.4 ± 7.8	20.4 ± 8.4	2.4 ± 1.1	4.9 ± 2.3
NCS-treated tumor bearer	+	13.4 ± 3.7	18.2 ± 10.2	4.0 ± 0.6	3.3 ± 0.9
	−	10.1 ± 2.2	11.3 ± 1.3*	3.8 ± 2.1	1.4 ± 0.4

* $P < 0.05$ (versus normal mice splenocytes cultured without stimulator)

inhibited by suppressor cells or suppressor factors derived from host or tumor cells; and the possibility that ZSS- and NCS-induced antitumor activity is based on the elimination of these suppressors has not been ruled out.

Rechallenged MethA was rejected without transient growth in mice that had been cured of MethA by pretreatment with ZSS or NCS (Table 4). The finding that the same MethA rejection was also observed in mice challenged about 7 weeks after MethA eradication (data not shown) proved that the augmented antitumor resistance lasted for a long time in mice.

In Balb/c *nu/nu* mice, MethA growth was significantly inhibited by pretreatment with ZSS or NCS (Fig. 4). These results suggest that, in addition to T lymphocytes, which are important for tumor eradication, ZSS or NCS may activate nonspecific antitumor effector cells such as NK cells, macrophages and LAK cells.

In cell-mediated cytotoxicity assay, splenocytes from ZSS- or NCS-pretreated mice did not significantly exhibit cytotoxic activity of NK cells, LAK-like cells or macrophages. In addition, splenic T cells, with tumor-neutralizing activity, showed no CTL activity. These results suggest that the antitumor effector cells induced by pretreatment with these drugs might eliminate the tumor cells by a mechanism other than direct cytotoxicity. Several laboratories have reported that noncytotoxic T cells can induce the eradication of tumors without CTL activity [3, 7, 8, 11]. It is possible that such noncytotoxic T cells also participate in tumor eradication in our system. Moreover, tumor necrosis factor α, interferon, and interleukin-2 all have in vivo anti-MethA activity [4, 13, 14, 25], and interferon production by SMANCS has been reported [31]. Such cytokine production by these cells might also be involved tumor suppression.

The tumor eradication activity of other anti-cancer chemotherapeutic agents such as cyclophosphamide, busulfan, melphalan and bleomycin has been investigated, and a number of related studies have been reported [1, 2, 5, 12, 20–24, 28–30, 35]. Pretreatment with cyclophosphamide and busulfan is possible, but the optimal intervals between tumor transplantation and their administration were in the range of a few days to about 1 week [28, 29]. ZSS and NCS are unique among these drugs, since then drugs can induce tumor eradication by a single injection as long as 4 weeks before tumor transplantation.

The antitumor effect of pretreatment with ZSS or NCS was recognized not only on MethA but also on Colon 26 and Sarcoma 180 (Fig. 3), suggesting that ZSS and NCS might induce antitumor immunity against various tumors. The optimal dose of ZSS in pretreatment, which was 0.625–2.5 mg/kg depending on the time of injection, was closely similar to the effective dose for therapeutic administration in tumor-bearing mice [26]. ZSS might therefore show both direct cytocidal activity and enhancement of antitumor immunity in therapy.

To understand better the antitumor immunity induced by ZSS and NCS, we also examined (a) the changes in the splenocyte population after administration of these drugs and tumor transplantation, (b) histological analysis of tumor-infiltrating cells, (c) in vivo cell depletion by antibody or carrageenan after pretreatment or post-treatment of these drugs, and so on. These results will be described in a forthcoming report.

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