Role of Natural Killer Cells and Macrophages in the Nonspecific Resistance to Tumors in Mice Stimulated with SMANCS, a Polymer-conjugated Derivative of Neocarzinostatin

Fujio Suzuki,2 Richard B. Pollard, Satomi Uchimura, Tetsuo Munakata, and Hiroshi Maeda

ABSTRACT

Copoly(styrene-maleic acid)-conjugated neocarzinostatin (SMANCS), a lipophilic derivative of the proteinaceous antitumor antibiotic neocarzinostatin, has been reported to stimulate a nonspecific resistance to tumors (NSRT) in solid tumor-bearing mice, in addition to its chemotherapeutic antitumor effect through the arrest of DNA synthesis by direct DNA strand scission. In the present study, splenic or peritoneal effector cells were used to investigate the ability of SMANCS to augment natural killer (NK) cell activity and to generate cytotoxic macrophages (A-MΦ). Splenic NK cell activity augmented by SMANCS was characterized by cytotoxicity to various target cells, nylon wool nonadherence, and sensitivity to treatment with anti-asialo-GM1 antiserum or monoclonal anti-Thy-1.2 antibody followed by complement. The A-MΦ generated by SMANCS stimulation were characterized by their adherence to a plastic surface coated with fetal calf serum and their ability to phagocytize carbonyl-Iron. The maximum level of NK cell activity in the spleens of mice was detected 3 days after i.v. injection of SMANCS, and the highest activity of the peritoneal A-MΦ was demonstrated in mice 4 days after SMANCS treatment. On the other hand, the NSRT of mice stimulated with SMANCS was not detectable in mice treated with carrageenan or trypan blue, whereas SMANCS-stimulated NSRT was observed in mice treated with anti-asialo-GM1 antiserum. The NSRT that was stimulated with SMANCS was also demonstrated in mice homzygous for the beige mutation and their non-beige littermates, when NK cell-resistant EL-4 thymoma was used as a tumor target. These results suggest that the expression of NSRT of mice stimulated with SMANCS may require the function of A-MΦ, although NK cell activity was also augmented in spleens of mice by administration of SMANCS.

INTRODUCTION

NCS3 is an antitumor protein antibiotic (M, 12,000) that is well characterized chemically and biologically (1). NCS has been reported to exert a striking antitumor activity against lymphatic leukemia (e.g., murine leukemia SN-36, P388, and L-1210) in experimental animals (2–4). Limited clinical efficacy of NCS has been established in patients with leukemia, lymphoma, and bladder cancer (5–8). The molecular mode of action of NCS is to arrest DNA synthesis by direct strand scission brought about by its prosthetic group (9–11). This type of strand scission of DNA leads to inhibition of DNA synthesis in bacteria (10) and mammalian cells (11). Recently, a lipophilic derivative of NCS, designated SMANCS, was chemically synthesized by conjugation of poly(styrene-co-maleic acid) deriva-

tives and NCS (12). The chemical reaction was achieved by reacting the two amino groups in NCS with the anhydride group of the partially hydrolyzed poly(SMA) in alkaline solution (13). Since SMANCS is a conjugate of two molecules of SMA (M, 2000) and a molecule of NCS, the final molecular weight of the conjugate is 16,000 (12). The physical, chemical, and biochemical properties of SMANCS have been well documented (12–16). Since, like parental NCS, SMANCS retains properties for arresting the DNA synthesis of mammalian cells by direct DNA strand scission (15), an antitumor activity of the conjugate against various experimental tumors in vivo and in vitro has been demonstrated (12, 14, 16). Compared with NCS, however, the conjugate has been reported to have several considerable pharmacological advantages, including decreased toxic side effects, extension of biological half-life, enhanced tropism for tumor tissues, and increased hydrophobicity (12–16). Its therapeutic activity in various human malignancies, particularly liver cancer, has also been reported (17–21). Because lipophilic SMANCS can be solubilized in a lipid contrast medium (Lipiodol) (20), Konno and co-workers (17–19) have successfully administered SMANCS dissolved in this medium to patients with liver cancer, by catheterizing the respective feeding arteries under X-ray monitoring. By this method, SMANCS could be delivered and deposited in the tumor tissues over a prolonged period without producing major side effects (21).

Conversely, after conjugation of SMA with NCS, SMANCS has displayed various immunomodulating activities, such as MΦ activation (22), induction of IFN-γ production (23), and stimulation of NSRT of mice bearing syngeneic tumors (24). Because the NCS or SMA molecule itself has not been shown to induce IFN (23), activate MΦ (22), or stimulate NSRT of mice (24), this would suggest that the immunomodulating activities of the compound were produced by the conjugation. It is well known that polycarboxylates have a variety of biological activities including IFN induction (25). However, the SMA used in the present study has not been shown to possess the capability to induce host resistance (22–24), even though it is one of the polycarboxylates with a low molecular weight (12). The SMA conjugated with bovine serum albumin has been shown to stimulate A-MΦ generation (22), SMA conjugated with human serum albumin, human immunoglobulin, or mouse immunoglobulin has been shown to induce IFN,4 and SMA conjugated with bovine or human serum albumin has been shown to be able to generate cytotoxic T-lymphocytes from Epstein Barr virus-sensitized human peripheral blood lymphocytes (26). These facts suggest that the host resistance produced by stimulation with SMANCS may be established by the SMA molecule but not the NCS molecule.

It has been reported (23) that circulating IFN could be stimulated by the conjugate. The IFN demonstrated in circu-
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loration of SMANCS-treated mice contained about 60% IFN-γ and 40% α/β interferon (23), and SMANCS required Mφ, Lyt-2+ T-cells, or both for the production of IFN-γ (23). The NSRT of mice bearing syngeneic solid tumors was also stimulated by administration of SMANCS (24). Because NSRT of mice was not developed when tumor-bearing mice were treated by the combination of SMANCS and anti-IFN-γ antisera, but not anti-α/β interferon antisera, it was suggested that the NSRT in SMANCS-treated mice was expressed by the IFN-γ produced by SMANCS stimulation (24). It is known (27–30) that three major effector killer cells (A-Mφ, NK cells, and cytotoxic T-lymphocytes) are considered to play a role in the antitumor activity following IFN exposure, in addition to their direct inhibitory effect on tumor cell growth (31). Therefore, it would seem possible that the NSRT of SMANCS-stimulated mice could be expressed through these effector cells, augmented or activated by SMANCS-induced IFN in tumor-bearing mice.

In the present study, the ability of SMANCS to generate A-Mφ from resting Mφ and to augment splenic and peritoneal NK cell activities in vivo was investigated. Further, the requirement for A-Mφ or NK cells in the expression of NSRT of mice stimulated with SMANCS was examined when these effector cell activities were increased by administration of the compound.

MATERIALS AND METHODS

Mice. Eight-week-old inbred BALB/c and C57BL/6 mice of both sexes were obtained from the Shizuoka Agricultural Cooperative for Experimental Animals (Hamamatsu, Japan) and The Jackson Laboratory (Bar Harbor, ME) (24). C57BL/6-6/bg/bg (beige) and their littermates (bg+−) were obtained from The Jackson Laboratory.

Tumors. RL11 leukemia, maintained by i.p. inoculation into BALB/c mice serially in an ascites form, was used as a target tumor of the NSRT assay in mice treated with SMANCS and/or blockers of NK cells or Mφ. EL-4 thymoma, serially passed in C57BL/6 mice in an ascites form, was used as a target tumor of the NSRT assay in mice treated with SMANCS and/or blockers of NK cells. The tumors used as target cells in the NSRT assay in mice treated with SMANCS and/or blockers of NK cells were incubated at 37°C in an atmosphere of 5% CO₂ in air, and viable leukemia cells were counted daily, with a hemocytometer, by the trypan blue dye exclusion test (32).

Reagents. NCS was obtained from the Kayaku Co., Ltd. (Tokyo, Japan). SMANCS was synthesized by Kuraray Co., Ltd. (Kurashiki, Japan), as described previously (23). SMA was prepared as described previously (23). SMA was prepared as described previously (23). SMA was prepared as described previously (23).

Preparation of Effector Cells for the Assay of Mφ Cytotoxicity. Peritoneal cell preparations from mice treated with saline, NCS, SMA, or SMANCS were used as the effector cells for the A-Mφ cytotoxic assay. As the target cell, EL-4 thymoma was used. For the preparation of effector cells, peritoneal cells were isolated by peritoneal lavage. The cells were then washed and resuspended in complete medium. These cells were used as the effector cells.

Preparation of Effector Cells for the Assay of NK Cell-mediated Cytotoxicity. Splenic mononuclear and peritoneal cells from mice treated with saline, NCS, SMA, or SMANCS were used as the effector cells for the NK cell-mediated cytotoxicity assay. As the target cell, EL-4 thymoma was used. For the preparation of effector cells, splenic mononuclear and peritoneal cells were isolated by splenic lavage. The cells were then washed and resuspended in complete medium. These cells were used as the effector cells.

Preparation of Effector Cells for the Assay of A-Mφ Cytostasis. To detect a growth-inhibitory effect of plastic-adherent cells from SMANCS-treated mice against co-cultured target tumor cells, the method of Schultz and co-workers (38) described previously was utilized (34). Approximately 1 × 10⁶ adherent cells were seeded in 3.5-cm plastic dishes (Falcon) in 1 ml of complete medium. RL11 cells were washed against 10×10⁶ cells/ml with complete medium, and 2-ml aliquots were immediately admixed with the plastic-adherent cultures. Cultures were incubated at 37°C in an atmosphere of 5% CO₂ in air, and viable leukemia cells were counted daily, with a hemocytometer, by the trypan blue dye exclusion test (32).

Cell suspensions were serially passed in C57BL/6 mice in an ascites form, was used as a target tumor of the NSRT assay in mice treated with SMANCS and/or blockers of NK cells. The tumors used as target cells were incubated at 37°C in an atmosphere of 5% CO₂ in air, and viable leukemia cells were counted daily, with a hemocytometer, by the trypan blue dye exclusion test (32). The ratio of adherent cells to target cells was determined by a standard 4-h 51Cr-release assay, as described previously (34). All cultures were performed in triplicate.

Summary. The present study demonstrated that SMANCS-induced IFN in tumor-bearing mice stimulated the NSRT of mice bearing syngeneic solid tumors. The NSRT of mice bearing syngeneic solid tumors was also stimulated by administration of SMANCS (24). Because NSRT of mice was not developed when tumor-bearing mice were treated by the combination of SMANCS and anti-IFN-γ antisera, but not anti-α/β interferon antisera, it was suggested that the NSRT in SMANCS-treated mice was expressed by the IFN-γ produced by SMANCS stimulation (24). It is known (27–30) that three major effector killer cells (A-Mφ, NK cells, and cytotoxic T-lymphocytes) are considered to play a role in the antitumor activity following IFN exposure, in addition to their direct inhibitory effect on tumor cell growth (31). Therefore, it would seem possible that the NSRT of SMANCS-stimulated mice could be expressed through these effector cells, augmented or activated by SMANCS-induced IFN in tumor-bearing mice.

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(6 × 10⁶ cells/ml) was seeded into each well of a 96-well microtiter plate (round-bottomed) and incubated at 37°C in 5% CO₂ in moist air. Two h after incubation, RL21 target cells were added to each well (0.1 ml of the target cell suspension; 6 × 10⁴ cells/ml). Twenty-four h after cocultivation, 1 μCi of [³H]thymidine was added to each well for 24 h. Cells were collected on a glass filter by using a multiple cell harvester. The cytostasis of the effector cells was estimated by measuring the inhibition of [³H]thymidine incorporation into target cells in triplicate cultures. The radioactivity of [³H]thymidine incorporated into the tumor cells was determined with a liquid scintillation counter (Beckman LS6800). Percentage of SMANCS-induced cytostasis was calculated by the following formula:

\[
\text{% Cytostasis} = \frac{\text{cpm}_{\text{control}} - \text{cpm}_{\text{experiment}}}{\text{cpm}_{\text{control}}} \times 100
\]

where \(\text{cpm}_{\text{control}}\) equals target cell alone and \(\text{cpm}_{\text{experiment}}\) equals target cells and adherent cells. Spontaneous cytostasis was calculated as above, where \(\text{cpm}_{\text{control}}\) equals target cells alone and \(\text{cpm}_{\text{experiment}}\) equals target cells and adherent cells obtained from normal mice.

Antitumor Assay. The NSRT stimulated by SMANCS was examined in mice depleted of Mϕ functions or NK cell activity. To abolish NK cell activity from tumor-bearing mice, BALB/c mice were given a 5-mg/kg i.v. dose of anti-asialo-GM1 antiserum 4 times, at 5-day intervals beginning 1 day before the tumor inoculation (40). Control mice were treated with a 1:15 dilution of normal rabbit serum. This treatment with the antiserum has been shown to abolish NK cell activity from mice 12 h after injection (36). Because it has been previously reported (41, 42) that the neutral glycolipid asialo-GM1 was expressed on A-Mϕ and cytotoxic T-lymphocytes, a possibility arose that the treatment of mice with anti-asialo-GM1 antiserum resulted in the elimination of these activated cells, as well as NK cells. To avoid this confusion, congenitally NK cell-deficient beige mice and their litters were also used in the additional experiments (43). The beige mutation arose spontaneously as a recessive coat color mutant in the C57BL/6 mouse strain. Substantial experimental evidence indicated that this mutation in the homozygous condition resulted in a marked deficiency of NK cell activity (43). Trypan blue (25 mg/kg; Sigma Chemical Co., St. Louis, MO) or carrageenan (50 mg/kg; Sigma) was administered i.p. to BALB/c mice to remove Mϕ activity in vivo (37, 40). The effectiveness of these treatments in the depletion of Mϕ function has been previously described (37). It has also been reported that an i.v. injection of carrageenan into mice caused a prompt and substantial decline of splenic NK cell activities (44). The inhibitors of Mϕ were administered 2 days before and 1 day after tumor inoculation and once weekly for 2 weeks (total of 4 treatments) (40). RL21 leukemia (BALB/c mice) or EL-4 thymoma (beige mice and their litters) was implanted s.c., at 1 × 10⁶ cells/mouse, into the shaved left side of the abdomen of mice depleted of Mϕ or NK cells. To detect NSRT of mice stimulated with SMANCS, mice were pretreated with a 1.6-mg/kg i.v. dose of SMANCS 24 h before the tumor was implanted (24). Because SMANCS administered i.v. has been shown to be completely cleared within 90 min (15) and tumor cells were implanted into mice 24 h after SMANCS treatment, the possibility of direct interactions between tumor cells and SMANCS would seem negligible (24). Therefore, as described previously (24), in this system the demonstrated inhibition of growth of tumors by SMANCS administration should be expressed through the NSRT of mice. The NSRT of mice stimulated with SMANCS was evaluated by comparisons of tumor sizes of tested mice through the NSRT of mice. The NSRT of mice stimulated with SMANCS-augmented NK cells is shown in Table 1. In addition to YAC-1 lymphoma, SMANCS-mediated augmentation of NK cell activity in splenic mononuclear cells was observed against RL21 leukemia. However, no consistent increases in NK cell-mediated cytotoxicity were detected against Meth A fibrosarcoma or L-5178Y lymphoma cells.

Characterization of NK Effector Cells. Cell types other than NK cells might have been responsible for the cellular cytotoxicity, because splenic mononuclear as well as peritoneal cells treated with a 0.5-mg/kg i.v. dose of NCS or a 20-mg/kg i.v. dose of SMA did not have detectable levels of NK cell activity against YAC-1 target cells (Fig. 1). Similarly, treatment with various doses of NCS (0.01–1 mg/kg) or SMA (2.7–25 mg/kg) also did not augment splenic or peritoneal NK cell cytotoxicities (data not shown). Injection of a 1.6-mg/kg i.v. dose of SMANCS into BALB/c mice augmented NK cell activity (approximately 5- to 6-fold) of both effector cells obtained from SMANCS-treated mice (Fig. 1), as compared with the control. Examination of the cytotoxic potential of splenic mononuclear cells from SMANCS-treated mice at various effector to target cell ratios (Fig. 2) revealed a slightly augmented NK cell activity at an effector to target cell ratio of 12.5:1, although the levels of cytotoxicity were negligible. Significant increases in NK cell activity of splenic mononuclear cells from SMANCS-treated mice were demonstrated at a 25:1 or 50:1 effector to target cell ratio (P < 0.001). The levels of NK cell cytotoxicity observed at different effector to target cell ratios were almost equal in splenic mononuclear cells from mice treated with NCS, SMA, or saline.

The susceptibility of various target cells to the cytotoxicity of SMANCS-augmented NK cells is shown in Table 1. In addition to YAC-1 lymphoma, SMANCS-mediated augmentation of NK cell activity in splenic mononuclear cells was observed against RL21 leukemia. However, no consistent increases in NK cell-mediated cytotoxicity were detected against Meth A fibrosarcoma or L-5178Y lymphoma cells.

RESULTS

Effect of SMANCS on NK Cell Cytotoxicity. In the first series of experiments, the effect of SMANCS, administered to normal BALB/c mice, on splenic or peritoneal NK cell cytotoxicity was investigated. Splenic mononuclear cells from BALB/c mice were also assayed in the same system as controls.
be eliminated by treatment of effector cells with anti-asialo-GM1 antiserum and complement, splenic effector cells were treated with the antiserum plus complement before being subjected to the NK cell assay. As shown in Table 3, treatment of splenic mononuclear cells from SMANCS-treated mice with anti-asialo-GM1 antiserum and complement completely abolished their killing activities, as measured against YAC-1 and RL61 target cells, while no decrease of NK cell activity was found following treatment with normal rabbit serum and complement. These results suggest that the effector cells mediating the SMANCS-inducing cellular cytotoxicity possess asialo-GM1 antigen on their surface, as do NK cells. Since activated T-cells like cytotoxic T-lymphocytes are susceptible to the treatment with anti-asialo-GM1 antiserum plus complement (42), there is a possibility that the SMANCS-induced cytotoxic effector cell is a T-cell. To preclude the possibility, splenic mononuclear cells from SMANCS-treated mice were also treated with monoclonal anti-Thy-1* antibody, followed by complement. As shown in Table 3, the monoclonal antibody treatment had no effect on the cytotoxic activity of the effector cells.

Effect of SMANCS on the Generation of A-MΦ in Mice. In the next experiment, the ability of plastic-adherent cells from mice treated with SMANCS, NCS, SMA, or saline to inhibit the growth of RL61 target cells in vitro was studied. The adherent cells, harvested from mice 4 days after treatment, were cocultured with RL61 target cells at an effector to target cell ratio of 10:1, and the growth of target cells was determined by the trypan blue dye exclusion test. As shown in Fig. 3, plastic-adherent cells from SMANCS-treated mice significantly inhibited the growth of RL61 target cells when they were cocultured in vitro. Compared with controls (RL61 cells cocultured with the adherent cells from mice treated with saline), however, the growth of tumor cells was not significantly inhibited when target cells were cocultured with adherent cells from mice treated with SMA or NCS. The cytostatic capacity of adherent cells from SMANCS-treated mice was also measured using [3H]thymidine-labeled target cells. As shown in Fig. 4, plastic-adherent cells from SMANCS-treated mice had a significant increase in cytostatic capacity (44 ± 7.5%) against RL61 target cells, when compared with that of adherent cells harvested from control mice (8 ± 4% cytostasis). No increase in the cytostatic activity was seen in the adherent cells from mice treated with SMA or NCS. Because the administration of SMANCS to mice generated tumor-cytostatic MΦ, kinetic studies of A-MΦ appearance in the peritoneal cavity of SMANCS-treated mice were performed. The cytostasis of plastic-adherent cells prepared from SMANCS-treated mice were used as effector cells in the preceding experiments. Therefore, nylon-woven nonadherent splenic mononuclear cells, which retain NK activity but have decreased numbers of B-cells and MΦ, were tested for their ability to mediate cellular toxicity. As shown in Table 2, nylon nonadherent splenic mononuclear cells had cytotoxic activities against both YAC-1 and RL61 target cells that were comparable to the activities of whole spleen cell or splenic mononuclear cell populations. The fact that these data suggest that the cellular cytotoxicity of splenic mononuclear cells prepared from SMANCS-treated mice was not mediated by B-cells or MΦ would appear consistent with the activity being NK-cell-mediated.

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Fig. 3. Growth inhibition of RL61 target cells by peritoneal effector cells from mice treated with SMANCS. The plastic-adherent peritoneal cells prepared from mice 4 days after treatment with SMANCS (1.6 mg/kg, i.v.) (O), NCS (0.5 mg/kg, i.v.) (△), SMA (20 mg/kg, i.v.) (•), or saline (0.2 ml/mouse, i.v.) (Θ) were co-cultured with RL61 target cells at an effector to target cell ratio of 10:1. Numbers of viable target cells were determined daily by trypan blue dye exclusion test. The mean scores obtained from triplicate cultures were plotted in the figure.

Fig. 4. Cytostasis of peritoneal effector cells from BALB/c mice treated with SMANCS. The plastic-adherent peritoneal cells from mice treated with saline (0.2 ml/mouse, i.v.), NCS (0.5 mg/kg, i.v.), SMA (20 mg/kg, i.v.), or SMANCS (1.6 mg/kg, i.v.) were examined for their cytostatic capacity against [JH]thymidine-labeled Kl 'I leukemia cells, at an effector to target cell ratio of 10:1. Incorporation of [3H]thymidine by tumor cells was calculated, as well as its inhibition in the presence of M0.

Fig. 5. Cytostatic capacity of plastic-adherent peritoneal effector cells from BALB/c mice various days after SMANCS treatment. The adherent cells were harvested from mice at various days after treatment with a 1.6-mg/kg i.v. dose of SMANCS and assayed for their cytostatic activity against RL61 target cells, at an effector to target cell ratio of 10:1.

Table 4 Some properties of peritoneal effector cells from mice treated with SMANCS

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<tr>
<th>Effector cells</th>
<th>Cytostasis (%)</th>
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<tr>
<td>PC</td>
<td>42.0 ± 6.6</td>
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<tr>
<td>PAC</td>
<td>41.3 ± 7.8</td>
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<tr>
<td>PAC treated with C</td>
<td>42.5 ± 8.1</td>
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<tr>
<td>PAC treated with anti-asialo-GMI antiserum + C*</td>
<td>40.9 ± 9.5</td>
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<tr>
<td>PAC treated with monoclonal anti-Thy-1.2 antibody + C*</td>
<td>46.8 ± 9.6</td>
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<tr>
<td>PC treated with carbonyl-iron*</td>
<td>4.1 ± 2.4</td>
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* Cytostatic capacity of effector cells was determined by the [3H]thymidine incorporation inhibition technique, at an effector to target cell ratio of 10:1. Procedures for antibody and carbonyl-iron treatments are described in the text.

The cytostatic activity gradually decreased until 9 days after SMANCS administration.

Characterization of MΦ Effector Cells. To further characterize this cellular activity, plastic-adherent cells were (a) treated with monoclonal anti-Thy-1.2 antibody plus complement to eliminate T-cells, (b) treated with anti-asialo-GM1 antisera plus complement to abolish NK cells, and (c) incubated with carbonyl-iron to eliminate phagocytic cells. The effector cells were then assayed for their cytostatic capacities against RL61 target cells, at an effector to target cell ratio of 10:1. Characterization of MΦ effector cells showed that the cytostatic activity of adherent cells remained in the adherent population and it was reduced by elimination of phagocytic cells (carbonyl-iron treatment). However, the activity of adherent cells was unchanged after depletion of asialo-GM1+-cells and Thy-1+ T-lymphocytes. These results indicate that peritoneal effector cells generated in mice by SMANCS stimulation had the characteristics of MΦ.

Role of NK Cells in the NSRT Developed in SMANCS-treated Mice. To determine the role of NK cells in the NSRT of mice stimulated with SMANCS, mice bearing solid tumors were depleted of these cells by treatment with anti-asialo-GM1 antisera in vivo. This anti-asialo-GM1 treatment provides one of the most popular methods available to remove NK cells from mice (36). However, in vivo treatment of mice with anti-asialo-
GM1 antiserum may cause the depletion of cytotoxic T-lymphocytes (42). Therefore, the NSRT of mice stimulated with SMANCS was also examined in congenital NK cell-deficient beige mice and their non-beige littermates, against NK cell-resistant EL-4 target cells (43). To demonstrate that both groups of mice were defective in NK function, the splenic NK cell activity in a 4-h cytotoxicity assay against 51Cr-labeled YAC-1 target cells was measured. Consistent with previous reports (37, 39), the in vivo treatment of mice with anti-asialo-GM1 antiserum almost completely abolished splenic NK activity, and the cytotoxicity of splenic NK cells prepared from beige mutant mice was <10% of that observed in normal litters (data not shown). Because NK cell activity was clearly suppressed in both groups of mice, the NSRT of these mice stimulated with SMANCS, against syngeneic solid tumors, was next evaluated.

As shown in Fig. 6, there was no significant difference in tumor growth after mice received SMANCS alone or SMANCS plus anti-asialo-GM1 antiserum. Following SMANCS stimulation, tumor growth was significantly decreased in mice treated with anti-asialo-GM1 antiserum, as compared with that of the controls. The data in Table 5 demonstrated that, when tumor-bearing beige mice and their non-beige littermates were treated with SMANCS, no significant difference in the NSRT of the two groups of mice treated with SMANCS was observed. The NSRT of mice stimulated with SMANCS was equal in NK cell-deficient mice (beige mice) and normal mice (non-beige littermates). These results suggest that the function of NK cells was not involved in the development of the NSRT of mice stimulated with SMANCS, although the activity of splenic and peritoneal NK cells could be augmented.

SMANCS-stimulated NSRT in Mice Depleted of Mφ. Because A-Mφ were observed to be generated in mice following

![Fig. 6. Effect of anti-asialo-GM1 antiserum treatment on the NSRT of mice stimulated with SMANCS. A group of 10 mice bearing 1 × 10⁶ RL1 solid tumor cells were treated with 0.5 ml of saline (i.p.) (A), 5 mg/kg anti-asialo-GM1 antiserum (i.v.) (B), 1.6 mg/kg SMANCS (i.v.) (C), or the same amount of SMANCS and the antiserum together (D). Antiserum treatment began 1 day before tumor inoculation and was repeated 4 times at 5-day intervals. SMANCS and saline were administered once 24 h before tumor inoculation.](#)

![Fig. 7. Effect of carrageenan treatment on the NSRT of mice stimulated with SMANCS. Groups of 10 mice bearing 1 × 10⁶ RL1 solid tumor cells were treated with saline (i.v., 0.2 ml/mouse) (A), 50 mg/kg carrageenan (i.p.) (B), 1.6 mg/kg SMANCS (i.v.) (C), or the same amount of SMANCS and carrageenan (D). Carrageenan was administered to mice 2 days before and 1 day after tumor inoculation and once weekly for 2 weeks. SMANCS and saline were administered to mice 24 h before tumor inoculation.](#)

<table>
<thead>
<tr>
<th>Table 5 Suppression of tumor growth in beige mice and their non-beige littermates treated with SMANCS</th>
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<tr>
<td><strong>Mice</strong></td>
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<td>Beige</td>
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<tr>
<td>Non-beige littermate</td>
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<td>Non-beige littermate</td>
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*6, 10, and 14 days after implantation, tumor size was measured by a microcaliper. Tumor sizes were expressed as means of squares that were determined from the largest diameter (a) and the smallest diameter (b) of solid tumors, i.e., ab mm².

SMANCS administration, the role of these peritoneal effector cells in the development of the NSRT of mice bearing syngeneic solid tumors was examined next. To evaluate the role of A-Mφ in the expression of the NSRT, Mφ were depleted from mice bearing RL1 solid tumors by treatment with the Mφ blockers trypsin blue and carrageenan (37, 40). Fig. 7 shows that, when tumor-bearing mice were treated with SMANCS and carrageenan, no NSRT resulted and the level of tumor growth in Mφ-depleted mice treated with SMANCS did not differ significantly from that of controls. Treatment of tumor-bearing mice with trypsin blue produced similar results (Fig. 8). Thus, the elimination by carrageenan or trypsin blue of functioning Mφ from mice bearing RL1 solid tumors resulted in suppression of the NSRT of mice treated with SMANCS. These results suggest that Mφ which were stimulated by SMANCS were
NONSPECIFIC RESISTANCE TO TUMORS INDUCED BY SMANCS

DISCUSSION

In addition to its direct antitumor effect against tumor cells through DNA strand scission (1, 9-11), SMANCS induced a NSRT in mice bearing syngeneic solid tumors (24). The IFN-inducing capability of the agent has also been demonstrated in mice (23). Since the SMANCS-induced IFN response can be abrogated in mice treated with MΦ blockers or monoclonal anti-Thy-1.2 and Lyt-2.2 antibodies (23), this suggests that MΦ and/or T-cells with a Lyt-2+ phenotype participated in the production of the IFN. Further, SMANCS was not able to stimulate the expression of NSRT in mice treated with multiple administrations of anti-IFN-γ antiserum (24). These results suggest that the induction of IFN was required for the development of NSRT in mice treated with SMANCS. Many reports (for review, see Ref. 30) have suggested that IFNs have a major role in host defenses, including the development of the NSRT in tumor-bearing hosts. The well known activities of IFN that might be important in development of NSRT of mice include (a) increased NK cell activity, which could be one of the first barriers of defense; (b) activation of MΦ against tumor cells; (c) increased expression of major histocompatibility complex class II antigens, thereby increasing antigen presentation capabilities; and (d) increased expression of major histocompatibility complex class I antigens, which might make target cells more susceptible to lysis by cytotoxic T-cells (30, 45).

In the present study, the activity of NK cells in splenic mononuclear cells and peritoneal cells prepared from SMANCS-treated mice was significantly augmented compared to that of saline-treated mice. However, augmentation of NK cell activity was not demonstrated in splenic and peritoneal effector cells prepared from NCS- or SMA-treated mice. The NK cell activity reached a peak in spleens of mice 3 days after SMANCS administration. The YAC-1 lymphoma and RLC1 leukemia cells, but not Meth A fibrosarcoma and L-5178Y lymphoma cells, were killed by NK cells prepared from SMANCS-treated mice. The SMANCS-augmented splenic NK cell activity was eliminated in vitro by treatment with anti-asialo-GM1 antiserum followed by complement, but not by monoclonal anti-Thy-1.2 antibody plus complement, and remained in nylon wool-nonadherent cell populations. These results indicated that the effector cells demonstrated in spleens and peritoneal cavities of SMANCS-treated mice were NK cells. A-MΦ were also detected in the peritoneal cavities of SMANCS-treated mice. Plastic-adherent cells prepared from mice 4 days after SMANCS administration clearly inhibited growth of RLC1 target cells in vitro. The cytostatic activity found in the peritoneal cavity of SMANCS-treated mice was abrogated in vitro by treatment of cells with carbonyl-iron, but not with anti-asialo-GM1 antiserum or monoclonal anti-Thy-1.2 antibody followed by complement. These findings indicated that A-MΦ were generated from resting MΦ by the stimulation with SMANCS.

In the next step, solid tumor-bearing mice were treated with both SMANCS and blockers of NK cells (anti-asialo-GM1 antiserum) or MΦ (trypan blue, carrageenan), and tumor growth was measured by microcaliper. It has been reported that i.v. injections of anti-asialo-GM1 antiserum to mice abolished the NK cell activity in spleens of athymic nude mice and enhanced the growth of transplanted solid tumors (36). Activated T-cells and a portion of MΦ may be also inactivated by in vivo treatment of mice with the antiserum (41, 42). Treatment of mice with trypan blue and carrageenan has been shown to inactivate the activity of peritoneal cells and spleens within a half day after injection (37, 40). Carrageenan has also been shown to block NK cell activity in vivo (44). When tumor-bearing mice were depleted of NK cell activity by treatment with anti-asialo-GM1 antiserum, the antitumor activity of SMANCS was unchanged, suggesting that NK cells did not play a significant role in the development of the NSRT of SMANCS-stimulated mice. In addition, the NSRT of mice stimulated with SMANCS was examined using the genetically NK cell-deficient beige mice and their non-beige littermates that were inoculated with NK cell-resistant EL-4 solid tumors. Thus, like anti-asialo-GM1 antiserum-treated mice, beige mice and their non-beige littermates bearing EL-4 thymoma were treated with SMANCS, at a dose of 1.6 mg/kg, 1 day before tumor inoculation. As a result of this experiment, an equal decrease in tumor growth in both groups treated with SMANCS was demonstrated. However, the depletion of MΦ from tumor-bearing mice by the administration of blockers resulted in complete abrogation of the NSRT of mice stimulated with SMANCS. These results would suggest that functioning MΦ, but not NK cells, appeared to be involved in the developed NSRT of SMANCS-stimulated mice. The mechanism by which SMANCS activated MΦ, as well as how these MΦ express their antitumor activity, remains unknown. However, it has been previously reported that the induction of IFN-γ was required for the development of the NSRT in mice following SMANCS administration (24). It is also known that IFN-γ is an important lymphokine which activates MΦ to express cytotoxic properties in vitro and in vivo (46). Therefore, the A-MΦ generated from resting MΦ by SMANCS-induced IFN-γ might play an important role in the expression of NSRT in mice bearing syngeneic solid tumors. Further investigations
will be required to determine the role of cytotoxic T-lymphocytes in the development of the NSRT of mice stimulated with this agent.

REFERENCES

Role of Natural Killer Cells and Macrophages in the Nonspecific Resistance to Tumors in Mice Stimulated with SMANCS, a Polymer-conjugated Derivative of Neocarzinostatin


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