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## Induction of Suppressor T Cells by Neuraminic Acid Derivatives

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We investigated the effects of  $\alpha$ - and  $\beta$ -methylglycosides of *N*-acetylneuraminic acid and its disaccharide derivatives on the proliferation and immunological functions of murine lymphocytes. The Con A-induced increase of deoxyribonucleic acid (DNA) synthesis was enhanced by some of these neuraminic acid derivatives when they were added to a culture of murine spleen lymphocytes, and the disaccharide nucleosides, 5-fluoro-2',3'-isopropylidene-5'-*O*-(4-*N*-acetyl-2,4-dideoxy-3,6,7,8-tetra-*O*-acetyl-1-methoxycarbonyl- $\beta$ -glycero- $\alpha$ - $\beta$ -galactooctapyranosyl)uridine (compound 9) and 2',3'-di-*O*-acetyl-4-*N*-acetyl-2,4-dideoxy-3,6,7,8-tetra-*O*-acetyl-1-methoxycarbonyl- $\beta$ -glycero- $\alpha$ - $\beta$ -galactooctapyranosyl)inosine (compound 10), were especially effective.

The above disaccharide nucleosides and their starting materials, 5-fluoro-2',3'-isopropylidene uridine (FIU) and 2',3'-di-*O*-acetylinoine (DAI), suppressed *in vitro* primary antibody response toward sheep red blood cells (SRBC). *In vivo* antibody response toward SRBC was also suppressed when compound 9 or 10 was injected intraperitoneally into mice with SRBC. Moreover, lymphocytes incubated with compound 9 or 10 showed suppressor activity on primary anti-SRBC antibody response *in vitro*. On the other hand, FIU and DAI did not induce the suppressor activity of murine lymphocytes *in vivo* or *in vitro*. The induction of suppressor cells by compounds 9 and 10 was abolished by pretreatment of the lymphocytes with anti Thy-1 antiserum plus complement. These results suggest that these disaccharide nucleosides can induce suppressor T cells.

**Keywords**—neuraminic acid; derivatives of neuraminic acid; disaccharide nucleoside; lymphocyte activation; antibody production; suppressor T cell

## Introduction

Neuraminic acid generally occupies the terminal positions of sugar chains of glycoproteins and glycolipids on the cell surface. Although the role of terminal sugars on the cell surface of mammalian cells has not been fully elucidated, interest in neuraminic acid and neuraminyltransferase has been increased by the finding that neoplastic transformation and differentiation of cells are accompanied by alterations in the composition and metabolism of cellular sialoglycoconjugates.<sup>1,2)</sup> In addition, recent studies have suggested that there is a close relationship between neuraminic acid or neuraminyltransferase, and the properties of immune competent cells. For example, the homing phenomenon of lymphocytes to lymphoid organs seems to depend on the presence or absence of neuraminic acid residues on the cell surface.<sup>3)</sup> The expression of neuraminyltransferase activity and the addition of neuraminic acid residues to cell membrane oligosaccharides may play an important role in the differentiation of T cells,<sup>4)</sup> and neuraminyltransferase can be utilized as a marker enzyme for T cell differentiation.<sup>5)</sup> Furthermore, activation of lymphocytes with mitogens is accompanied by an increase of cell-surface-associated neuraminyltransferase activity.<sup>6)</sup>

The aim of this communication is to present data on the effect of certain neuraminic acid derivatives on the immunological functions of murine lymphocytes.

## Materials and Methods

**Reagents**—*N*-Acetyl-D-neuraminic acid was obtained from edible birds-nest substance by the method of Czarniecki and Thornton.<sup>7)</sup>  $\alpha$ - and  $\beta$ -Methylglycosides of *N*-acetylneuraminic acid, its disaccharide derivatives, 5-fluoro-2',3'-isopropylidene uridine (FIU) and 2',3'-di-*O*-acetylinosine (DAI) were synthesized according to the methods described previously.<sup>8,9)</sup> The structures of *N*-acetylneuraminic acid and its derivatives are shown in Chart 1 (compounds 1–6) and the synthetic pathways to disaccharide nucleosides (compounds 9 and 10) in Chart 2. Concanavalin A (Con A) was purified according to the method of Agrawal and Goldstein.<sup>10)</sup> Anti Thy-1,2 antiserum and guinea pig complement were from Miles (Elkhart, Ind., U.S.A.) and Denka Seiken Co., Ltd. (Tokyo, Japan), respectively. [6-<sup>3</sup>H]Thymidine was purchased from New England Nuclear (Boston, Mass., U.S.A.), RPMI 1640 medium from Grand Island Biological Co. (Grand Island, NY., U.S.A.) and fetal calf serum (FCS) from Granite Diagnostics, Inc. (Burlington, NC., U.S.A.).

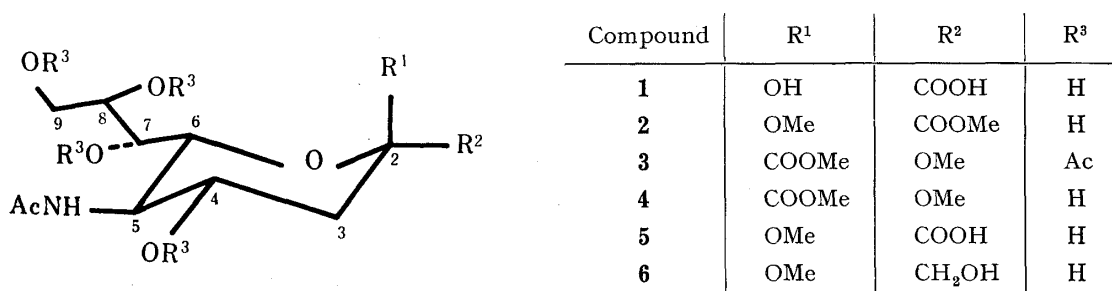


Chart 1

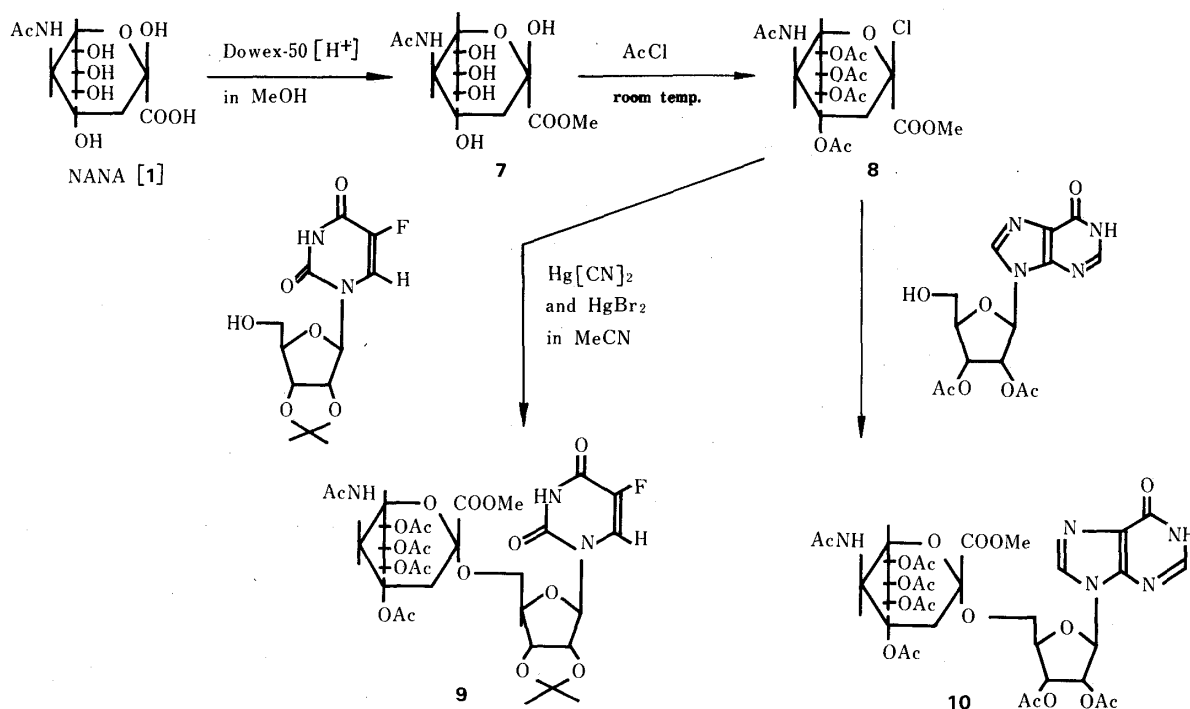


Chart 2

**Cells**—Splenic lymphocytes (SPC) were obtained from specific pathogen-free BALB/c strain female mice (7 to 8 weeks of age, Charles River Japan, Kanagawa, Japan) by the Ficoll-Urografin technique.<sup>11)</sup>

**Cell Cultures for Assay of Deoxyribonucleic Acid (DNA) Synthesis and for Suppressor Cell Induction**—SPC were suspended in RPMI 1640 tissue culture medium supplemented with 5% FCS at the concentration of  $2 \times 10^6$  cells/ml, and cultured in a microtiter plate with neuraminic acid derivatives ( $10^{-6}$ – $10^{-4}$  M) in the presence or absence of Con A (2  $\mu$ g/ml). Cultures were maintained in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C for 2 d.

**Cell Cultures for *in Vitro* Assay of Primary Anti-SRBC Plaque Forming Cells (PFC)**—Two  $\times 10^6$  SPC and  $10^6$  sheep red blood cells (SRBC, Funabashi Farm Co., Chiba, Japan) were suspended in 0.4 ml of RPMI 1640 medium supplemented with 10% FCS, 4 mM L-glutamine, 1 mM pyruvic acid,  $5 \times 10^{-5}$  M 2-mercapto-

ethanol and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.2), and were cultured with neuraminic acid derivatives ( $10^{-6}$ – $10^{-4}$  M) or SPC preculture with neuraminic acid derivatives in the presence or absence of Con A in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C for 5 d. At the termination of cultures, cells were washed with RPMI 1640 medium, and then the number of PFC was determined according to the methods of Cunningham and Szenberg.<sup>12)</sup>

**Assay of DNA Synthesis**—[6-<sup>3</sup>H]Thymidine (1 μCi) was added to each culture ( $4 \times 10^5$  cells/well) 18 h before the termination of culture. After culture for 2 d, cells were harvested on a glass fiber filter with a multiple cell harvester (Labo Science Co., Ltd., Tokyo, Japan), and total [6-<sup>3</sup>H]thymidine incorporated into the cells was determined by the method of Toyoshima *et al.*<sup>13)</sup> using a liquid scintillation spectrometer (Aloka Co., Ltd., Tokyo, Japan).

**Depletion of T Cells from SPC**—SPC were treated with anti Thy-1,2 antiserum (final dilution, 1:5) at 4°C for 45 min and then with guinea pig complement at 37°C for 45 min. These antiserum-treated cells contained less than 5% Thy-1, 2 positive T cells as determined by direct immunofluorescence using fluorescein-conjugated anti Thy-1, 2 antiserum (Miles).

**Immunization of Mice with SRBC**—Mice were immunized with an intraperitoneal injection of  $4.5 \times 10^8$  SRBC. Five hundred μg of compound 9 or 10 was simultaneously injected with SRBC to investigate the *in vivo* effect of these compounds on anti-SRBC PFC response.

## Results

### Effect on DNA Synthesis

As shown in Table I, the Con A-induced increase of DNA synthesis of lymphocytes was significantly enhanced by compound 9 ( $10^{-5}$  M) and compound 10 ( $10^{-4}$  M). FIU, a component of compound 9, and DAI, a component of compound 10, did not induce any increase of DNA synthesis in Con A-stimulated lymphocytes. Almost complete inhibition of [6-<sup>3</sup>H]thymidine incorporation was observed in FIU ( $10^{-5}$  M)-treated culture. Compound 8, a common com-

TABLE I. Effect of Neuraminic Acid Derivatives on the DNA Synthesis of Lymphocytes cultured with Con A or without Con A

Derivatives	Concentration (M)	Stimulation index of [6- <sup>3</sup> H] TdR incorporation <sup>a)</sup>	
		With Con A	Without Con A
None		1.00	1.00
1	$10^{-4}$	1.08	1.01
2	$10^{-6}$	1.39	1.29
	$10^{-4}$	1.03	1.10
3	$10^{-4}$	0.94	0.78
4	$10^{-4}$	1.04	0.69
5	$10^{-4}$	0.89	0.86
6	$10^{-4}$	0.94	0.68
7	$10^{-4}$	1.21	0.99
8	$10^{-5}$	1.03	1.23
	$10^{-4}$	1.10	0.93
9	$10^{-5}$	1.57	1.26
	$10^{-4}$	0.01	0.43
10	$10^{-5}$	1.15	2.21
	$10^{-4}$	1.81	1.24
FIU	$10^{-5}$	0.02	0.47
	$10^{-4}$	0.01	0.27
DAI	$10^{-5}$	0.87	1.22
	$10^{-4}$	1.07	1.24
8+FIU	$10^{-5}$	0.02	0.30
	$10^{-4}$	0.01	0.27
8+DAI	$10^{-5}$	0.92	1.17
	$10^{-4}$	1.02	1.33

a) These are the results of a typical experiment.

ponent of compounds **9** and **10**, seemed to induce some increase of DNA synthesis, but the increase was not statistically significant. Furthermore, a mixture of compound **8** ( $10^{-5}$  M) and FIU ( $10^{-5}$  M), or a mixture of compound **8** ( $10^{-4}$  M) and DAI ( $10^{-4}$  M) exerted the same effect as FIU or DAI alone on the DNA synthesis of Con A-stimulated lymphocytes. Compounds **2**, **9** and **10** enhanced the DNA synthesis of lymphocytes even in cultures without Con A (Table I).

#### Effect on *in Vitro* Primary PFC Response to SRBC

When disaccharide nucleosides (compounds **9** and **10**) and their starting materials were simultaneously added to the primary culture with SRBC, they (except **8**) induced a decrease of PFC response to SRBC (Table II). Since cell viability as checked by the trypan blue exclusion test was not affected by these compounds, it seemed that the suppression of PFC response was not due to their toxicity to lymphocytes.

TABLE II. Effect of Disaccharide Nucleosides and Their Starting Materials on the Primary PFC Response to SRBC *in Vitro*

Treatment	PFC/ $2 \times 10^6$ cultured lymphocytes <sup>a)</sup>
None (control)	$57 \pm 4$
FIU ( $10^{-5}$ M)	$11 \pm 3$
DAI ( $10^{-4}$ M)	$28 \pm 6$
<b>8</b> ( $10^{-4}$ M)	$43 \pm 11$
<b>9</b> ( $10^{-5}$ M)	$16 \pm 2$
<b>10</b> ( $10^{-4}$ M)	$25 \pm 17$

a) Mean  $\pm$  S.E. of triplicate cultures.

#### Effect on Suppressor Activity of Con A-Stimulated and Non-Stimulated Lymphocytes

As described previously,<sup>14)</sup> Con A-stimulated lymphocytes showed suppressor activity on the primary PFC response to SRBC. This induction of suppressor cells by Con A was significantly enhanced by the presence of compound **9** ( $10^{-5}$  M) or compound **10** ( $10^{-4}$  M) during the incubation with Con A. Furthermore, SPC precultured with compound **9** or **10** in the absence Con A also exhibited suppressor activity. On the other hand, FIU, DAI and compound **8** did not induce suppressor activity of the cells (Table III).

TABLE III. Effect of the Treatment of Con A-stimulated and Non-stimulated Cells with Disaccharide Nucleosides on the Suppressor Activity of the Cells

Treatment <sup>a)</sup>	PFC/ $2 \times 10^6$ cultured lymphocytes <sup>b)</sup>		
	Exp. 1	Exp. 2	Exp. 3
None (control)	$103 \pm 17$	$111 \pm 4$	$130 \pm 16$
FIU ( $10^{-5}$ M)	—	—	$108 \pm 10$
DAI ( $10^{-4}$ M)	—	—	$108 \pm 8$
<b>8</b> ( $10^{-4}$ M)	—	—	$145 \pm 24$
<b>9</b> ( $10^{-5}$ M)	$83 \pm 16$	$54 \pm 2$	$72 \pm 5$
<b>10</b> ( $10^{-4}$ M)	$67 \pm 9$	$34 \pm 7$	$89 \pm 17$
Con A ( $2 \mu\text{g/ml}$ )	$68 \pm 3$	$65 \pm 8$	—
<b>9</b> ( $10^{-5}$ M) + Con A	$51 \pm 6$	$39 \pm 13$	—
<b>10</b> ( $10^{-4}$ M) + Con A	$29 \pm 9$	$27 \pm 8$	—

a)  $2 \times 10^5$  lymphocytes were incubated with disaccharide nucleosides and their starting materials in the presence or absence of Con A at  $37^\circ\text{C}$  for 45 h, washed with RPMI 1640 medium and then co-cultured with normal fresh spleen cells ( $2 \times 10^6$  cells) plus SRBC ( $1 \times 10^6$  cells).

b) Mean  $\pm$  S.E. of triplicate cultures.

### Effect of T Cell Depletion on Disaccharide Nucleoside-induced Suppressor Activity

Disaccharide nucleosides (compounds **9** and **10**) did not induce suppressor cells after treatment of SPC with anti Thy-1,2 antiserum plus complement (Table IV). This result suggests that the disaccharide nucleosides induce suppressor T cells.

TABLE IV. Effect of the Treatment of Murine Lymphocytes with anti Thy-1 Serum Plus Complement on the Suppressor Activity of the Cells

Treatment <sup>a)</sup>	PFC/ $2 \times 10^6$ cultured lymphocytes <sup>b)</sup>	
	Exp. 1	Exp. 2
None (control)	153 $\pm$ 18	56 $\pm$ 12
FIU ( $10^{-5}$ M)	122 $\pm$ 11	—
DAI ( $10^{-4}$ M)	134 $\pm$ 18	—
<b>8</b> ( $10^{-4}$ M)	148 $\pm$ 11	44 $\pm$ 6
<b>9</b> ( $10^{-5}$ M)	136 $\pm$ 22	54 $\pm$ 14
<b>10</b> ( $10^{-4}$ M)	130 $\pm$ 11	40 $\pm$ 1

a) Lymphocytes treated with anti Thy-1 serum plus complement were incubated in the same manner as in Table III.

b) Mean  $\pm$  S.E. of triplicate cultures.

### Effect on *in Vivo* Primary PFC Response to SRBC

When 500  $\mu$ g/head of compound **9** or **10** was intraperitoneally injected into mice with SRBC, *in vivo* primary antibody response was also suppressed (Table V).

TABLE V. Effect of Disaccharide Nucleosides on the Primary PFC response to SRBC *in Vivo*<sup>a)</sup>

Treatment	PFC/ $2 \times 10^6$ lymphocytes <sup>b)</sup>	
	Exp. 1	Exp. 2
Saline (control)	574 $\pm$ 49	616 $\pm$ 188
<b>9</b> (0.5 mg/mouse)	368 $\pm$ 41	387 $\pm$ 70
<b>10</b> (0.5 mg/mouse)	358 $\pm$ 44	255 $\pm$ 56

a) Splenic lymphocytes were obtained from mice 5 d after the intraperitoneal injection of compound **9** or **10** with SRBC.

b) Each group in Exp. 1 and 2 consisted of 5 mice. Each value in this table is the mean  $\pm$  S.E. of triplicate experiments.

### Discussion

The disaccharide nucleosides, compounds **9** and **10**, enhanced the DNA synthesis of Con A-stimulated lymphocytes (Table I). This enhancing activity was not found with compound **8**, DAI or their mixture. However, FIU ( $10^{-5}$  M) strongly inhibited DNA synthesis. Thus, the suppressive effect of FIU on Con A response of lymphocytes was diminished by the introduction of a neuraminic acid residue into FIU and the resulting compound **9** actually enhanced the response.

Furthermore, compounds **9** and **10** suppressed *in vitro* primary antibody response to SRBC, and enhanced the suppressor cell activity of Con A-stimulated lymphocytes (Tables II and III). Lymphocytes precultured with the disaccharide nucleosides in the absence of Con A also exhibited suppressive activity against the primary antibody response to SRBC. After treatment of the lymphocytes with anti Thy-1,2 antiserum and complement, these disaccharide nucleosides did not induce suppressive activity of the cells (Table IV). These results suggest that compounds **9** and **10** can induce suppressor T cell activity toward the primary antibody

response. On the other hand, FIU and DAI suppressed primary antibody response but could not induce suppressor cells. The mechanism of suppression observed in FIU and DAI, therefore, seemed to be different from that of compounds **9** and **10**. Since *in vivo* primary antibody response was also suppressed by the administration of these disaccharide nucleosides (Table V), they may be useful as therapeutic agents for immune diseases caused by defect in suppressor T cells.

Recent studies have shown that the expression of neuraminyltransferase activity and the addition of neuraminic acids to cell membrane oligosaccharides may be an important event in the differentiation of T cells,<sup>4)</sup> and Con A-induced suppressor T cells are selectively labeled with peanut agglutinin, which binds to neuraminic acid-free  $\beta$ -galactosyl residues.<sup>15,16)</sup> Furthermore, some nucleotides and nucleotide sugars have been found to inhibit neuraminyltransferase activity on cell membranes.<sup>17,18)</sup> Although the mechanism of induction of suppressor T cells by the disaccharide nucleosides remains obscure, it may be worthwhile to investigate the effect of the disaccharide nucleosides on neuraminyltransferases of lymphocytes.

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