ANTITUMOR EFFECT OF POLYSACCHARIDE GRIFOLAN NMF-5N ON SYNGENEIC TUMOR IN MICE

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Antitumor activity of grifolan NMF-5N, a β -1,3-glucan obtained from mycelia of *Grifola frondosa*, was examined. Grifolan NMF-5N showed antitumor activities in allogeneic and syngeneic murine tumor systems. In the allogeneic tumor system, a potent antitumor activity over 95% was observed against the solid form of sarcoma 180 when grifolan NMF-5N was injected intraperitoneally (i.p.) at 25–200 μ g/mouse daily for 10 successive days. In the syngeneic tumor systems, significant antitumor activities were observed against Meth A fibrosarcoma and MM 46 carcinoma by injection at 100 μ g/mouse daily for 5 successive days, especially i.p. injection at day 7–11, when the tumor cells were inoculated subcutaneously (s.c.) on day 0. Moreover, when grifolan NMF-5N was injected i.p. every other week, significant antitumor activity was also observed. In addition, a single treatment with grifolan NMF-5N at 500 μ g/mouse showed antitumor activities. Grifolan NMF-5N exhibited antitumor activities against these two syngeneic tumors by intravenous (i.v.) injection. However, a marked inhibitory activity was observed by intratumorous (i.t.) injection against Meth A fibrosarcoma but not against MM46 carcinoma. These results suggest that antitumor activities of grifolan NMF-5N in murine syngeneic tumor systems depend on not only dosage but also injection routes and timing.

Keywords— *Grifola frondosa*; grifolan; 6-branched β -glucan; antitumor activity; Meth A fibrosarcoma

INTRODUCTION

Grifola frondosa is an edible mushroom which is obtainable by cultivation. Recently, we reported that 1) a polysaccharide fraction (GF-1) extracted from the fruit bodies of *G. frondosa* possesses antitumor activity in an allogeneic tumor system,¹⁾ 2) GF-1 can modulate the murine immunological responses,²⁾ 3) hot water, cold alkali and hot alkali extracts, which were obtained from the fruit bodies of *G. frondosa*, also exhibited marked antitumor activities.³⁾ The main structure for the antitumor activity of these fractions is a 6-branched β -1,3-glucan. The detailed structure and antitumor activities against allogeneic tumor are described elsewhere.⁴⁾ On the other hand, the matted mycelia of the fruit body were discarded away as useless materials of cultured *G. frondosa*. We found that a polysaccharide which possesses antitumor activity exists in the cold alkali extract of the matted mycelia. An antitumor 6-branched- β -1,3-glucan was purified from the extract and was designated as grifolan NMF-5N.⁵⁾ It is suggested that some physicochemical properties of

grifolan NMF-5N are different from other known antitumor β -1,3-glucans.⁶⁾

In this paper, an antitumor activity of grifolan NMF-5N against allogeneic and syngeneic tumors will be reported.

MATERIALS AND METHODS

Mice — Male 6–8 weeks old ICR, BALB/c, C3H/HeN mice obtained from Shizuoka Laboratory Animal Center, Hamamatu, were used.

Tumors — Sarcoma 180 cells were maintained serially in ascites form by weekly passage in ICR mice. Similarly, Meth A fibrosarcoma and MM 46 carcinoma were maintained in BALB/c and C3H/He mice, respectively.

Isolation of Polysaccharide Fraction — The polysaccharide fraction was prepared from matted mycelia of *G. frondosa* as described previously.⁵⁾ Briefly, the procedure was: the mycelia were washed with water, lyophilized and then, pulverized to a powder. The powder was extracted with water using an autoclave (1 kg/cm², 121 °C) for 1 h. The extraction was repeated 7 times.

The residue of the water extraction was then extracted by stirring with 10% NaOH containing 5% urea for 24 h at 4 °C. The extract was neutralized with acetic acid and dialyzed extensively against tap and distilled water. The precipitates produced during dialysis were discarded. The supernatant fluid was concentrated to a small volume and the polysaccharide fraction was precipitated by adding 4 volumes of ethanol. The polysaccharide fraction was designated as NMF-5. The extraction was repeated 3 times.

Purification of Grifolan NMF-5N — NMF-5N was purified in accordance with the previous description.⁵⁾ NMF-5 dissolved in water was applied to a DEAE-Sephadex A-25 (HCO₃⁻) column. The column was washed with water and the fractions collected were dialysed. The dialysed fraction was digested with α -amylase in 50 mM Tris-HCl buffer pH 6.9. After 24 h, the reaction mixture was dialysed against water. The dialysed fraction was applied to a SP-Sephadex C-25 (H⁺) column and the column was washed with water. The resulting solution, NMF-5N, was precipitated with 1 volume of ethanol.

Evaluation of Antitumor Activity — Viable tumor cells in 0.05 ml of saline were inoculated s.c. into the right groin of mice (day 0). Test samples were injected several times from 1 d after the tumor inoculation. The perpendicular diameters were measured (mm) with slide calipers every 7 days and tumor size was calculated

as tumor area (mm²). Five weeks after tumor inoculation, the mice were sacrificed. The inhibition ratio was calculated as follows;

$$\left(1 - \frac{\text{average tumor weight of the treated group}}{\text{average tumor weight of the control group}}\right) \times 100\%$$

The significance (*p* value) of the difference between the control and treated groups was evaluated according to Student's *t* test. Complete regression indicates the ratio of the number of mice showing complete regression to the number of mice tested.

RESULTS

Effect of Grifolan NMF-5N against Allogeneic Tumors

To examine the effective dose of grifolan NMF-5N against the solid form of sarcoma 180, ICR mice were administered i.p. with various doses of grifolan NMF-5N daily for 10 successive days from the day following tumor inoculation. High effectiveness, over 95% of inhibition ratio, was shown at 25–200 μ g (Table I). The administration of 50 μ g particularly was most effective resulting in 98% inhibition ratio and 59% complete regression.

The kinetics of these tumor growths are shown in Fig. 1. The suppression of the tumor growths was observed clearly at 3 weeks after

TABLE I. *Antitumor Effect of Grifolan NMF-5N against Sarcoma 180*^{a)}

Sample ^{b)}	Dose $\times$ 10 (μ g/mouse)	Tumor weight (g, mean $\pm$ S.D.) ^{c)}	Inhibition ratio (%) ^{d)}	C.R. ^{e)}	<i>p</i> ^{f)}
Grifolan NMF-5N	12.5	0.62 $\pm$ 0.70	86	4/9	< 0.001
	25	0.10 $\pm$ 0.10	98	2/10	< 0.001
	50	0.09 $\pm$ 0.14	98	5/10	< 0.001
	75	0.09 $\pm$ 0.13	98	4/9	< 0.001
	100	0.18 $\pm$ 0.24	96	3/9	< 0.001
	125	0.07 $\pm$ 0.06	99	2/9	< 0.001
	200	0.20 $\pm$ 0.13	96	0/6	< 0.01
	300	0.73 $\pm$ 1.34	84	1/7	< 0.01
Nil	(Saline)	4.54 $\pm$ 2.74	—	0/11	—

^{a)} Sarcoma 180 (5×10^6) cells were inoculated subcutaneously (s.c.) on day 0. ^{b)} Grifolan NMF-5N was administered as saline solution by intraperitoneal (i.p.) injection daily for 10 successive days (day 1–10). ^{c)} Arithmetic mean weight of tumor $\pm$ standard deviation. ^{d)} Inhibition ratio was determined at 35th day after tumor inoculation. ^{e)} Complete regression = number of mice with complete regression/number of treated mice. ^{f)} The significance was evaluated according to Student's *t*-test.

FIG. 1. *Effect of Grifolan NMF-5N on Growth of Sarcoma 180*

ICR mice were administered i.p. with various doses of grifolan NMF-5N daily for 10 successive days after tumor inoculation (day 0).

○, 12.5 µg; △, 50 µg; □, 100 µg; ▲, 300 µg; ●, nil (saline).

tumor inoculation.

Effect of Grifolan NMF-5N against Syngeneic Tumors

To examine the effect of grifolan NMF-5N on the growth of syngeneic tumors, Meth A fibrosarcoma and MM 46 carcinoma were ino-

culated to BALB/c and C3H/He mice, respectively on day 0. Grifolan NMF-5N was administered by i.p. injection daily for 5 successive days (day 1–5). As shown in Table II, significant antitumor activities were shown against Meth A fibrosarcoma with 100 and 200 µg of grifolan NMF-5N. In the case of MM 46 carcinoma, 100 µg of grifolan NMF-5N significantly inhibited this tumor (Table III).

Effect of Various Administration Schedules of Grifolan NMF-5N against Meth A Fibrosarcoma and MM 46 Carcinoma

To examine the relation between the timing of the administration of grifolan NMF-5N and antitumor activity against Meth A fibrosarcoma and MM 46 carcinoma, the following experiments were performed: 1) One hundred micrograms of grifolan NMF-5N were administered i.p. daily for 5 successive days on days 1–5, 7–11, and 14–18 (experiment 1) and 2) 500 µg of grifolan NMF-5N were administered i.p. on days 1, 7, or 14 after tumor inoculation on day 0 (experiment 2). As shown in Table IV, the most effective timing against Meth A fibrosarcoma was administration on days 7–11 in experiment 1, and on day 7 in experiment 2. The manner of the tumor growth in the experiment 1 is shown in Fig. 2. Significant suppression was shown by injections of grifolan NMF-5N on days 7–11 and 14–18. The most suppressive effect against MM 46 carcinoma was observed at days 7–11 involving one treatment per day (100 µg) for 5 d and at day 14 involving one treatment (500 µg) (Table V). When grifolan NMF-5N was injected every other week, signifi-

TABLE II. *Antitumor Effect of Grifolan NMF-5N against Meth A Fibrosarcoma^{a)}*

Sample ^{b)}	Dose × 5 (µg/mouse)	No. of mice	Tumor weight (g, mean ± S.D. ^{c)}	Inhibition ratio (%) ^{d)}	<i>p</i> ^{e)}
Grifolan NMF-5N	25	8	1.17 ± 0.79	6	n.s. ^{f)}
	50	9	0.81 ± 0.71	35	n.s.
	100	10	0.52 ± 0.61	58	< 0.02
	200	8	0.54 ± 0.29	57	< 0.02
	400	9	0.79 ± 0.60	37	n.s.
Nil	(Saline)	12	1.25 ± 0.68	—	—

^{a)} Meth A fibrosarcoma (5×10^5) cells were inoculated s.c. on day 0. ^{b)} Grifolan NMF-5N was administered as saline solution by i.p. injection daily for 5 successive days (day 1–5). ^{c)} Arithmetic mean weight of tumor ± standard deviation. ^{d)} Inhibition ratio was determined at 35th day after tumor inoculation. ^{e)} The significance was evaluated according to Student's *t*-test. ^{f)} n.s. = not significant.

TABLE III. Antitumor Effect of Grifolan NMF-5N against MM46 Carcinoma^{a)}

Sample ^{b)}	Dose $\times$ 5 (μ g/mouse)	No. of mice	Tumor weight (g, mean $\pm$ S.D.) ^{c)}	Inhibition ratio (%) ^{d)}	<i>p</i> ^{e)}
Grifolan NMF-5N	25	10	1.52 $\pm$ 0.91	31	n.s. ^{f)}
	50	10	2.05 $\pm$ 0.55	7	n.s.
	100	10	1.24 $\pm$ 0.61	44	< 0.05
	200	10	1.46 $\pm$ 0.66	34	n.s.
	400	10	1.76 $\pm$ 0.57	19	n.s.
Nil	(Saline)	13	2.20 $\pm$ 1.18	—	—

^{a)} MM46 carcinoma (4×10^6) cells were inoculated s.c. on day 0.
^{b)} See footnotes b–f) of Table II.

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TABLE IV. Antitumor Effect of Grifolan NMF-5N with Various Treatment Schedules against Meth A Fibrosarcoma^{a)}

Exp. No.	Sample ^{b)}	Dose (μ g/mouse)	Treatment schedules Day(s) Time(s)	No. of mice	Tumor weight (g, mean $\pm$ S.D.) ^{c)}	Inhibition ratio (%) ^{d)}	<i>p</i> ^{e)}
1.	Grifolan NMF-5N	100	1–5 $\times$ 5	10	1.78 $\pm$ 1.00	37	< 0.01
		100	7–11 $\times$ 5	10	0.42 $\pm$ 0.33	85	< 0.001
		100	14–18 $\times$ 5	10	0.66 $\pm$ 0.59	76	< 0.001
	Nil	(Saline)	1–5 $\times$ 5	13	2.84 $\pm$ 0.75	—	—
2.	Grifolan NMF-5N	100	1,7,14,21,28 $\times$ 5	9	0.30 $\pm$ 0.21	70	< 0.01
		500	1 $\times$ 1	9	0.68 $\pm$ 0.61	33	n.s. ^{f)}
		500	7 $\times$ 1	9	0.31 $\pm$ 0.25	69	< 0.01
		500	14 $\times$ 1	10	0.63 $\pm$ 0.74	38	n.s.
	Nil	(Saline)	1–5 $\times$ 5	13	1.01 $\pm$ 0.58	—	—

^{a, c–f)} See footnotes a, c–f) of Table II.
^{b)} Grifolan NMF-5N was administered as saline solution by i.p. injection.

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TABLE V. Antitumor Effect of Grifolan NMF-5N with Various Treatment Schedules against MM46 Carcinoma^{a)}

Dose ^{b)} (μ g/mouse)	Treatment schedules Day(s) Time(s)	No. of mice	Tumor weight (g, mean $\pm$ S.D.) ^{c)}	Inhibition ratio (%) ^{d)}	<i>p</i> ^{e)}
100	1–5 $\times$ 5	10	2.37 $\pm$ 1.04	22	n.s. ^{f)}
100	7–11 $\times$ 5	9	1.89 $\pm$ 0.75	37	< 0.01
100	14–18 $\times$ 5	10	2.03 $\pm$ 0.56	33	< 0.01
100	1,7,14,21,28 $\times$ 5	10	1.76 $\pm$ 0.35	42	< 0.001
500	1 $\times$ 1	10	3.07 $\pm$ 1.12	–2	n.s.
500	7 $\times$ 1	10	2.45 $\pm$ 0.61	19	n.s.
500	14 $\times$ 1	10	2.11 $\pm$ 0.34	30	< 0.001
Nil(Saline)	1–5 $\times$ 5	14	3.02 $\pm$ 0.72	—	—

^{a, c–f)} See footnotes a, c–f) of Table III.
^{b)} Grifolan NMF-5N was administered as saline solution by i.p. injection.

^{b)} Grifolan NMF-5N was administered as saline solution by i.p. injection.

FIG. 2. Effect of Various Administration Schedules of Grifolan NMF-5N on Growth of Meth A Fibrosarcoma

BALB/c mice were administered i.p. with 100 μ g of grifolan NMF-5N for 5 successive days after tumor inoculation (day 0).

○, day 1–5; △, day 7–11; □, day 14–18; ●, nil (saline). a) $p < 0.05$, b) $p < 0.001$.

cant antitumor activity was observed against both syngeneic tumors.

Effect of Administration Routes of Grifolan NMF-5N against Meth A Fibrosarcoma and MM 46 Carcinoma

To examine whether antitumor activity is affected by the administration routes, grifolan NMF-5N was administered i.v. or i.t. daily for 5 successive days at days 1–5 and 7–11. Saline was injected into the control group on days 1–5 against Meth A fibrosarcoma (Table VI). An intravenous injection of NMF-5N on days 7–11 was highly effective against Meth A fibrosarcoma but an intratumorous injection was not effective against MM 46 carcinoma (Table VII).

DISCUSSION

In this report, the antitumor activity of grifolan NMF-5N, which was obtained from matted mycelia of *G. frondosa*, was examined. In the allogeneic tumor system, grifolan NMF-5N exhibited potent antitumor activity against the solid form of sarcoma 180. In the kinetics study of this tumor, marked suppression of the tumor growth was observed 3 weeks after tumor inoculation. In the syngeneic tumor system, a significant antitumor activities against Meth A fibrosarcoma and MM 46 carcinoma were observed. Lentinan, which is a 6-branched β -1,3-glucan is known to be ineffective against Meth A fibrosarcoma.^{7,8)} It is interesting that grifolan NMF-5N, which has a similar primary structure as lentinan, is effective against Meth A fibrosarcoma.

Grifolan NMF-5N was effective as a single treatment. The examination of administration timing demonstrated that administration 1 or 2 weeks after tumor inoculation was more effective than that after 1 d after inoculation. Lentinan is reported to be effective when it is given after tumor growth attains a certain volume.⁹⁾ In

TABLE VI. Antitumor Effect of Grifolan NMF-5N Administered by Intravenous or Intratumorous Injection against Meth A Fibrosarcoma^{a)}

Routes	Dose $\times$ 5 ^{b)} (μ g/mouse)	Treatment schedules	No. of mice	Tumor weight (g, mean $\pm$ S.D.) ^{c)}	Inhibition ratio (%) ^{d)}	p ^{e)}
i.v.	100	1–5	10	0.51 $\pm$ 0.36	45	< 0.05
	100	7–11	10	0.75 $\pm$ 0.45	26	n.s. ^{f)}
	Nil (Saline)	1–5	11	0.93 $\pm$ 0.40	—	—
i.t.	100	1–5	10	0.02 $\pm$ 0.04	97	< 0.001
	100	7–11	9	0.53 $\pm$ 0.44	31	n.s.
	Nil (Saline)	1–5	9	0.77 $\pm$ 0.42	—	—

a, c–f) See footnotes a, c–f) of Table II.

b) Grifolan NMF-5N was administered as saline solution daily for 5 successive days.

TABLE VII. Antitumor Effect of Grifolan NMF-5N Administered by Intravenous or Intratumorous Injection against MM46 Carcinoma^{a)}

Routes	Dose $\times 5$ ^{b)} ($\mu\text{g}/\text{mouse}$)	Treatment schedules	No. of mice	Tumor weight (g, mean $\pm$ S.D.) ^{c)}	Inhibition ratio (%) ^{d)}	p ^{e)}
i.v.	100	1—5	11	2.75 ± 0.75	22	n.s. ^{f)}
	100	7—11	11	2.35 ± 1.04	34	<0.02
	Nil (Saline)	1—5	11	3.54 ± 1.11	—	—
i.t.	100	1—5	10	3.19 ± 0.87	—6	n.s.
	100	7—11	10	2.38 ± 0.62	21	n.s.
	Nil (Saline)	1—5	10	3.00 ± 0.93	—	—

a, c—f) See footnotes a, c—f) of Table III.
 a) Grifolan NMF-5N was administered as saline solution daily for 5 successive days.

b) Grifolan NMF-5N was administered as saline solution daily for 5 successive days.

our study, the same phenomenon was observed with grifolan NMF-5N. In the experiment of administration timing, tumor growth was suppressed significantly when grifolan NMF-5N was administered on days 7—11 or 14—18. This observation suggests that effector cells may be activated by grifolan NMF-5N during these periods. Grifolan NMF-5N was found to be effective by i.v. or i.t. injection against syngeneic tumors.

The antitumor effect against Meth A fibrosarcoma was different from that against MM 46 carcinoma. Grifolan NMF-5N was more effective against Meth A fibrosarcoma on day 7 whereas it was more effective on day 14 against MM 46 carcinoma. In the experiment of administration routes, grifolan NMF-5N was effective by i.v. and i.t. injections against Meth A fibrosarcoma, but only i.v. injection was effective against MM 46 carcinoma.

Currently, the mechanism of antitumor activity of grifolan NMF-5N is being studied using effective dosage, timing and routes of administration.

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REFERENCES

- 1) I. Suzuki, T. Itani, N. Ohno, S. Oikawa, K. Sato, T. Miyazaki, and T. Yadomae: Antitumor activity of a polysaccharide fraction extracted from cultured fruit bodies of *Grifola frondosa*, *J. Pharmacobio-Dyn.*, **7**, 492—500 (1984).
- 2) I. Suzuki, T. Itani, N. Ohno, S. Oikawa, K. Sato, T. Miyazaki, and T. Yadomae: Effect of a polysaccharide fraction from *Grifola frondosa* on immune response in mice, *J. Pharmacobio-Dyn.*, **8**, 217—226 (1985).
- 3) N. Ohno, I. Suzuki, S. Oikawa, K. Sato, T. Miyazaki, and T. Yadomae: Antitumor activity and structural characterization of glucans extracted from cultured fruit bodies of *Grifola frondosa*, *Chem. Pharm. Bull.*, **32**, 1142—1151 (1984).
- 4) K. Iino, N. Ohno, I. Suzuki, T. Miyazaki, and T. Yadomae: Structural characterization of a neutral antitumor β -D-glucan extracted with hot sodium hydroxide from cultured fruit bodies of *Grifola frondosa*, *Carbohydr. Res.*, **141**, 111—119 (1985).
- 5) N. Ohno, K. Iino, T. Takeyama, I. Suzuki, K. Sato, S. Oikawa, T. Miyazaki, and T. Yadomae: Structural characterization and antitumor activity of the extracts from matted mycelium of cultured *Grifola frondosa*, *Chem. Pharm. Bull.*, **33**, 3395—3401 (1985).
- 6) K. Iino, N. Ohno, I. Suzuki, K. Sato, S. Oikawa, and T. Yadomae: Structure-function relationship of antitumor β -1,3-glucan obtained from matted mycelium of cultured *Grifola frondosa*, *Chem. Pharm. Bull.*, **33**, 4950—4956 (1985).
- 7) G. Chihara: The antitumor polysaccharide Lentinan: an overview, "Manipulation of Host Defence Mechanism," ed. by T. Aoki, I. Urushizaki, and E. Tsubura, Excerpta Medica, North Holland, 1981, pp.1—16.
- 8) T. Sasaki and N. Takasuka: Further study of the structure of lentinan, and antitumor polysaccharide from *Lentinus edodes*, *Carbohydr. Res.*, **47**, 99—104 (1976).
- 9) Y. Akiyama, S. Kashima, T. Hayami, M. Izawa, K. Mitsugi, and J. Hamuro: Immunological characteristics of antitumor polysaccharides, Lentinan and its analogues, as immune adjuvants, "Manipulation of Host Defence Mechanisms," ed. by T. Aoki, I. Urushizaki, and E. Tsubura, Excerpta Medica, North Holland, 1981, pp. 227—238.