Antitumor Effects of Pronase-Treated Fragments, Glycopeptides, from Ovomucin in Hen Egg White in a Double Grafted Tumor System

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Antitumor effects of fragments (220 and 120 kDa, highly glycosylated peptides) separated from Pronase-treated hen egg white ovomucin were analyzed in a double grafted tumor system. BALB/c mice received simultaneous inoculations of Meth-A fibrosarcoma cells on the right flank (1×10^6 cells) and left flank (2×10^5 cells) on day 0. The two fragments (100 μg/mouse/day) were injected into the right tumor on days 3, 4, and 5, and mice were raised for 21 days. Both fragments cured directly and entirely the right (treated) tumor and inhibited indirectly and slightly the growth of the left (distant) one. Examinations of desialylated 120 kDa fragment indicated that the sialic acid residues in the 120 kDa fragment are not necessarily essential for direct antitumor activity but might be indispensable for regression of distant tumors. The noninhibitory activities in a single tumor system, in which mice received intradermal inoculation of tumor cells only in the left flank, and the increase of immunosuppressive acid protein in serum suggested the slight activation of the immune system.

Keywords: *Ovomucin; antitumor effect; glycopeptide*

INTRODUCTION

Hen egg white ovomucin (OVM) is the fibriform and highly viscous glycoprotein of the macromolecule, which accounts for approximately 3.5% of egg white protein. It consists of an α-subunit [apparent molecular mass (AMM) ~220 kDa] containing 10−15% carbohydrate and a β-subunit (AMM ~400 kDa) containing 50−65% carbohydrate, with a macromolecular structure bound by disulfide bonds between their subunits (Donovan et al., 1970; Kato et al., 1970; Itoh et al., 1987). Our previous studies on the relationships between biological function and molecular characteristics of OVM revealed some differences in the affinities with viruses such as bovine rotavirus, hen newcastle disease virus, and human influenza virus. This indicated that a specific molecular structure and carbohydrate chain of OVM are required for their affinities and participation of disulfide bonds in the reaction between antigen and antibody of OVM (Tsuge et al., 1996a,b, 1997a,b). We also demonstrated that Pronase treatment for the prepared gel-like OVM would be a good method for solubilizing the protein, which was difficult to solubilize with distilled water and some buffers. There was no significant loss of biological activity, so the treatment was effective to obtain fragments from the β-subunit which were composed of O-glycoproteins, containing more or less clustered sialic acid moieties (Tsuge et al., 1997a). All sialic acid present in OVM was described as *N*-acetylneuraminic acid (NeuAc) (Robinson and Monsey, 1971).

Various substances that are active against tumor cells are still being discovered from biological products in nature. Among them, high molecular weight glycoproteins with antineoplastic activity from biological sources and fungal or bacterial origin have been also described. For example, in vitro and in vivo antitumor activities have been reported on some lectins such as phytohemagglutinin and concanavalin A (Kim et al., 1993), aplysianin E (Kisugi et al., 1987) from *Aplysi kurodai* eggs (sea hare), and PSK, one of the biological response modifiers (BRMs), a protein-bound polysaccharide preparation isolated from *Coriolus versicolo* (Tsukagoshi et al., 1984; Ebina et al., 1989; Ebina and Murata, 1992). Furthermore, mucin-like glycoprotein in human milk fat globule membrane was found to inhibit growth of BALB/c 3T3 cells (Shimizu et al., 1990).

β-Subunit and its protease-digested glycoprotein fragments should be expected to show potential antitumor activity similar to that the glycoproteins as described above. In our preliminary paper (Ohami et al., 1993), β-subunit from OVM was shown to have a cytotoxic effect on the cultured tumor cells through scanning electron microscopy. The present paper describes the antitumor effect of the fragments, glycopeptides from Pronase-treated OVM, using a double grafted tumor system, which is an experimental model [evaluations of direct action of agents and their indirect (distant) action via the host's immune system] of an antitumor effect reported by Ebina et al. (1986) and Ebina and Kohya (1988). The relationship between the antitumor effect and the molecular characteristic of OVM fragment is also discussed.

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3034 *J. Agric. Food Chem.,* Vol. 46, No. 8, 1998

Watanabe et al.

MATERIALS AND METHODS

Materials. OVM from fresh egg white (White Leghorn hens) was prepared as a gel-like precipitate according to the method of Kato et al. (1970). The prepared OVM was digested with Pronase from *Streptomyces griseus* (Kaken Kagaku, Tokyo), under the following conditions: OVM/Pronase = 1:1/50 (w/w); 10 mM phosphate buffer (pH 8.4); 37 °C for 24 h. The two main fractions of P1 and P2 from its digest were separated by gel filtration (Sephacryl S-400, Pharmacia LKB Products, Uppsala, Sweden), dialyzed against deionized water, and lyophilized. These procedures were also carried out according to the method described in a previous paper (Tsuge et al., 1997a). The main components in P1 and P2 were gel filtrated again under the same procedures. The desialylation of the preparation from fraction P2 was carried out as follows: Neuraminidase (7.6×10^{-3} units) from *Clostridium perfringens* (Sigma Chemical Co., St. Louis, MO) was added to samples (184 μg/mL) and solubilized with 10 mM acetate buffer (pH 5.0). The mixture was incubated at 37 °C for 24 h, heated at 100 °C for 3 min to stop the digestion, dialyzed against 50 mM carbonate buffer (pH 9.6), and lyophilized.

Analytical Methods. The determinations of content and composition of protein and carbohydrate, content of NeuAc residue in the preparations, and the method of SDS−polyacrylamide gel electrophoresis (SDS−PAGE) were carried out as described in a previous paper (Tsuge et al., 1997a).

Mice and Tumors. Six-week-old male BALB/c mice were obtained from Shizuoka Laboratory Animal Center, Hamamatsu. Meth-A, a methylcholanthrene-induced fibrosarcoma, was administered to syngeneic BALB/c mice in solid form by intradermal inoculation.

Double Grafted Tumor System. Mice received simultaneous intradermal inoculations of Meth-A tumor cells in both the right (1×10^6 cells) and the left (2×10^5) flanks, according to the method described previously (Ebina et al., 1986; Ebina and Kohya, 1988). Lyophilized samples (1 mg/mL) were solubilized with physiological saline, filtered with an Ekicorodisk filter (pore size = 0.45 μm), and 100 μL (100 μg) solutions were injected into the right-flank tumor [direct (treated) region] on days 3, 4, and 5 after the incubation (from day 3, when the tumor was already palpable). Both right and left [indirect (nontreated and distant) region] tumors were observed for 21 days. Meth-A tumor-bearing mice treated with physiological saline served as the control group.

Single Tumor System. The single tumor system, as a control, in which mice received intradermal inoculations of 2×10^5 Meth-A tumor cells only in the left flank, was used (Ebina et al., 1986; Ebina and Ishikawa, 1989). A sample from the P2 fragment (100 μg/mouse/day) as described above was injected into the right flank on days 3, 4, and 5, and the left tumor was observed for 21 days.

Assessment of Antitumor Activity. Antitumor activity was assessed in terms of tumor weight and tumor diameter. On day 21, the animals were sacrificed, and each tumor was weighed to obtain the mean value (grams) ± standard deviation (SD). The tumor diameter was serially measured with calipers to estimate tumor size, as calculated by the following formula: square root of long diameter × short diameter (mm). Each experimental and control group consisted of seven mice.

Determination of Mouse Immunosuppressive Acidic Protein. Mice were inoculated intradermally with 1×10^6 Meth-A tumor cells only in the right flank. A sample from the P2 fragment (100 μg/mouse/day) described above was injected intratumorally into the right flank on days 3, 4, and 5, under comparison with examinations with BRMs of PSK (5 mg × three times) and lentinan [100 μg × three times (LNT), β-1,3-glucan with average molecular weight of 500 kDa from fruit bodies of *Lentinus edodes*). Blood was collected from all mice (day 6, 1 day after the intratumoral injection of samples) before they were sacrificed, and sera were separated by centrifugation. IAP, a marker protein of activated macrophages and neutrophils, levels in serum were measured with a single radical immunodiffusion method using rabbit anti-mouse IAP serum (mouse IAP plate, Sanko Junyaku, Tokyo)

(Shibata et al., 1983; Ebina et al., 1994). Each group consisted of three mice. The calibration curve with purified IAP was linear between 30 and 1500 μg/mL.

RESULTS

The main components in P1 and P2 fractions separated by gel filtration from Pronase-treated OVM in this study were recognized to be glycopeptides having AMMs of 220 kDa (220-GP) and 120 kDa (120-GP) by SDS−PAGE, respectively, derived from β-subunit in OVM, in analogy with the results in a previous paper (Tsuge et al., 1997a). The purified 220-GP and 120-GP appeared in a broad but single band stained with periodate−Schiff reagent on SDS−PAGE for the detection of carbohydrate, respectively.

When the purified 220-GP was again digested with Pronase in a manner similar to that above, the formation of 120-GP, of which further digestion to the much smaller fragments with Pronase under the condition used is difficult, was observed in the patterns of SDS−PAGE of its digests. This phenomenon might be due to protection by the presence of carbohydrate chain bound to peptide. Thus, 120-GP was regarded as a part of 220-GP (Tsuge et al., unpublished data). The characteristics of those glycopeptides isolated from both their fractions in this study were confirmed to be almost the same as described in a previous paper (Tsuge et al., 1997a). Briefly, 220-GP was the carbohydrate-rich peptide (carbohydrate content = 88.0% containing NeuAc of 10.3%) with the main part O-linked oligosaccharides and the minor part N-linked oligosaccharides, whereas 120-GP was also a carbohydrate-rich peptide (carbohydrate content = 93.5% containing NeuAc of 28.5%) with only O-linked oligosaccharides. No NeuAc residue was found in the prepared desialylated 120-GP.

In the double grafted tumor system, antitumor effects of samples (220-GP, 120-GP, and desialylated 120-GP) were observed on the right (treated) and left (distant) tumors for 21 days (Table 1). Sample 220-GP was found to inhibit significantly the growth of the right (treated) tumor. All seven mice were tumor-free on day 21 (Table 1A). The right (treated) tumor sizes scarcely increased after treatment with 220-GP and successively reduced as each day progressed, in contrast to the right control tumor, which increased each day (Figure 1A). Thus, complete tumor regression in tumor size and weight was obtained when 220-GP was given by intratumoral administration. On the other hand, 220-GP slightly, but not significantly, inhibited growth of the left (distant) tumor; one of seven mice was tumor-free on day 21. The average tumor size and weight in the left (distant) tumor decreased slightly, in contrast to the control at 21 days (Table 1A). The diameter in the left (distant) tumor in mice treated with and without 220-GP successively increased with each day, respectively, showing some suppressing effects in rising growth curve in the latter part of the experiment days in the treated mice (Figure 1B). As a result, 220-GP was found to have a higher direct antitumor activity for the right (treated) tumor and a lower indirect activity for the left (distant) tumor.

Under similar conditions, the antitumor effect of 120-GP was examined. 120-GP also inhibited the growth of the right (treated) tumor. That is, two of seven mice were tumor-free, and the average tumor size and weight

Table 1. Antitumor Effects of 220-GP, 120-GP, and Desialylated (D) 120-GP in a Double Grafted Tumor System[a]

		right tumor (1×10^6 cells)			left tumor (2×10^5 cells)		
	sample	tumor-free/ tested	tumor diam (mm ± SD)	tumor wt (g ± SD)	tumor-free/ tested	tumor diam (mm ± SD)	tumor wt (g ± SD)
A	control	0/7	25.4 ± 2.2	4.8 ± 0.9	0/7	13.2 ± 4.0	1.5 ± 0.7
	220-GP	7/7	0.0 ± 0.0	0.0 ± 0.0	1/7	10.7 ± 6.6	1.0 ± 1.0
B	control	0/7	24.3 ± 1.6	5.0 ± 0.9	0/7	14.7 ± 3.9	1.5 ± 0.9
	120-GP	2/7	8.5 ± 6.1	0.7 ± 0.8	0/7	12.7 ± 3.6	1.1 ± 0.8
C	control	0/7	24.9 ± 2.2	5.1 ± 0.6	0/7	16.4 ± 1.8	2.0 ± 0.7
D	120-GP	5/7	1.9 ± 3.2	0.1 ± 0.1	0/7	16.4 ± 2.1	2.0 ± 0.6

[a] BALB/c mice received simultaneous intradermal inoculations of Meth-A fibrosarcoma cells in both the right and the left flanks on day 0. 220-GP, 120-GP, and desialylated 120-GP (100 μg/mouse/day, days 3, 4, and 5) were injected into the right (treated) tumor followed by a 21-day observation period. SD: standard deviation.
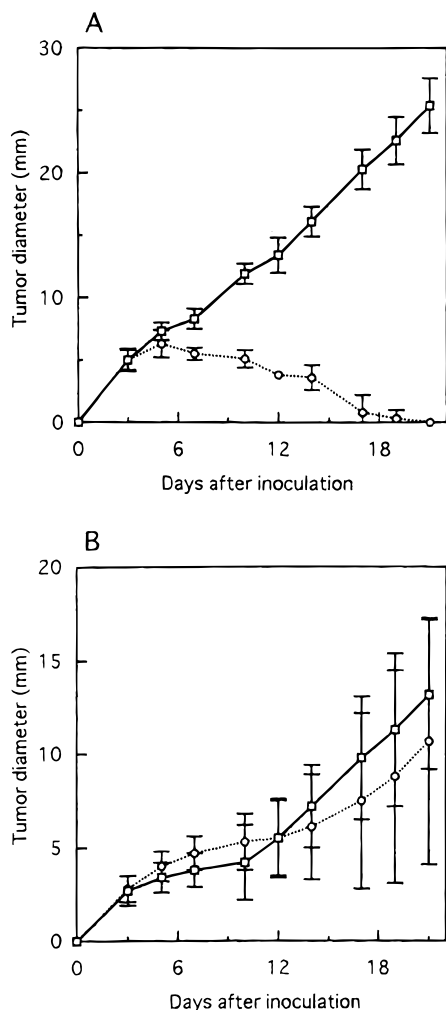

Figure 1. Antitumor effect of intratumoral injection of 220-GP in the double grafted tumor system: (A) right (treated) tumor; (B) left (distant) tumor; (□) control; (○) 220-GP. BALB/c mice received simultaneous intradermal inoculations of Meth-A tumor cells in both the right (1×10^6 cells) and the left (2×10^5 cells) flank. Sample was injected into the right flank tumor on days 3, 4, and 5 (100 μg/mouse/day), and both tumor diameters were observed for 21 days. Values are means of seven mice per group.

on the 21st day in the right (treated) tumor were significantly less than in the control tumor (Table 1B). Tumor sizes scarcely grew after the treatment with 120-GP, in contrast to those in the control mice (Figure 2A). On the other hand, 120-GP slightly, but not significantly, inhibited the growth of the left (distant) tumor, although none of the seven mice was tumor-free (Table
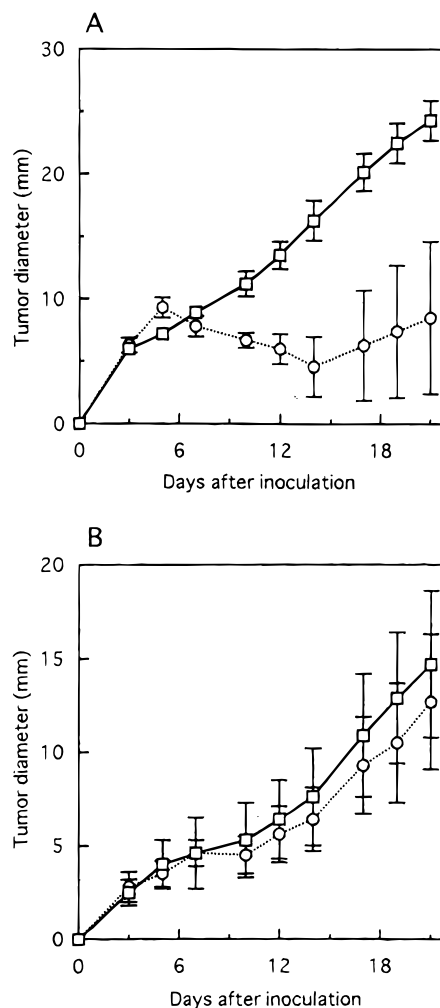

Figure 2. Antitumor effect of intratumoral injection of 120-GP in the double grafted tumor system: (A) right (treated) tumor; (B) left (distant) tumor; (□) control; (○) 120-GP. The experimental methods were the same as in Figure 1.

1B). The average tumor size and weight in the seven mice were slightly less than in the control mice (Figure 2B).

Intratumoral administration of 120-GP at the dose of 20 μg/mouse/day showed the reduction of both the tumor size and weight in the right (treated) tumor on day 21 at relative ratios from 15 to 20%, compared with the control group (data not shown). There was no effect against the left (distant) tumor. These inhibitory activities at the dose level of 20 μg × three times were much lower than at 100 μg × three times described above (Table 1B and Figure 2). Thus, it was shown that the

3036 *J. Agric. Food Chem.,* Vol. 46, No. 8, 1998
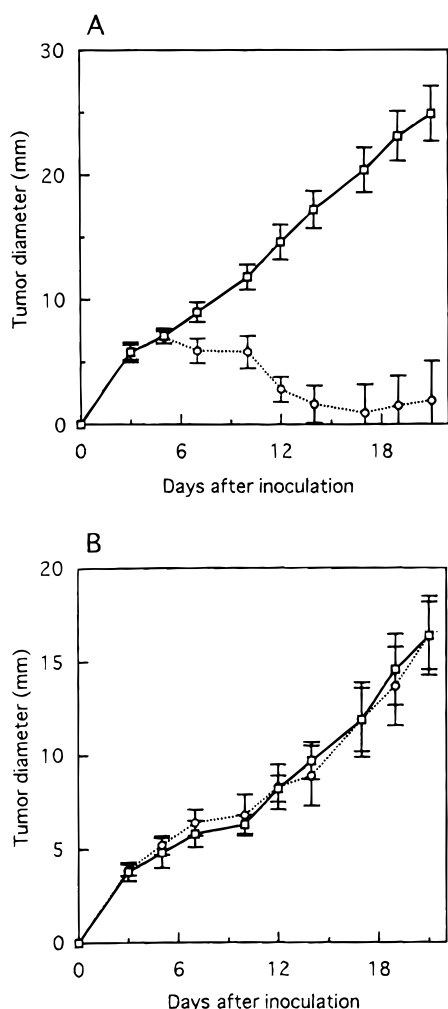
Watanabe et al.

A

B

Figure 3. Antitumor effect of intratumoral injection of desialylated 120-GP in the double grafted tumor system: (A) right (treated) tumor; (B) left (distant) tumor; (□) control; (○) desialylated 120-GP. The experimental methods were the same as in Figure 1.

sample at a level more than 20 μg might be dose-dependently required to induce the antitumor activity on the treated tumor by 120-GP.

To see the effect on antitumor activity of the NeuAc residues in the carbohydrate chain bound to peptide in 120-GP, desialylated 120-GP was examined in the same manner as described above. As shown in Table 1C and Figure 3, five of seven mice were free of the tumor on the right (treated) side on day 21. However, desialylated 120-GP did not significantly inhibit the growth of the left (distant) tumor, indicating that NeuAc residue in the used glycopeptides might act as recognition signals in the immune system.

The above-mentioned results on the antitumor effect on the left (distant) tumor did not exclude the possibility that 220-GP and 120-GP inoculated into the right (treated) tumor might directly affect the left (distant) tumor via the blood stream. Therefore, the antitumor effect of 120-GP was examined by the single grafted tumor system. As shown in Table 2 and Figure 4, 120-GP scarcely inhibited growth of the left tumor, indicating that 120-GP itself might not directly affect the opposite left flank. Thus, the results in the double grafted tumor system described above suggested that administration of 120-GP in the right (treated) tumor affected tumor growth in the left (distant) region of the
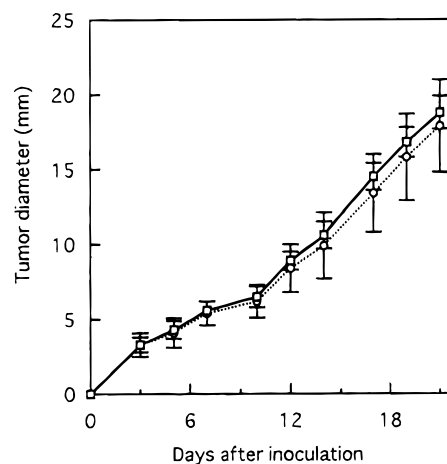

Figure 4. Antitumor effect of intratumoral injection of 120-GP in the single grafted tumor system: (□) control; (○) 120-GP. BALB/c mice received intradermal inoculations of 2×10^5 Meth-A tumor cells only in the left flank. Samples were injected into the right flank on days 3, 4, and 5 (100 μg/mouse/day) and the left tumor diameters were observed for 21 days. Values are means of seven mice per group.

Table 2. Antitumor Effect of 120-GP in a Single Grafted Tumor System

	left tumor (2×10^5 cells)		
sample	tumor-free/ tested	tumor diam (mm ± SD)	tumor wt (g ± SD)
control	0/7	18.8 ± 1.1	3.0 ± 0.4
120-GP	0/7	17.9 ± 3.1	2.5 ± 0.8

Table 3. Induction of IAP in Serum by 120-GP, PSK, and LNT[a]

sample	IAP (μg/mL)
control	153 ± 4
120-GP (100 μg × 3)	460 ± 50
PSK (5 mg × 3)	930 ± 77
LNT (100 μg × 3)	189 ± 42

[a] One day after intradermal injections of samples, serum IAP levels in BALB/c mice were assayed.

immune system, although its antitumor activities were at a low level.

It has been shown that the induction of IAP in serum was an important trigger of the antitumor effect caused by the intratumoral injection of BRMs and that IAP increased dramatically soon after their intradermal injections (Ebina et al., 1994). Therefore, IAP in serum of Meth-A-bearing mice treated with and without 120-GP 1 day after the final injection was measured in comparison with PSK- or LNT-treated mice (Table 3). The serum IAP level in 120-GP-treated Meth-A-bearing mice significantly increased in comparison with that of the control mice. This value in the mice treated with 120-GP was intermediate between those with PSK and LNT. It was also mentioned that PSK greatly induced IAP and suppressed the tumor growth, whereas LNT scarcely induced IAP and suppressed the tumor growth (Ebina et al., 1994). Thus, 120-GP was found to activate moderately the macrophage and neutrophils in comparison with the two BRMs.

DISCUSSION

The immunopotentiating and immunomodulating agents, which may modify the relationship between tumor and host by modifying the biological responses

Antitumor Effect of Glycopeptides from Ovomucin

J. Agric. Food Chem., Vol. 46, No. 8, 1998 **3037**

of the host of tumor cells, are broadly termed as BRM. Antitumor effects of various BRMs, for example, immunopotentiators such as PSK, OK-32 (a *Streptococcus* preparation), and LNT, cytokines such as IL (interleukin)-1, -2, and -6, IFN (interferon), and TNF (tumor necrosis factor), have been evaluated in a double grafted tumor system. Compared with chemotherapeutics such as mitomycin (MMC) and neocarzinostain (NCS) as a control, notable differences were observed in the antitumor effect among BRMs (Ebina et al., 1986, 1994). PSK, IL-1, and IFN cured not only the right (treated) tumor but also the left (distant). OK-432 and MMC cured the treated tumor and inhibited growth of the distant tumor, whereas TNF and NCS cured the treated tumor only. LNT, IL-2, and IL-6 inhibited neither the treated tumor nor the distant one. Antitumor effects of both 220-GP and 120-GP in this study were found to be similar to groups of OK-432 and MMC rather than ones of TNF and NCS.

When PSK, which has been available on the market in Japan as an antitumor agent (Krestin) (Tsukagoshi et al., 1984), was given three times at the level of 5 mg/mouse/day by intratumoral administration to inoculated Meth-A cells in the same manner described in this study, the antitumor activities were as follows: In the right (treated) tumor, four of eight mice became tumor-free, with an average tumor weight of 0.7 ± 0.52 (g ± SD) (control = 4.5 ± 0.79). In the left (distant) tumor, two of eight mice were tumor-free, with an average tumor weight of 1.4 ± 0.74 (g ± SD) (control = 3.0 ± 0.42) (Ebina et al., 1986). When the results on the antitumor activities of 220-GP and 120-GP tested at 100 μg in this study (Figures 1 and 2; Table 1) are compared with the rather high concentration of 5 mg for the results of antitumor activities of PSK, the characteristics in our samples were found to have higher direct antitumor activities at their lower levels, although their indirect antitumor activity was lower, as seen in the chemotherapeutics such as MMC.

Antagonistic activities of PSK against tumor cells through the immune mechanisms have been investigated from various standpoints. For example, PSK efficiently induced protein production of cytokines such as IL-1β, IL-6, IFN-γ, and TNF-α, augmented macrophage cytotoxicity and T cell-mediated immune responses, and enhanced the induction of apoptosis (trigger of programmed cell death) of Meth-A tumor cells (Ebina et al., 1989; Ebina and Murata, 1991). As distinct from PSK, it is possible that when 220-GP and 120-GP were given intratumorally, they came into close contact with tumor cells and developed a direct antitumor action. This mechanism was probably dependent on the induction of either apoptosis or necrosis of the tumor cells rather than a sequential antitumor immune cascade reaction. Thus, the intratumoral administration caused local inflammatory responses but did not greatly affect the tumor cells at a distant site. However, from comparisons between the results of the single and double grafted tumor systems and between the IAP levels in serum of mice treated with 120-GP and two BRMs, glycopeptides from ovomucin can be concluded to have some host-mediated activity. The fact that one of seven mice treated with 220-GP was tumor-free on day 21 in the distant tumor in the double grafted system supports the concept of host-mediated activity. Thus, there was a possibility that intratumoral therapy for a direct tumor with 120-GP and 220-GP stimulated the mild systemic immune response to distant tumors.

It was found in this study that the direct antitumor activities against Meth-A tumor cells in BALB/c mice were roughly in the order 220-GP, desialylated 120-GP, and 120-GP. On the other hand, the indirect ones were apparently in the order 220-GP, 120-GP, and desialylated 120-GP (desialylated 120-GP scarcely induced the indirect activity). Thus, NeuAc residues are not necessarily essential for direct antitumor activity but might be indispensable for indirect activity of the kind. This result is similar to the fact that sialic acid removal was reported to enhance the biological function of certain molecules while destroying the activities of others (Pilatte et al., 1993).

120-GP was described above to be a part of 220-GP. The fact that 120-GP showed somewhat lower direct and indirect antitumor activities than 220-GP suggested that there were basic active sites in 120-GP even in the unit of 220-GP and that the larger molecular size of 220-GP effectively induced development of the antitumor activities. It must be clarified hereafter how the regions with carbohydrate chain containing the highest NeuAc residue in 120-GP are related to antitumor activities and how the fragment of 120-GP arranges in 220-GP and so on. Direct growth inhibition of glycopeptides with and without NeuAc suggested that their samples might adhere specifically to tumor cells as described in a previous paper (Ohami et al., 1993) and lead to their death. However, their detailed mechanisms are unknown at present.

In conclusion, the present findings indicate that glycopeptides from OVM developed complete rejection of direct (treated) Meth-A tumors and slight growth inhibition of distant tumors in syngeneic mice, as seen in the general chemotherapeutic agents. Confirmatory studies of the possible mechanisms of action are underway.

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3038 *J. Agric. Food Chem.,* Vol. 46, No. 8, 1998

Watanabe et al.

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