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Purification and Characterization of Mouse α_1-Acid Glycoprotein and Its Possible Role in the Antitumor Activity of Some Lichen Polysaccharides

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Administration of some antitumor lichen polysaccharides (ALP) increased the serum level of α_1-acid glycoproteins (α_1-AG) in mice, possibly as a result of liver cell necrosis or inflammation induced by those polysaccharides.

Ascites tumor-bearing mice were treated with ALP, and ascitic fluid having a high α_1-AG level was harvested. Mouse α_1-AG was obtained from this source in quantity, and fractionated into α_1-AG-1 and -2.

The time courses of the serum level of α_1-AG and of the growth of subcutaneously implanted tumor were found to correlate well in ALP-treated mice, and purified α_1-AG-1 inhibited the growth of tumor cells *in vitro* at a concentration normally observed in animals with inflammation. These results suggest that α_1-AG plays a significant role in the antitumor activity exhibited by ALP.

Keywords——partially acetylated pustulan (GE-3); lichenan; anti-tumor activity; mouse α_1-acid glycoprotein

Introduction

Some lichen polysaccharides with β-glycosidic linkages were reported to have antitumor activity, which was claimed to be host-mediated.[1-4] However, the mode of tumor growth suppression by these antitumor lichen polysaccharides (ALP) appeared somewhat different from that with so-called host-mediated antitumor polysaccharides. When lentinan, an antitumor $\beta(1\rightarrow3)$ glucan from the fruit body of *Lentinus edodes* (BERG.) Sing,[5,6] for example, was administered to tumor-bearing mice, the tumor continued to increase in size until day 10—14 of the polysaccharide injection, when it started decreasing.[7] Lentinan was less active at a dose of 25 mg/kg/d than at a dose of 5 mg/kg/d.[5]

We observed that, when ALP was administered to tumor-bearing mice, suppression of the tumor growth or decrease in tumor size followed the injection without delay. Such an immediate reaction suggested that a more direct reaction was involved, such as immediate production of direct-acting substances.

To elucidate the mechanism involved in the antitumor action of ALP, three types of ALP, partially acetylated pustulan (GE-3), urea-treated GE-3 (UGE-3) and lichenan (L), were examined for their systemic effect in mice, with special reference to the early phase reactions.

In this paper, we also discuss a possible relationship between the antitumor activity of ALP and the pathological changes induced by ALP, especially the increased serum level of an acidic serum glycoprotein or mouse α_1-acid glycoprotein (α_1-AG), which corresponds to human α_1-acid glycoprotein in its isoelectric point (IEP) and chemical composition.[8]

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Experimental

Animals——Six- to seven-week-old female mice of ddY strain, weighing 22—26 g, were used.

Polysaccharide Solutions——Three lichen polysaccharides, GE-3 (Partially acetylated $\beta(1\rightarrow6)$glucan from thallus of *Gyrophora esculenta* MIYOSHI), UGE-3 prepared from GE-3 according to the method described for the preparation of urea-treated pachyman[6,9] and L ($\beta(1\rightarrow3)(1\rightarrow4)$glucan from *Cetraria islandica* (L.) ACH. and *C. richardsonii* HOOK.), all prepared in our laboratory, were used in the present experiments. The polysaccharide solutions in physiological saline were passed through a Millipore filter (0.45 μm) and injected at a dose of 15—70 mg/kg/0.1 ml/d.

Examination of Systemic Effect——Polysaccharide solutions were injected intraperitoneally into normal mice at a daily dose of 15, 30 or 50 mg/kg for 10 d.

Blood samples (about 50 μl) were collected from the retro-orbital plexus into heparinized capillary tubes. The numbers of leucocytes were counted, leucograms were prepared and the serum was assayed for α_1-AG by the single radial diffusion technique.[8]

Animals were then sacrificed, and the liver and spleen were removed, weighed and fixed in 10% formalin. Tissue sections for microscopic observation were prepared in the usual manner and stained with hematoxylin and eosin.

***In Vivo* Antitumor Effect Assay**——About 5×10^6 Ehrlich carcinoma cells were inoculated subcutaneously into mice and a polysaccharide solution was injected i.p. for 10 d. Antitumor effect was assessed in terms of the volume of the tumor, $S^2L/2$, where S and L are two perpendicular diameters of the tumor.

Preparation of Mouse α_1-AG——About 2×10^6 Ehrlich carcinoma cells were inoculated into mouse peritoneal cavity. On day 7 when ascitic fluid began to accumulate, 60 mg/kg/d of UGE-3 was subcutaneously injected for 4 d. Some 20 h after the final polysaccharide injection, the ascitic fluid, which was often almost clear, was collected and centrifuged. About 10 ml/mouse on average of cell-free ascitic fluid was obtained (α_1-AG titer about 800 μg/ml).

Ammonium sulfate was added to the fluid at 60% saturation and the precipitate was discarded. Solid ammonium sulfate was further added to 90% saturation. The precipitate was dialyzed against distilled water and applied to a diethyl aminoethyl (DEAE) cellulose column equilibrated with 0.05 M acetate buffer, pH 5.0. The column was then eluted in the usual way with a linear NaCl gradient (0.05 M acetate buffer, pH 5.0, and 0.5 M NaCl containing 0.05 M acetate buffer, pH 5.0).

The fractions eluted with 0.27—0.37 M NaCl-containing buffer were collected and subjected to preparative isoelectric focusing using Ampholine pH 2.5—4.5 (LKB Products) on a BIO RAD model 1404 electrophoresis cell. This fraction was regarded as α_1-acid glycoprotein (α_1-AG) on the basis of its physical and chemical properties.[8]

Preparation of Anti Mouse α_1-AG Serum——Mouse α_1-AG was injected into footpads and the back of a New Zealand white rabbit weighing about 3 kg as an emulsion in liquid paraffin. About 500 μg of α_1-AG was injected each time, and the administration was repeated five times at 10-d intervals.

Standard Calibration Curve of α_1-AG and Estimation of α_1-AG Content in Samples——A standard calibration curve was prepared by using the single radial immunodiffusion technique. The square of the diameter of the precipitin rings obtained after 48 h incubation was plotted against the amount of α_1-AG, and the α_1-AG contents in various samples were estimated by the same technique using this standard calibration curve.

Assay of the Effect of α_1-AG on the Growth of L 1210 Cells *in Vitro*——α_1-AG was assayed for effect on the growth of tumor cells *in vitro* in the usual way using L 1210 mouse leukemia cells. The cells (5×10^4 cells) were inoculated in RPMI-1640 (GIBCO) medium (1 ml/tube) supplemented with 5% fetal bovine serum, 5 μM 2-hydroxyethyl disulfide, 100 units of penicillin G and 100 μg of streptomycin, in the presence of various amounts of α_1-AG. The inoculated media were incubated at 37 °C for 48 h in an atmosphere containing 5% CO_2 with continuous exposure to the glycoprotein. The result is expressed in terms of the growth rate of the cells, where the number of cells in the control culture is taken as 100%.

Results

Effect of the Polysaccharides on Tumor Growth *in Vivo*

As shown in Fig. 1, the polysaccharides inhibited the tumor growth effectively and promptly at all the stages tested. GE-3 and L were more effective than UGE-3.

Pathological Changes

(a) Liver and Spleen——The most obvious macroscopic change noted in the polysaccharide-treated mice was hepatosplenomegaly. When UGE-3, the agent inducing the most pronounced pathological effect, was administered at a dose of 50 mg/kg/d, the liver on day 2 showed extensive disseminated focal liver cell necrosis heavily infiltrated by neutrophils (Fig. 2). Histiocytic proliferation occurred around the necrotic lesions a few days later,

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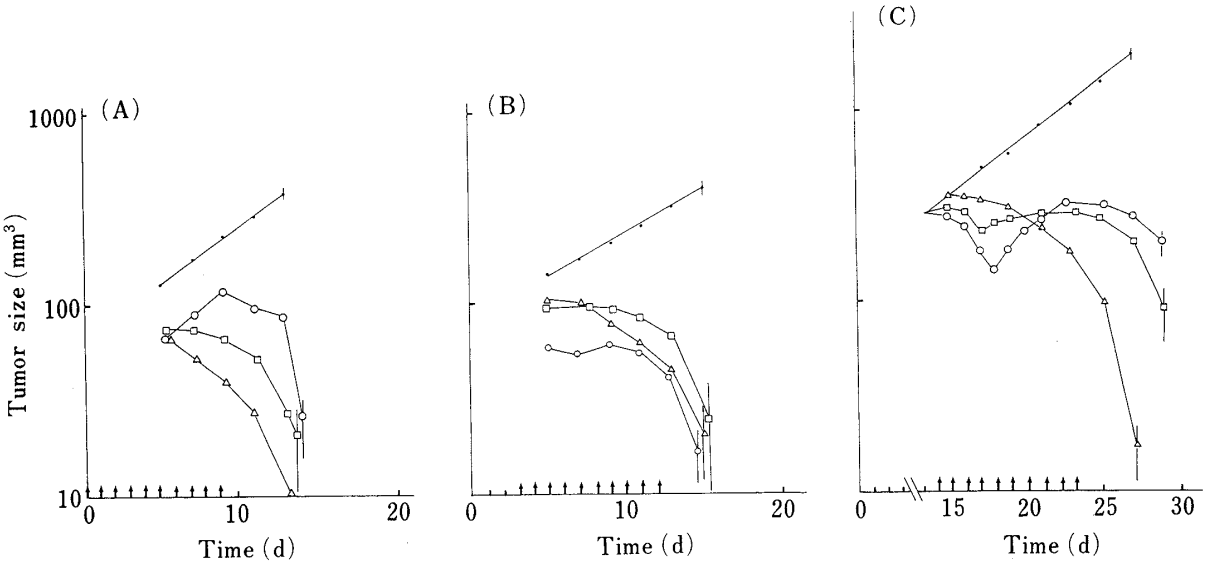

Fig. 1. Effect of Intraperitoneal Administration of GE-3 (30 mg/kg/d), UGE-3 (40 mg/kg/d) and L (30 mg/kg/d) on Tumor Growth *in Vivo*

Injection was started on day 0 (A), day 3 (B) or day 14 (C) after the subcutaneous inoculation of Ehrlich carcinoma cells (5×10^6 cells/mouse).
Small arrows denote polysaccharide injection. Each spot represents an average of at least five specimens.
△—△, GE-3; ○—○, UGE-3; □—□, L; ●—●, control.

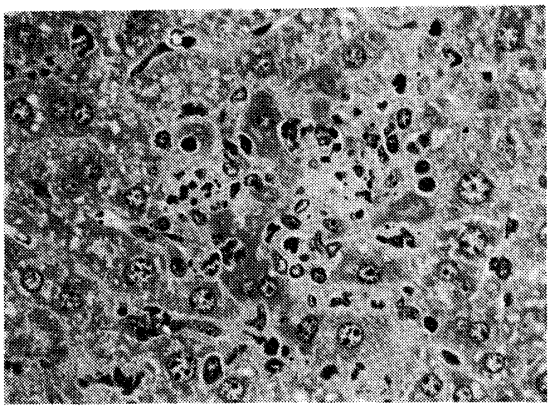

Fig. 2. Photomicrograph of a Microabscess Including Infiltrated Neutrophils and Liver Cell Nuclear Debris in the Liver of a UGE-3 (50 mg/kg/d)-Treated Mouse (20 h after the Second Injection)

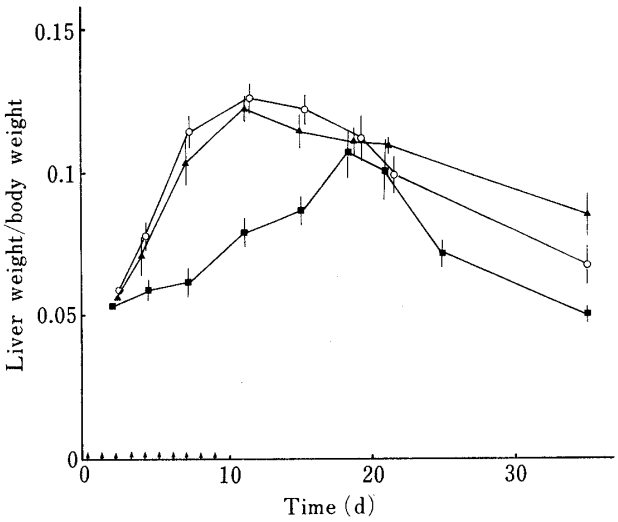

Fig. 3. Time Course of Liver Weight/Body Weight Ratio in Polysaccharide-Treated Mice

The ratio for normal mice was 0.051 ± 0.001.
Small arrows denote i.p. injection of polysaccharide at a dose of 50 mg/kg/d. (▲), GE-3; (○), UGE-3; (■), L. Each spot represents an average of five or six specimens, and is given with the S.E.

forming histiocytic clusters including multinucleate giant cells on days 9—11, when the liver weight was maximum (Fig. 3). The histiocytic aggregates then decreased gradually in size and number, but were still observable at the end of the 5-week experimental period.

In the spleen, enlarged lymph follicles with increased cell density were surrounded by a clear zone of proliferating histiocytes on day 4. On day 11, germinal centers appeared in enlarged lymph follicles with histiocytic fringes in the marginal sinus. Histiocytic clusters

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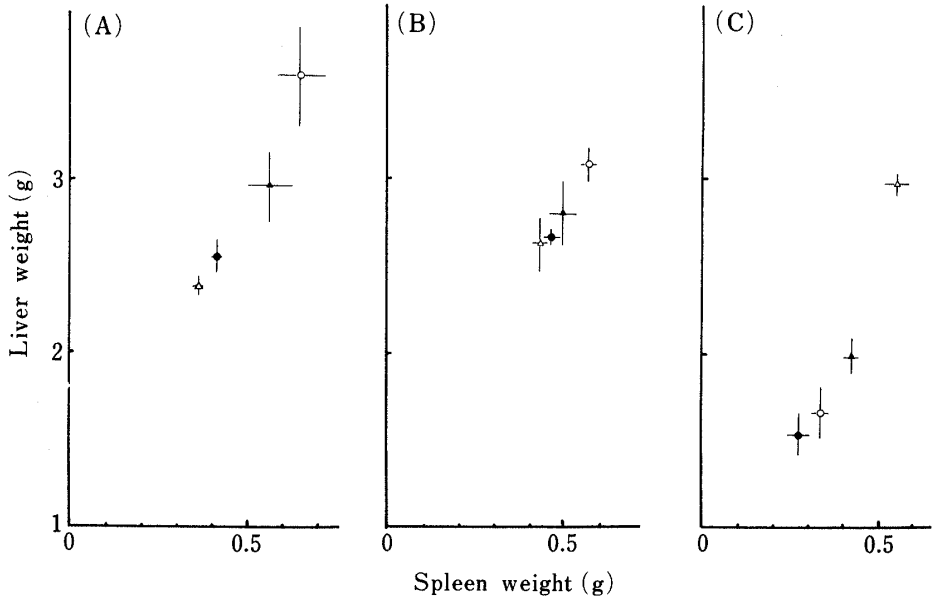

Fig. 4. Relationship between Liver Weight and Spleen Weight of Polysaccharide-Treated Mice at Various Stages

(A), GE-3; (B), UGE-3; (C), L.
Polysaccharide was injected i.p. from day 0 to day 9 at a dose of 50 mg/kg/d. Measurements were made on day 9 (●), day 11 (○), day 14 (▲) and day 19 (△). Each spot represents an average of six specimens and is given with the S.E.

similar to those seen in the liver were observed around the lymph follicles on day 18.

Time course changes in liver weight/body weight ratios are shown in Fig. 3 and the liver weight-spleen weight relation is shown in Fig. 4.

(b) Production of α_1-AG and Effect on the Leucogram——Time courses of the number of leucocytes and the serum level of α_1-AG are shown in Fig. 5 for mice receiving 50 mg/kg/d of polysaccharide. The maximum serum levels of α_1-AG (g/ml serum) on administration of 15 mg/kg/d of the polysaccharides were 1024 ± 63 for GE-3 (day 6), 1182 ± 32 for UGE-3 (day 3) and 752 ± 68 for L (day 9), and the value in normal mice was 256 ± 20.

GE-3, UGE-3 and L all induced leukopenia followed by leukocytosis, splenic lymph follicle enlargement and granulomatous cluster in liver and spleen though there were differences in the extent of induction. UGE-3 and GE-3 had more pronounced effects than L, and at higher doses the effects were more evident. In the liver of L-treated mice (15 mg/kg/d) neutrophil infiltration in the early lesions was less distinct and the formation of histiocytic clusters was delayed. Changes in leucograms were likewise less distinct and slightly delayed at lower doses.

All these effects appeared to be dose-dependent in the range of 10—50 mg/kg/d. No further enhancement in the effect was induced at doses higher than 50 mg/kg, and the effect was often not clear below 10 mg/kg.

Preparation and Physical and Chemical Characterization of Mouse α_1-AG

Preparative isoelectric focusing produced two α_1-AG fractions, α_1-AG-1 with an IEP of 3.2 and α_1-AG-2 with an IEP of 3.4. The yield and recovery of α_1-AG during the purification process are shown in Table I. Analytical isoelectric focusing on a polyacrylamide gel plate containing Pharmalite pH 2.5—5.0 (Pharmacia Fine Chemicals) and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of α_1-AG-1 and α_1-AG-2 showed that they were each homogeneous. (Fig. 6(A) and (B)). Analytical data for each of the α_1-AG fractions are summarized in Table II. Sialic acid content was determined by the cresol–periodate

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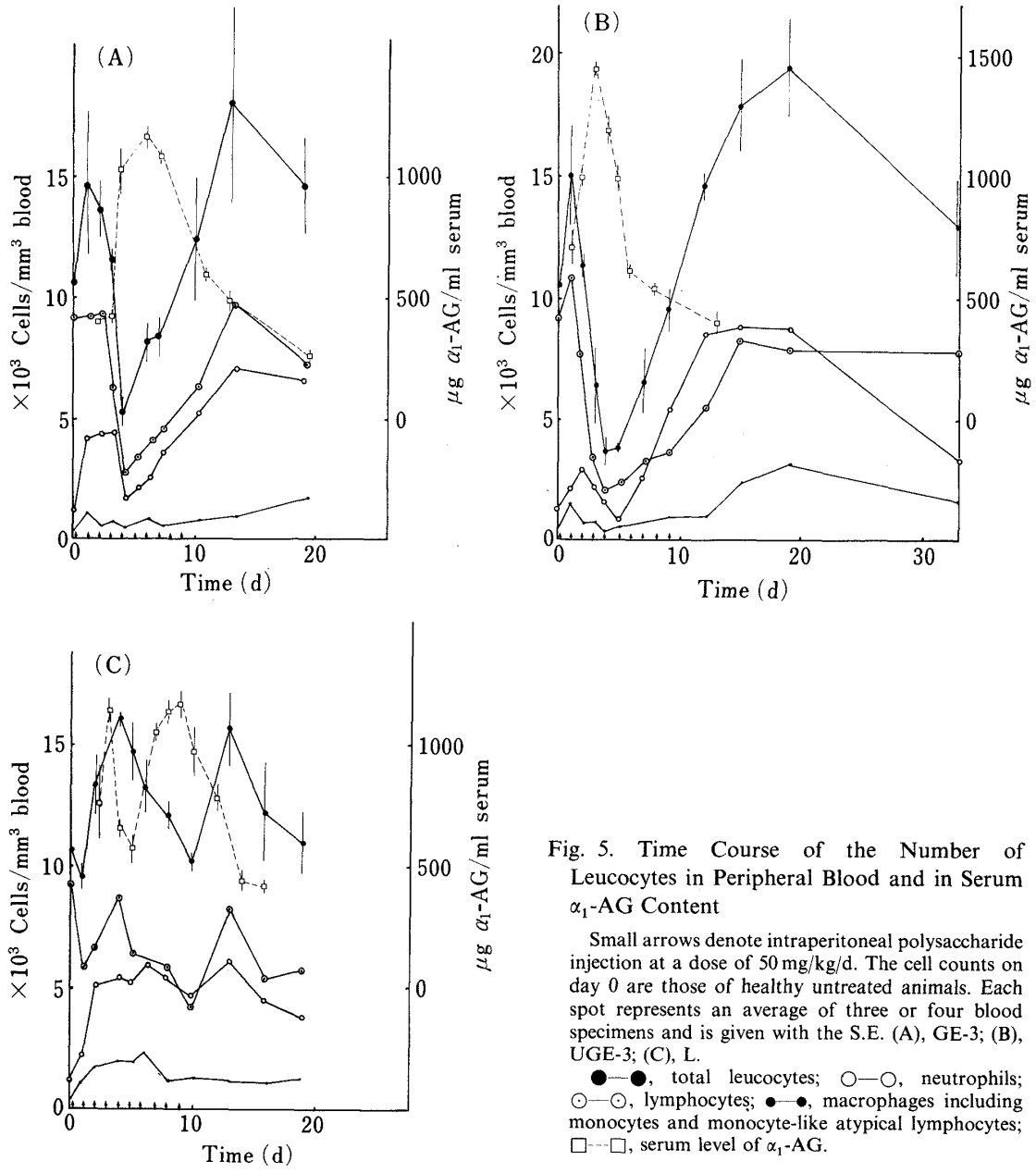

Fig. 5. Time Course of the Number of Leucocytes in Peripheral Blood and in Serum α_1-AG Content

Small arrows denote intraperitoneal polysaccharide injection at a dose of 50 mg/kg/d. The cell counts on day 0 are those of healthy untreated animals. Each spot represents an average of three or four blood specimens and is given with the S.E. (A), GE-3; (B), UGE-3; (C), L.

●—●, total leucocytes; ○—○, neutrophils; ⊙—⊙, lymphocytes; •—•, macrophages including monocytes and monocyte-like atypical lymphocytes; □--□, serum level of α_1-AG.

method,[10] hexose content by the phenol–sulfuric acid method,[11] amino sugar content by the 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) method,[12] and protein content by the micro biuret method.[13] For amino acid analysis, protein was hydrolyzed with 6 N HCl in a sealed tube at 110 °C for 20 h.

α_1-AG-1 and α_1-AG-2 induced rabbit antiserum of the same immunological characteristics and about the same titer (about 8—16 according to the Ouchterlony technique), and reacted with either of the antisera to produce an identical precipitin line, showing that they possess the same antigenic determinants.

Discussion

Intraperitoneally administered ALP produced liver cell necrosis immediately. The necrosis induced infiltration of neutrophils to form microabscesses which subsequently took

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TABLE I. Recovery of α_1-AG during Preparation from Mouse Ascitic Fluid

Procedures	Cell-free ascitic fluid	90% $(NH_4)_2SO_4$ precipitate	DEAE cellulose chromatography	Isoelectric focusing
Recovery of α_1-AG weight	1000 ml 800 μg/ml 800 mg[a]	700 mg[a]	480 mg[a]	α_1-AG-1 35 mg α_1-AG-2 25 mg α_1-AG-3 28 mg[b] Total 88 mg
Per cent	100%	87.5%	60%	11.0%

a) Quantity estimated by the single radial immunodiffusion technique with rabbit antiserum. *b*) Non α_1-AG proteinous contaminants were detected on SDS-PAGE, and no further studies were made on the sample.

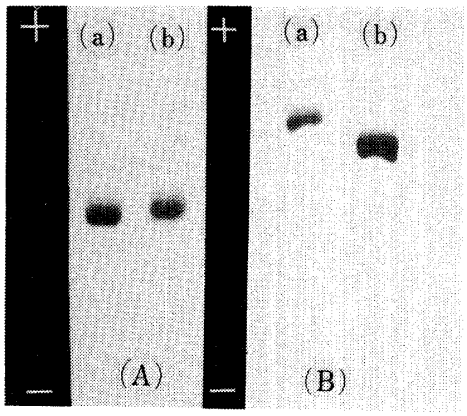

Fig. 6. (A) Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Samples were incubated with 1% SDS and mercaptoethanol at 100 °C for 3 min. (a), α_1-AG-1; (b), α_1-AG-2. Bands were stained with Coomassie Blue.

(B) Isoelectric Focusing on Polyacrylamide Gel Containing 1.2% Pharmalite, pH 2.5—5.0 (Pharmacia Fine Chemicals)

(a), α_1-AG-1; (b), α_1-AG-2.

the form of granulomatous clusters consisting of proliferating histiocytes and infiltrated mononuclear cells.

Predominant and immediate incorporation of intravenously injected [^{3}H]lentinan into liver (70%) and spleen (10%), and its selective retention in reticuloendotherial system cells were reported by Ohara.[14] Similar results were reported for yeast glucan in a review on zymosan by Fizpatrick and DiCarlo.[15] Granuloma formation in the liver of lichen polysaccharide-treated mice was noted by Tokuzen *et al.*,[16] and dose-dependent hepatosplenomegaly and granulomatous foci in the liver and spleen of rats treated with lentinan were reported.[17,18] Such pathological changes observed in mice treated with ALP are similar to those induced by the administration of lentinan or zymosan, and are apparently the result of common biological reactions to the introduction of poorly metabolizable polysaccharides, as suggested by Ohara.[14]

Another marked change induced by the administration of ALP was an immediate and marked decrease in the number of leucocytes in circulation, involving both neutrophils and lymphocytes. The leukopenia was then followed by leukocytosis. Administration of ALP also increased the serum content of an acidic serum glycoprotein having IEP 3.0—3.5, or mouse α_1-AG. The peak of α_1-AG content always preceded leucocytosis as shown in Fig. 5.

Human α_1-AG is a well-defined acute-phase serum glycoprotein[8,19] having characteristic IEP and chemical composition, comprising several fractions with similar IEP and identical antigenic determinants.[20] Components similar to human α_1-AG (having corresponding IEP and chemical characteristics) have been isolated from the serum of several species of animals.[21,22] However, there are few papers describing mouse α_1-AG. Normal mouse serum contains α_1-AG having IEP 3.0—3.5 in the range of 200—300 μg/ml, with little intra- or inter-individual variation. Subcutaneously implanted Ehrlich carcinoma had little effect on serum α_1-AG level before it grew to 500 mm^3.

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TABLE II. Characterization of Mouse α_1-AG

(A)

	M_r[a)]	IEP	Sialic acid[9)]	Hexose[10)]	Amino[11)] sugars	Protein[12)]
α_1-AG-1	40000	3.2	12.5% (16)	12.2% (27)	11.4% (25)	64.0%
α_1-AG-2	35300	3.4	11.4% (13)	15.1% (30)	12.0% (23)	61.6%

(B)

AA	Amino acid (AA) composition residues/mol α_1-AG-1	residues/mol α_1-AG-2
Asp	12	12
Thr	18	14
Ser	10	9
Glu	29	16
Gly	11	12
Ala	8	7
Val	8	6
Cys	1	1
Met	3	4
Ileu	10	8
Leu	18	17
Tyr	5	4
Phe	13	13
Lys	18	17
His	6	5
Arg	9	9
Pro	6	5

a) Molecular weight was estimated by the sucrose density-gradient centrifugation technique using bovine serum albumin, chymotrypsinogen A, ovalbumin and myoglobin as reference proteins. The molecular weights of α_1-AG-1 and α_1-AG-2 based on the mobility in SDS-PAGE are 40000 and 38000, respectively, and those based on the analytical data are 39500 and 34200, respectively. Figures in parentheses refer to the number of residues per mol of glycoprotein.

As regards human α_1-AG, micro-heterogeneity or differences in chemical composition were reported among α_1-AG samples from healthy individuals and from neoplastic patients[23-28)] and samples from different organs or tissue specimens.[29,30)] Differences in their functions have also been proposed.

In the present experiment, mouse α_1-AG was separated into α_1-AG-1 with IEP 3.2 and α_1-AG-2 with IEP 3.4 by preparative isoelectric focusing. Mouse α_1-AG-1 and α_1-AG-2 differ slightly from each other in chemical composition and molecular weight, but possess identical antigenic determinants (Table II). On sodium dodecyl sulfate polyacrylamide gel electrophoresis α_1-AG-1 and α_1-AG-2 each produced a single band with a slight difference in the mobility (Fig. 6(A)), but on polyacrylamide gel isoelectric focusing each fraction apparently contained more than one band (Fig. 6(B)).

A possible tumor-suppressing effect of α_1-AG is suggested by the changes in tumor size in ALP-treated mice and in the serum level of α_1-AG. Tumor growth curves in ALP-treated mice apparently consisted of two phases, as shown in Fig. 1, the first phase being clearly related to the serum level of α_1-AG. Administration of UGE-3 or L produced a rapid increase in the

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serum α_1-AG level (Fig. 5(B) and (C)) and an immediate decrease in the tumor size (Fig. 1(C)). In UGE-3-treated mice, the serum α_1-AG level showed only a very transient increase, and a renewed increase in the size of tumor was observed on day 5 or 6 when the serum level of α_1-AG had decreased. In L-treated mice, the first transient α_1-AG peak was closely followed by the second peak, and the renewed increase in tumor size was not as pronounced as in UGE-3 treated mice. In GE-3-treated mice, the increase in α_1-AG level was slightly delayed (Fig. 5(A)), and the decrease in tumor size was also delayed. However, in this case, the higher α_1-AG level was maintained for a longer period than in UGE-3-treated mice, and renewed increase in the tumor size as observed in UGE-3- or L-treated mice was avoided.

Human α_1-AG is known to be abundantly produced by actively multiplying cells, such as human breast epithelial cells,[31] or normal human lymphocytes, granulocytes and monocytes, especially by activated and proliferating lymphocytes.[32] Higher titers in the serum of cancer patients were also reported, and the clinical and diagnostic significance of this was discussed.[33-35]

As to the biological function of α_1-AG, a number of papers have referred to the immunosuppressive effect of the glycoprotein. For example, suppression of immunoglobulin (Ig) production, especially of IgM, by α_1-AG,[36,37] suppression of spleen cell response to Con A and lipopolysaccharide,[38] and inhibition of mitogen-induced lymphoproliferation by α_1-AG[39] were demonstrated.

According to Onda and Yoshikawa[40-42] α_1-AG is a hepatocyte-specific primary mitotic inhibitor. The cell-division of adult stage hepatocytes is inhibited by intracellular α_1-AG accumulated over a critical concentration, and the excretion of α_1-AG from the hepatocytes, resulting in a decrease of the intracellular concentration of the primary mitotic inhibitor below a critical level, allows the cell-division of hepatocytes. As mentioned above, various cells including leucocytes and tumor cells are known to produce α_1-AG, and α_1-AG may function as a mitotic inhibitor to all of these cells. Immunosuppression may be a result of suppression of leucocyte multiplication by α_1-AG.

In ALP-treated mice the drastic increase in serum α_1-AG level on administration of ALP may be explained as follows. A sudden decrease in the number of leucocytes in circulation on administration of ALP gives rise to a multiplication signal to relevant cells, and those leucocytes or their precursors receiving the signal release their intracellular cell division suppressor, α_1-AG, before they start multiplication. When the number of cells involved is very large, α_1-AG would be produced in quantity and its serum level would be very high.

As suggested by Onda,[41,42] a lower α_1-AG level in the environment facilitates excretion of intracellular α_1-AG with α_1-antitrypsin as a secondary factor to encourage cell division, whereas a higher environmental α_1-AG level suppresses it and suppresses the subsequent cell division. α_1-AG functions analogously as a mitotic regulator for Ehrlich's carcinoma cells. An unusually high α_1-AG level in the circulation induced by the administration of ALP inhibits the division of tumor cells to suppress their growth.

TABLE III. Effect of α_1-AG on the Growth of L1210

	μg α_1-AG/ml medium	Growth rate (%)
α_1-AG-1	0 (Control)	100
	100	108.6
	200	62.6
	400	19.7
	800	15.6
α_1-AG-2	2000	53.2

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This assumption was supported by the fact that α_1-AG-1 inhibited the growth of L 1210 cells *in vitro* at concentrations above 200 μg/ml. The content of α_1-AG-1 in biological fluids could not be individually determined quantitatively, but polyacrylamide gel isoelectric focusing showed that the serum of ALP-treated mice contained a sufficient amount of α_1-AG-1 for the suppression of tumor cell growth. Therefore, it is probable that α_1-AG-1 plays a significant role in the anti-tumor activity of ALP in mice.

The role of α_1-AG in the antitumor activity of ALP in the later stage, leading to complete regression of the tumor, is not clear. Suppression of the cell growth by α_1-AG in the earlier stage may affect the cell growth in the later stage, or may facilitate the normal immunological attack on the cells, or totally different mechanisms may be involved.

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