Identification of a Serum Glycoprotein Whose Content is Increased in PSK-Treated Mice as Haptoglobin

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A serum glycoprotein whose content is increased in mice by the administration of an antitumor agent, PSK, and which migrates to the so-called LC region on polyacrylamide gradient gel electrophoresis, was purified by repeated ion-exchange column chromatography on DEAE-Sephadex A-50, chromatofocusing and gel filtration.

The purified protein was identified as mouse haptoglobin, since its chemical composition, isoelectric point (4.1), molecular weight (94000) and subunit structure were found to be very similar to those of human haptoglobin. Furthermore, the purified protein formed a complex with hemoglobin and increased the peroxidase activity of hemoglobin.

Keywords——serum protein; haptoglobin; mouse; PSK

Maeda *et al.* reported[1] a marked increase of three serum components (LA, LB and LC) in serum of mice to which an antitumor polysaccharide, lentinan, had been administered. Later, Mizuno reported[2] that there was a close relationship between the increase of protein components after administration of various polysaccharides and their antitumor activity of the polysaccharides in most cases.

We have already identified purified LB as mouse hemopexin[3] and shown that it exhibits growth inhibitory activity against sarcoma-180 cells *in vivo*[4] and an enhancing effect on tumor cell killing by OK-432-elicited macrophages *in vitro*.[5] We also identified LA as mouse ceruloplasmin.[6] This serum glycoprotein was also found to have growth inhibitory activity against sarcoma-180 *in vivo*.[7] Furthermore, studies on the mechanism of the antitumor activity of ceruloplasmin revealed that the glycoprotein has a neutralization effect against various toxohormone-like activities of the basic protein isolated from Ehrlich carcinoma cells[7] and a restorative effect on the decrease of both delayed hypersensitivity and helper T-cell activity in sarcoma-180 bearing mice, and moreover, it enhanced alloreactive cytotoxic cells *in vitro*.[8]

In this paper, we report the purification and characterization of LC.

Materials and Methods

Mice——Female ICR-JCL mice (CLEA Japan Inc., Tokyo), 7 to 9 weeks old, were used in this study.

Antitumor Agents——PSK is a protein-bound polysaccharide obtained from Basidiomycetes, and it was kindly supplied by Kureha Chemical Industries Co., Ltd. (Tokyo). Five percent PSK in saline was prepared for injection.

Tumor Cells——Sarcoma-180, a transplantable sarcoma, was used throughout this study. The tumor cells were passaged weekly in the peritoneal cavity of ICR mice. These cells were washed three times with saline and used for transplantation.

Preparation of Serum from Mice Injected with PSK——ICR-JCL mice injected *i.p.* with PSK were bled from the heart and the blood samples were allowed to clot at room temperature for 1 to 2 h. The serum was obtained by

centrifugation at 2000 rpm for 5 min.

Polyacrylamide Gradient Gel Electrophoresis——Four to 25% polyacrylamide gradient gels were run in 0.1 M Tris-borate buffer, pH 8.35, containing 25 mM ethylene diamine tetracetic acid (EDTA) for 15 h at 125 V on a GE-4 gel electrophoresis apparatus (Pharmacia, Uppsala, Sweden). The gels were stained for 45 min in 1% Amido Black 10 B in 7.5% acetic acid or for 2 h in 0.1% Coomassie brilliant blue G-250 in 50% trichloroacetic acid. They were destained in 7.5% acetic acid.

Staining Solution for the Haptoglobin–Hemoglobin Complex (Hp–Hb)——Benzidine reagent was prepared by adding 1 ml of 5% hydrogen peroxide to 30 ml of a saturated solution of benzidine in glacial acetic acid according to the method of Neal et al.[9] When a gel was soaked in this reagent, an intense blue color developed within a few seconds in the presence of hemoglobin (Hb) or any heme-containing derivative. This blue color changed within the next few minutes to blue-black and the initially white background became light brownish-yellow.

Leuco malachite green reagent was prepared by mixing 1 g of Leuco malachite green in 100 ml of acetic acid, 150 ml of H_2O and 5 g of zinc powder followed by boiling until the solution became colorless. When a gel was soaked in this reagent for 10 min and then in 2% hydrogen peroxide, a green color developed in the presence of peroxidase.

Assay Procedures——Total protein was determined by the method of Lowry et al.[10] Amino acids were determined with a Hitachi model 835 amino acid analyzer according to the method of Spackman et al.[11] after hydrolysis of protein for 24 h with 6 M HCl at 110 °C in a sealed, evacuated tube. Sialic acid was determined by the resorcinol method of Jourdian et al.[12] Individual neutral sugars were determined by gas liquid chromatography after reduction to the corresponding alditols, followed by acetylation according to the method of Spiro,[13] hydrolysis being performed with 2 N HCl at 100 °C for 4 h. Amino sugars were determined with a Hitachi model 835 amino acid analyzer after hydrolysis at 100 °C for 5 h with 4 N HCl.

Isoelectric Point Estimation——Isoelectric point estimation by isoelectric focusing was carried out using a Pharmacia analytical flat bed IEF kit with 4.5% polyacrylamide gel.

Molecular Weight Determination——The molecular weight was estimated by sedimentation equilibrium analysis using a Beckman-Spinco model E analytical ultracentrifuge equipped with an electronic scanner at 8700 and 12500 rpm at 20 °C in 10 mM sodium phosphate buffer, pH 7.3, containing 0.15 M NaCl. The protein concentration was varied from OD_{280}^{1cm} 0.10—0.55. The partial specific volume was calculated to be 0.72 ml/g from the chemical composition. The molecular weight of the subunit of the protein after treatment with 2-mercaptoethanol was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis by comparing migration distances with those of standard proteins (obtained from a Pharmacia electrophoresis calibration kit for high molecular weight compounds).[14]

Quantitative Estimation of Haptoglobin——For quantitative estimation of haptoglobin, the peroxidase activity of the sample was measured after addition of a standard amount of human methemoglobin by the method of Owen et al.[15]

Results

Purification of LC

LC-rich sera were prepared from ICR-JCL mice which had been injected i.p. with 12.5 mg/mouse PSK once. The sera were generally obtained from blood 24 h after PSK injection. These conditions were found to be best for obtaining serum which was richest in LC in the cases of all antitumor drugs, PSK, lentinan and OK-432. At the first step, about 75 ml of LC-rich serum which had been dialyzed against the starting buffer was applied to a DEAE-Sephadex A-50 column (5.0 × 60 cm) equilibrated with 0.1 M Tris-HCl buffer, pH 8.3, and then the column was eluted with a linear NaCl gradient, formed from 2 l of 0.1 M Tris-HCl buffer, pH 8.3, in the mixing vessel and 2 l of the same buffer containing 0.25 M NaCl in the reservoir (Fig. 1). Fractions of 19 ml were collected and pooled as indicated in Fig. 1. Polyacrylamide gradient gel electrophoresis of the pooled fractions revealed the presence of a protein band, designated as LC, in fractions b, e, h and i (Fig. 2). The most LC-rich fraction among these four was fraction h, so we further purified LC from this fraction. Thus, fraction h was concentrated on an ultrafilter (Tokyo Ultrafilter UK-20), and dialyzed against the starting buffer.

Then the concentrated fraction h was applied to a DEAE-Sephadex A-50 column (3.2 × 40 cm) equilibrated with 17.8 mM sodium phosphate buffer, pH 6.7, containing NaCl ($\mu=0.2$) and eluted with a gradient formed from 1.0 l of 17.8 mM sodium phosphate buffer, pH 6.7, containing NaCl ($\mu=0.2$) in the mixing vessel and 1.0 l of 31.0 mM sodium
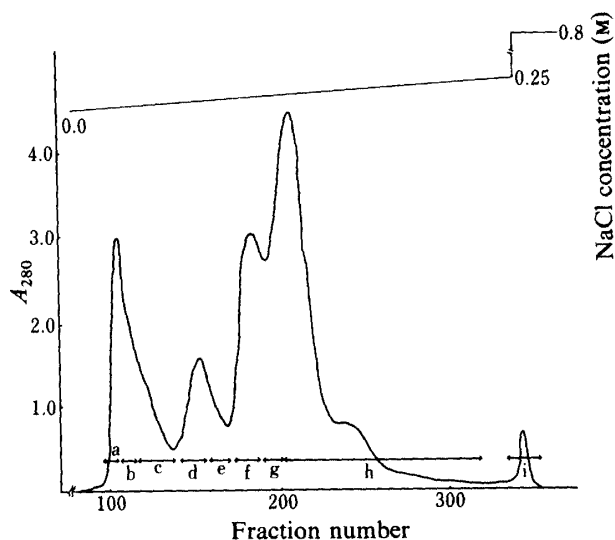
Fig. 1. Ion Exchange Chromatography of PSK-Treated Mouse Serum on a Column of DEAE-Sephadex A-50 (the First Step)
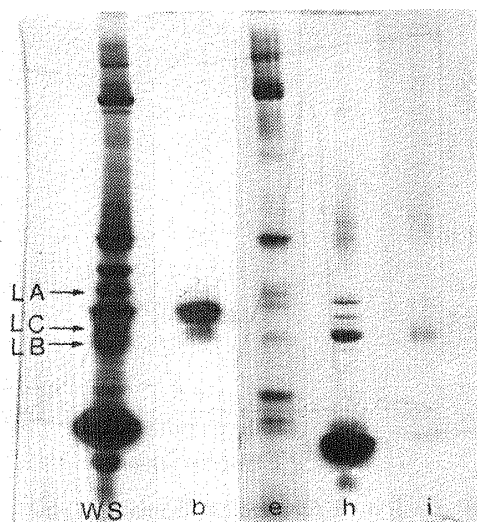
Experimental details are given in the text.

Fig. 2. Polyacrylamide Gradient Gel Electrophoresis of Serum Sample and Fractions in Fig. 1

WS: Whole serum from mice injected with PSK. b: Fraction b in Fig. 1. e: Fraction e in Fig. 1. h: Fraction h in Fig. 1. i: Fraction in Fig. 1.

phosphate buffer, pH 6.0, containing NaCl ($\mu = 0.2$) in the reservoir, followed by elution with a linear NaCl gradient formed from 1.0 l of 31.0 mM phosphate buffer, pH 6.0, containing NaCl ($\mu = 0.2$) in the mixing vessel and 1.0 l of the same buffer, containing NaCl ($\mu = 0.4$) in the reservoir (Fig. 3; the second step of purification). Fractions of 15 ml were collected. After concentration of a part of every 5—10th fraction on a Centriflo (Amicon CF 25), the concentrates were subjected to polyacrylamide gel electrophoresis on a gradient gel. It was found that fractions 180 to 258 contained LC (Fig. 6). These fractions were pooled as indicated in Fig. 3, concentrated on an ultrafilter and dialyzed against the starting buffer.

The concentrated fraction was applied again to a DEAE-Sephadex A-50 column (3.2 × 40 cm) equilibrated with 31.0 mM phosphate buffer, pH 6.0, containing NaCl ($\mu = 0.2$). Then the column was eluted with 500 ml of the same buffer, followed by elution with a linear NaCl gradient formed from 500 ml of 31.5 mM phosphate buffer, pH 6.0, containing NaCl ($\mu = 0.2$) in the mixing vessel and 500 ml of the same buffer, containing NaCl ($\mu = 0.4$) in the reservoir (the third step of purification). Fractions of 11.5 ml were collected. After examination of the fractions by polyacrylamide gradient gel electrophoresis, fractions 116 to 146 were pooled, concentrated in a collodion bag (Sartorius Membranfilter GmbH), dialyzed against the starting buffer and then filtered through a membrane filter (0.45 μ; Toyo Roshi) in order to remove precipitates.

The fourth step of purification was carried out by application of the above concentrated fractions to a PEB 94 chromatofocusing column (1.6 × 40 cm) (Pharmacia Fine Chemical) equilibrated with 0.025 M piperazine-HCl buffer, pH 5.3, containing 0.05 M NaCl. Elution was performed with a pH gradient formed from 960 ml of 10% poly buffer 74-HCl (Pharmacia), pH 3.6, containing 0.5% Pharmalyte, pH 2.5—5.0, and 0.05 M NaCl (Fig. 4). LC containing fractions (71—126 in Fig. 6) were pooled, concentrated in a collodion bag and filtered through a membrane filter (0.45 μ; Toyo Roshi).

Finally the concentrated fraction was applied to a Bio-Gel A-1.5 m column (1.54 × 138 cm) in 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS) (Fig. 5). LC-containing fractions (64—82 in Fig. 5) were collected and concentrated in a collodion bag. Polyacrylamide gradient gel electrophoresis (Fig. 6) of this fraction revealed the presence
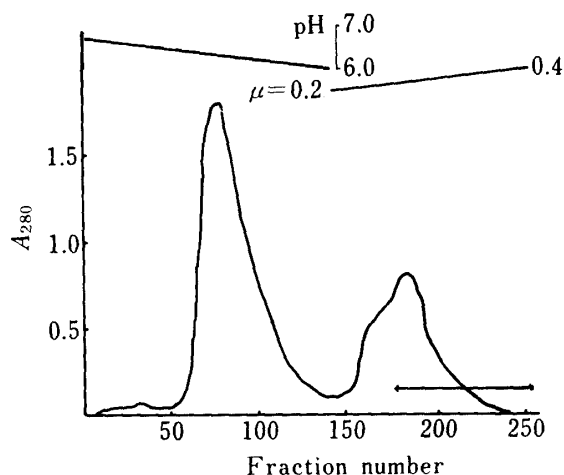
Fig. 3. Ion Exchange Chromatography of Fraction h in Fig. 1 on a Column of DEAE-Sephadex A-50 (the Second Step)

The fractions indicated by a double-headed arrow were pooled and subjected to the next purification step. Experimental details are given in the text.
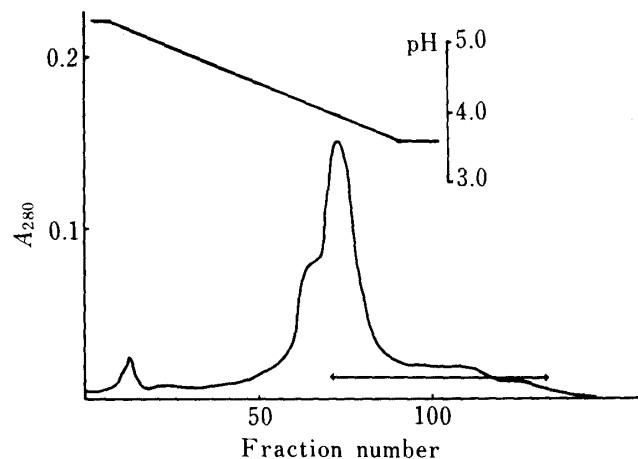

Fig. 4. Chromatofocusing of LC-Rich Fractions (No. 116—146) in Fig. 4 on a Column of PEB 94 (the Fourth Step)

The fractions indicated by a double-headed arrow pooled and subjected to the next purification step.
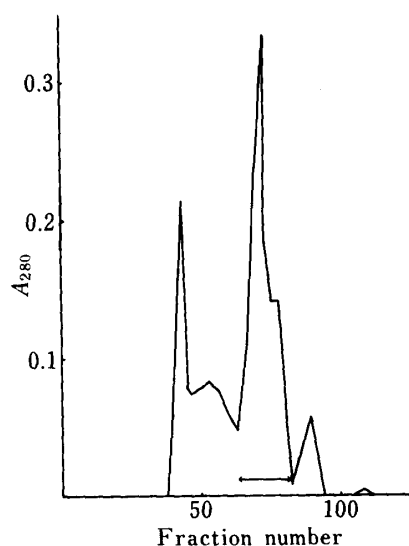

Fig. 5. Gel-Filtration of LC-Rich Fractions (No. 71—126) in Fig. 5 on a Column of Bio-Gel A-1.5 m (the Fifth Step)

The fractions indicated by a double-headed arrow were pooled and used as purified LC.
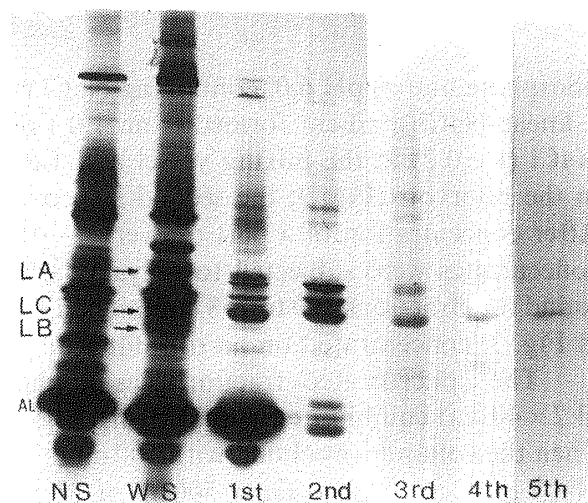

Fig. 6. Polyacrylamide Gradient Gel Electrophoresis of the LC-Rich Fraction at Each Purification Step

NS: Normal mouse serum. WS: Whole serum from mice injected with PSK. 1st—5th: LC-rich fraction at each the indicated purification step.

of LC as a single protein band.

Characterization of LC

The molecular weight of LC was estimated to be approximately 94000 by sedimentation equilibrium analysis (Table I). The isoelectric point of LC was estimated to be 4.1 by isoelectric electrophoresis (Table I). On the other hand, the molecular weight and isoelectric point of human haptoglobin 1-1 were reported to be 100000 and 4.1, respectively, by Herman–Boussier, et al.[16]

The molecular weights of the subunits of LC were estimated to be approximately 41000 and less than 10000 by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Table I).

On the other hand, the molecular weights of the subunits of human haptoglobin 1-1 were previously reported to be 40000 and 8900 by Cheftel *et al.*[17] and Connel *et al.*[18]

Chemical Analysis of LC

The results on the amino acid and carbohydrate compositions of LC as well as those of

TABLE I.   Physicochemical Properties of LC

	LC	Human haptoglobin 1-1[a]
Molecular weight	94000	100000
Molecular weights of subunits	41000 and < 10000	40000 and 8900
Isoelectric point	4.1 ± 0.03	4.1
E_{280} 1% 1 cm	11.8	12.1

a)   Values reported by Herman-Boussier *et al.*[16]

TABLE II.   Chemical Composition of LC

Constituent	g/100 g			
	LC	Human haptoglobin 1-1[a]	Rabbit haptoglobin[b]	Rat haptoglobin[b]
Lys	5.9	8.2	6.4	8.2
His	3.9	3.1	3.0	2.8
Arg	4.0	2.2	3.7	4.0
Asp	8.9	9.5	8.3	8.1
Thr	4.4	4.4	4.5	5.4
Ser	2.7	3.5	4.6	4.7
Glu	8.2	9.7	10.1	11.5
Pro	4.4	4.2	3.3	4.3
Gly	3.3	2.9	2.4	2.9
Ala	4.2	3.5	3.7	4.1
Cys	> 1.4	2.0	0.8	1.0
Val	6.3	6.3	5.1	5.3
Met	> 0.06	1.0	1.1	1.8
Ile	4.4	3.4	3.4	3.2
Leu	6.4	6.0	6.4	6.5
Tyr	4.9	5.6	5.9	4.7
Phe	2.8	2.4	3.7	3.6
Trp	3.7	2.6	2.2	3.0
Total (Amino acids)	80.6	80.5	78.6	85.1
Galactose	4.4	2.8	6.6[c]	4.7[c]
Mannose	4.6	2.8		
Glucosamine	6.1	5.3[d]	5.6[d]	4.6[d]
Galactosamine	0.3			
Sialic acid	3.7	5.3	5.4	4.4
Total (Carbohydrates)	19.3	16.4	17.9	14.4

a)   Values for amino acids were reported by Heimburger *et al.*[19] and those for carbohydrates were reported by Dobryszycka *et al.*[21]
b)   Values reported by Lombart *et al.*[20]
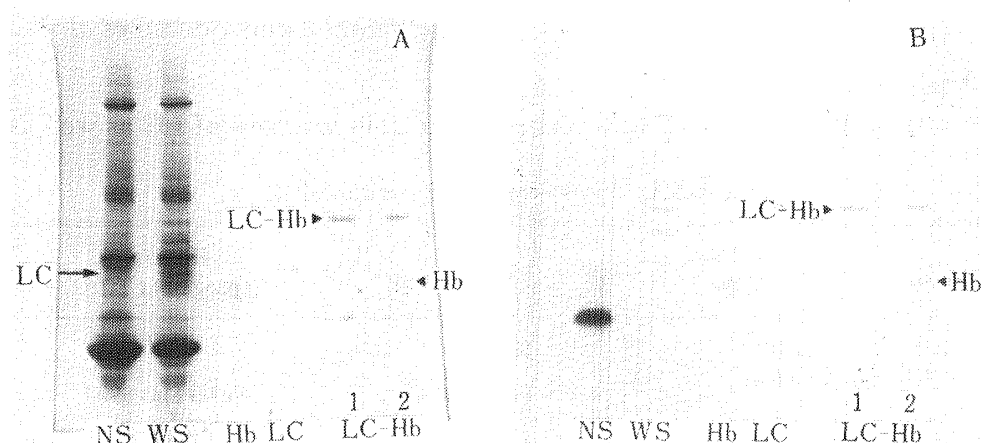c)   Content of neutral sugars.
d)   Content of hexosamines.

Fig. 7. Polyacrylamide Gradient Gel Electrophoresis of Mouse Serum and LC-Hemoglobin Complex

(A) Staining with Coomassie brilliant blue. (B) Staining with Leuco malachite green. NS: Normal mouse serum. WS: Whole serum from mice injected with PSK. Hb: Human hemoglobin. LC: Purified LC. LC–Hb 1: A mixture of LC and hemoglobin (1 : 1). LC–Hb 2: A mixture of LC and hemoglobin (1 : 2).

human, rabbit and rat haptoglobins are listed in Table II.

The most notable feature of the amino acid composition is the high contents of asparagic acid, glutamic acid and leucine. LC was found to contain 19.3% carbohydrate; galactose, mannose, glucosamine, galactosamine and sialic acid were the component sugars. This chemical composition is remarkably similar to those of human, rabbit and rat haptoglobins.

Hemoglobin Binding Capacity of LC

Sera from normal and PSK injected mice, purified LC, human hemoglobin and mixtures of LC and human hemoglobin [(1 : 1) and (1 : 2)] were subjected to polyacrylamide gradient gel electrophoresis and the gel was stained with benzidine reagent or Leuco malachite green reagent, both of which are specific for the Hp-Hb complex. In another experiment, the gel was stained with Coomassie brilliant blue for protein (Fig. 7). Since LC was not stained by the reagents specific for the Hp–Hb complex, LC itself did not have any peroxidase activity, but when LC was mixed with hemoglobin, the resulting complex moved on gradient gel electrophoresis differently from LC or hemoglobin. Furthermore, the peroxidase activity of hemoglobin appeared to be enhanced after complexing with LC, because the spot of LC-hemoglobin complex on the gel was stained more intensely than that of hemoglobin itself. The spot corresponding to the LC–Hb complex was also observed when the serum of PSK-injected mouse was subjected to gradient gel electrophoresis, but was not observed on electrophoresis of normal mouse serum.

Increase of Haptoglobin in PSK Serum

In order to confirm the increase of the serum level of haptoglobin in PSK-treated mice, the rate of increase of serum peroxidase activity was studied by measuring OD_{470}, and the results suggest that the level of haptoglobin increased in the PSK-treated mouse serum to as much as ten times that in normal mouse serum.

Discussion

LC is a protein whose content is increased in mouse serum after treatment with certain

antitumor agents, and it has been defined only by gradient gel electrophoresis;[1] the protein corresponding to the LC band on the electrophoresis has not been characterized. In this study, we fractionated the sera of PSK-injected mice by ion-exchange chromatography on a column of DEAE-Sephadex A-50 and found that the fraction which corresponded to LC on electrophoresis was actually composed of four kinds of proteins.

We purified and characterized one of these four proteins which showed the maximum increase in level in the PSK-treated mouse serum (fraction h, Fig. 1) and designated it as purified LC in this paper. The isoelectric point of the purified LC was 4.1 and its molecular weight was estimated to be 94000. These physicochemical properties are quite similar to those of human Hp 1-1 reported by Herman–Boussier et al.[16] Furthermore, comparison of the chemical composition and the subunit structure of the purified LC with those of human Hp 1-1 also indicated the possibility that the purified LC is mouse haptoglobin. Since haptoglobin is known to form a complex specifically with hemoglobin, LC was tested to determine whether it forms a higher molecular weight complex with hemoglobin, and we found the formation of an LC-hemoglobin complex which moved differently on electrophoresis from LC itself. Moreover, the peroxidase activity of hemoglobin appeared to be increased after the complex formation with the purified LC. These results are in good agreement with the properties reported for the haptoglobin–hemoglobin complex[22] and suggest that the purified LC is actually mouse haptoglobin.

The biological significance of the increase of haptoglobin concentration in serum is not yet known. Nakahara and Fukuoka[23,24] reported that depression of catalase activity in the liver and a decrease of hemoglobin concentration in serum can generally be seen in tumor-bearing animals. We also observed[7] a decrease of the plasma iron level in mice which had been administered the basic protein from Ehrlich carcinoma cells. These decreases of the plasma iron level and the catalase activity in liver tumor-bearing animals may result from the rapid breakdown of hemoglobin in these animals. However, since haptoglobin binds with hemoglobin, it might prevent the breakdown of hemoglobin and thus maintain the plasma iron level. Thus, the increased level of haptoglobin in serum might lead to the normalization of the utilization of iron in tumor-bearing animals.

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