IDENTIFICATION OF A MOUSE SERUM PROTEIN INCREASED BY ADMINISTRATION OF AN ANTITUMOR POLYSACCHARIDE, PSK, AS A VARIANT OF MOUSE TRANSFERRIN AND SOME OF ITS BIOLOGICAL ACTIVITIES

EISUKE KOJIMA, TOHRU MITSUNO, AND TOSHIKAI OSAWA

Division of Chemical Toxicology and Immunology, Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo, 113, Japan

(Received June 5, 1985)

One of the so-called LC components, the content of which is increased in the serum of PSK (antitumor polysaccharide) treated mice, was purified by repeated ion-exchange column chromatography on DEAE-Sephadex A-50.

The purified protein, designated as LC-2, was identified as a variant of mouse transferrin, which is a serum β2-globulin having an iron-binding capacity. The absorption spectrum and ultraviolet circular dichroism (CD) curve of LC-2 were identical with those of mouse transferrin. The molecular weight, isoelectric point and amino acid composition of LC-2 were in good agreement with the results obtained previously for mouse transferrin. However, the carbohydrate content of LC-2 was different from that of mouse transferrin.

This protein restored the depressed spleen cell response of tumor-bearing mice to concanavalin A (Con A) and promoted the metabolism of proteose-peptone induced peritoneal macrophages obtained from tumor-bearing mice. Furthermore, it had weak but definite antitumor activity against Sarcoma 180 cells in vivo.

Keywords: mouse transferrin; mouse transferrin variant; antitumor activity; antitumor polysaccharide PSK

INTRODUCTION

PSK and lentinan, antitumor polysaccharides, were found to suppress the growth of certain lines of transplantable tumor cells.1-3) Furthermore, it has been shown that there is a marked increase in three kinds of serum proteins after i.p. administration of these polysaccharides to mice. The three serum protein components were separated by polyacrylamide gradient gel electrophoresis and designated as LA, LB and LC. Moreover, there was a clear parallel between the antitumor activity of the polysaccharides and this unique increase in these serum protein components.4)

We have already identified the major components of LA, LB and LC as mouse ceruloplasmin, hemopexin and haptoglobin, respectively.5-7) We have also reported that these serum proteins actually represent a part of the effector molecules in the tumor-defense mechanism.8-12)

In a previous paper,7) we showed that the fraction which corresponded to LC by electrophoresis was composed of four kinds of proteins. Furthermore, we have purified one of these four proteins (LC-1 in this paper) which showed a maximum increase in level in PSK treated mouse serum and have identified it as haptoglobin.

In this study, we have identified one of the remaining three LC components (LC-2) as a variant of transferrin and have investigated some of its biological activities.

MATERIALS AND METHODS

Mice — Female ICR;JCL mice (CLEA Japan, Inc., Tokyo, Japan), 7-9 weeks old, and female DBA/2 mice (Charles River Japan Inc., Kanagawa, Japan), 8-12 weeks old, were used in this study.

Tumor Cells — Sarcoma 180 and P815 cells were maintained as an ascites form in ICR and DBA/2 mice, respectively, by serial transplantation, and were used for tumor inoculation.

Preparation of LC Rich Serum — The detailed procedure for the preparation of LC rich serum was reported previously.7)

Polyacrylamide Gradient Gel Electrophoresis — Four to 25% polyacrylamide gradient gels were used with 0.1 M Tris-borate buffer, pH 8.5, containing 25 mM ethylenediamine tetracetic acid for 15 h at 125 V in a GE-4 gel electrophoresis apparatus (Pharmacia, Uppsala, Sweden). The gels were stained for 2 h in 0.1% Coomassie brilliant blue G-250 in 50% trichloro-
roacetic acid, and then destained in 7.5% acetic acid.

**Absorption Spectrophotometry** — Ultraviolet and visible spectra of LC-2 were recorded with a Hitachi model 200-10 spectrophotometer with a Hitachi 056 recorder.

**Circular Dichroism (CD) Measurements** — The CD spectra of LC-2 were obtained with an automatic recording spectropolarimeter (J-40A, JASCO, Tokyo, Japan) at 25 °C. Samples were examined in 1-cm fused quartz cells at a concentration with which the dynode voltage could be maintained below 400 V.

**Molecular Weight Determination** — The molecular weight was determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis according to the method of Laemmli. The following molecular weight standards (Pharmacia) were used: Phosphorylase b (94000), bovine serum albumin (67000), ovalbumin (45000), carbonic dehydratase (30000) soybean trypsin inhibitor (20100) and α-lactalbumin (14400). The gels were stained with 0.2% Coomassie Blue R-250 and destained with 10% acetic acid.

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

**Assay Procedures** — Protein was measured by the method of Lowry et al. The amino acid composition was determined with a Hitachi model 835 amino acid analyzer according to the method of Spackman et al., after hydrolysis of the protein for 24 h with 6 N HCl at 110 °C in a sealed, evacuated tube. Sialic acid was determined by the resorcinol method of Jourdian et al. Neutral sugars were determined by gas liquid chromatography after reduction to their corresponding alditols, followed by acetylation according to the method of Spiro, 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.

**Concanavalin A (Con A) Response** — The Con A response of spleen cells from P815 bearing mice was measured as described previously in the presence of LC-2.

**Glucose Consumption of Proteose-Peptone Induced Macrophages** — The glucose content of culture supernatants of PP-macrophages (proteose-peptone induced macrophages) from normal or P815 bearing mice was determined with a “Glucose B-Test-Wako” kit (Wako Pure Chemical Industries, Co., Osaka, Japan) as described previously in the presence of LC-2.

**Antitumor Polysaccharides** — PSK, a protein-bound polysaccharide obtained from bacidiomycetes, was kindly supplied by Kureha Chemical Industries, Co. (Tokyo, Japan). Lentinan, a β-(1→3)-glucan isolated from a popular edible mushroom grown in Japan, *Lentinus edodes* (Berk) Sing., was kindly supplied by Ajinomoto Co.

**Evaluation of Antitumor Activity** — The antitumor activity of LC-2 was studied in vivo. Washed Sarcoma 180 cells (2×10⁶) were inoculated subcutaneously into the right groin of ICR mice on day 0. LC-2 or mouse hemopexin (600 μg/mouse) was injected intravenously on days 1–5. Lentinan (300 μg/mouse) was injected intraperitoneally on days 1 and 5. Tumors were extirpated and weighed on day 28.

**RESULTS**

**Purification of LC-2**

LC rich sera were prepared from ICR mice which had been injected intraperitoneally with 12.5 mg PSK/mouse once. The sera were generally obtained from blood 24 h after the PSK injection. As the first step, about 15 ml of LC rich serum, which had been dialyzed against the starting buffer, was applied to a DEAE-Sephadex A-50 column (4.5×40 cm) equilibrated with 0.1 M Tris–HCl buffer, pH 8.3, and the column was eluted with a linear NaCl gradient, formed from 1 l of 0.1 M Tris–HCl buffer, pH 8.3, in the mixing vessel and 1 l of the same buffer containing 0.2 M NaCl in the reservoir. Fractions of 13.5 ml were collected and pooled as indicated in Fig. 1. As shown in Fig. 2, polyacrylamide gradient gel electrophoresis of the pooled fractions revealed that LC was contained in four fractions (a, b, c and d). As judged from the intensities of the bands corresponding to LC by electrophoresis, we estimated that the LC-contents of these fractions were in the order of c, a, b and d. Since LC in fraction c (LC-1) had already been purified and identified as mouse haptoglobin, we attempted to purify LC in fraction a (LC-2). Actually the increase of LC-2 in sera of PSK-treated mice was ascertained by comparison of the electrophoretic pattern of fraction a obtained from
PSK-treated mice with that of fraction a obtained from normal mice.

The concentrated fraction a was dialyzed against the starting buffer and then applied to a DEAE-Sephadex A-50 column (3.6 × 48 cm) equilibrated with 0.1 M Tris-HCl buffer, pH 8.3. The column was eluted with a linear NaCl gradient, formed from 1 l of the buffer described above in the mixing vessel and 1 l of the same buffer containing 0.2 M NaCl in the reservoir. Fractions of 17 ml were collected (Fig. 3). Examination of the fractions by polyacrylamide gradient gel electrophoresis revealed that fractions 88 to 92 contained a single protein band of LC-2 (Fig. 4). These fractions were used as purified LC-2.

Purification of Mouse Transferrin

Since human transferrin was reported to migrate between hemopexin and ceruloplasmin on polyacrylamide gradient gel electrophoresis, the protein corresponding to an intense band, which moved just ahead of LC-2 in Fig. 2 and did not increase even after administration of PSK, was isolated by pooling the fractions (Nos. 59–72) obtained on DEAE-Sephadex A-50 column chromatography (Fig. 3). The pooled fractions showed a single protein band on electrophoresis (Fig. 4) and were used as standard mouse transferrin in this study.
Characterization of LC-2

The absorption spectrum of LC-2 was found to have a faint shoulder at 470 nm. This spectrum is similar to that of mouse transferrin (Fig. 5). Furthermore, the CD spectrum of LC-2 was almost identical with that of mouse transferrin (Fig. 6).

As shown in Table I, the molecular weight of LC-2 was estimated to be approximately 68000 by SDS polyacrylamide gel electrophoresis. Reduction of LC-2 with 2-mercaptoethanol indicated that this protein was composed of a single polypeptide chain (data not shown). The isoelectric point of LC-2 was estimated to be 5.3 by isoelectric electrophoresis. The iron content of LC-2 was determined to be 0.1 μg/mg protein or 0.16 atoms/mol by the bathophenanthroline direct method using a Fe B-Test Wako (Wako). Except for the iron content of LC-2, all the results were in agreement with those for mouse transferrin.

Chemical Analysis of LC-2

The results of the amino acid and carbohydrate compositions of LC-2, as well as those of mouse, rat and human transferrins, are listed in Table II. The most notable features of the amino acid composition of LC-2 are the high contents of aspartic acid, glutamic acid, leucine and lysine. The major characteristics of the amino acid composition of LC-2 are in good agreement with those of the mouse, rat and human transferrins. Furthermore, LC-2 was found to contain 6.0% carbohydrate with galactose, mannose, glucosamine and sialic acid as component sugars. Although a small amount of

![Near-Ultraviolet and Visible Spectra of LC-2](image5.png)

![CD Spectra of LC-2 (A, 200—260 nm; B, 250—320 nm)](image6.png)

**TABLE I. Physicochemical Properties of LC-2**

<table>
<thead>
<tr>
<th></th>
<th>LC-2</th>
<th>Mouse transferrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>68000</td>
<td>68000</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>5.3</td>
<td>5.4</td>
</tr>
<tr>
<td>Iron content</td>
<td>0.10</td>
<td>0.37</td>
</tr>
<tr>
<td>μg/mg protein</td>
<td>0.16</td>
<td>0.60</td>
</tr>
<tr>
<td>atoms/mol</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
galactosamine was found in mouse transferrin, this amino sugar was not detected in LC-2. The contents of all component sugars were lower than those in mouse transferrin.

With the Ouchterlony two-dimensional diffusion technique, goat antiserum raised against mouse serum showed a single precipitin line with LC-2, and this line completely fused with that formed between anti-mouse serum and mouse transferrin (data not shown). After removal of iron ions from LC-2 by Aisen’s method, the protein was colorless. Furthermore, it was found that the apo-LC-2 bound 2.05 iron atoms per protein molecule as determined by Roop’s method. These results confirm that LC-2 is a variant of mouse transferrin.

Restoration of the Depressed Con A Response of Spleen Cells from P815 Bearing Mice

Twelve days after subcutaneous inoculation of $2 \times 10^6$ P815 cells, spleens were removed and a cell suspension was prepared as described previously. The cells were cultured with LC-2 and Con A (2.5 $\mu$g/ml) for 42 h and then labeled with 0.5 $\mu$Ci [3H]-TdR for the final 18 h culture. As shown in Fig. 7, the response of spleen cells obtained from tumor-bearers to Con A was much weaker than that of spleen cells from normal controls. Addition of LC-2 to the culture restored the depressed Con A response of spleen cells of tumor-bearers to an almost normal level.

**Activation of Metabolism of PP-Macrophages by LC-2**

Twelve days after subcutaneous inoculation of P815 (2 $\times 10^6$ cells/mouse), DBA/2 mice were injected intraperitoneally with 1.5 ml of a 10% proteose-peptone (PP) solution. Four days after the PP injection, peritoneal exudate cells were harvested and cultured with LC-2 for 24 h. A 10 $\mu$l aliquot of the culture supernatant was removed and the glucose content was determined. As shown in Fig. 8, PP-macrophages from the tumor-bearing mice showed a marked decrease in glucose consumption when compared with that of PP-macrophages from normal mice. This depressed glucose consumption of PP-macrophages from tumor-bearing mice could be

---

**TABLE II. Chemical Composition of LC-2**

<table>
<thead>
<tr>
<th>Constituent</th>
<th>LC-2</th>
<th>Mouse transferrin</th>
<th>Rat serum transferrin&lt;sup&gt;a)&lt;/sup&gt;</th>
<th>Human serum transferrin&lt;sup&gt;b)&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>11.2</td>
<td>11.3</td>
<td>14.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Thr</td>
<td>5.3</td>
<td>5.8</td>
<td>6.5</td>
<td>3.9</td>
</tr>
<tr>
<td>Ser</td>
<td>5.8</td>
<td>7.5</td>
<td>6.2</td>
<td>4.4</td>
</tr>
<tr>
<td>Glu</td>
<td>12.0</td>
<td>11.0</td>
<td>13.7</td>
<td>10.7</td>
</tr>
<tr>
<td>Gly</td>
<td>5.0</td>
<td>5.3</td>
<td>6.7</td>
<td>3.6</td>
</tr>
<tr>
<td>Ala</td>
<td>6.0</td>
<td>5.0</td>
<td>7.6</td>
<td>5.3</td>
</tr>
<tr>
<td>Cys/2</td>
<td>1.1</td>
<td>1.2</td>
<td>5.3</td>
<td>5.3</td>
</tr>
<tr>
<td>Val</td>
<td>6.8</td>
<td>6.3</td>
<td>7.2</td>
<td>5.7</td>
</tr>
<tr>
<td>Met</td>
<td>1.7</td>
<td>1.4</td>
<td>1.0</td>
<td>1.4</td>
</tr>
<tr>
<td>Ile</td>
<td>3.9</td>
<td>4.1</td>
<td>4.0</td>
<td>2.2</td>
</tr>
<tr>
<td>Leu</td>
<td>10.2</td>
<td>10.9</td>
<td>11.2</td>
<td>8.6</td>
</tr>
<tr>
<td>Tyr</td>
<td>3.2</td>
<td>3.1</td>
<td>3.7</td>
<td>4.8</td>
</tr>
<tr>
<td>Phe</td>
<td>6.6</td>
<td>5.9</td>
<td>7.7</td>
<td>5.3</td>
</tr>
<tr>
<td>Lys</td>
<td>9.9</td>
<td>9.0</td>
<td>11.3</td>
<td>10.1</td>
</tr>
<tr>
<td>His</td>
<td>3.4</td>
<td>3.7</td>
<td>3.9</td>
<td>3.5</td>
</tr>
<tr>
<td>Arg</td>
<td>4.7</td>
<td>4.8</td>
<td>6.0</td>
<td>5.4</td>
</tr>
<tr>
<td>Pro</td>
<td>3.3</td>
<td>3.7</td>
<td>6.8</td>
<td>4.0</td>
</tr>
<tr>
<td>Galactose</td>
<td>1.5</td>
<td>4.6</td>
<td>1.0</td>
<td>0.74</td>
</tr>
<tr>
<td>Mannose</td>
<td>1.5</td>
<td>3.3</td>
<td>1.5</td>
<td>1.85</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>2.1</td>
<td>2.5</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>Galactosamine</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
<td>2.0</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>0.9</td>
<td>2.1</td>
<td>0.95</td>
<td>1.3</td>
</tr>
</tbody>
</table>

<sup>a)</sup> Values reported by Skinner et al.<sup>19</sup> <sup>b)</sup> Values reported by Jamieson.<sup>20</sup>
restored by the addition of LC-2 to an almost normal level. Furthermore, the glucose consumption of PP-macrophages from normal mice could be augmented above the normal level by the addition of LC-2.

**Antitumor Activity of LC-2**

After subcutaneous inoculation of $2 \times 10^6$ Sarcoma 180 cells (day 0), LC-2, mouse transferrin or mouse hemopexin was injected intravenously on 5 consecutive days, days 1—5. Lentinan was injected intraperitoneally 2 times on days 1 and 5. The results are summarized in Table III. LC-2 and mouse hemopexin partially inhibited the growth of Sarcoma 180 cells, whereas standard mouse transferrin did not show any inhibitory activity. Lentinan also showed a marked inhibitory effect against this tumor.

**DISCUSSION**

The results presented here indicate that the LC-2 component that increased in the sera of PSK treated mice is a variant of mouse transfer-

---

**FIG. 7. Restoration of the Con A Resonse of Spleen Cells Obtained from Tumor (P815)-Bearing Mice by LC-2**

DBA/2 mice were inoculated with $2 \times 10^6$ P815 cells subcutaneously. Twelve days after the inoculation, spleen cells were cultured with LC-2 and Con A (2.5 µg/ml) for 42 h and labeled with 0.5 µCi [3H]-TdR for the final 18 h. Each value is the mean for triplicate cultures with the standard error of the mean.

**FIG. 8. Enhancement of Glucose Consumption of Proteose Peptone-Elicited Peritoneal Macrophages Obtained from Tumor (P815)-Bearing Mice by LC-2**

Macrophages from tumor (P815)-bearing mice (16 d after P815 inoculation) were cultured with LC-2. After 24 h, the glucose contents of culture supernatants were determined. Each value is the mean for triplicate cultures with the standard error of the mean. a) Significantly different from control, $p<0.005$.

**TABLE III. Antitumor Activity of LC-2**

<table>
<thead>
<tr>
<th>Dose (µg/mouse)</th>
<th>Tumor weight (g) Mean ± S.E.</th>
<th>%Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>6.28 ± 0.40</td>
</tr>
<tr>
<td>LC-2</td>
<td>600</td>
<td>4.47 ± 0.46</td>
</tr>
<tr>
<td>Hemopexin</td>
<td>600</td>
<td>4.25 ± 0.86</td>
</tr>
<tr>
<td>Mouse transferrin</td>
<td>560</td>
<td>5.69 ± 1.22</td>
</tr>
<tr>
<td>Lentinan</td>
<td>300</td>
<td>1.85 ± 1.09</td>
</tr>
</tbody>
</table>

ICR mice were inoculated subcutaneously with Sarcoma 180 cells ($2 \times 10^6$ cells/mouse) on day 0. LC-2, hemopexin and mouse transferrin were injected intravenously on 5 consecutive days, days 1—5. Lentinan was injected intraperitoneally 2 times, on days 1 and 5. Tumors were extirpated and weighed on day 28. Each group consisted of five animals. a), b) significantly different from control, $p<0.025$ and 0.010, respectively.
rin. Firstly, the absorption spectrum and ultraviolet CD curve of LC-2 showed various characteristics identical to those of mouse transferrin. Secondly, the molecular weight and isoelectric point of LC-2 were similar to those of mouse transferrin. Thirdly, the amino acid composition of LC-2 was in good agreement with that of mouse transferrin. Fourthly, LC-2 contained iron atoms and apo-LC-2 bound 2.05 iron atoms per protein molecule.

Transferrin is involved in iron transport in the blood of vertebrates and supplies iron for hemoglobin synthesis and other metabolic requirements. Apo-transferrin, which is normally colorless, is salmon-pink in color when complexed with ferric ions. The absorption maximum for such a complex is in the vicinity of 470 nm. Rabbit transferrin has a single polypeptide chain and generally exists in various genetically controlled forms.

Hudson et al. have attributed the marked differences that they found in the carbohydrate compositions of cattle, rabbit, horse, and pig serum transferrins to variations in the number of carbohydrate chains. They proposed that there is one chain in cattle transferrin, two chains in rabbit and horse transferrins, and four chains in pig transferrin. On the other hand, Graham et al. reported one chain in pig transferrin and two chains in cattle transferrin. Thus, serum transferrins studied to date show heterogeneity with respect to carbohydrate composition.

We found a variant of mouse transferrin that differs in the carbohydrate content. The different electrophoretic mobilities of LC-2 and mouse transferrin on gradient gel electrophoresis may be due to their different carbohydrate contents. This assumption was apparently strengthened by the finding of Kornfeld that the removal of sialic acid or galactose from transferrin changed its electrophoretic mobility.

The biological significance of the fact that only one of the transferrin variants increases in serum is not yet known. However, it is of interest that LC-2, which increased upon administration of antitumor polysaccharides, has weak but definite antitumor activity whereas standard mouse transferrin, which does not apparently increase after such treatment, has no antitumor activity. The reason for this is not clear at present, but one possibility may be that iron-deficient LC-2 can block cell surface receptor sites for iron-rich transferrin which is needed for active proliferation of tumor cells. Further investigation on the binding of LC-2 to tumor cells and also on the effects of LC-2 and transferrin on the immune system are necessary to substantiate this assumption.

REFERENCES


