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ISOLATION AND CHARACTERIZATION OF A RECEPTOR LECTIN SPECIFIC FOR GALACTOSE/*N*-ACETYLGALACTOSAMINE FROM MACROPHAGES*†

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ABSTRACT

Rat-peritoneal macrophages are shown to be able to take up glycoproteins terminated by galactose as well as those by mannose and/or *N*-acetylglucosamine. A lectin responsible for the uptake of galactose-terminated glycoproteins was isolated by affinity chromatography on a column of Sepharose 4B–asialoorosomucoid. The macrophage lectin isolated shared many properties in common with the well-established[1] hepatic lectin specific for galactose/*N*-acetylgalactosamine. Thus, the lectin bound to asialoglycoproteins specifically, and the binding was inhibited by galactose and *N*-acetylgalactosamine. The lectin had a single major component of mol. wt. 42,000 as well as two minor components of 60,000 and 65,000, and required calcium for binding. In addition, the macrophage lectin was immunologically crossreactive with the hepatic lectin. Despite these similarities, however, the macrophage lectin was differentiated from the hepatic lectin in molecular size, relative preponderance of minor components, and titration profile with the antibodies raised against the hepatic galactose/*N*-acetylgalactosamine-specific lectin.

INTRODUCTION

Beginning with the pioneering work of Ashwell and associates[1], five distinct carbohydrate-recognition systems responsible for the pinocytosis of glycoproteins have been described: (*i*) Gal/GalNAc recognition by hepatocytes[1] and by bone-marrow cells[2], (*ii*) Man/L-Fuc/GlcNAc recognition by Kupffer cells[3–5] and various macrophages[6], (*iii*) (1→3)-α-L-Fuc recognition by hepatocytes[7], (*iv*) Man-6-phosphate recognition by fibroblasts[8,9], and (*v*) GlcNAc recognition by avian hepatocytes[10,11]. During the course of a study on carbohydrate recognition by

*Dedicated to Roger W. Jeanloz.

†Abbreviations used: Man, D-mannose; Gal, D-galactose; GlcNAc, 2-acetamido-2-deoxy-D-glucose; GalNAc, 2-acetamido-2-deoxy-D-galactose; Fuc, fucose; ASOR, asialo-orosomucoid; AGOR, agalacto-orosomucoid; and BSA, bovine serum albumin.

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macrophages, we recently found that rat-peritoneal macrophages incorporated Gal-terminated glycoproteins as well as Man/L-Fuc/GlcNAc-terminated glycoproteins[12]. A pertinent question raised by this observation was whether these two types of recognition indicated the occurrence of two distinct lectins with their respective specificities, or a single lectin having dual specificity. A relevant question was whether a Gal-specific lectin on macrophages, if present, was identical to the hepatic lectin having the same sugar specificity or not. Experiments were performed to answer these questions with the results described in this paper.

EXPERIMENTAL

Materials. — Specific Pathogen Free (SPF) Wistar strain rats (male) weighing 140–160 g were purchased from Kitayama LABES Co. Ltd., Kyoto. $Na^{125}I$ (carrier free) was obtained from the Radiochemical Centre (Amersham, U.K.). Mannan was prepared as described previously[5] from baker's yeast (*Saccharomyces cerevisiae*) purchased from Oriental Yeast Co., Tokyo, Japan. Asialo-orosomucoid (ASOR) and agalacto-orosomucoid (AGOR) were prepared as described previously[5] from orosomucoid provided by Dr. M. Wickerhauser of the American Red Cross Research Center, Bethesda, Maryland. Asialoglycopeptides were prepared from orosomucoid, human ceruloplasmin, and human transferrin by exhaustive digestions with pronase followed by acid hydrolysis in 0.05M H_2SO_4 at 80°. Man–BSA and GlcNAc–BSA containing 33 mol and 43 mol of the respective sugars per mol of protein were kindly provided by Dr. Y. C. Lee of Johns Hopkins University, Baltimore, Maryland. Affinity resins of Sepharose 4B–mannan and Sepharose 4B–ASOR were prepared as described previously[13–15]. Antiserum against the Gal/GalNAc specific lectin of rat liver was prepared in rabbits by multiple muscular and subcutaneous injections of an emulsified mixture of an aqueous solution of the purified lectin (0.1 mg), prepared by essentially the same procedure as used for the isolation of rabbit-liver lectin[14], and Freund's complete adjuvant (Difco Laboratories Inc.) 4 times every 4 weeks. The IgG fraction was recovered from antiserum as described previously[15]. Eagle's minimum essential medium supplemented with kanamycin was purchased from Nissui Seiyaku Co., Tokyo, Japan.

Methods. — *Iodination.* Glycoproteins were iodinated by a modification of the procedure of Greenwood *et al.*[16].

Assay conditions. The uptake or binding activity of intact cells and the binding activity of the solubilized lectins towards various glycoproteins were determined as described previously[5], except that each tube contained 300 ng of ^{125}I-labeled glycoprotein. For the uptake assay, cells ($1–20 \times 10^6$) were incubated with labeled glycoproteins in 0.5 mL of Eagle's minimum essential medium buffered to pH 7.4 with 20mM Hepes at 37° on a gyrotatory shaker. After 30 min, the incubation was terminated by the addition of cold medium and the cells were recovered by centrifugation. Radioactivity taken up into the cells was determined

with a Beckman Auto-Gamma spectrophotometer, Gamma-5500. For the binding assay in a solubilized system, the solubilized lectin was incubated with labeled glycoproteins in 0.5 mL of the binding-assay mixture, containing 0.1% Triton X-100, 0.6% BSA, 50mM imidazole · HCl, pH 7.8, M NaCl, and 50mM $CaCl_2$. After 15 min at room temperature, the lectin–glycoprotein complex formed was precipitated by the addition of an equal volume of cold, saturated $(NH_4)_2SO_4$ and the radioactivity in the precipitate was determined. Nonspecific binding was estimated by the addition of a 100-fold excess of unlabeled ligand or EDTA (at the final concentration of 5mM) to the incubation mixture. One uptake or binding unit is defined as a nanogram of labeled ligand taken up or bound under the conditions. Specific activity refers to uptake units or binding units per 10^7 cells or μg of protein.

Isolation of peritoneal macrophages. Macrophages were prepared from SPF rats 4 days after injection of 10 mL of 3% thioglycolate medium I (Wako Pure Chemical Industries, Ltd., Osaka, Japan) by peritoneal lavage with 30 mL of Eagle's minimum essential medium. Cell yield, viability, and the cell composition were routinely tested. Approximately 70% of the fluid cells were macrophages, as determined by staining with Giemsa's solution. The isolated cells were finally washed in 0.9% NaCl and were stored at −20° before use.

Isolation of a Gal/GalNAc specific lectin from macrophages. Approximately 6.9×10^9 cells obtained from 76 rats were homogenized with Biomixer (Nippon Seiki Co., Tokyo, Japan) in an extracting buffer consisting of 0.02M imidazole · HCl, pH 7.8, 0.4M KCl, and 0.5mM EDTA. After addition of 10% Triton X-100 to give a final concentration of 2%, the homogenate was stirred for 20 min at 4° and the solubilized lectins were recovered by centrifugation. The extraction was repeated once more. The combined extract was made to 0.02M $CaCl_2$ and mixed with 50 mL of Sepharose 4B–mannan that had been equilibrated with a loading buffer consisting of 0.02M imidazole · HCl, pH 7.8, 0.02M $CaCl_2$, 1.25M NaCl, and 0.5% Triton X-100. After stirring for 30 min at 4°, affinity resins were separated by centrifugation, whereby Man/GlcNAc specific lectin(s) in the extract were removed with the resins. The supernatant solution was applied to 25 mL of Sepharose 4B–ASOR in a column. After washing the column with the loading buffer, the Gal/GalNAc-specific lectin was eluted with an eluting buffer (eluting buffer *I*), consisting of 0.02M imidazole · HCl, pH 7.8, 1.25M NaCl, 2mM EDTA, and 0.5% Triton X-100. The eluate was made to 0.02M $CaCl_2$ and then applied to and eluted from a second affinity column of Sepharose 4B–ASOR (5 mL). The eluate from the second affinity column was adjusted to 0.02M $CaCl_2$ and applied to the third affinity column (1 mL). After washing the column with the loading buffer, the lectin was eluted with 0.02M imidazole · HCl, pH 7.8, 0.02M $CaCl_2$, 1.25M NaCl, 0.01M Gal, and 0.5% Triton X-100. Fractions containing binding activity were pooled, dialyzed against eluting buffer *I*, and stored at 4°.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed with 10% acrylamide gels according to the procedure of Laemmli[17]. Protein bands were stained with silver

nitrate as described by Morrissey[18]. The following proteins were used as the mol. wt. reference standards: phosphorylase b (94,000), BSA (67,000), ovalbumin (43,000), and carbonic anhydrase (30,000).

Protein determination. Protein was determined with fluorescamine by the procedure of Nakai *et al.*[19], using BSA as a standard.

RESULTS AND DISCUSSION

As shown in Table I, peritoneal macrophages took up glycoproteins having terminal Man or GlcNAc, namely, Man–BSA, GlcNAc–BSA, mannan, and AGOR. This is a common property shared by tissue macrophages, as previously shown with rat-alveolar macrophages[20,21], mouse macrophages from the peritoneal cavity and bone marrow[22,23], rat-liver macrophages (Kupffer cells)[4,5,24], and lymph-node macrophages[25]. Interestingly, however, peritoneal macrophages showed in addition a marked ability to take up ASOR, a Gal-terminated glycoprotein. This has not been observed with other macrophages from lung[20], liver[4,5,24], and lymph nodes[25] and appears to be characteristic to peritoneal macrophages. These findings prompted us to identify lectin(s) involved in the Gal-specific uptake by macrophages. For this purpose, we first used the antibodies (IgG) raised against the Gal/GalNAc-specific lectin of rat hepatocytes. When macrophages were treated with a small amount of the anti-IgG, incorporation of ASOR was completely inhibited, whereas under the same conditions, uptake of Man–BSA took place without any alteration (see Fig. 1). These results clearly indicate that the Gal-recognizing system on macrophages is immunologically identical or closely related to that on hepatocytes, and that two glycoprotein-uptake systems on peritoneal macrophages are immunologically distinct and functionally independent from each other. Close similarities between the Gal recognition by macrophages and that by hepatocytes were further demonstrated by the following studies. Uptake by macrophages depended on the presence of calcium and was inhibited reversibly by the addition of EDTA, as was the case with the hepatic lectin. The value of K uptake for ASOR by macrophages as estimated by Lineweaver–Burk type

TABLE I

UPTAKE OF VARIOUS GLYCOPROTEINS BY RAT-PERITONEAL MACROPHAGES[a]

Glycoprotein	*Uptake (units/10^7 cells/30 min)*
AGOR	0.8
Mannan	0.9
GlcNAc–BSA	1.2
Man–BSA	4.2
ASOR	8.7

[a]Cells (2×10^7) were incubated for 30 min at 37° as described in the Experimental section. Values are the mean of two experiments.

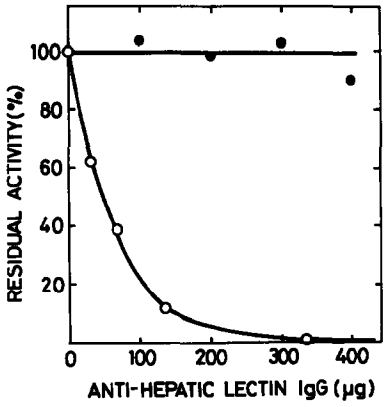

Fig. 1. Neutralizing-antibody titration of cellular uptake. Cells (1.5×10^7) were incubated with increasing amounts of anti-hepatic Gal/GalNAc-specific lectin IgG in Eagle's minimum essential medium (0.5 mL) with the labeled ligands omitted. After 60 min at room temperature, the uptake activity was determined with the addition of ^{125}I-ASOR (○) and ^{125}I-Man-BSA (●), respectively, as described in the Experimental section.

analysis of the uptake data of ASOR (see Fig. 2) was 23nM, being very similar to that by hepatocytes (10nM)[26].

Realizing that uptake by macrophages was very similar to the Gal/GalNAc-specific uptake by hepatocytes, we attempted to isolate a presumptive lectin involved in the ASOR uptake from peritoneal macrophages, by the procedure used for the isolation of the hepatic lectin with minor modifications. Extraction of the lectin with Triton X-100 was followed by three successive affinity chromatographies on Sepharose 4B–ASOR columns; the first two utilized the dependence of binding on calcium and the last one utilized the sugar specificity of the lectin. This sequence

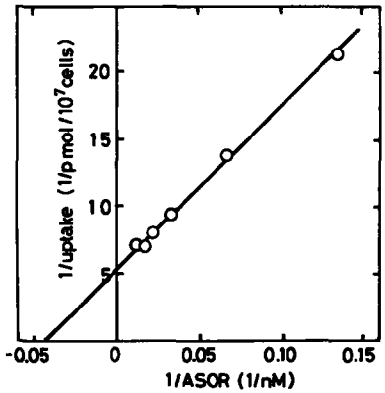

Fig. 2. Estimation of K uptake for ASOR by a Lineweaver–Burk plot. Cells (1×10^7) were incubated with increasing amounts of ^{125}I-ASOR ranging from 150–1500 ng in Eagle's minimum essential medium for 30 min at 37° as described in the Experimental section. Nonspecific uptake was estimated by the addition of EDTA at the final concentration of 5mM and the values so obtained were subtracted from the experimental values.

TABLE II

SPECIFICITY OF BINDING AS MEASURED BY INHIBITION ASSAY[a]

Sugar	*Inhibition (%)*
N-Acetylgalactosamine	67.1
Galactose	61.7
Mannose	0.1
L-Fucose	0.1
N-Acetylglucosamine	0.0
N-Acetylmannosamine	0.0
Glucosamine	0.0
Mannosamine	0.0

[a]The assay was carried out as described in the Experimental section in the presence of 14.8 units of the purified binding protein and 15mM of the sugars listed in the table. Values are the mean of two experiments. Sugars are of the D configuration unless otherwise stated.

resulted in the purification of a lectin with a specific activity of 52 units/μg of protein, the value being almost identical to that of the hepatic lectin (40–60 units/μg of protein)[13,14]. By this procedure, ~17 μg of the purified lectin was obtained from 6.9 $\times$ 10^9 cells. Although recovery of the lectin throughout the isolation procedure could not be assessed because of the low activity of the original Triton X-100 extract, it was estimated that total recovered activity (888 units) corresponded to 198% of the lectin detectable on the surface of the cells, namely, the amount of ASOR bound to the cells at 4° (0.65 unit/10^7 cells). This value indicates that more than one-half of the lectin was present inside the cells and the remainder on the cell surface, being consistent with the Gal/GalNAc-specific lectin in hepatocytes[27,28].

The binding specificity of the macrophage lectin was tested by hapten-inhibition studies with various sugars for binding to ^{125}I-ASOR (see Table II). Among the sugars tested at a concentration of 15mM, GalNAc and Gal inhibited the binding most potently, whereas Man, GlcNAc, and L-Fuc did not show any inhibitory activity. In order to know the effects of the number of "antennae" of oligosaccharide chains on binding, asialoglycopeptides were prepared from orosomucoid (a mixture composed mainly of tri- and tetra-antennary oligosaccharides)[29], ceruloplasmin (tri- plus bi-antennary)[30], and transferrin (mostly bi-antennary)[31], and were tested also as haptenic inhibitors. In the presence of a 1,000-fold excess of asialoglycopeptides from orosomucoid, ceruloplasmin, and transferrin, the binding of ASOR to the macrophage lectin was inhibited by 49, 20, and 0%, respectively, indicating a strong preference of binding for tri- and tetra-antennary oligosaccharides. Similarly, the hepatic Gal/GalNAc-specific lectin was inhibited by these asialoglycopeptides by 63, 8, and 6%, respectively, under the same conditions, being consistent with the previous studies[32]. In contrast, the Gal/GalNAc-specific lectin in bone-marrow cells is suggested to prefer bi-antennary oligosaccharides[2].

Polyacrylamide-gel electrophoresis of the isolated lectin with sodium dodecyl

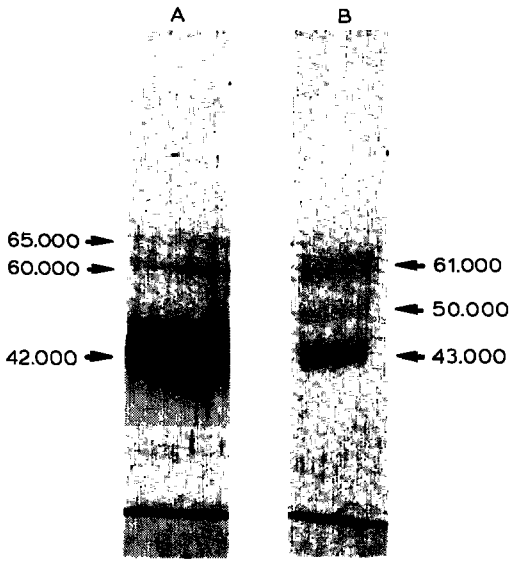

Fig. 3. Polyacrylamide-gel electrophoresis of Gal/GalNAc-specific lectins in the presence of sodium dodecyl sulfate. Electrophoresis was carried out according to the procedure of Laemmli[17] in 10% acrylamide gel. Protein bands were stained with silver nitrate[18]. The figures refer to the estimated molecular weight. Lane A; the macrophage lectin (1.7 μg), Lane B; the hepatic lectin (1.0 μg).

sulfate under reducing conditions, showed a predominant band of mol. wt. 42,000 as well as very minor bands of 60,000 and 65,000 (see Fig. 3, A). The molecular size of the major component was almost identical to that of the hepatic Gal/GalNAc-specific lectin. In contrast, the minor components of the macrophage lectin were distinct from those of the hepatic lectin. Thus, the molecular weights of the macrophage components (60,000 and 65,000) were slightly larger than those of the hepatic lectin (50,000 and 61,000) and the ratios of two minor components to the major component of the macrophage lectin were much lower than those of the hepatic lectin[33,34] (Fig. 3, B). Although the significance of the minor components in terms of the binding ability and the receptor function remains to be elucidated in both systems, components of mol. wt. 50,000 and 61,000 were shown to be immunologically relevant to the 43,000 component[34], and their partial amino acid sequences have been reported[35].

In order to examine further the relationship between macrophage and hepatic lectins, the effects of anti-hepatic Gal/GalNAc-specific lectin IgG on the binding of the purified binding proteins were studied. As the amount of the anti-IgG added to the purified binding proteins was increased, the activity toward ASOR decreased gradually for either binding protein. However, there were clear differences in their responses to the anti-IgG, as shown in Fig. 4. Thus, for the hepatic binding protein, 30 μg of anti-IgG was enough to cause 50% inhibition, whereas 100 μg of anti-IgG was required for the macrophage lectin. Furthermore, in the presence of more than

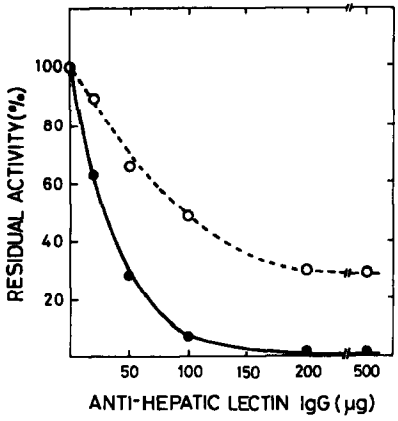

Fig. 4. Neutralizing-antibody titration of isolated lectins. The Gal/GalNAc-specific lectins from macrophages (12 units), (○) and hepatocytes (11 units), (●) were incubated separately with increasing amounts of the anti-hepatic Gal/GalNAc-specific lectin IgG in the binding-assay mixture (0.5 mL) with the labeled ligand omitted. After 30 min at room temperature, the binding activity was determined with the addition of ^{125}I-ASOR, as described in the Experimental section.

200 μg of anti-IgG, the hepatic lectin lost its binding activity completely, whereas the macrophage lectin still retained approximately one-third of the original binding activity. These results clearly indicate that the macrophage lectin is closely related, but not identical, to the hepatic lectin in its antigenicity, and therefore, in its primary structure. The incomplete inhibition of binding by an excess of anti-IgG was in sharp contrast to the complete inhibition of ASOR uptake by the same anti-IgG (see Fig. 1). A plausible explanation for this apparent discrepancy may be as follows. The major component (42,000) of the macrophage lectin, which probably shares some common epitopes with the 43,000 component of the hepatic lectin, may be the component involved in the uptake process, whereas the minor components (60,000 and 65,000), which are immunologically distinct from the hepatic lectin, may not be associated with the uptake process. Alternatively, the receptor function of the lectin may depend on the association of the three components, and inactivation of one of them (42,000) by the anti-IgG may result in the total inactivation of the whole functional unit, whereas in the solubilized system, each component expresses its own binding activity independently.

Kolb and associates have suggested the occurrence of a calcium-dependent Gal/GalNAc-specific receptor on the surface of Kupffer cells and peritoneal macrophages of the rat[36–39]. This receptor lectin was, however, shown to mediate endocytosis specifically of such particles as desialylated erythrocytes or Gal–BSA coupled to colloidal gold, but not of such glycoproteins as ASOR[4,5,23]. Therefore, it may be reasonable to infer that the lectin isolated in this study is not the presumptive particle-receptor, although a final conclusion must wait further studies. Similar, but apparently different, lectin-like molecules have also been reported to be isolable from the surface of mouse-peritoneal macrophages[40]. These

molecules have larger molecular size (77,000–79,000) and more complex sugar specificity.

The present paper describes for the first time the isolation of a novel lectin specific for Gal/GalNAc from rat-peritoneal macrophages. In addition to its *in situ* function as a receptor, the lectin may, because of its limited distribution, be important as a parameter of functional differentiation of macrophages.

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