Human Mannose-Binding Lectin Preferentially Binds to Human Colon Adenocarcinoma Cell Lines Expressing High Amount of Lewis A and Lewis B Antigens

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The binding of mannose-binding lectin (MBL) to human colon adenocarcinoma cell lines and leukemia cell lines was analyzed by flow cytometry using specific antibodies against MBL. MBL binding was observed in 3 of 7 colon adenocarcinoma cell lines (Colo205, Colo201 and DLD-1), but not in any of 3 leukemia cell lines tested. The binding of MBL to these cell lines was sugar-specific and calcium-dependent, since it was almost completely inhibited in the presence of 10 mM EDTA or 50 mM mannosne. The MBL binding to Colo205 cells was more strongly reduced by the pretreatment of the cells with an O-linked glycosylation inhibitor, benzyl-2-acetamido-2-deoxy-α-galactopyranoside (Bz-α-GalNAc), rather than an N-linked glycosylation inhibitor, tunicamycin. The degree of MBL binding was well correlated with the expression of Lewis A and Lewis B antigens on these cell lines. Moreover, MBL binding to Colo205 cells was inhibited by anti-Lewis A and anti-Lewis B antibodies. These results suggest that MBL could bind to some human colon adenocarcinoma cell lines through their Lewis A and Lewis B moieties.

Key words mannose-binding lectin; Colo205; Lewis A; Lewis B; ligand

Mannose-binding lectin (MBL), which has been reported in various mammalian sera1–5 sometimes called mannan-binding protein (MBP), is a calcium-dependent C-type lectin recognizing mannosne (Man), N-acetylgalactosamine (GalNAc) and fucose (Fuc). MBL is synthesized in the hepatocytes and secreted into circulating blood as oligomers consisting of 9–15 identical subunits of about 31 kDa. The multimeric structure of MBL resembles that of C1q, a complement component. After binding to carbohydrate ligands, MBL activates the complement via MBL-associated serine proteases MASP-16,7 and MASP-2.8 This pathway is antibody- and C1q-independent, as is thus called the lectin pathway.9,10 Since MBL exhibits complement-dependent bactericidal activity and acts directly as an opsonin, MBL is thought to be an important constituent of the innate immune system.9,11

A variety of microorganisms express manno-oligosaccharide structures on their surface, while mature mammalian cells generally do not. For this reason, MBL was thought to specifically recognize only exogenous microorganisms. However, it has been reported that MBL can bind to some mammalian cells, such as virus-infected cells12 and glioma cells13 in which high mannose-type oligosaccharides are expressed. This suggests that MBL can recognize and eliminate the abnormally glycosylated endogenous cells, since cells surface sugar chains are altered during virus infection or malignant transformation.14,15

Contrary to the above experiments using whole cells, MBL has also been demonstrated in vitro to bind to sugar structures containing galactose (Gal), Fuc and GlcNAc, such as Galβ1–3(Fucα1–4)GlcNAc and Fucα1–2Galβ1–3(Fucα1–4)GlcNAc, called Lewis A and Lewis B antigens respectively, by a binding assay using neoglycolipids and thin-layer chromatography (TLC).7

Therefore, we examined the binding of human serum MBL to various human tumor cell lines and report here that MBL can bind to some colon adenocarcinoma cell lines, and that Lewis A and Lewis B antigens on their cell surface could be ligands for MBL.

MATERIALS AND METHODS

Cell Lines Seven human colon adenocarcinoma cell lines and 3 leukemia cell lines were used in this study. Colon adenocarcinoma cell lines Colo205, HT-29, Colo320DM and SW48 were obtained from American Type Culture Collection (ATCC, U.S.A.), Colo201, WiDr and DLD-1 were from Japanese Cancer Research Resources Bank/Health Science Research Resources Bank (JCRB/HSSRB, Japan). Three Leukemia cell lines, HL-60 (acute promyeloctic leukemia), Daudi (Burkitt’s lymphoma) and Jurkat (acute T cell leukemia) were from JCRB/HSSRB. Unless otherwise stated, these cell lines were cultured in RPMI 1640 medium (Nissui Seiyaku, Japan) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10% heat-inactivated fetal bovine serum (JRH Biosciences, U.S.A.). All cultures were performed at 37°C in a humidified atmosphere containing 5% CO₂.

Purification of Human MBL. Human MBL was purified from normal human serum (Anapure Biosciences Co., China) using an affinity column of mannan-agarose (Sigma Chemical Co., U.S.A.), according to the method of Lu et al.8 with a slight modification as follows. Polyelectrolyte gycocol 6000 was added to 1000 ml of human serum at a concentration of 7% and then the precipitate was dissolved in 100 ml of 50 mM Tris–HCl, 1 mM NaCl, 20 mM CaCl₂ and 0.05% NaN₃, pH 7.8 (starting buffer). After removing undissolved materials by centrifugation and passing them through a filter with a 0.45 μm pore size, the supernatant was applied onto a first mannan-agarose column (30 ml packed volume). The column was washed with 150 ml of the starting buffer and then the bound proteins were eluted with a buffer containing EDTA (50 mM Tris–HCl, 1 mM NaCl, 10 mM EDTA and 0.05% NaN₃, pH 7.8). The calcium concentration of the eluate was adjusted to 25 mM with 1 mM CaCl₂ and the pH was ad-
justed to 7.8. Then, the eluate was applied onto a second
mannan-agarose column (3 ml packed volume) and washed
in the same manner as the first mannan-agarose column. The
bound proteins were eluted with the starting buffer contain-
ing 50 mM mannose. The purity of MBL was confirmed by
sodium dodecyl sulfate-polyacrylamide gel electrophoresis
(SDS-PAGE) under reducing conditions in 10% polyacry-
lamide gels. After removing mannose and replacing the
buffer to 10 mM Tris–buffered saline (pH 7.4) containing
5 mM CaCl₂ and 0.1% NaN₃ (TBS/Ca/NaN₃) by subsequent
centrifugation using Ultrafree-MC Membrane, 10000 MW
Filter Unit (Millipore Co., U.S.A.), the solution thus obtained
was used as the source of purified human serum MBL in
the following experiments.

Preparation of Anti-Human MBL Antibody To obtain
specific antibodies against human MBL, we used recombi-
nant human MBL as an antigen. Recombinant MBL was pre-
pared as follows. MBL cDNA was amplified by a two-step
polymerase chain reaction (PCR) using the specific primers
for human MBL gene.¹⁹,²⁰ The target cDNA was reverse
transcribed from total RNA prepared from the human
hepatoma cell line HuH-7 (JCRB/HSRRB) as a template. The
specific primers for MBL were: 5'-ATGTGCACTATATCAGCA-
AGGAGGCGCTGAGTGC-3' for the first PCR, and 5'-CTG
GGATCCCCGCACTGTGACCTGTGAGATGC-3' and 5'-
TCAGCTTTACGGAGAATCTCACAGACAGGC-3' for the
second PCR. Restriction sites for BamHI and HindIII in-
troduced in the second PCR primers are marked by
underlining. The second PCR products, which contain a whole
molecule region of mature MBL except for the signal peptide
region, were ligated into a TA cloning vector pCR2.1 (Invitro-
gen, U.S.A.). The plasmids were digested with BamHI and
HindIII. The resulting MBL cDNA fragment was purified
by gel electrophoresis and subcloned into the BamHI and
HindIII sites of expression vector pQE30 (Qiagen, Ger-
many), which contain the sequence coding 6 histidines and
enable the introduction of 6 histidine-residues into the N-
terminal of the MBL, amino acid sequence. To induce the
expression of the MBL gene, transformed E. coli M15 was
 treated with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG,
Nacalai Tesque, Japan) for 1 h at 30°C. The cells were dis-
rupted by sonication, and recombinant MBL with a histidine-
tag was purified using a Ni-NTA column (Qiagen, Ger-
many) according to the manufacturer's instruction.

To produce anti-MBL antibody, 250 µg of recombinant
MBL was mixed with Freund's complete adjuvant (Difco,
U.S.A.) and injected to JW rabbit (female, Sankyo Labo
Service Co., Japan). At 2-week intervals after the first injection,
the recombinant MBL mixed with Freund's incomplete adju-
vant (Wako Pure Chemicals, Japan) was injected four times
into the same rabbit. The serum obtained two weeks after the
final injection of MBL was used as the source of polyclonal
anti-MBL antibodies (anti-MBL serum).

The specificity and titer of the antiserum against human
MBL were ascertained by Western blotting and ELISA using
purified human serum MBL. The titer of this antiserum was
estimated to be over 100000, since 1:100000 diluted anti-
s serum still reacted with human MBL coated on a 96-well mi-
croplate. When human serum was separated by 10% SDS-
PAGE under a reduced condition, transferred to nitrocellu-
lose membrane, and stained with anti-MBL serum in combi-
nation with peroxidase-labeled anti-rabbit IgG (Wako, Japan)
and Konica Immunostaining HRP-1000 (Konica, Japan),
only one band with 31 kDa corresponding to the monomeric
MBL was detected.

Assessment of MBL Binding Binding of MBL to vari-
ous tumor cell lines was assessed by flow cytometry using
the rabbit anti-MBL serum obtained as above. Prior to the incu-
bation with MBL, tumor cells were washed 3 times with
10 mM Tris–buffered saline (TBS, pH 7.4) containing 10 mM
EDTA to remove bovine collections involved in the culture
medium, and then resuspended in TBS containing 5 mM
CaCl₂, 0.1% NaN₃, and 1% bovine serum albumin (1%BSA/
TBS/Ca/NaN₃). The cells (5×10⁵ cells) were first treated
with 1 µg of purified serum MBL in 50 µl of 1%BSA/TBS/
Ca/NaN₃ for 1 h on ice, then incubated with 50 µl of a 1:100
dilution of rabbit anti-MBL serum for 45 min, and then
stained with 50 µl of 10 µg/ml fluorescein isothiocyanate-
(FITC)-labeled goat anti-rabbit IgG (Wako, Japan) for an
additional 45 min on ice. The cells were washed twice with
1%BSA/TBS/Ca/NaN₃ between each reaction by centrifuga-
tion at 4°C, and finally suspended in TBS/Ca/NaN₃. Flow
cytometry was performed with CytoACE-300 (Jasco Corp.,
Japan), and the data obtained from 5000 cells were expressed
as histograms or mean fluorescence intensities (MFI). For
the evaluation of MBL binding, MFI obtained in the absence
of MBL was subtracted from the MFI obtained in the presence
of MBL.

Modification of Cell Surface Carbohydrates To exam-
ine the effects of glycosylation inhibitors on MBL bind-
ing, Colo205 cells were incubated for 3 d in the presence of
2 mM benzyl-2-acetamide-2-deoxy-α-galactopyranoside (Bz-
α-GalNAc, Sigma, U.S.A.) or 0.25 µg/ml of tunicamycin
(Sigma, U.S.A.). To remove the cell surface sialic acids,
Colo205 cells (6×10⁶ cells) were suspended in 1 ml phos-
phate buffered saline (PBS) and treated with 300 µM neu-
raminidase from Streptococcus 6646K (Seikagaku Corp.,
Tokyo) for 60 min at 37°C. MBL binding to these cells was
assessed by flow cytometry as above.

Expression of Carbohydrate Antigens The expression of
carbohydrate antigens on tumor cell lines was also ana-
yzed by flow cytometry. Mouse monoclonal antibodies
(MAbs) against Lewis A (clone 7LE, IgG1), Lewis B (clone
2-25-LE, IgG1) and sialyl Lewis A (clone 1H4, IgG3) were
purchased from Seikagaku, and an antibody against sialyl
Lewis X (clone SNH-3, IgM) was from Wako. Control
mouse IgM was purchased from Inter-Cell Technologies,
Inc., U.S.A., and control mouse IgG (clone MOPC-21, IgG1)
was prepared in our laboratory from the culture supernatant
of mouse plasmacytoma P3X63Ag8 (JCRB/HSRRB). The
cells (5×10⁵ cells) were stained with 0.5 µg of mouse MABS
against specific carbohydrate antigens for 45 min on ice and
then with 0.5 µg of FITC-labeled goat anti-mouse IgG
(Wako, Japan) or FITC-labeled goat anti-mouse IgM (Caltag
Lab., U.S.A.) for 45 min on ice. After washing, the cells were
analyzed by flow cytometry as above.

Inhibition of MBL Binding with Carbohydrate-Specific
Antibodies The cells (5×10⁵ cells) were treated with 2.5
µg of MABS or corresponding control IgG/IgM for 45 min on
ice prior to the addition of MBL, and then treated with 1 µg
MBL as above. In the subsequent staining steps to detect the
MBL bound to the cells, mouse anti-MBL MAb (clone D8.18, IgG1), which was produced in our laboratory and biotinylated, was used instead of rabbit anti-MBL serum, since nonspecific staining with rabbit antisera increased after the treatment of Colo205 cells with control IgG or IgM. The hybridoma secreting D8.18 was produced by the cell fusion of mouse plasmacytoma P3/NS1/Ag4-1 (JCRB/HSRBB, Japan) and BALB/c mouse (Sankyo Labo, Japan) spleen cells immunized with purified human serum MBL. Details of the production and characterization of D8.18 will be described elsewhere.

The cells treated with anti-carbohydrate antibodies and MBL as above were incubated with 5 μg of biotinylated mouse anti-MBL MAb D8.18 for 45 min on ice, and then stained with 0.5 μg of FITC-labeled avidin (Zymed Laboratories, Inc., U.S.A.) for 45 min on ice. After washing, the cells were analyzed by flow cytometry as above.

RESULTS

Binding of MBL to Various Tumor Cell Lines To examine whether MBL generally binds to various human cell lines, the binding of MBL to 7 colon adenocarcinoma cell lines and 3 leukemia cell lines was analyzed by flow cytometry. As shown in Fig. 1, MBL binding was observed in 3 of 7 colon adenocarcinoma cell lines (Colo205, Colo201 and DLD-1), but not in any of the 3 leukemia cell lines tested. The binding of MBL to Colo205 cells was sugar-specific and calcium-dependent, since it was almost completely inhibited in the presence of 10 mM EDTA or 50 mM mannose (Fig. 2).

Effect of Glycosylation Inhibitors on the Binding of MBL to Colo205 Cells To elucidate the contribution of high-mannose type oligosaccharides and other sugar structures on the binding of MBL to Colo205 cells, the cells were treated with an O-glycosylation inhibitor, Bz-α-GalNAc, or an N-glycosylation inhibitor, tunicamycin, for 3 d, and then the binding of MBL to these cells was analyzed. As shown in Fig. 3, MBL binding disappeared almost completely (reduced at about 10% of control) after the Bz-α-GalNAc treatment, whereas the tunicamycin treatment reduced the MBL binding at about 60% (60.3±9.8%) of control. This suggests that MBL ligands on Colo205 cells are mainly expressed on O-linked sugar chains rather than N-linked sugar chains. Although the inhibitory effect of tunicamycin on the MBL binding is not negligible, it is difficult to conclude that N-linked glycoproteins on Colo205 cells are also necessary for the MBL binding since tunicamycin also affected the cell growth (reduced at about 50%) as well as N-glycosylation (reduced at about 50% assessed by concanavalin A (Con A) binding) (data not shown).

Correlation between MBL Binding and the Expression of Lewis Antigens We assumed that Lewis blood antigens on Colo205 cells might be MBL ligands, since these antigens were highly expressed on colon adenocarcinoma and are considered tumor associated carbohydrate antigens. Thus we examined the relationship between MBL binding and the ex-
Fig. 4. Expression of Lewis A, Lewis B, Sialyl Lewis A and Sialyl Lewis X Antigens on Various Colon Adenocarcinoma Cell Lines

Cells were stained with MAb specific for Lewis A, Lewis B, sialyl Lewis A, sialyl Lewis X (solid line) or control IgG/IgM (dotted line) followed by FITC-labeled anti-mouse IgG/IgM, then analyzed by a flow cytometer.

expression of Lewis antigens on 7 colon adenocarcinoma cell lines (Fig. 4). Lewis A and Lewis B antigens were highly expressed on Colo205, Colo201 and DLD-1 cells, moderately expressed on WiDr and HT-29 cells, and undetectable on SW-48 and Colo320DM cells. The former 3 cell lines were shown to be recognized by MBL in Fig. 1, while the latter 4 cell lines were not. MFLs of WiDr and HT-29 cells stained with anti-Lewis A or anti-Lewis B antibodies were less than 20% of Colo205 cells. The expression of sialyl Lewis A was almost similar to that of Lewis A or Lewis B. Sialyl Lewis X, however, was detected in all cell lines, and furthermore, the expression was higher on WiDr and HT-29 cells than on Colo205 cells. Therefore, the degree of MBL binding seems to be correlated with the expression of Lewis A, Lewis B and

sialyl Lewis A antigens, but not with that of the sialyl Lewis X antigen. In addition, neuraminidase treatment of Colo205 cells did not reduce MBL binding (Fig. 5). These results suggest that Lewis A and Lewis B antigens, but not sialyl Lewis A and sialyl Lewis X antigens, seem to be ligands for MBL on Colo205 cells.

Inhibition of MBL Binding by Anti-Lewis A or Anti-Lewis B Antibodies To confirm that Lewis A and Lewis B moieties on Colo205 cells could be ligands for MBL, the cells were treated with antibodies specific for Lewis antigens, then MBL binding was assessed by flow cytometry using bi-
otinylated anti-MBL MAb D8.18 and FITC-labeled avidin. Antibodies against Lewis A and Lewis B antigens were able to inhibit the MBL binding effectively, while antibodies against sialyl Lewis A antigens had no effect (Fig. 6). These results suggest that MBL binds to Colo205 cells through the Lewis A or Lewis B moieties on these cells. Since Lewis A and Lewis B antigens are highly expressed on Colo205 cells, they may exist closely each other. Therefore, the antibodies bound to Lewis A antigens may mask the Lewis B antigens close to the Lewis A antigens and affect the approach of MBL. This may be the reason why anti-Lewis A antibodies completely inhibited the binding of MBL. Anti-sialyl Lewis X antibody reduced the MBL binding at about 60% (59.3 ± 23.7%) of the control (Fig. 6), however, this might be explained by the bulky effect of the anti-sialyl Lewis X antibody SNH-3, since the class of this antibody is IgM, but others are all IgG class antibodies. That is, the IgM antibody bound to the cell surface might prevent the access of large molecules such as MBL.

DISCUSSION

In this study, we showed that human MBL could bind to some human colon adenocarcinoma cell lines, and suggested that Lewis A and Lewis B antigens on them could be ligands for MBL. This is the first report to show that Lewis A and Lewis B antigens on mammalian cells could function as ligands for MBL as well as high-mannose type oligosaccharides.

Concerning the functional role of MBL, it has already been reported that MBL bound to mammalian cells could activate the complement system. Ohta and Kawasaki reported that BHK cells modified to express high mannose-type oligosaccharides were recognized by MBL and lysed through complement activation. Fujita et al. reported that MBL bound to human glioma cell lines was able to activate the complement pathway and led to C4 and C3 consumption, but not final lysis. Recently, we observed the deposition of complement components, C4 and C3, on Colo205 cells after treatment with MBL. This indicates that MBL binds to Colo205 cells through Lewis A and Lewis B antigens could also activate the complement pathway in a similar manner as in the above reports.

On the other hands, Lewis A and Lewis B antigens, which were demonstrated to be ligands for MBL in this study, are also expressed on normal gastrointestinal tissues and erythrocytes. However, we could not detect MBL binding to the erythrocytes obtained from Lewis A-positive or Lewis B-positive individuals (our preliminary observation).

In addition, we could not detect MBL binding to WiDr or HT-29 cells, although they express these antigens. The reason why we could not detect MBL binding to these latter two cell lines may be explained by the lower expression of Lewis A and Lewis B antigens on these cells, since the mean fluorescence intensities of these cells stained with anti-Lewis A or anti-Lewis B antibodies were less than 20% of Colo205 cells. There is another possibility that MBL may be able to bind only to colon adenocarcinoma cells which express these antigens as highly clustered forms on the cell surface, since the cluster-like array of multiple sites can achieve significant functional avidity when MBL binds to the cell surface with many repeating clusters of ligand sugar chains, although the affinity of each carbohydrate recognition domain of MBL is weak. Furthermore, extended unique Lewis A related structures, Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-3(Fucα1-4)GlcNAc (dimeric Lewis A) or Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-3(Fucα1-4)GlcNAc (Lewis B-Lewis A), have been reported to exist on Colo205 cells. These extended structures might be more preferentially recognized by MBL than simple structures of Lewis A and Lewis B antigens. However, further investigations are required to answer the question why MBL could not bind to WiDr or HT-29 cells as well as normal erythrocytes.

Recently, Uemura et al. reported that mouse immature thymocytes were also recognized by MBL and that the ligand protein for MBL was identified as CD45 with high-mannose type oligosaccharides. In our preliminary experiment, we observed that MBL could bind to a high-molecular weight fraction obtained from the Colo205 culture supernatant. MUC1 and CD43 mucins are known to be major O-glycosylated proteins that are expressed on Colo205 cells and secreted into culture supernatants. At the present time, however, there is no evidence that these mucins express Lewis A or Lewis B antigens and function as ligands for MBL. As we have shown in this paper, O-glycosylated proteins could function as MBL ligands. The physiological and pathological importance of O-glycosylated proteins has already been reported in other biological systems, such as the adhesion of leukocytes and cancer cells to endothelial cells. Identification of the ligand glycoproteins for MBL will contribute to the understanding of their physiological and pathological roles. Therefore, we are now attempting to identify the ligand glycoproteins for MBL.

REFERENCES