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High-level and effective production of human mannan-binding lectin (MBL) in Chinese hamster ovary (CHO) cells

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Abstract

We have developed a high-expression system of recombinant human mannan-binding lectin (MBL) with CHO cells. Geneticin-resistant transformants harboring human MBL cDNA in the expression vector pNOW/CMV-A were screened by immunoblot analysis for secretion of recombinant MBL. Cloning and selection by both geneticin and methotrexate resulted in the production of recombinant MBL to a final concentration of 128.8 μg/ml in media after four days of culture. SDS-PAGE and gel-filtration analyses showed that recombinant MBL is characterized by two lower-order oligomeric structures (apparent molecular weights: 1150 kDa and 300 kDa) compared to native MBL (apparent molecular weight: 1300 kDa). The recombinant human MBL has both sugar-binding and complement activation activity and, like native MBL, can inhibit hemagglutination of influenza A virus. Lectin blots with recombinant MBL indicate that it can bind such microorganisms as HIV and influenza virus suggesting that it might inhibit their infection of hosts. This high-level expression of human MBL with the full range of biological activity will be useful for studies on the immunological role of MBL in humans.

Keywords: Mannan-binding lectin (MBL); CHO expression system; Anti-viral activity; Complement activation

Abbreviations: CRD, carbohydrate recognition domain; HI, hemagglutination inhibition; HIV, human immunodeficiency virus; MBL, mannan-binding lectin; MTX, methotrexate; PAGE, polyacrylamide gel electrophoresis; rh MBL, recombinant human MBL; SDS, sodium dodecyl sulfate; SP-A, surfactant protein A; SP-D, surfactant protein D; VC, vitamin C

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1. Introduction

The collectins are characterized by a collagen-like sequence and a carbohydrate recognition domain (CRD), and are members of the vertebrate C-type lectin superfamily (Drickamer, 1988). Mannan-binding lectin (MBL) is one of the collectin group and is found in serum and liver (Kawasaki et al., 1978, 1983). MBL has a structure similar to C1q (Thiel and Reid, 1989) and is associated with the activation of complement in the absence of immunoglobulin (Ikeda et al., 1987). It can also act as an opsonin due to its interaction with a collectin receptor (Malhotra et al., 1990). Various reports suggest that MBL deficiency is associated with recurrent infections in infants and adults (Super et al., 1989; Summerfield et al., 1995). Therefore, much attention is currently focused on mammalian lectins, especially on their role in innate immunity. The concentration of MBL in serum is determined by the allelic forms associated with MBL gene mutations in codons of exon 1 encoding the collagen-like domain (Sumiya et al., 1991; Lipscombe et al., 1992; Madsen et al., 1994) and sequence polymorphisms in the promoter region (Madsen et al., 1995).

MBL binds to carbohydrate moieties on several viral antigens, such as HIV-gp120 (Ezekowitz et al., 1989) and influenza neuraminidase (NA) (Malhotra et al., 1994). It may interfere with HIV infection in vitro and it activates complement upon binding to gp120 of HIV-1 and gp110 of HIV-2 (Haurum et al., 1993). Hartshorn et al. (1993) showed that human MBL and conglutinin are opsonic for influenza A virus. In our experiments, we found that the bovine collectin, conglutinin, inhibits both infection and hemagglutination by influenza A virus (Wakamiya et al., 1992; Eda et al., 1996). Our preliminary study showed that human MBL can directly neutralize influenza virus without the involvement of complement.

Two recent reports suggest that variant alleles of MBL result in susceptibility to HIV infection and progression to AIDS (Nielsen et al., 1995; Garred et al., 1997). These investigations of the in vivo activities of MBL in humans suggest that it plays an important role in defense against microbial infections.

In this paper, we describe an effective expression system for human MBL in CHO cells and characterize it for possible future clinical applications.

2. Materials and methods

2.1. Construction of an expression vector and expression of human MBL in CHO cells

The 0.8-kbp cDNA for human MBL was amplified using the polymerase chain reaction (PCR) method with the primers 5′-AAGCGGCCGCATGTCCCTGTTTCCATCACTC-3′ and 5′-CCTCTAGATCAGATAGGGAACTCACAGAC-3′. The PCR was performed in a TP-cycler (Toyobo, Osaka, Japan), and consisted of 30 cycles of 1 min at 94°C for denaturation, 1 min at 55°C for annealing and 2 min at 72°C for extension, followed by 5 min at 72°C for the final extension. The PCR product was digested with the restriction endonucleases *Not*I and *Xba*I and cloned into the expression vector pNOW/CMV-A (construction to be presented elsewhere) which was then designated pNOW-MBL. Transfection of CHO DG44 was accomplished using a DOTAP (Boehringer Mannheim, Mannheim, Germany) transfection reagent. The cells were plated at a density of 2×10^5 cells/6 ml in 60-mm plastic culture dishes in Iscove's modified Dulbecco's Medium (I-MDM; GIBCO BRL, Life Technologies, MD, USA) supplemented with 100 mM hypoxanthine (GIBCO BRL), 10 mM thymidine (GIBCO BRL) and 10% dialyzed FCS (JRH Biosciences, KS, USA, Lot. 6E2117) for 20 h prior to transfection. They were then transfected with pNOW-MBL complexed with DOTAP. Geneticin-resistant (400 μg/ml) transfectants were identified after two weeks and selected colonies were subcloned in 96-well microtiter plates. The transformed CHO DG44 cell line with the highest level of MBL production was subjected to increasing concentrations of MTX (Sigma-Aldrich, MO, USA) from 5 nM to 50 nM in I-MDM supplemented with 10% FCS (dialyzed) and 400 μg/ml G 418 (GIBCO BRL). Cells, after selection with MTX were cultured with I-MDM containing 10% FCS (dialyzed) and 100 μg/ml of vitamin C. The recombinant MBL content of culture fluid was determined by an ELISA procedure as described in Section 2.4.

2.2. Purification of native and recombinant human MBL

To purify MBL from human serum, the serum (1000 ml) was first adjusted to 10 mM calcium with $CaCl_2$ and was incubated overnight at 4°C with mannan–agarose (50 ml) (Sigma) which had been equilibrated with TBS (10 mM Tris–HCl, 150 mM NaCl, pH 7.4) containing 10 mM $CaCl_2$. In the case of recombinant human MBL, the culture supernatant of stable transformants was dialyzed against TBS and TBS/Ca (TBS containing 10 mM $CaCl_2$). The dialyzed culture supernatant was incubated with mannan–agarose as above and then both gels were packed in columns and washed extensively with TBS/Ca. The columns were eluted with 10 ml of TBS/E (TBS containing 10 mM EDTA) and both eluates were adjusted to 10 mM calcium and mixed again with mannan–agarose (10 ml) at 4°C overnight. The gels were packed in columns and washed with TBS/Ca. Finally, the MBL preparations were eluted with TBS/Ca containing 100 mM D-mannose and were stored at 4°C.

2.3. Biochemical characterization of recombinant human MBL

Protein samples treated with sodium dodecyl sulfate (SDS), with SDS and 2-mercaptoethanol, or left untreated were separated by polyacrylamide gel electrophoresis and detected by staining with Coomassie Brilliant Blue R250. Gel permeation chromatography of MBL (40 μg) was performed with a Superose 6 HR 10/30 column (Pharmacia Biotech, Tokyo, Japan) at a flow rate of 0.5 ml/min with TBS/E. Each eluted fraction monitored at 280 nm was recalcified with 10 mM $CaCl_2$ and used in the biological assays. The column was calibrated using the Bio Rad Gel Filtration Standard mixture containing thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), and equine myoglobulin (17 kDa) (Bio-Rad Lab., CA, USA). Rabbit MBL (2300 kDa) (Kawai et al., 1998) was used as another high-molecular weight marker, and the gel permeation study was repeated several times. Following repeated dialysis against water of the purified native and recombinant MBL from several cell clones, amino acid analyses were performed using the Waters Pico-Tag system (Suzuki et al., 1997).

2.4. ELISA procedures

Two ELISA procedures were developed to determine the expression level of recombinant MBL and its sugar specificities. A sandwich ELISA procedure used for quantitative analysis employed two rabbit polyclonal antibodies. The coating antibody was raised in a New Zealand White rabbit by serial injection of a recombinant human MBL expressed in CHO cells as described in the present report. The detection antibody was developed in another NZW rabbit by injection of recombinant human MBL-CRD produced in *Escherichia coli* (Eda et al., 1998). Microtiter plates were coated with rabbit anti-recombinant human MBL antibody (IgG fraction) at a concentration of 10 μg/ml in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.05% NaN_3, pH 9.6) by incubation overnight at 4°C. TTBS/Ca (10 mM Tris–HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.05% Tween 20, pH 7.4) was used in all of the washing steps. After washing, the plates were blocked with Block Ace (a blocking solution made from skim milk, purchased from Dainippon Pharmaceuticals, Tokyo, Japan) and washed again. The plates were then incubated at 37°C for 1 h with culture fluid diluted with TTBS (10 mM Tris–HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.4) with or without $CaCl_2$ or EDTA at concentrations of 5 mM. The plates were washed and incubated with biotinylated rabbit anti-recombinant human MBL-CRD antibody (IgG fraction) at a concentration of 1 μg/ml in TTBS/Ca. After washing, the plates were incubated at 37°C for 1 h with VECTASTAIN *Elite* ABC kit (Vector Lab., CA, USA). Finally, 100 μl of TMB substrate solution were applied to each well (TMB Microwell Peroxidase Substrate, Kirkegaard and Perry Lab. (KPL), MD, USA). After the addition of 100 μl of 1 M phosphoric acid, the absorbance at 450 nm was read with a Model 450 Microplate Reader (Bio-Rad Lab.). We used an in-house native MBL preparation as the standard and this was validated against a plasma standard kindly provided by Prof. J. Jensenius (Aarhus University). The results using the two ELISA procedures showed less than 10% divergence ($r = 0.993$, $P < 0.01\%$).

The other ELISA procedure was a saccharide binding assay using microtiter plates coated with mannan (Sigma) at a concentration of 10 μg/ml in the same coating buffer as above. After washing with TTBS and blocking with Block Ace, the plates were then incubated at 37°C for 1 h with native and recombinant MBLs diluted with TTBS with or without $CaCl_2$ or EDTA at concentrations of 5 mM. The plates were washed, incubated and developed as above.

For the assay of sugar selectivity, the microtiter plates were coated with mannan (10 μg/ml) and the recombinant or native MBL (50 ng/ml) was incubated with increasing concentrations of sugars. The concentration of sugar which inhibited binding by 50% was defined as the I_{50} as previously described (Lu et al., 1992).

2.5. Assays of biological function

Hemagglutination inhibition (HI) tests were performed with a standard microtiter assay procedure in 96-well microplates with 0.5% (v/v) chicken erythrocytes (Wakamiya et al., 1992). The level of inhibition of virus-mediated agglutination of chicken erythrocytes by native and recombinant human MBL was determined after 1 h of incubation at 4°C. To inhibit the activity of human MBLs, some sugars and EDTA were added after the incubation of viruses with the MBL. The HI titer was expressed as the lowest concentration of human MBL causing HI (Eda et al., 1996).

Complement-dependent passive hemolysis was performed with sheep erythrocytes (SRBC) coated with mannan (Sigma) by the chromium chloride method (Ikeda et al., 1987). The SRBC–mannan was washed with gelatin veronal buffer saline (GVB) and then used for hemolysis assays with guinea pig complement (5 CH_{50}) (ICN Pharmaceuticals, CA, USA). The guinea pig complement had already been passed through a mannan–agarose column to remove the guinea pig MBL. SRBC–mannan suspensions were incubated with each fraction for 15 min at room temperature. They were then centrifuged and the absorbance of the supernatant was determined at 541 nm in a Ubest-30 (JASCO, Tokyo, Japan) spectrophotometer. Specific lysis was calculated from the absorption of an equivalent amount of cells lysed in

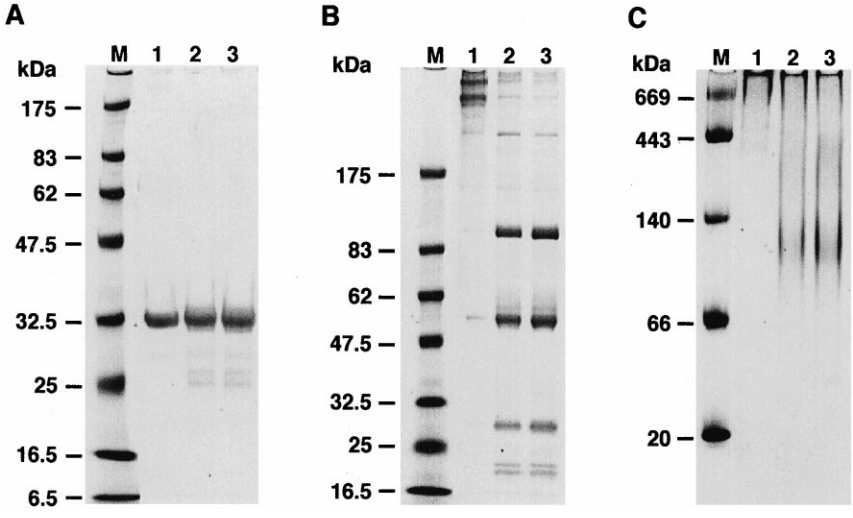

Fig. 1. Polyacrylamide gel electrophoresis analyses of recombinant MBLs and native MBL. (A) Samples were treated with SDS and 2-mercaptoethanol and loaded onto a 10–20% polyacrylamide gel. (B) Samples were treated with SDS and loaded onto a 4–20% polyacrylamide gel. (C) Samples were directly loaded onto a 2–15% polyacrylamide gel. Lane 1, native MBL; lane 2, recombinant MBL produced in the presence of vitamin C (VC +); lane 3, native MBL produced without vitamin C (VC −).

water (Kawai et al., 1997). The guinea pig complement on its own caused no hemolysis (0.01%).

Ten micrograms of HIV-1 III B gp120 (Advanced Biotechnologies, MD, USA), HIV-1 III B gp160 (Advanced Biotechnologies), and hemagglutinin (HA) and neuraminidase (NA) antigens of influenza virus A/Yamagata/32/89 (H1N1) and A/Beijing/352/89 (H3N2) were dissolved in SDS-sample buffer, separated by SDS-PAGE and transferred to BioBlot-NC membranes (Corning Costar Japan, Tokyo, Japan) by standard procedures (Towbin et al., 1979). After blocking unoccupied sites on the membranes with Block Ace in TTBS (10 mM Tris–HCl, 150 mM NaCl, 0.05% Tween 20), the membranes were incubated with human recombinant MBL (VC+) (3 μg/ml) with TTBS with or without $CaCl_2$ or EDTA at a concentration of 5 mM for 60 min at room temperature, washed in TTBS, and then incubated with rabbit anti-recombinant human MBL-CRD IgG (1 μg/ml) in TTBS/Ca for 60 min at room temperature. After washing in TTBS/Ca, the membranes were incubated with donkey anti-rabbit IgG-conjugated alkaline phosphatase 1/5000 (CHEMICON International, CA, USA), and stained with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (BCIP-NBT system; GIBCO BRL).

3. Results

3.1. Transformation and amplification of human MBL cDNA and purification of recombinant MBL

The expression vector pNOW/CMV-A which we used contains the cytomegalovirus strong promoter adjacent to the polylinker *Hin*dIII–*Not*I–*Xba*I–*Apa*I, aph gene for selection by G418, and the dihydrofolate reductase gene for gene amplification. The CHO DG44 cell line used for the expression of recombinant MBL was grown in 48 wells of ten 96-well microtiter plates seeded with 3×10^3 cells/well. Cells from 5 out of 84 recombinant MBL-producing wells were cloned, including the two showing the highest production. The concentrations of recombinant MBL secreted by these five lines were 4.8, 6.6, 13.2, 14.5, and 23.3 μg/ml after the four-day culture with 3×10^6 cells/3 ml/25 cm^2 flask. The 3A5 and 10F7 cell lines (23.3, 13.2 μg/ml) were subjected to selection with increasing concentrations

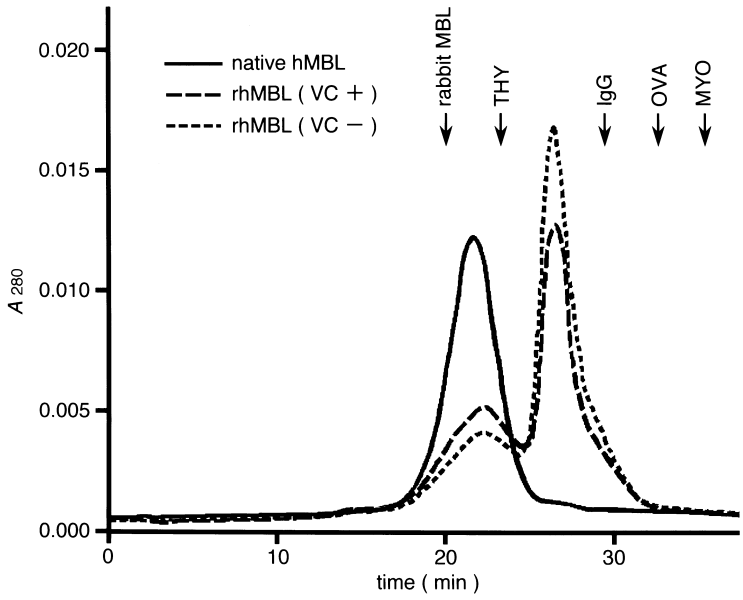

Fig. 2. Gel-filtration chromatography of recombinant MBLs and native MBL on a Superose 6 column. Gel-filtration chromatography was performed under native conditions as described in Section 2. The column was calibrated as described in Section 2 and the elution positions of molecular weight standards are as indicated.

Table 1
Amino acid composition of native and recombinant MBL

	Native MBL		Recombinant MBL	
	cDNA[a]	Present study[b]	+VC[b]	−VC[b]
Asx	25	23.6	23.2	22.8
Thr	15	12.0	11.6	11.4
Ser	13	10.0	9.4	10.8
Glx	27	28.5	28.9	29.2
Pro	18	16.3	16.4	16.1
HyPro		3.7	3.4	2.7
Gly	31	29.1	29.5	29.9
Ala	16	16.0	16.0	16.0
Val	10	9.5	8.9	8.4
Met	2	2.1	2.0	1.9
Ile	8	7.0	6.8	6.7
Leu	17	16.3	16.5	16.0
Tyr	1	1.1	0.9	0.9
Phe	9	8.6	8.4	8.4
His	1	1.4	1.2	1.5
Lys	19	18.0	18.0	17.9
HyLys		3.2	2.5	2.1
Arg	6	6.7	5.5	5.3

[a]Calculated from the predicted amino acid sequence of human MBL deduced from the cDNA from DDBJ, EMBL and GenBank Data Libraries.
[b]Determined by amino acid analysis of purified MBL.
The value shows the number of amino acid residues. Abbreviations: +VC, culture with vitamin C; −VC, culture without vitamin C.

of MTX from 5 nM to 50 nM. These selections resulted in a six-fold increase in the concentration of secreted recombinant MBL to a level of 128.8 and 77.6 μg/ml, respectively. The recombinant MBL was isolated from the culture medium of the MTX-amplified 3A5 line using affinity chromatography on mannan–agarose following which we generally obtained more than 1 mg of recombinant MBL from 10 ml of media.

3.2. Biochemical characterization of recombinant MBL

Polyacrylamide gel electrophoresis (PAGE) of the native and recombinant MBL under reducing conditions revealed a single 32-kDa band (Fig. 1A), which was identified as human MBL by immunoblot analysis (data not shown). The N-terminal amino acid sequences of the 32-kDa protein of native and recombinant MBL were identical (data not shown). Analyses of native and recombinant MBL by nonreducing PAGE (Fig. 1B) and by native PAGE (Fig. 1C) show that native MBL is characterized by higher-order oligomeric structures compared to recombinant MBL. Recombinant MBLs produced with or without vitamin C, have the same oligomeric structure by PAGE analysis (Fig. 1B,C). Gel-filtration analysis suggested that these recombinant MBLs exist in two major forms, with peaks corresponding to apparent molecular weights of 1150 kDa and 300 kDa, while native MBL eluted with a peak corresponding to an apparent molecular weight of 1300 kDa (Fig. 2). MBL from cells cultured in the presence of vitamin C had more of the first peak and less of the second peak than MBL from cells grown without vitamin C. Amino acid analyses of the native and recombinant MBLs showed that a minor increase in hydroxylation of proline and lysine was observed in the presence of vitamin C (Table 1). The hydroxylation of native MBL was higher than that of recombinant MBLs. The binding of mannan to recombinant MBL produced in the presence or absence of vitamin C was inhibited by D-mannose in a dose-dependent manner and with almost identical kinetics (data not shown). The sugar-binding specificities of recombinant MBLs produced in the presence or absence of vitamin C were almost similar to that of

Table 2
Sugar specificities of recombinant MBLs and native MBL

Sugars	I_{50} (mM)[a]		
	Recombinant MBL		Native MBL
	+VC[b]	−VC[b]	
N-Acetyl-D-glucosamine	3.6	3.8	2.7
L-Fucose	4.4	8.7	4.2
D-Fucose	39.1	86.7	33.5
D-Mannose	6.7	6.8	3.7
Maltose	6.4	7.4	7.4
N-Acetyl-D-mannosamine	6.9	6.9	4.4
Glucose	2.0	2.4	3.2
Galactose	29.8	36.7	24.2
N-Acetyl-D-galactosamine	>100	>100	>100
Lactose	46.6	59.4	36.0

[a]The value shows the concentration of sugars required for 50% inhibition of binding.
[b]Abbreviations: +VC, culture with vitamin C; −VC, culture without vitamin C.

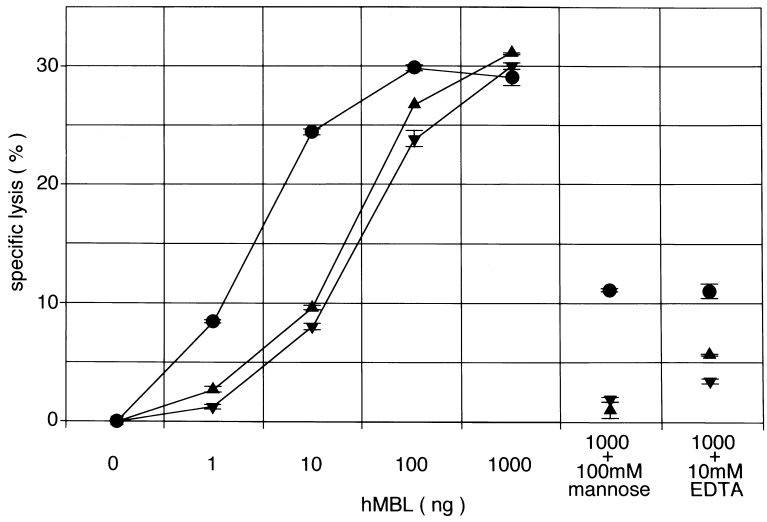

Fig. 3. Dose-dependence of the complement activation by the recombinant MBLs and native MBL. SRBC–mannan were sensitized with 1–1000 ng of recombinant MBLs (VC + / ▲, VC − / ▼) and native MBL (●), then lysed with complement (5 CH_{50}). Results are shown as the means of triplicates and the bars indicate standard errors of the mean.

native MBL (Table 2). The affinity of recombinant MBL for L-fucose and D-fucose was higher if the MBL was derived from cells incubated with vitamin C rather than from cells incubated without vitamin C.

3.3. Biological activities of recombinant MBL

Two biological activities of native and recombinant MBL were examined using microtiter plates assay systems. Both MBLs were able to lyse SRBC–mannan in a dose-dependent manner using guinea pig complement (Fig. 3). Their activities were inhibited by 10 mM EDTA or 100 mM D-mannose. The hemolytic activity of recombinant MBL (27% lysis/100 ng) was about 10-fold lower than that of native MBL (25% lysis/10 ng). After gel-filtration, the recombinant MBL fraction of 1150 kDa produced 53% lysis/100 ng and the 300-kDa fraction gave 10% lysis/100 ng. The high-molecular weight MBL exhibited higher hemolytic activity than the

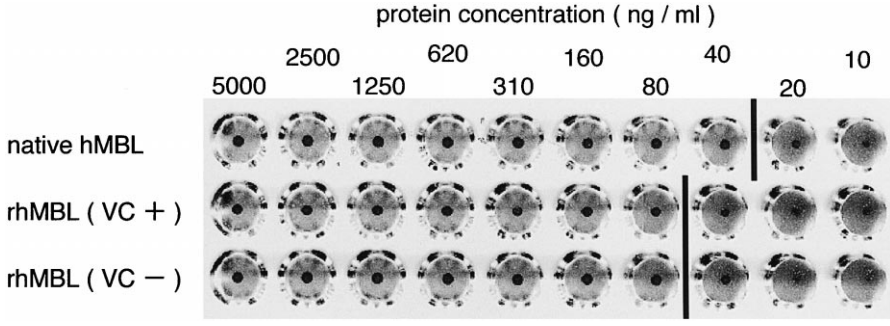

Fig. 4. Inhibition of hemagglutination of influenza A virus (H3N2) by incubation with recombinant MBLs and native MBL. The HI test was performed with two-fold dilutions of three MBLs in 96-well V plates to which 16 HA units (final concentration) of influenza A virus (A/Ibaraki/1/90-H3N2) were then added.

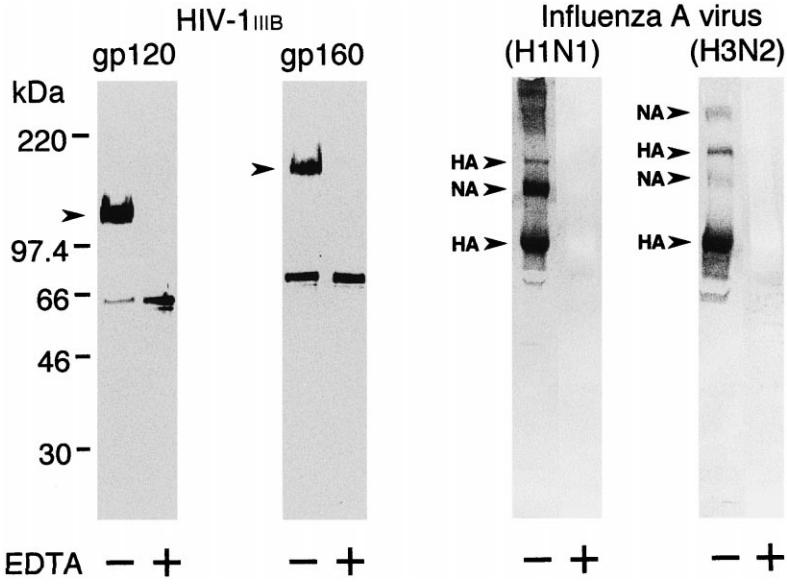

Fig. 5. Lectin blot of HIV gp120, gp160, and influenza A virus NA and HA. The viral proteins transferred on the membrane were treated with human recombinant MBL(VC +). Lectin blots were prepared as described in Section 2.

low-molecular weight MBL. The MBL from cells cultured with vitamin C showed slightly higher hemolytic activity than MBL from cultures without vitamin C.

The minimum concentrations required for inhibition of hemagglutination by native and recombinant MBLs, using H3N2 type influenza A virus were 40 and 80 ng/ml, respectively (Fig. 4). Following gel-filtration, the minimum concentration of the 1150-kDa fraction corresponded to about 30 ng/ml, whereas over 500 ng/ml of the 300-kDa fraction was required. The two forms of MBL (vitamin C ±) had identical specific activities in the HI tests. A lectin blot showed that human recombinant MBL was able to bind to both HA and NA of influenza A virus subtypes H3N2 and H1N1, and HIV-gp120 and gp160 (Fig. 5), all of which were inhibited by EDTA or D-mannose (data not shown).

4. Discussion

We report the first demonstration that a recombinant human MBL with anti-viral and complement activating activity can be produced in CHO cells. Previously, expression systems using *E. coli* were evaluated for the production of MBL and other collectins. We and several other groups made truncated collectins lacking collagen domains which had sugar-binding activity and some of the biological activities of native collectins (Lim et al., 1994; Kishore et al., 1996; Eda et al., 1996, 1997). However, there are no reports of the expression of a full-length collectin with a collagenous domain in *E. coli*. Physiological studies and possible clinical applications of human MBL require eukaryotic expression systems. Previously, we developed a high-expression system for bovine conglutinin using pNOW/CMV-A in CHO cells (Suzuki et al., 1997). In this study, we have attempted to establish a high-level expression system for human MBL with retention of full biological activity.

The recombinant MBLs reported here migrate in a manner similar to native MBL on reduced SDS-PAGE. Gel-filtration analysis shows that recombinant MBL has two smaller oligomeric structures (apparent molecular weights: 1150 and 300 kDa) than native MBL (apparent molecular weight: 1300 kDa). Other groups have made recombinant MBLs in different eukaryotic expression systems (Super et al., 1992; Ma et al., 1997). Their MBLs showed oligomeric structures consisting of 670, 520, and 300

kDa (MBL of Super et al.) and 1200, 540, 270, and 90 kDa (MBL of Ma et al.). Our recombinant MBL is mainly composed of two protein species (approx. 1150 and approx. 300 kDa), both of which bind to mannan efficiently. The addition of vitamin C to the culture medium was found to increase the amount of the 1150-kDa recombinant MBL and amino acid analysis of the recombinant MBLs suggested that post-translational hydroxylation of proline and lysine occurs even in the absence of vitamin C in the culture medium although this increases when vitamin C is present. However, the hydroxylation of native MBL is higher than that of recombinant MBLs and these results indicate that this high-level expression system might require more vitamin C or other factors for full hydroxylation.

The sugar-binding activity of recombinant MBL was very similar to that of native MBL. Similarly, the hemolytic activity of recombinant MBL was dose-dependent and inhibited by EDTA and mannose. Growing the cells with vitamin C increased this activity slightly. Ma et al. (1997) reported that recombinant MBL with post-translational modification, was assembled into high-molecular weight oligomer complexes which had high hemolytic activity. These results indicate that the further hydroxylation and glycosylation of proline and lysine in the collagen-like domain in the presence of vitamin C might augment the biological activities and add stability to the structure by increasing the proportion of higher oligomeric forms.

Like native MBL, the recombinant protein was able to inhibit hemagglutination by influenza virus. However, this HI activity was not affected by presence or absence of vitamin C in the culture medium. Post-translational modifications induced by the inclusion of vitamin C occur in the collagen-like domain, but not in the carbohydrate recognition domain (CRD). These results indicate that the HI activity is mainly determined by the CRD. Our preliminary studies suggest that recombinant MBL has several biological activities (neutralizing and viral growth inhibition activities) in common with native MBL (data not shown).

The lectin blots show that recombinant MBL binds to HIV-gp120 and gp160 as well as to influenza envelope proteins. Recent reports indicate that the low levels of MBL due to mutations in the MBL gene are associated with susceptibility to HIV infection (Nielsen et al., 1995) and progression in AIDS (Garred et al., 1997). We did not examine the direct neutralizing activity of recombinant MBL, but it is possible that the recombinant MBL might be used as a viral inhibitor. The increase of anti-influenza virus activity and complement activation induced by vitamin C in our results suggest that vitamin C may augment host immunity. Although vitamin C treatment against influenza virus has no good scientific basis, further post-translational modification in the collagen-like domain may increase higher oligomeric structures and prolong the half-life of MBL and its effects. In summary, a functional MBL has been successfully expressed in CHO DG44 cells. This system should provide a useful model for studying MBL synthesis and secretion and permit further structural and functional studies employing molecular mutagenesis. In addition, this MBL should be useful for studies on the immunological role of MBL in vivo.

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References

Drickamer, K., 1988. Two distinct classes of carbohydrate-recognition domains in animal lectins. J. Biol. Chem. 263, 9557.

Eda, S., Suzuki, Y., Kase, T., Kawai, T., Ohtani, K., Sakamoto, T., Kurimura, T., Wakamiya, N., 1996. Recombinant bovine conglutinin, lacking the N-terminal and collagenous domains, has less conglutination activity but is able to inhibit haemagglutination by influenza A virus. Biochem. J. 316, 43.

Eda, S., Suzuki, Y., Kawai, T., Ohtani, K., Kase, T., Fujinaga, Y., Sakamoto, T., Kurimura, T., Wakamiya, N., 1997. Structure of

a truncated human surfactant protein D is less effective in agglutinating bacteria than the native structure and fails to inhibit haemagglutination by influenza A virus. Biochem. J. 323, 393.

Eda, S., Suzuki, Y., Kawai, T., Ohtani, K., Kase, T., Sakamoto, T., Wakamiya, N., 1998. Characterization of truncated human mannan-binding protein (MBP) expressed in *Escherichia coli*. Biosci. Biotechnol. Biochem. 62, 1326.

Ezekowitz, R.A., Kuhlman, M., Groopman, J.E., Byrn, R.A., 1989. A human serum mannose-binding protein inhibits in vitro infection by the human immunodeficiency virus. J. Exp. Med. 169, 185.

Garred, P., Madsen, H.O., Balslev, U., Hofmann, B., Pedersen, C., Gerstoft, J., Svejgaard, A., 1997. Susceptibility to HIV infection and progression of AIDS in relation to variant alleles of mannose-binding lectin. Lancet 349, 236.

Hartshorn, K.L., Sastry, K., White, M.R., Anders, E.M., Super, M., Ezekowitz, R.A., Tauber, A.I., 1993. Human mannose-binding protein functions as an opsonin for influenza A viruses. J. Clin. Invest. 91, 1414.

Haurum, J.S., Thiel, S., Jones, I.M., Fischer, P.B., Laursen, S.B., Jensenius, J.C., 1993. Complement activation upon binding of mannan-binding protein to HIV envelope glycoproteins. AIDS 7, 1307.

Ikeda, K., Sannoh, T., Kawasaki, N., Kawasaki, T., Yamashina, I., 1987. Serum lectin with known structure activates complement through the classical pathway. J. Biol. Chem. 262, 7451.

Kawai, T., Suzuki, Y., Eda, S., Ohtani, K., Kase, T., Fujinaga, Y., Sakamoto, T., Kurimura, T., Wakamiya, N., 1997. Cloning and characterization of a cDNA encoding bovine mannan-binding protein. Gene 186, 161.

Kawai, T., Suzuki, Y., Eda, S., Ohtani, K., Kase, T., Sakamoto, T., Uemura, H., Wakamiya, N., 1998. Molecular and biological characterization of rabbit mannan-binding protein (MBP). Glycobiology 8, 237.

Kawasaki, N., Kawasaki, T., Yamashina, I., 1983. Isolation and characterization of a mannan-binding protein from human serum. J. Biochem. 94, 937.

Kawasaki, T., Etoh, R., Yamashina, I., 1978. Isolation and characterization of a mannan-binding protein from rabbit liver. Biochem. Biophys. Res. Commun. 81, 1018.

Kishore, U., Wang, J.Y., Hoppe, H.J., Reid, K.B., 1996. The alpha-helical neck region of human lung surfactant protein D is essential for the binding of the carbohydrate recognition domains to lipopolysaccharides and phospholipids. Biochem. J. 318, 505.

Lim, B.L., Wang, J.Y., Holmskov, U., Hoppe, H.J., Reid, K.B., 1994. Expression of the carbohydrate recognition domain of lung surfactant protein D and demonstration of its binding to lipopolysaccharides of gram-negative bacteria. Biochem. Biophys. Res. Commun. 202, 1674.

Lipscombe, R.J., Sumiya, M., Hill, A.V., Lau, Y.L., Levinsky, R.J., Summerfield, J.A., Turner, M.W., 1992. High frequencies in African and non-African populations of independent mutations in the mannose binding protein gene. Hum. Mol. Genet. 1, 709.

Lu, J., Willis, A.C., Reid, K.B., 1992. Purification, characterization and cDNA cloning of human lung surfactant protein D. Biochem. J. 284, 795.

Ma, Y., Shida, H., Kawasaki, T., 1997. Functional expression of human mannan-binding proteins (MBPs) in human hepatoma cell lines infected by recombinant vaccinia virus: post-translational modification, molecular assembly, and differentiation of serum and liver MBP. J. Biochem. 122, 810.

Madsen, H.O., Garred, P., Kurtzhals, J.A., Lamm, L.U., Ryder, L.P., Thiel, S., Svejgaard, A., 1994. A new frequent allele is the missing link in the structural polymorphism of the human mannan-binding protein. Immunogenetics 40, 37.

Madsen, H.O., Garred, P., Thiel, S., Kurtzhals, J.A., Lamm, L.U., Ryder, L.P., Svejgaard, A., 1995. Interplay between promoter and structural gene variants control basal serum level of mannan-binding protein. J. Immunol. 155, 3013.

Malhotra, R., Haurum, J.S., Thiel, S., Sim, R.B., 1994. Binding of human collectins (SP-A and MBP) to influenza virus. Biochem. J. 304, 455.

Malhotra, R., Thiel, S., Reid, K.B., Sim, R.B., 1990. Human leukocyte C1q receptor binds other soluble proteins with collagen domains. J. Exp. Med. 172, 955.

Nielsen, S.L., Andersen, P.L., Koch, C., Jensenius, J.C., Thiel, S., 1995. The level of the serum opsonin, mannan-binding protein in HIV-1 antibody-positive patients. Clin. Exp. Immunol. 100, 219.

Sumiya, M., Super, M., Tabona, P., Levinsky, R.J., Arai, T., Turner, M.W., Summerfield, J.A., 1991. Molecular basis of opsonic defect in immunodeficient children. Lancet 337, 1569.

Summerfield, J.A., Ryder, S., Sumiya, M., Thursz, M., Gorchein, A., Monteil, M.A., Turner, M.W., 1995. Mannose binding protein gene mutations associated with unusual and severe infections in adults. Lancet 345, 886.

Super, M., Thiel, S., Lu, J., Levinsky, R.J., Turner, M.W., 1989. Association of low levels of mannan-binding protein with a common defect of opsonisation. Lancet 2, 1236.

Super, M., Gillies, S.D., Foley, S., Sastry, K., Schweinle, J.E., Silverman, V.J., Ezekowitz, R.A.B., 1992. Distinct and overlapping functions of allelic forms of human mannose-binding protein. Nat. Genet. 2, 50.

Suzuki, Y., Eda, S., Kawai, T., Ohtani, K., Kase, T., Sakamoto, T., Wakamiya, N., 1997. Characterization of recombinant bovine conglutinin expressed in a mammalian cell. Biochem. Biophys. Res. Commun. 238, 856.

Thiel, S., Reid, K.B., 1989. Structures and functions associated with the group of mammalian lectins containing collagen-like sequences. FEBS Lett. 250, 78.

Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350.

Wakamiya, N., Okuno, Y., Sasao, F., Ueda, S., Yoshimatsu, K., Naiki, M., Kurimura, T., 1992. Isolation and characterization of conglutinin as an influenza A virus inhibitor. Biochem. Biophys. Res. Commun. 187, 1270.