Protein Cell 2010, 1(7): 664–674
DOI 10.1007/s13238-010-0088-9

Protein & Cell

RESEARCH ARTICLE

Specific interaction of hepatitis C virus glycoproteins with mannan binding lectin inhibits virus entry

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Received June 9, 2010 Accepted June 24, 2010

ABSTRACT

Mannan-binding lectin (MBL) is a soluble innate immune protein that binds to glycosylated targets. MBL acts as an opsonin and activates complement, contributing to the destruction and clearance of infecting microorganisms. Hepatitis C virus (HCV) encodes two envelope glycoproteins E1 and E2, expressed as non-covalent E1/E2 heterodimers in the viral envelope. E1 and E2 are potential ligands for MBL. Here we describe an analysis of the interaction between HCV and MBL using recombinant soluble E2 ectodomain fragment, the full-length E1/E2 heterodimer, expressed *in vitro*, and assess the effect of this interaction on virus entry. A binding assay using antibody capture of full length E1/E2 heterodimers was used to demonstrate calcium dependent, saturating binding of MBL to HCV glycoproteins. Competition with various saccharides further confirmed that the interaction was via the lectin domain of MBL. MBL binds to E1/E2 representing a broad range of virus genotypes. MBL was shown to neutralize the entry into Huh-7 cells of HCV pseudoparticles (HCVpp) bearing E1/E2 from a wide range of genotypes. HCVpp were neutralized to varying degrees. MBL was also shown to neutralize an authentic cell culture infectious virus, strain JFH-1 (HCVcc). Furthermore, binding of MBL to E1/E2 was able to activate the complement system via MBL-associated serine protease 2. In conclusion, MBL interacts directly with HCV glycoproteins, which are present on the surface of the virion, resulting in neutralization of HCV particles.

KEYWORDS hepatitis C virus, neutralization, mannose binding lectin, chronic viral infection

INTRODUCTION

Hepatitis C virus (HCV) is the causal agent of hepatitis C, an infection causing more than 170 million chronic infections worldwide (Anon, 1999). HCV infection has been proven to cause liver fibrosis, cirrhosis and liver cancer, as well as digestive tract hemorrhage. Approximately 1.4 million deaths in 2001 were caused by chronic liver disease, of which 280,000 were attributed to HCV infection (Kim, 2002). It is estimated that some 20% of infected patients are able to spontaneously clear the virus (Zhang et al., 2006). A further 50%–60% of patients have a sustained response following antiviral treatment. The response to treatment is dependent on the genotype of the infecting virus (Pawlotsky, 2006). This might be a result of differing efficacy of a sustained immune response against these viruses. Indeed, results of animal and clinical studies highlight a potential role for neutralizing antibody responses in both acute and chronic infection (Farci et al., 1994; Ishii et al., 1998; Steinmann et al., 2004; Pestka et al., 2007). Furthermore, the strength and quality of T cell responses have been associated with viral clearance or

persistence (Gerlach et al., 1999; Thimme et al., 2001). There currently is no effective vaccine and a substantial proportion of treated patients do not achieve sustained response to treatment.

Although adaptive immunity is directly correlated with viral clearance, the capacity for the innate immune system to prime adaptive responses also contributes to the quality of the immune response (Pulendran and Ahmed, 2006; Sanders et al., 2006). Further evidence is gathering as to the importance of the innate immune system in combating HCV disease, for example, NK cells (Takehara and Hayashi, 2005; Golden-Mason et al., 2008) and intracellular innate immunity (Meyer et al., 2002). One innate immune factor linked with HCV disease progression and response to treatment is mannan binding lectin (MBL) (Matsushita et al., 1998a, b; Sasaki et al., 2000). MBL is an acute-phase reactant protein secreted from the liver into the blood (Thiel et al., 1992; Bouwman et al., 2005). It is a soluble pattern recognition receptor (PRR), which is able to recognize carbohydrate patterns associated with infectious non-self surfaces. MBL exists in complexes with MBL-associated serine proteases (MASPs) (Tan et al., 1996; Thiel et al., 1997), which activate the complement cascade to limit the spread of infection. MBL is a C-type lectin that contains the EPN motif, which confers specificity for mannose-type sugars (Weis et al., 1992). MBL polymorphisms resulting in a deficiency of MBL have been linked with an increased risk of bacterial infections (Kilpatrick et al., 2003). More recently MBL deficiency has been identified as an important factor in the defense against hepatitis B virus infection (Chong et al., 2005). MBL has previously been shown to interact with a range of viruses, including HIV (Ying et al., 2004; Ji et al., 2005a), influenza (Anders et al., 1990; Malhotra et al., 1994) and Ebola (Ji et al., 2005b) viruses. Previous studies have identified MBL polymorphisms as factors that influence the course of HCV infection (Matsushita et al., 1998b; Sasaki et al., 2000; Koutsounaki et al., 2008). MBL has also been implicated in the pathogenesis of HCV infection (Endo et al., 2001; Brown et al., 2007a). Other studies have found no correlation between low serum levels of MBL and susceptibility to hepatitis C virus (Kilpatrick et al., 2003; Vallinoto et al., 2009).

To date there have been no reports of the biochemical interaction between MBL and HCV. Here we show for the first time that MBL can interact directly with the E1 and E2 glycoproteins of HCV, and that this interaction results in neutralization of entry in the HCVpp and HCVcc models of infection, as well as activation of the lectin-dependent complement fixation pathway.

RESULTS

Although MBL has been linked with disease progression and response to treatment, its interaction with hepatitis C virus structural proteins has not yet been demonstrated. Our study aimed to investigate the binding of MBL to HCV glycoproteins, and subsequently find whether this interaction inhibits virus entry.

Binding of MBL to HCV sE2 and E1/E2

To determine the binding of MBL to the glycoproteins of HCV, an immunosorbent assay was employed with a fixed concentration of target molecules and a dilution series of purified MBL. MBL was shown to bind to HCV H77c sE2 in a dose-dependent, saturable manner (Fig. 1A). The sE2 bound less MBL than the equivalent mass of pure mannan. Binding was demonstrated to be dependent on the presence of calcium ions, and was completely abrogated by EDTA, a chelator of calcium ions. Denaturing the sE2 had only a minor effect on binding of MBL, suggesting that the tertiary structure of the virus glycoprotein is not required for the presence of MBL-specific molecular patterns. There was no binding to BSA under these conditions.

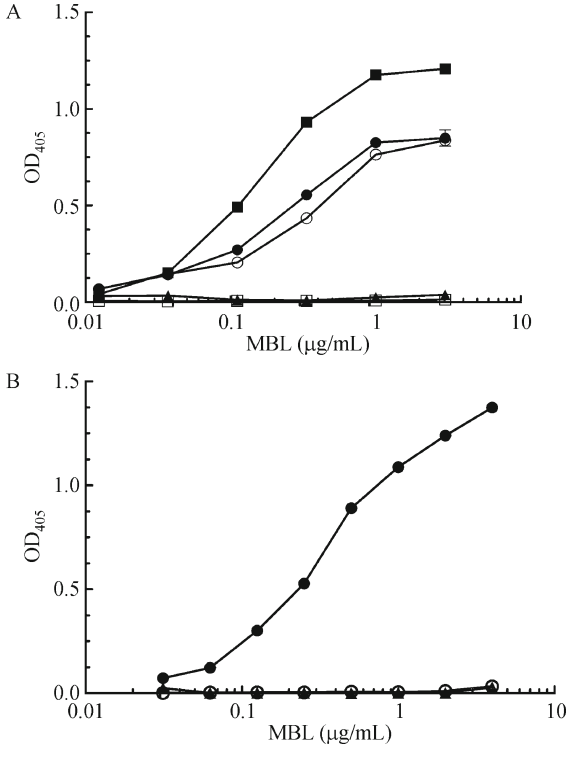

Figure 1. Binding of MBL to HCV glycoproteins, purified $sE2_{661}$ and E1/E2. (A) Purified human MBL was applied to wells coated with 20 μg/mL mannan (■), BSA (▲), native HCV $sE2_{661}$ in the presence of 5 mM $CaCl_2$ (●) or 5 mM EDTA (□), or denatured HCV $sE2_{661}$ (○). (B) MBL was applied to wells with E1/E2 captured from HEK 293FT cell lysate by monoclonal antibody ALP98 in the presence of 5 mM $CaCl_2$ (●) or 5 mM EDTA (○). Control lysates containing no E1/E2 were included (▲). In each case, MBL binding was detected by ELISA.

MBL binding to HCV glycoproteins was further investigated with E1/E2 heterodimers. Specificity in this assay was achieved by capturing E1/E2 from cell lysates via the monoclonal antibody ALP98, directed to a linear epitope between amino acids 644–651 (referenced to the polyprotein of strain H77c) in the E2 protein. To ensure that this antibody did not interfere with the binding of MBL, a comparison was made with another anti-E2 monoclonal antibody AP33, directed to a distinct epitope between amino acids 413–420 of E2 (Tarr et al., 2006). Capture of E1/E2 via either of these antibodies allowed subsequent detection of MBL binding, although a stronger signal was obtained when capturing the E1/E2 complex with ALP98 (data not shown). MBL bound to E1/E2 from cell lysates in a dose-dependent manner. This binding was also Ca^{2+}-dependent, as shown by abrogation of binding in the presence of EDTA (Fig. 1B). A negative control lysate from untransfected cells was used to show the specificity of this interaction.

Saccharide competition of MBL binding to HCV E1/E2

To further investigate the nature of binding of MBL to HCV E1/E2, a saccharide competition assay was performed. Saccharides were selected that were good representatives of those with which MBL has been shown to interact strongly (GlcNAc, D-mannose), with intermediate strength (maltose, galactose) and weakly or not at all (GalNAc and L-mannose). These saccharides were incubated with MBL for 30 min prior to addition to the microtiter plate containing ALP98-captured E1/E2. The binding of MBL to E1/E2 was inhibited by GlcNAc, D-mannose, maltose and galactose with IC_{50}s of 18.4 mM, 22.8 mM, 34 mM and 119 mM, respectively (Fig. 2). Inhibition of binding of MBL to E1/E2 by these saccharides was dose dependent. Inhibition of 50% binding was not achieved for GalNAc or L-mannose.

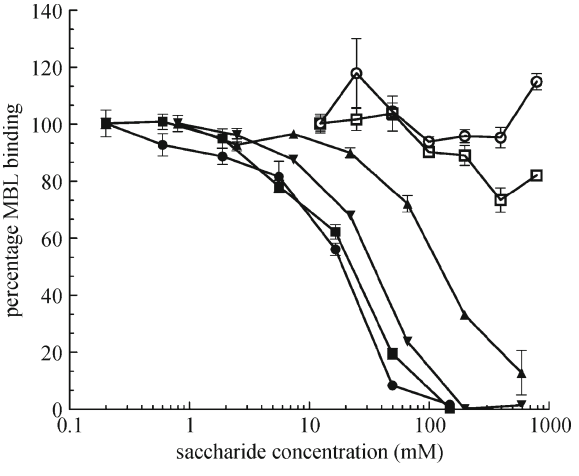

Figure 2. Effect of saccharides on the binding of MBL to E1/E2. HCV E1/E2 derived from clone H77.20 and generated in HEK 293FT cells was captured on plates by monoclonal antibody ALP98. MBL was pre-incubated with the indicated concentrations of N-acetylglucosamine (●), D-mannose (■), maltose (▼), galactose (▲), N-acetylgalactosamine (○), and L-mannose (□) prior to addition to the plate. Results are consistent with previously published avidity series for MBL (see text).

MBL Binding to E1/E2 from diverse HCV genotypes

HCV as a species exists as a number of different genotypes, each differing from the others by more than 30% of the nucleotide sequence (Simmonds et al., 2005). Some of these differences in sequence affect the glycosylation potential of E1 and E2. This could affect the ability of MBL to bind to proteins derived from different HCV genotypes. Using the ALP98 capture assay, we tested MBL binding to functional E1/E2 heterodimers expressed from a representative panel of HCV genotypes, which had been previously characterized as facilitating infection of HCV pseudoparticles (Lavillette et al., 2005; Owsianka et al., 2005; Tarr et al., 2006). The proteins in our study have between 13 and 16 sequons for N-linked sugars (Table 1). MBL bound to E1/E2 proteins derived from all the genotypes of HCV tested (Fig. 3). Binding was MBL-dose-dependent and approached saturation, in most cases with 4 μg/mL MBL. The greatest increase in binding to each genotype of E1/E2 occurred between 0.25 and 0.5 μg/mL MBL. Our data showed no correlation between number of sequons and MBL binding.

Amino acid numbering is referenced to the polyprotein of the H77c strain of HCV (Genbank accession number AF011751). Virus strains included are molecular clones H77c and JFH1, as well as clones from the well-characterized panel of patient-derived E1E2 clones from the University of Nottingham Trent Cohort (Owsianka et al., 2005, 2006; Tarr et al., 2006).

Neutralization of HCV pseudoparticles and cell culture infectious HCV

Neutralization of viral particles by MBL in a retrovirus-based pseudoparticle assay was performed (Fig. 4). The ability of MBL to inhibit virus infectivity in Huh-7 cells was observed at various concentrations of MBL. Inhibition was incomplete under all conditions tested, with representative genotypes shown (Fig. 4A). Pseudoparticles expressing different genotypes of E1/E2 were inhibited to different degrees by MBL. Representative data from three experiments shows that at 20 μg/mL, MBL reduces the infectivity of the different genotypes by up to 50% (Fig. 4B). The amount of inhibition of infectivity may bear some relation to the different genotypes, although this relationship is not clear cut. For example, all of the genotype 2 clones are of intermediate susceptibility to MBL in this assay (range 58% to 66% infectivity). However, E1/E2 clones representing genotype 1

Table 1 Locations of potential N-linked glycosylation sites on the E1 and E2 glycoproteins of HCV

virus	glycosylation site at amino acid residue																		total
	E1						E2												
	197	210	235	251	300	306	418	424	431	449	477	533	541	557	577	582	624	646	
H77C	+	+	+			+	+	+	+	+	+	+	+	+	+		+	+	15
1A14.38	+	+	+			+	+	+	+	+	+	+	+	+	+		+	+	15
1A20.8	+	+	+			+	+	+	+	+		+	+	+	+		+	+	14
1B5.23	+	+	+	+		+	+	+	+	+		+	+	+	+		+	+	15
1B12.16	+	+	+	+		+	+	+	+	+		+	+	+	+		+	+	15
JFH-1	+	+	+			+	+	+	+	+	+	+	+	+	+		+	+	15
2A1.2	+	+	+			+	+	+	+	+	+	+	+	+	+		+	+	15
2A2.4	+	+	+			+	+	+	+	+	+	+	+	+	+		+	+	15
2B1.1	+	+	+		+	+	+	+	+	+	+	+	+	+	+		+	+	16
2B2.8	+	+	+		+	+	+	+	+	+	+	+	+	+	+		+	+	16
3A1.28	+	+	+			+	+	+	+	+	+	+		+	+		+	+	14
3A13.6	+	+	+			+	+	+	+	+	+	+		+	+	+	+	+	15
4.11.1	+	+	+			+	+	+	+	+	+	+	+	+	+		+	+	15
4.21.16	+	+	+			+	+	+	+	+	+	+	+	+	+		+	+	15
5.14.4	+	+	+			+	+	+	+	+	+	+	+	+	+		+	+	15
5.15.7	+	+	+			+	+	+		+		+	+	+	+		+	+	13
6.5.340	+	+	+		+	+	+	+	+	+	+	+		+	+		+	+	15
6.5.8	+	+	+		+	+	+	+	+	+	+	+		+	+		+	+	15

display the broadest range of inhibition by MBL, with clone UKN1B5.23 showing no susceptibility with 98.0%± 4.0% infectivity and clone UKN1A14.38 showing 50.0%± 1.5% infectivity at 20 μg/mL MBL.

Following demonstration of neutralization by MBL in the pseudoparticle assay we determined whether MBL was capable of neutralizing a cell culture infectious clone of HCV (HCVcc). HCVcc pre-incubated with various concentrations of MBL was used to infect naïve Huh-7 cells and the relative quantity of HCVcc RNA was assessed by real-time PCR. MBL was capable of neutralizing 95% of HCVcc infectivity at a concentration of 200 μg/mL (Fig. 5A) and about 75% neutralization at 20 ug/mL. Furthermore, blocking the interaction of MBL with HCVcc by the addition of mannose restored HCVcc infectivity (Fig. 5B). Restoration of infectivity was complete at 100 μg/mL mannose.

Activation of MBL/MASP-2 complexes on binding to E1/E2

To determine whether the binding of MBL to HCV glycoproteins induced activation of the MBL associated serine protease 2 (MASP-2), MBL from pooled human serum (HCV negative) was applied to dilutions of H77.20 E1/E2 immobilized to microtiter wells by capture with monoclonal antibody ALP98. A C4b deposition assay showed specific increase in C4b deposition in the presence of E1/E2 as compared with negative cell lysates (Fig. 6). Further dilution of lysate resulted in deposition of C4b at no more than the level of the negative lysate. This indicated that the E1 and E2 proteins are capable of activating MBL/MASP-2 complexes only when captured at sufficient density on the microtiter plate surface. This is consistent with the role of MBL as a pattern recognition receptor, requiring an array of target carbohydrates for high avidity binding.

DISCUSSION

As a factor in innate immunity, MBL has been implicated in the control of a range of infectious diseases including viral hepatitis caused by HBV and HCV (Chong et al., 2005; Brown et al., 2007b; Alves Pedroso et al., 2008; Koutsounaki et al., 2008). The precise role played by MBL in these diseases is unclear. Several possibilities arise as the functions of MBL in innate immunity include opsonization, direct blocking of viral entry and complement activation (Krarup et al., 2007). Studies of the relationship between HCV pathogenesis and MBL have examined correlations between MBL levels in circulation, *MBL2* haplotype and disease severity or response to treatment. Overall, these studies indicate the potential for a role of MBL in HCV disease (Alves Pedroso et al., 2008; Koutsounaki et al., 2008). To date there have been no

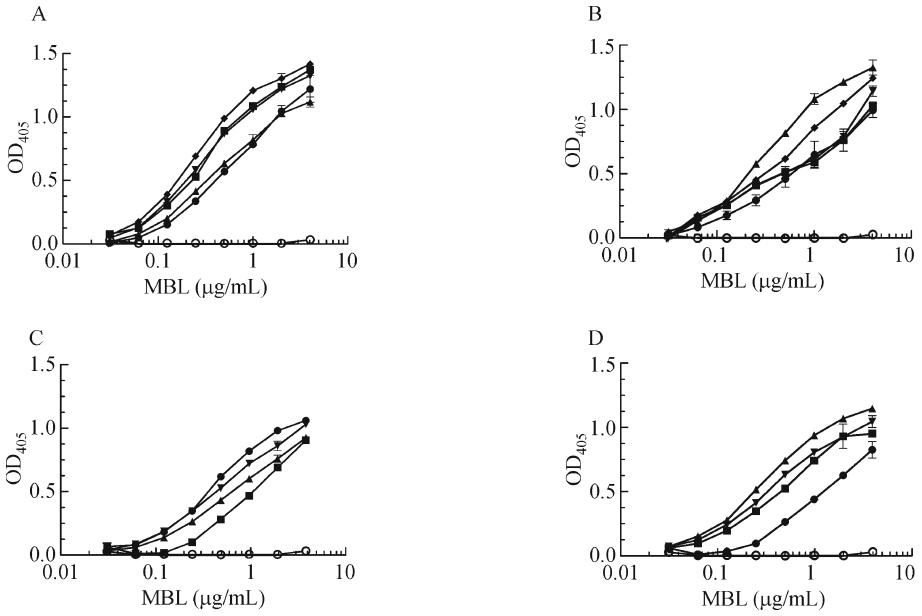

Figure 3. Analysis of the binding of MBL to HCV E1/E2 from a diverse range of viral genotypes. Lysates of HEK 293FT cells containing viral glycoproteins derived from genotypes 1a, 1b (A), 2a, 2b (B), 3a, 3b, 4 (C), 5 and 6 (D) or negative lysates (○) were captured in wells by monoclonal antibody ALP98 or AP33 as appropriate. Clones were selected on the basis that they formed functional glycoproteins as determined by the pseudotype assay described previously (Lavillette et al., 2005; Owsianka et al., 2005; Tarr et al., 2006). (A) Genotype 1 clones were H77.20 (■), UKN1A14.38 (●), UKN1A20.8 (▼), UKN1B5.23 (▲), and UKN1B12.16 (◆). (B) Genotype 2 clones were JFH-1 (■), UKN2A1.2 (●), UKN2A2.4 (▼), UKN2B1.1 (▲) and UKN2B2.8 (◆). (C) Genotype 3 and 4 clones were UKN3A1.28 (■), UKN3A13.6 (●), UKN4.11.1 (▼) and UKN4.21.1 (▲). (D) Genotype 5 and 6 clones were UKN5.14.4 (■), UKN5.15.7 (●), UKN6.5.340 (▼) and UKN6.5.8 (▲). MBL was applied as indicated in a twofold dilution series and was detected by ELISA.

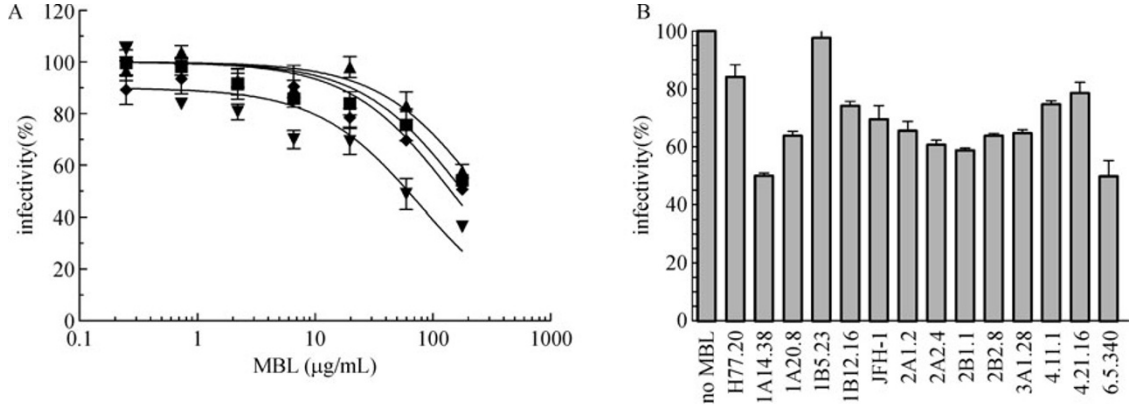

Figure 4. Neutralization of HCV pseudoparticles (HCVpp) derived from diverse genotypes by MBL. (A) HCVpp derived from clones H77 (■), JFH-1 (▼), UKN 1B5.23 (▲) and UKN 4.21.16 (◆) were pre-incubated with MBL at the indicated concentrations prior to infection of Huh-7 cells. The neutralization activity of MBL is expressed as percentage of inhibition of the infectious titers. (B) A comparison of the sensitivity of HCVpp, displaying glycoproteins derived from different genotypes, to MBL at 20 μg/mL.

published studies on the biochemical interaction of MBL with HCV and only one measuring MBL function in relation to disease (Brown et al., 2007a). Here we demonstrate MBL binding to glycoproteins derived from diverse genotypes of HCV and show that such binding is sufficient to inhibit virions from infecting cells.

The binding of MBL to target surfaces relies on the interaction of its lectin domains with an array of carbohydrates across different N-linked glycans. The conformation of lectin domains in native MBL favors high avidity binding only to such arrays as are found on microbial surfaces and not on host glycoproteins (Hart et al., 2003). MBL has a preference for

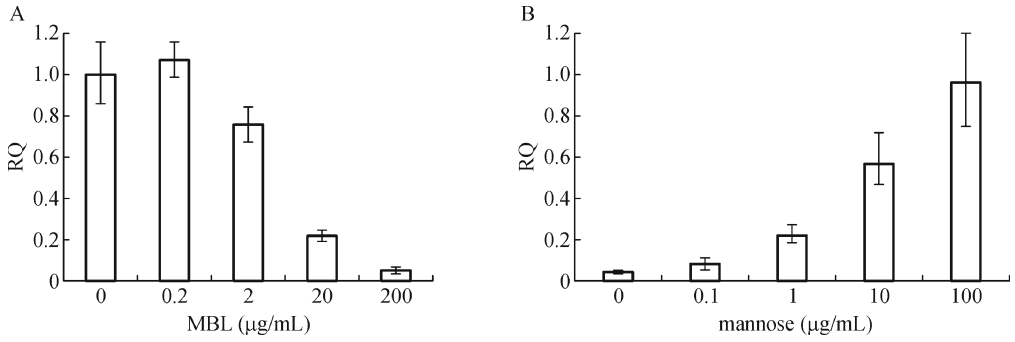

Figure 5. Inhibition of HCVcc by MBL. (A) Purified MBL was incubated with HCVcc virions before adding to target cells. Inhibition was determined for serial dilutions of MBL as determined by the reduction in relative quantity (RQ) of HCVcc RNA. (B) Using 20 μg/mL MBL to neutralize entry of HCVcc, infectivity was restored with the addition of mannose, in a dose-dependent manner.

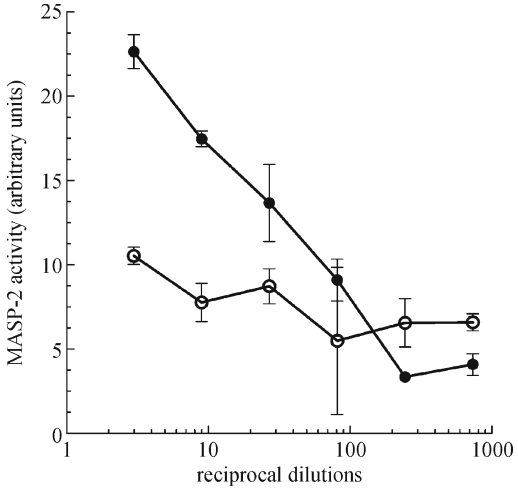

Figure 6. Activation of MBL/MASP-2 complexes was determined by the level of C4b deposited after binding to E1/E2 (•) or negative lysate (○). MBL/MASP-2 complex activity is expressed in arbitrary units based on C4b deposition as measured by ELISA.

binding to terminal mannose and N-acetyl glucosamine residues. The precise avidity of MBL binding to a target therefore depends upon both the carbohydrate structures present and their spatial orientation. We have demonstrated that the glycoproteins derived from different HCV genotypes all bind MBL, but to differing extents. Differences in binding occurred between glycoproteins derived from two viruses of the same genotype that differed in the number of N-linked glycan sequons present. This was highlighted by the two genotype 3 isolates and the two genotype 5 isolates (Fig. 3). However, there was no consistent pattern to the relative levels of binding based on the number of glycosylation sequons. Production of glycoproteins with individual glycan knockout mutations would permit characterization of binding in comparison to wild-type proteins. This may provide further evidence for binding of MBL to differentially glycosylated glycoproteins. Differences in the number of potential glycosylation sites occupied by glycans have been linked to HCV cell entry and the accessibility of neutralizing antibodies to epitopes on E2 (Goffard and Dubuisson, 2003; Helle et al., 2007). MBL may preferentially bind glycoproteins where the number, or positions, of glycans present inhibit antibody neutralization.

Binding of MBL to HCV glycoproteins, and subsequent inhibition of infectivity in *in vitro* models demonstrates that essential interactions in the entry steps of the virus are blocked by MBL. This inhibitory effect has also been observed for the bacterial lectin cyanovirin-N (Helle et al., 2006), which blocks the interaction with the CD81 entry receptor. It remains to be determined which interactions are specifically blocked by MBL. Our current data demonstrates that MBL at a level within the range observed in HCV patients *in vivo* (Brown et al., 2007a) is capable of blocking infectivity in all the genotypes tested to varying degrees, and as such must target a conserved step in the entry process. It was previously shown that neutralization of influenza A by MBL was related to the number of potential glycosylation sites present (Reading et al., 1997). A clearly defined relationship between numbers of N-linked glycan sequons and MBL neutralization does not exist for the HCV pseudoparticles tested here. However, there does seem to be a trend toward those more highly glycosylated on E1 being more susceptible to MBL neutralization. The lack of a clear association between number of potential glycosylation sites and neutralization potentially indicates a difference between MBL binding to recombinant glycoproteins as opposed to virus particles. There is likely to be a more subtle effect of overall spatial arrangement of glycans on the surface of the virion as opposed to soluble proteins. Furthermore, it is possible that target glycans are presented in different arrays on the surface of HCVpp and HCVcc. For JFH-1 envelope sequences, MBL was able to neutralize HCVcc better than HCVpp. This difference has been observed for neutralizing monoclonal antibodies (Owsianka et al., 2008). Subtle differences between proteins in the context of sE2, recombinant E1/E2,

virus like particles or pseudoparticles have previously been demonstrated (Owsianka et al., 2001; Clayton et al., 2002).

All potential glycosylation sequons in the envelope glycoproteins of HCV have been described as being occupied by glycans, except for a C-terminal position in E1 which is followed by a proline residue (Goffard and Dubuisson, 2003). These glycans display microheterogeneity, with the majority being occupied by high mannose, and a minority having complex oligosaccharides (Iacob et al., 2008). However, these studies only examined individual strains of HCV. Comparisons between different genotypes with equal numbers of N-linked glycan sequons, as used in the present study, are difficult to interpret as there is limited information on which of the sequons in each strain are modified, and to what extent. The fine structure of these proteins is likely to be subtly different. Studies to determine precise structures of glycans present on both E1 and E2, together with the spatial arrangements of these glycans, would aid understanding of the role of MBL in binding to HCV. It would be informative to look at different combinations of glycosylation in the context of the same polypeptide backbone, to determine the specific glycans required for MBL binding. It also remains to be determined if the different glycosylation states of the glycans modulates MBL binding. For influenza, glycans attached neuraminidase (Malhotra et al., 1994) or to the globular head of HA conferred sensitivity to collectins (Reading et al., 1997).

We have demonstrated here that binding of MBL to HCV glycoproteins is sufficient to activate the complement system via deposition of C4b. Complement was previously shown to enhance antibody neutralization of HCVpp (Meyer et al., 2002), suggesting that MBL-mediated deposition of complement could be involved in clearance of viral particles. C4 activity has also been shown to correlate with successful response to therapy (Dumestre-Perard et al., 2002). This indicates that the role of MBL in HCV disease may include blocking viral entry and activation of the complement system. Complement has been implicated in clearance of HIV virions (Aasa-Chapman et al., 2005). It was also recently demonstrated that L-ficolin, a protein structurally and functionally related to MBL, can activate complement on binding to HCV (Liu et al., 2009). This conserved function of collagenous, complement-activating proteins is likely to be an important element of the immune response to viral infections.

Previous studies concerning MBL and HCV have focused on clinical data. They have linked MBL polymorphisms to disease severity or potential response to treatment, associating mutant alleles with more aggressive disease or a poor response to therapy (Matsushita et al., 1998a, b; Sasaki et al., 2000; Alves Pedroso et al., 2008; Koutsounaki et al., 2008). MBL polymorphisms affect circulating levels of the protein and the number of lectin domains per molecule. Some studies examined the relationship of serum levels of MBL were with disease outcomes (Kilpatrick et al., 2003; Brown et al., 2007a; Alves Pedroso et al., 2008; Koutsounaki et al., 2008). These studies implicate MBL in the pathogenesis of chronic HCV disease. However, these findings might be indicative of other underlying factors influencing HCV disease. Polymorphisms of MBL have been linked with susceptibility to hepatocellular carcinoma or cirrhosis in individuals infected with hepatitis B virus (Chong et al., 2005). This may be linked to an inability to clear virus as MBL activates complement on binding to HBV surface antigen. Further studies are required to confirm the role of MBL in HCV disease.

Taken together, our results support the idea that MBL may play a role in HCV disease, through blocking entry of virions into host cells and activating the complement system to clear viral particles and recruit other elements of the immune system. The precise nature of this activity is likely to be influenced by the extent of glycosylation of the viral glycoproteins and the spatial arrangement of the N-linked glycans; factors which differ across and within genotypes. Further studies are warranted to investigate the result of MBL binding to viral particles in terms of complement activation and opsonization of viral particles.

MATERIALS AND METHODS

Cell culture

Human hepatoma cells Huh-7 (Nakabayashi et al., 1982) and human epithelial kidney (HEK) 293FT cells (Invitrogen) were grown in Dulbecco' s modified Eagle's medium, (Invitrogen) supplemented with 10% fetal calf serum, 5% non-essential amino acids, and 2 mM glutamine (Sigma).

Viral glycoproteins

HCV E2 was produced and purified as previously described, with some modifications (Yamada et al., 2005). Briefly, HEK 293FT cells in OptiMEM (Invitrogen) were transfected with plasmid expressing soluble E2 fused to 6 × his tag ($sE2_{661}$ or sE2) using Lipofectamine 2000 (Invitrogen). Supernatant was harvested after 72 h and 20 mM iodoacetamide was added. Supernatants were centrifuged to remove debris, adjusted to 300 mM NaCl and loaded onto a HisTrap column (1 mL: GE Healthcare). HCV sE2 was eluted with 150 mM imidazole following a 50 mM imidazole wash. Fractions were concentrated on a Vivaspin column (Vivascience). Concentrated sE2 was subjected to size exclusion chromatography on a TSK 3000SWXL column (7.5 × 300 mm: TosoHaas). Fractions of monomeric sE2 were retained for binding studies.

HCV E1/E2 heterodimer was produced in HEK 293FT cells as previously described (Owsianka et al., 2005). E1/E2 clones are named here using the nomenclature of the well-defined UKN glycoprotein panel (Lavillette et al., 2005; Owsianka et al., 2005; Tarr et al., 2006). Transiently transfected cells were harvested after 72 h by lysis in 50 mM Tris-HCl, 150 mM NaCl, 20 mM Iodoacetamide, 1% NP-40, pH 7.6. A sample of cell lysate was analyzed for E1/E2 content. The remainder of the cell lysate was stored frozen at −80°C until assayed.

Antibodies

Polyclonal anti-human MBL was raised in rabbits against recombinant MBL and was adsorbed to remove anti-mannan antibodies as described previously (Arnold et al., 2004). Mouse monoclonal anti-HCV E2 antibodies ALP98 and AP33 were raised against recombinant soluble E2 as previously described (Clayton et al., 2002).

Mannan binding lectin

Human MBL-MASPs complexes were purified from 1 L of pooled citrated plasma (HD Supplies), free from anti-HCV antibodies, using a method adapted from Tan et al. (Tan et al., 1996). Briefly, serum was generated from the plasma, then PEG 3350 (Sigma) added to a final concentration of 7% (*w/v*) and the mixture stirred overnight at 4°C. Precipitated proteins were dissolved in ice-cold TBS-TCa^{2+} Buffer (50 mM Tris-HCl, 1 M NaCl, 20 mM $CaCl_2$, 0.05% Tween, pH 7.8) and incubated with 25 mL mannan-agarose (Sigma) for 2 h. Bound MBL was eluted with TBS-EDTA Buffer (50 mM Tris-HCl, 1 M NaCl, 10 mM EDTA, pH 7.6). Contaminating IgG and IgM were removed using a HiTrap Protein G HP column (GE Healthcare) and anti-human IgM agarose (Sigma) respectively. MBL purity was assessed by SDS-PAGE and quantified by ELISA as previously described (Arnold et al., 2004). MASPS are not separated from MBL by these purification procedures. MBL without MASPs was obtained by dialyzing the preparation into gel filtration running buffer (0.1 M sodium acetate, 0.2 M NaCl, 5 mM EDTA, pH 5.0) and running on a Superose 6 gel filtration column (GE Healthcare). The fractions judged to contain MBL, were analyzed by SDS-PAGE and pooled. The resulting MBL was pure and was used as a standard to quantify MBL by ELISA (Arnold et al., 2004).

Detection of MBL binding to HCV sE2 by enzyme-linked immunosorbent assay (ELISA)

ELISA plates (Maxisorp, Nunc) were coated with 50 ml HCV sE2, mannan or BSA (all at 20 μg/mL) in coating buffer (0.1 M NaHCO3 pH 9.5) for 1 h at 37°C. After washing 3 times with phosphate buffered saline (PBS) 0.05% (*v/v*) Tween-20 (PBS-T) wells were incubated with 200 μL blocking buffer (PBS-T containing 5% (*w/v*) milk powder) for 1 h at 37°C. The plates were washed 3 times with wash buffer (10 mM HEPES, 1 M NaCl, 5 mM $CaCl_2$, 0.05% (*v/v*) Tween-20, pH 7.6). MBL was diluted in wash buffer or EDTA Buffer (10 mM HEPES, 1 M NaCl, 5 mM EDTA, 0.05% (*v/v*) Tween-20, pH 7.6) in a 3-fold serial dilution (3 mg/mL to 12 ng/mL) and 50 μL was added to the appropriate wells. Plates were incubated with MBL overnight at 4°C. Washing was repeated and 50 μL of anti-rMBL, diluted 1/700 in wash buffer, was added per well for incubation at 37°C for 1 h. Wells were washed and incubated with 50 μL of 1.3 μg/mL alkaline phosphatase (AP)-conjugated monoclonal anti-rabbit IgG (Sigma) in wash buffer. Following 3 final washes, 50 μL of pNPP (ρ-Nitrophenyl Phosphate) substrate (Sigma) was added to each well. The OD at 405 nm was determined after 30 min incubation at room temperature.

Detection of MBL binding to HCV E1/E2 by capture ELISA

Each well of a 96 well microtiter plate (Nunc) was coated with 50 μL of 10 μg/mL mouse monoclonal antibody ALP98 in coating buffer and incubated for 1 h at 37°C. Blocking was then performed with 200 μL per well of PBS-T overnight at 4°C. Three washes per well were then performed with wash buffer. Next, 50 μL of H77c (47) E1/E2 lysates were added to each well, at 1/10 dilution in PBS-T, then incubated for 1 h at 37°C. Wells were washed again three times with wash buffer. Two fold serial dilutions of MBL were made in wash buffer, or EDTA buffer to give final concentrations of MBL from 4 μg/mL to 31 ng/mL. MBL dilutions were added in triplicate to each appropriate well and incubated at 37°C for 1 h (50 μL/well). Bound MBL was detected as described for the sE2 assay.

Binding of MBL to representative clones of each HCV genotype of E1/E2 was performed by the above method. Equivalent concentrations of each E1/E2 were calculated experimentally as previously described (Clayton et al., 2002). Briefly, proteins were captured with *Galanthus nivalis* agglutinin (GNA; Sigma) and bound proteins detected with ALP98 followed by anti-mouse IgG AP conjugate and pNPP substrate. The dilution of lysate at which equivalent ODs were obtained was subsequently used in the MBL assay.

Saccharide competition assay

To test for the inhibition of binding by saccharides, E1/E2 glycoproteins were captured on microtiter plates by monoclonal antibody ALP98 as described above. MBL (1 μg/mL) was incubated with various concentrations of each saccharide for 30 min at 20°C before addition to the plate. Bound MBL was detected as above. IC_{50}s were determined from inhibition curves.

HCVpp infection and neutralization assays

Full-length E1/E2 (representing amino acid residues 170 to 746 of the HCV open reading frame referenced to strain H77c (Yanagi et al., 1997)) clones were generated and their nucleotide sequence determined as previously described (Lavillette et al., 2005). HCVpp were produced essentially as previously described (Bartosch et al., 2003) by cotransfection of plasmids expressing the full-length E1/E2, murine leukemia virus (MLV) Gag-Pol and the MLV transfer vector carrying either the green fluorescent protein (GFP) or the firefly luciferase gene under the control of the human cytomegalovirus promoter. MBL-mediated neutralization of HCVpp was carried out in a similar manner to that previously described for antibody neutralization (Owsianka et al., 2005). MBL was pre-incubated with HCVpp for 30 min at 37°C in 3-fold serial dilutions from 180 μg/mL to 0.25 μg/mL and then the particles were used to infect Huh-7 cells to observe inhibition of infectivity. Infectivity was expressed as percentage of reporter gene activity compared with uninhibited control.

Production of cell culture infectious HCV (HCVcc) and neutralization assays

HCVcc was generated essentially as described by Wakita et al. (2005). Briefly, the plasmid pJFH1-pUC carrying the full-length cDNA of the genotype 2a HCV strain JFH-1 was linearized with XbaI and treated with mung bean nuclease. The linearized construct was then used as a template to generate viral genomic RNA by *in vitro* transcription. Approximately 10 μg of *in vitro* synthesized RNA was electroporated into Huh-7 cells in a 0.4 cm Gene Pulser cuvette (Bio-Rad) and pulsed once at 960 μF and 270V using the GenePulser

Xcell (Bio-Rad) electroporator. The transfected cells were immediately mixed with cell medium and seeded into a tissue culture dish or flask. Following incubation at 37°C for 4 d, the medium containing the infectious virus progeny was clarified by brief centrifugation to remove cell debris, filtered through 0.45 μm pore-sized membrane, and used to infect naïve Huh-7 cells.

To perform MBL-mediated neutralization of HCVcc, the filtered medium containing HCVcc was mixed with appropriate amounts of MBL and incubated for 1 h at 37°C prior to application to the naïve Huh-7 cells. Following incubation at 37°C for 4 d, cells were investigated for HCVcc RNA by quantitative RT-PCR (qRT-PCR). Total RNA was isolated using an RNeasy mini kit (Qiagen). For Real-Time analysis of total RNA from infected cells, a relative quantification (RQ) reaction was performed, where each sample was normalized to an endogenous control gene (GAPDH). First, cDNA was generated by reverse transcription of the total RNA using TaqMan Reverse Transcription Reagents (Applied Biosystems), with random primers. Each cDNA was analyzed in triplicate in a singleplex reaction to detect either HCV target cDNA with a Fluorescein-CE Phosphoramidite (6-FAM) labeled probe (250 nM) and HCV specific primers (900 nM each), or GAPDH with a pre-validated endogenous control VIC-probe/primer mix (Applied Biosystems). The HCV probe sequence was 6-FAM-AAAGGCCTTGTGGTACTG-MGB (synthesized by Applied Biosystems), and primer sequences were: 5'-TCTGCGGAACCGGTGAGTAC-3', forward, and 5'- GCACTCG-CAAGCACCCTATC-3', reverse (Sigma). Real-time reactions were run using TaqMan Fast Universal PCR Master Mix, without ampErase UNG, (Applied Biosystems) under Fast Universal conditions on a 7500 Fast Real-Time PCR machine and data analyzed using 7500 Fast System Software (SDS v1.3.1) (Applied Biosystems).

The specificity of MBL-mediated inhibition of virus infection was confirmed by pre-incubating 20 μg/mL MBL with 10-fold dilution series of mannose (Sigma) starting from 0 to 100 μg/mL at 37°C for 45 min. This mix was then incubated with the virus for a further 45 min at 37°C before infecting the cells and the levels of infectivity were determined by measuring viral RNA by qRT-PCR.

Activation of MBL/MASP-2 complex on binding to E1/E2

H77 E1/E2 was captured in the ALP98-coated wells of a Microfluor 2 white microtiter plate (Thermo Labsystems) as described above. Wells were blocked with 5% skimmed milk powder in PBS-T. Following three washes with PBS-T, wells were incubated with serum diluted 1:1 in 40 mM HEPES, 2 M NaCl, 10 mM $CaCl_2$, pH 7.4. Plates were incubated for 1 h on ice. MBL/MASP-2 complex activity was assayed as previously described (Mayilyan et al., 2006) with modifications. Wells were washed twice at 37°C with 20 mM HEPES, 1 M NaCl, 5 mM $CaCl_2$, 0.1% (*v/v*) Tween-20, pH 7.4 and twice with 20 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$, pH 7.4 (wash buffer). The use of 1 M NaCl prevents C1q binding to the antibody on the plate. The wells were incubated with 4 μg/mL purified human complement component C4 diluted in wash buffer then washed 3 times in the same buffer. Rabbit anti-C4 (Sigma) diluted 1:1000 in wash buffer was applied to the wells and incubated for 1 h at 20°C. After three washes, alkaline-phosphatase conjugated goat anti-rabbit IgG (Sigma), diluted 1:1000, was added to the wells. After three washes with wash buffer, wells were developed with 200 μL of 1 mM 4-methylumbelliferyl-phosphate (Calbiochem), in 5 mM 2-(N-cyclohexylamino) ethanesulfonic acid (CHES), 1 mM $MgCl_2$, pH 9.8. The fluorescent product was measured by excitation at 355 nm and emission at 460 nm using a microtiter plate reader (Fluoroskan, ThermoLife Sciences). MBL/MASP-2 complex activity was calculated from the initial slope of the activity curve and expressed as arbitrary units per minute. All samples were tested in triplicate.

ACKNOWLEDGEMENTS

We would like to thank Takaji Wakita, Jens Bukh, Mark Harris and Francois-Loic Cosset for provision of reagents. Funding was provided by The Royal Society, The University of Nottingham, and the Medical Research Council (UK).

ABBREVIATIONS

CHES, 2-(N-cyclohexylamino) ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; E1/E2, HCV E1 and E2 glycoproteins; GNA, galanthus nivalis agglutinin; HCV, hepatitis C virus; HCVcc, cell cultured HCV; HCVpp, HCV pseudoparticle; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MASP-2, MBL-associated serine protease 2; MBL, mannan binding lectin; MLV, murine leukaemia virus; PBS-T, phosphate buffered saline with Tween-20; 6-FAM, fluorescein-CE phosphoramidite

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