Recognition of bacterial capsular polysaccharides and lipopolysaccharides
by the macrophage mannose receptor

Susanne Zamze1*, Luisa Martinez-Pomares2, Hannah Jones1, Philip R. Taylor2, Richard J.
Stillion2, Siamon Gordon2, and Simon Y.C. Wong1.

From the: 1 Edward Jenner Institute for Vaccine Research, Compton, Berkshire, RG20 7NN,
U.K. and the: 2 Sir William Dunn School of Pathology, University of Oxford, South Parks
Road, Oxford, OX1 3RE, U.K.

*To whom correspondence should be addressed: The Edward Jenner Institute for Vaccine
Research, Compton, Berkshire, RG20 7NN, UK. Tel: +44-(0)1635-577934; Fax: +44-
(0)1635-577901; E-mail: susanne.zamze@jenner.ac.uk.

Running title: Mannose receptor recognition of bacterial polysaccharides.
Summary

The in vitro binding of the macrophage mannose receptor to a range of different bacterial polysaccharides was investigated. The receptor was shown to bind to purified capsular polysaccharides from Streptococcus pneumoniae and to the lipopolysaccharides, but not capsular polysaccharides, from Klebsiella pneumoniae. Binding was Ca\(^{2+}\)-dependent and inhibitable with D-mannose. A fusion protein of the mannose receptor containing carbohydrate recognition domains 4-7 and a full length soluble form of the mannose receptor containing all domains external to the transmembrane region both displayed very similar binding specificities towards bacterial polysaccharides, suggesting that domains 4-7 are sufficient for recognition of these structures. Surprisingly, no direct correlation could be made between polysaccharide structure and binding to the mannose receptor, suggesting that polysaccharide conformation may play an important role in recognition. The full length soluble form of the mannose receptor was able to bind simultaneously both polysaccharide via the carbohydrate recognition domains and sulphated oligosaccharide via the cysteine-rich domain. The possible involvement of the mannose receptor, either cell surface or soluble, in the innate and adaptive immune responses to bacterial polysaccharides is discussed.
Introduction

The macrophage mannose receptor (MR) is considered an important molecule of innate immunity mediating the non-opsonic phagocytic uptake by macrophages (MØs) of a wide variety of microbes including yeast, fungi, protozoa and bacteria (1). It is a type I integral membrane glycoprotein expressed on most tissue macrophages (MØs), certain endothelial cells and in vitro-derived dendritic cells (DCs) (2-4). In addition to phagocytosis, the MR mediates the endocytosis of soluble glycoconjugates leading to enhanced uptake of ligands in both MØs and DCs (5).

The MR belongs to the Ca²⁺-dependent lectin family which bind sugars through their carbohydrate recognition domains (CRDs) (6). The MR itself has eight CRDs located on a single polypeptide chain. CRDs 4 and 5 of the MR are central to sugar binding and at least one further CRD proximal to the membrane is necessary for the binding and uptake of multivalent ligands (7,8).

The MR displays a mannose-type binding specificity recognising monosaccharides in the order of affinity D-mannose: (D-Man) = L-fucose (L-Fuc) > D-glucose (D-Glc) = D-N-acetyl glucosamine (D-GlcNAc) >>> D-galactose (D-Gal) (6). The binding of a monosaccharide by the CRD requires the presence of free equatorial hydroxyl groups at both the C₃ and C₄ positions of hexoses, and at the C₂ and C₃ positions of the 6-deoxyhexose, L-Fuc. With respect to microbial ligands, a high affinity of the MR for polysaccharides displaying numerous terminal D-Man residues, as exemplified by yeast mannan, is well documented. The recognition of other complex microbial polysaccharides, particularly those expressed by bacteria, however, has not been investigated in any depth.
In this study we investigate the binding of the MR to the capsular polysaccharides (CPSs) from different capsular serotype strains of *Streptococcus pneumoniae* and to the CPSs and lipopolysaccharides (LPSs) from different capsular and O-serotype strains of *Klebsiella pneumoniae*. Both bacterial species are major opportunistic pathogens and in both cases antibodies directed against their CPSs confer protection. Vaccines currently in use against *S. pneumoniae* are comprised of either CPS alone or are glycoconjugates based on the conjugation of CPS or oligosaccharides derived from CPS to a protein carrier.

In addition to binding through CRDs, the MR also has a second distinct lectin activity located in the N-terminal cysteine-rich (CR) domain of the molecule, this time with specificity for host sulphated oligosaccharides (9,10). Sulphated ligands for the CR domain are present in secondary lymphoid tissues where they are expressed on metallophilic marginal zone MØs in the spleen, subcapsular sinus MØs in lymph nodes and also on a sub-population of DCs (11,12).

A soluble form of the MR (sMR), released by constitutive proteolytic cleavage of the membrane bound form by an endogenous cell metalloprotease, is also present in serum (13). The CPSs of *S. pneumoniae* and *K. pneumoniae* are high molecular weight polysaccharides, typically $10^6$ Daltons and greater, and are composed of linear or branched repeating units containing from two to seven monosaccharides. *S. pneumoniae* CPSs variously contain D-glucuronic (D-GlcA) and D-galacturonic (D-GalA) acid residues, and may be substituted with O-acetyl, pyruvate acetal, and glycerol phosphate groups (14). Several resemble teichoic acids in containing ribitol-phosphate linked repeat units. They frequently contain amino-sugars which include different amino-hexoses and also N-acetyl-D-mannuronic acid. D-Gal, D-Glc and L-Rha are common components whereas D-Man is not found in any of these structures. All CPS preparations from *S. pneumoniae* contain from 1-10% by weight of common cell wall polysaccharide (CW-PS) which is the teichoic acid of this organism.
CPSs from *K. pneumoniae* commonly contain D-Glc, D-Man, D-Gal, and L-Rha. L-Fuc is found in only a very few serotype strains (15). They all contain one hexuronic acid residue in each repeat unit which is nearly always D-GlcA acid and are frequently substituted with pyruvate acetal or O-acetyl groups. Unlike CPSs from *S. pneumoniae*, phosphate groups and amino-sugars are absent from these polysaccharides. The uptake of *K. pneumoniae* by alveolar MØs mediated by recognition of CPS via the MR has been reported (16).

The structures of all of the CPSs used in the present study are shown in Table 1a & b (14,17-22).

In contrast to the CPS of *K. pneumoniae* the LPS O-antigens of this species display very limited structural variation with only nine recognised O-serotypes. Three main serotypes consist of linear polymers of α-linked D-mannose residues (serotype 03) and linear polymers of D-galacto-pyranose residues (galactan II) or both D-galacto-pyranose and D-galacto-furanose residues (galactan I) (serotypes 01 and 02) (Table 2a) (15). Other *K. pneumoniae* LPS types lack O-antigen (rough LPS).

In this study we investigate the interactions between bacterial polysaccharides and the MR *in vitro* using purified CPS and LPS and the following proteins: (a) CRDs 4-7 of the MR fused to human IgG1 Fc CRD(4-7)Fc (b) the CR domain of the MR fused to human IgG1 Fc (CR-Fc) and (c) the full length soluble form of the MR (sMR).

(INsert TABLES 1 & 2)
Experimental procedures

Preparation of monoclonal antibodies against the mouse mannose receptor

Monoclonal antibody MR5D3 was generated by subcutaneous immunisation of Fischer rats with 100 µg of CRD(4-7)Fc in complete Freund's adjuvant containing 10 mM Ca$^{2+}$, followed after 14 days by a similar booster with incomplete Freund's adjuvant. A final boost of 50 µg CRD(4-7)Fc in PBS with 10 mM Ca$^{2+}$ was given i.p. 4 days prior to fusion. The splenic B cells were fused with the Y3 myeloma cell line as described in (23).

Hybridoma supernatants were screened for the presence of anti-MR antibodies in ELISA using CRD(4-7)Fc coated plates. Of several monoclonal cell lines that were obtained, MR5D3 was chosen for further application due to its performance in immunohistochemistry, FACS, enzyme linked immunosorbent assays (ELISA) and immunoprecipitation (Martinez-Pomares et al., manuscript in preparation). MR5D3 was shown to be non-inhibitory for the binding of the MR to carbohydrate ligands (data not shown).

Proteins

The fusion proteins consisting of the cysteine-rich domain of the MR fused to human IgG$_1$ (CR-Fc) and carbohydrate recognition domains 4-7 fused to human IgG$_1$ (CRD(4-7)Fc) were obtained as previously described (11,24).

The sMR was purified from culture supernatants of NIH 3T3-derived MR transfectants (Martinez-Pomares et al., manuscript in preparation) by affinity chromatography using MR5D3 as follows. Transfected cells were grown in DMEM containing 10% (v/v) FCS, penicillin, streptomycin and glutamine under the selection of G418 (400 µg/ml). Once cells were confluent, this medium was replaced with the same medium containing 3% (v/v) FCS. After 13 days, the supernatants were harvested, centrifuged twice at 3,000 rpm for 15 min,
filtered and pre-cleared using Gammabind PLUS Sepharose (Amersham Pharmacia Biotech.). The sMR was purified from the medium on a MR5D3 affinity column, prepared by cross-linking of MR5D3 to Gammabind PLUS Sepharose with dimethyl pimelimidate (Sigma). After extensive washing in 0.01 M phosphate buffer, pH 7.4, 0.154 M NaCl (PBS), bound proteins were eluted with 0.5% (v/v) diethylamine and neutralised using 1 M Tris-HCl, pH 7.0.

Fractions containing sMR were detected by dot-blot analysis using MR5D3 (5 µg/ml) and anti-rat IgG horseradish peroxidase conjugate (Chemicon). Positive fractions were pooled, dialysed against 10 mM Tris-HCl, pH 8.0, containing 10 mM Ca\(^{2+}\) and 0.154 M NaCl, aliquoted and frozen at -20°C.

Bacteria

*K. pneumoniae* capsular serotype strains, K11, K17, K22, K27, K31, K46 and K60 were obtained from Dr Harry Deneer, University of Saskatchewan, Canada, and strains K1, K3, K26, K36, K40 and K52 from the Statens Serum Institute, Copenhagen, Denmark.

Antisera

Rabbit polyclonal antisera specific for the different CPS serotypes of *K. pneumoniae* and *S. pneumoniae* and for the *S. pneumoniae* CW-PS were obtained from the Statens Serum Institute.
Authentic polysaccharide ligands

Soluble multivalent monosaccharide or short oligosaccharide ligands attached to a soluble polyacrylamide support (Sugar-PAAs) were obtained from Syntosome, Munich, Germany. Yeast mannan from *Saccharomyces cerevisiae* was purchased from Sigma, Dorset, U.K.

Analytical methods

CPS from *K. pneumoniae* was quantified by measuring the total uronic acid content (one D-GlcA present/ polysaccharide repeating unit) (25). Protein content was measured by the Micro BCA™ assay (Pierce) and nucleic acid by UV absorbance. Total carbohydrate was measured by the phenol-sulphuric acid reaction (26). LPS content was measured by a kinetic turbidometric *Limulus* amoebocyte lysate (LAL) assay, using reagents from Charles River Endosafe and *E. coli* 055 LPS as standard according to the manufacturer's instructions.

SDS-PAGE

SDS-PAGE was carried out according to (27). LPS was resolved on 15% (w/v) gels and visualised by silver staining according to (28). CPS was resolved on 10% (w/v) gels and visualised by combined alcian blue and silver staining (29).

Bacterial capsular polysaccharides

Purified CPSs from *S. pneumoniae* were obtained from Dr Chris Jones, National Institute for Biological Standards and Controls (NIBSC), Hertfordshire, U.K. These polysaccharides contain less than 1% by weight protein and nucleic acid contaminants. *S. pneumoniae* CW-PS was obtained from the Statens Serum Institute. *K. pneumoniae* CPSs were isolated and purified as follows. Bacteria were grown overnight at 37 °C on lactose agar plates to maximise CPS production. The bacterial growth from 20-30
plates was suspended in 25-50 ml distilled water and heated for 10 min at 100 °C to release
capsular material. The CPS was precipitated with 80% (v/v) acetone at 4 °C and recovered
by spooling around a glass pasteur pipette. The precipitate was dried overnight at room
temperature, re-suspended in PBS and insoluble material removed by centrifugation twice at
30,000 x g for 30 min at 4 °C.

The supernatant containing CPS was then centrifuged for a further 8 h at 100,000 x g and 4
° C and the pellet containing LPS and protein discarded. CPS was dialysed against distilled
water and freeze dried.

In some cases the CPS was also digested with ribonuclease type II-B (30 µg/ml) and
deoxyribonuclease EC.3.1.2.1 (70 µg/ml) for 24 h, followed by the addition of subtilisin (70
µg/ml) and incubation for a further 24 h. Incubations were carried out in 0.01 M Tris-HCl,
 pH 7.4 containing 4 mM Mg^{2+} and 0.05% (w/v) thiomersal at an approximate concentration
of 500 µg/ml CPS at 37 °C with shaking. Following digestion, samples were dialysed
extensively against distilled water, 25 kDa cut-off membrane, and freeze dried.

A final purification step was carried out by gel filtration chromatography under dissociating
conditions on a TSK G5000 PW xl column (300 x 7.5mm). The buffer used was PBS
containing 0.25% (w/v) sodium deoxycholate. Chromatography was carried out at 60 °C at a
flow rate of 0.25 ml/min. Absorbance at 205 and 280 nm, and refractive index of the eluant
were monitored. Fractions were collected at 2 min intervals. Fractions containing
carbohydrate were pooled, dialysed against water, concentrated by freeze drying, and
resuspended in approximately 5 ml of water. Residual detergent was removed by
precipitation of CPS twice from 80% (v/v) ethanol at 4 °C.
Lipopolysaccharides

LPSs from *Haemophilus influenzae* type b strain Eagen, *Pseudomonas aeruginosa* strain PAC1, and *K. pneumoniae* were obtained by extraction with hot aqueous 45% (w/v) phenol (30). LPSs were dialysed extensively against distilled water followed by centrifugation at 100,000 x g for 18 h at 4 °C. The LPS pellet was resuspended in water and quantified by gravimetric measurement. *Salmonella typhimurium* rough LPS types, Rb2, Rb3, Rc, Rd1, Rd2 and Re were purchased from Accurate Chemical and Scientific Corporation, N.Y., USA. LPSs from *Neisseria meningitidis* serogroup B, immunotype L3 and mutant 4 type, were gifts from Dr.S.R.Andersen.

Mannose receptor binding assays

The binding of the mannose receptor to different polysaccharides was determined by ELISA, either directly by measuring binding to plates coated with different ligands or indirectly by inhibition assays as follows.

*Direct binding*

All washings and incubations were carried out in 10 mM Tris-HCl, pH 7.5, 10 mM Ca<sup>2+</sup>, 0.154 M NaCl and 0.05% (w/v) Tween 20.

Polysaccharides were coated onto the wells of ELISA plates (Nunc, Maxisorb) by incubation in 0.154 M NaCl overnight at 37 °C (50 µl/well). Plates were sealed with parafilm and incubated in a sealed damp box. Bacterial CPSs and LPSs were coated at 50 µg/ml, authentic sugar polyacrylamide substrates at 5 µg/ml and yeast mannan at 10 µg/ml. After coating, plates were washed five times. The coating efficiency of the different CPSs was confirmed by titration with anti-CPS specific antisera.

CRD<sub>(4-7)</sub>Fc, CR-Fc and sMR were incubated in the wells of coated plates at 2 µg/ml (50 µl/well) for 2 h at room temperature. Plates were washed five times. The MR fusion proteins
were detected by incubation with anti-human IgG Fc specific, alkaline phosphatase conjugate, species absorbed (Jackson Laboratories). Bound sMR was detected by incubation with MR5D3 at 10 µg/ml for 2 h at room temperature followed by detection with anti-rat IgG Fc specific, alkaline phosphatase conjugate, species absorbed (Jackson). Plates were washed five times and developed with p-nitrophenyl phosphate (pNPP) substrate (Sigma). Absorbance was measured at 405 nm after 30 min. Readings were measured against a blank of uncoated wells incubated with MR protein. Background readings were no more than 0.05 absorbance units higher than the reagent blank. All assays were carried out in duplicate or triplicate. Yeast mannan coated wells were used as a positive control in assays measuring CRD(4-7)Fc and sMR binding.

**Inhibition assays**

The inhibition of binding of CRD(4-7)Fc and sMR to ELISA plates coated with different ligands was measured. Inhibition was carried out in a high salt buffer consisting of 10 mM Tris-HCl, pH 7.5, containing 10 mM Ca^{2+}, 1 M NaCl and 0.05% (w/v) Tween 20. All incubations were at room temperature.

Either CRD(4-7)Fc or sMR were preincubated at 2 µg/ml with different concentrations of inhibitor, either monosaccharides or polysaccharides, for 30 min followed by incubation for 2 h in the wells of coated ELISA plates. The plates were washed five times and the detection of bound CRD(4-7)Fc and sMR was carried out as described above.
Results

Purification of the sMR

The sMR obtained by affinity chromatography was quantified by the BCA assay and analysed by SDS-PAGE and gel filtration chromatography. The sMR migrated as a single band on SDS-PAGE (Fig. 1a) with an apparent molecular weight of approximately 180 kDa. The protein similarly eluted as one main peak on gel filtration chromatography (Fig 1b, peak 2), with an apparent molecular weight just greater than the 158 kDa protein standard consistent with the presence of monomers of the full length soluble form of the molecule. The presence of a minor higher molecular weight component (peak 1) may represent a very small amount of sMR dimer or aggregate.

(PRESENT FIG 1).

Purification of CPS from K. pneumoniae

CPS extracts prior to gel filtration contained on average by weight 10% protein, 5% LPS and 1% or less nucleic acid. Extensive enzymic digestions with nuclease and protease followed by dialysis reduced the levels of protein and nucleic acid by approximately 50% suggesting a strong association of CPS with protein or peptide fragments. In order to further remove protein and also LPS, further purification was carried out by a gel filtration step under dissociating conditions. All CPSs gave very similar profiles (Fig. 2a). One main carbohydrate peak, (peak a), eluted close to the void volume of the column, molecular weight 2,000 kDa and greater, and a second minor peak of carbohydrate, (peak b), eluted, together with the majority of the protein, close to the total volume of the column.

Recovery of total carbohydrate was 80% and greater as measured by the phenol-sulphuric acid reaction. Carbohydrate was recovered from both pools as described in Experimental
procedures, and aliquots analysed by SDS-PAGE (Fig. 2 b&c). Carbohydrate in pool 1 migrated as a broad high molecular weight band which could be visualised only by using alcian blue prior to silver staining, confirming the presence and identity of CPS. The carbohydrate in pool 2 gave the characteristic banding pattern of LPS which was readily visualised by silver staining alone.

The CPS recovered in pool 1 was quantified by measuring the total uronic acid content. Protein content was no more than 1% and LPS content, estimated by the LAL assay, no more than 0.1% by weight. No nucleic acid was detectable. It was also confirmed that there was no reduction in the reaction of CPS with anti-CPS specific antisera following gel filtration, confirming that no degradation of CPS had taken place during purification. (INSERT FIG.2).

**Specificity of binding of CRD(4-7)Fc, CR-Fc and sMR for authentic standards**

The binding properties and specificities of the proteins, CRD(4-7)Fc, CR-Fc and sMR, towards authentic ligands were analysed prior to investigating binding to bacterial polysaccharides.

In direct binding assays both CRD(4-7)Fc and sMR displayed high affinity for terminal D-Man and L-Fuc residues, but no binding to D-Gal (Table 3). CR-Fc and sMR further recognised the sulphated ligands: SO_4^-3-β-D-Gal, SO_4^-3-β-D-GalNAc and SO_4^-4-β-D-GalNAc, none of which was bound by CRD(4-7)Fc. Neither CR-Fc nor sMR recognised monosaccharides sulphated at the C₆ positions, SO_4^-6-β-GalNAc or SO_4^-6-β-GlcNAc.

The measurements obtained in direct binding assays were directly proportional to the concentration of protein used in the assay (Fig.3) and all subsequent assays were standardised using 2 μg/ml of protein. (INSERT TABLE 3 & Fig. 3)

The specificity and affinity of binding were further investigated by monosaccharide inhibition assays (Fig.4). The sMR and CRD(4-7)Fc both showed the highest affinity towards D-Man and L-Fuc with lower affinity for D-Glc, D-GlcNAc and L-Rha. The $K_i$ values were
all in the mM range consistent with the known low affinity of binding of the MR to monovalent ligands. As in earlier studies (7,8), the inhibition assays reported here were carried out in buffer containing 1 M NaCl but very little difference was found when assays were performed using physiological saline during the present study (data not shown).

In conclusion, CRD(4-7)Fc and sMR both showed similar specificities towards different monosaccharides, which are consistent with the known sugar binding properties of the MR (7). In addition it was shown that the MR recognises the non-host, but common bacterial monosaccharide, L-Rha with similar affinity to that shown for D-Glc and D-GlcNAc. Binding to L-Rha most probably occurs through C3 and C4 equatorial hydroxyl groups.

(insert fig. 4)

Direct binding of the MR to CPSs from S. pneumoniae

The direct binding of CRD(4-7)Fc and sMR to pneumococcal CPSs is shown in Fig.5. The MR, both CRD(4-7)Fc and sMR, recognised all except three of the CPS structures, serotypes 1, 4, and 18C where binding was not detectable. The CW-PS, a minor component of most or all of the CPS samples, also did not act as a ligand for the MR, and therefore is unlikely to contribute to the observed binding towards CPS.

The relative binding of CRD(4-7)Fc and sMR to the different CPSs was very similar, although overall, a higher percentage binding relative to yeast mannan was obtained with the fusion protein. The affinity of binding to the different CPSs also appeared to be very similar. However three of the CPS types (2, 14 and 19F) consistently gave higher readings in direct binding. No binding was detected when Ca\(^{2+}\) was omitted from the buffer and CR-Fc did not recognise any CPS structure. (INSERT FIG 5).

To further investigate the specificity of the interaction with the MR, inhibition assays were carried out using different concentrations of D-Man and D-Glc (Fig.6). In all cases, binding
of CRD(4-7)Fc to CPS could be completely inhibited by incubation with 5 mM D-Man. A concentration of 0.5 mM D-Man resulted in approximately 50% inhibition. Incubation with 5 mM D-Glc resulted in inhibition from 30 to 55% and from 50 to 80% inhibition at 10 mM.

As expected, higher concentrations of both sugars were required to inhibit binding of the MR to yeast mannan. Inhibition was approximately half that obtained for binding to CPS coated plates at the same sugar concentrations. A concentration of 10 mM D-Man was required to obtain complete inhibition of binding to yeast mannan. (INSERT FIG. 6)

D-Gal tested at a 10 mM concentration had no effect on binding.

Inhibition of binding by S. pneumoniae CPSs in solution

To determine whether S. pneumoniae CPSs could act as ligands for the MR when in solution, as well as when surface bound, CPSs were tested for their ability to inhibit the binding of the MR to coated ELISA plates. In all cases where direct binding to CPS was observed the same CPSs inhibited binding in solution.

Each CPS was tested at 50 µg/ml for its ability to inhibit binding of CRD(4-7)Fc (2 µg/ml) to ELISA plates coated with the homologous CPS. In each case binding was inhibited between 70 and 90%. To test whether the CPS could also inhibit binding to heterologous CPS structures, type 14 CPS (50 µg/ml) was incubated with CRD(4-7)Fc (2 µg/ml) and binding to different CPSs measured. Again inhibition was from 60 to 90%, with inhibition of binding to yeast mannan lower at 40%.

The capacity of CPS to inhibit was further investigated by measuring the inhibition of binding of CRD(4-7)Fc to α-D-Man-PAA coated plates by different concentrations of CPS from 10 to 200 µg/ml (Fig 7). Little or no inhibition occurred at 10 µg/ml. At 50 µg/ml inhibition was less than that observed for binding to CPS, ranging from 10 to 40% reflecting
the higher affinity of the coating substrate for CRD(4-7)Fc. Inhibition at 100 µg/ml ranged from 30 to 60% and at 200 µg/ml from 50 to 80%.

Of the three CPS serotypes (1, 4 and 18C) that were not recognised by the MR in direct binding assays, two (1 and 4) also failed to inhibit in solution while 18C showed low inhibition at the higher concentrations. The CW-PS which was not recognised in direct binding assays also did not inhibit.

In contrast to CPS, incubation of CRD(4-7)Fc with yeast mannan resulted in 50% inhibition of binding to α-D-Man-PAA at a very low concentration of 50 ng/ml and 100% inhibition at 500 ng/ml. (INSERT FIG 7).

**Binding to *K. pneumoniae* CPSs**

When tested in direct binding assays, crude extracts of *K. pneumoniae* CPS prior to the gel filtration purification step all showed very low levels of binding to both CRD(4-7)Fc and sMR. This binding was much lower, approximately 10% that obtained with pneumococcal CPSs, and was inhibitable with D-Man (data not shown). These extracts, however, contained up to 5% by weight LPS which is also a potential ligand for the MR.

After further purification all except three of the fifteen CPSs tested showed no binding to either CRD(4-7)Fc or sMR. All binding activity was instead contained in the fractions from gel filtration containing protein and LPS. The three CPSs which still showed some binding to the MR were K3, K46 and K64. Binding was still weak compared with the pneumococcal CPSs, however, at approximately 10% relative to yeast mannan. Binding was again inhibitable with D-Man, Ca^{2+} dependent, and CR-Fc did not bind any of these structures.
Recognition of LPS by the MR

LPS is the second main potential ligand for the MR on the surface of Gram negative bacteria in addition to CPS. To test whether LPS could act as a ligand for the MR, LPSs were extracted from representative O-serotype strains of *K. pneumoniae*. The results for direct binding to *K. pneumoniae* LPSs as well as to LPSs from a number of other bacteria are shown in Table 4. The structures of the LPS O-antigens from *K. pneumoniae* and the LPSs from other bacterial species tested are shown in Table 2(a&b).

As expected, the poly-mannose O-serotype LPS was an excellent ligand. More unexpectedly however, both the rough *K. pneumoniae* LPS and poly-galactose O-serotypes of LPS were good ligands with binding being almost equivalent to that observed with yeast mannan for the rough LPS and O2 serotype. LPSs from other bacterial species: *Pseudomonas, Haemophilus* and *Neisseria* did not bind. Also, the LPS from *K. pneumoniae* strain K36 with a Rib/Gal O-antigen failed to bind. A series of rough mutant LPSs from *Salmonella* all showed a very low level of binding to the MR. As with CPS, the binding specificities of CRD_{4-7}Fc and sMR were similar, and binding could be inhibited by incubation with 10 mM D-Man and D-Glc but not D-Gal.

The percentages of inhibition of binding of CRD_{4-7}Fc to *K. pneumoniae* LPS by different concentrations of D-Man and D-Glc are shown in Fig.8. Except for serotype 01, higher concentrations of monosaccharide were required for inhibition than seen with *S. pneumoniae* CPS, consistent with a higher affinity for LPS recognition. In particular, the 02 serotype showed inhibition values similar to yeast mannan.

When tested in solution, incubation of *K. pneumoniae* LPSs at approximately 50 µg/ml with CRD_{4-7}Fc at 2 µg/ml resulted in 100% inhibition of binding of CRD_{4-7}Fc to α-D-Man-PAA, except for serotype 04, K36 LPS.
Again, as with CPS, CR-Fc did not bind to any of the LPSs tested, and no binding occurred in the absence of Ca$^{2+}$. (INSERT FIG. 8)

**Dual lectin activity of the MR**

To test whether the sMR could bind both to polysaccharide through its CRD domains and to ligand for the CR domain simultaneously, sMR was pre-incubated with either yeast mannan, pneumococcal CPS or LPS, then incubated in ELISA plate wells coated with SO$_4$-3-β-D-Gal-PAA. Bound sMR was detected with monoclonal antibody MR5D3 and bound CPS was detected with rabbit anti-CPS specific antiserum. The secondary antibodies used in both cases to detect bound MR5D3 and bound rabbit antibodies were species absorbed. In no instance did binding of the sMR to polysaccharide prevent subsequent recognition via the CR domain (Fig.9). In contrast, incubation of the sMR with polysaccharides known to be ligands for the CRD domains increased the amount of sMR detected bound to SO$_4$-3-β-D-Gal-PAA coated plates. Large polysaccharides, such as CPS, presumably display several binding sites for the sMR per molecule. Multiple attachment of sMR to polysaccharide may therefore increase the effective valency of the sMR and hence the affinity for subsequent binding to SO$_4$-3-β-D-Gal. LPS is similarly multivalent, existing as micelles in solution.

(INSERT FIG 9)
Discussion

In this paper we report the binding of the MR to CPSs from *S. pneumoniae* and to the LPS but not to the CPS from *K. pneumoniae*. The finding that the MR was able to recognise pneumococcal CPS was surprising as these polysaccharides have none of the structural features associated with known MR specificities. Polysaccharides binding the MR may be expected to display, for example, multiple terminal D-Man residues, or possibly terminal residues with lower affinity such as D-Glc or D-GlcNAc, or in the case of bacterial polysaccharides L-Rha. Other potential recognition sites lie within the polysaccharide chain where the appropriate hydroxyl groups of the monosaccharides are not substituted. To date, however, there is no information as to whether the MR can recognise non-terminal residues. CPSs from certain capsular serotype strains of *K. pneumoniae* are potential ligands for the MR according to these criteria but none were able to bind the MR in *in vitro* assays, other than possibly in three cases with very low affinity.

The specificity of the binding to CPS and LPS was demonstrated by complete inhibition with D-Man, partial inhibition with D-Glc, no inhibition with D-Gal and by Ca$^{2+}$- dependence. Since CRD$_4$ is the only CRD known to bind D-Man by itself (36), this strongly suggests that CRD$_4$ is also involved in binding to bacterial polysaccharides. The fusion protein containing CRD$_{4-7}$ behaved in a very similar manner to the full length sMR indicating that CRD$_{1-3}$ and CRD$_8$ are not involved directly in CPS binding. At least three CRDs (4, 5, and 7) are known to be required for high affinity and binding to multivalent ligands (7). Each CRD shows weak affinity for a single monosaccharide, with high affinity being achieved through multiple weak interactions.

In this study the monomeric form of the sMR was shown to be able to bind both ligands for the CRDs and CR domain. In contrast to the sMR, the CRD$_{(4-7)}$Fc fusion protein is dimeric.
due to dimerisation via the Fc region. As such, CRD\(_{(4-7)}\)Fc might be expected to have a higher affinity than the sMR. A direct comparison of the affinity of binding between the two molecules was not carried out as part of this investigation. However, in a separate experiment, not reported here, pre-incubation and cross-linking of CRD\(_{(4-7)}\)Fc with MR5D3 strongly indicated that increasing the valency of the MR increased the affinity of binding for CRD ligands. Higher affinity acquired by multimerisation of several MR molecules was also indicated for binding to CR-domain ligands as shown by the increased binding observed following attachment of the sMR to a large polysaccharide backbone. Cross-linking of the MR by high molecular weight polysaccharides may also occur on the cell surface if the relevant CRD domains are accessible. Recognition of pneumococcal CPSs by the MR shows that criteria other than the primary polysaccharide structure must be taken into account. One possible explanation is the recognition of conformational epitopes. According to NMR data pneumococcal CPSs have an extended, flexible, ribbon-like structure with no stabilisation of secondary structure (37,38). It is possible that this flexibility allows the positioning of hydroxyl groups between different monosaccharide residues at suitable positions to allow for binding to CRD domains. Thus, rather than contact between one CRD and one monosaccharide residue, one CRD may make contact with two closely adjacent monosaccharides. It is known that the repeating units of pneumococcal CPSs have preferred secondary conformations and that conformational epitopes within these polysaccharides are recognised by antibodies (39,40). The polysaccharide structural moieties recognised by the MR are present on both surface-bound CPS and soluble CPS as shown by direct binding and inhibition assays. Compared with yeast mannan, however, the pneumococcal CPSs are relatively weak ligands. This was shown by (a) the amount of MR detected directly bound to CPS coated plates, (b) by the concentrations of monosaccharide required to inhibit binding to CPS and (c) by the concentrations of CPS needed to inhibit in solution.
In comparison with *S. pneumoniae*, CPSs from *K. pneumoniae* showed no, or in three cases very poor binding to the MR. *K. pneumoniae* CPSs have well defined secondary helical conformations shown by X-ray diffraction analysis (41). If conformation is a main factor in CPS recognition then a more rigid secondary polysaccharide conformation may result in a less varied display of sugar hydroxyl groups and preclude MR binding. Where side-branches are present in the polysaccharide repeating unit these are usually displayed outwards from the main alpha-helical chain. K17 and K64 CPSs both have single residue branches of L-Rha, K46 has a short side-branch terminating in D-Glc, K27 a single D-Glc and K60 three single branch residues of D-Glc per repeating unit. If such branch sugars are suitably orientated they may act as sites of recognition for the MR. In accordance, both K64 and K46 CPSs showed weak binding to the MR, however no binding to K17, K27 or K60 could be detected, so this idea cannot be substantiated. The other CPS to show weak binding to the MR, K3, has a sidebranch of a single D-Man residue substituted with cyclic pyruvate. While pyruvlation should prevent recognition it is possible that substitution is only partial or that some de-pyruvlation has occurred during purification. It would be of interest to know whether the three CPSs that appear to support low affinity binding have any unique features in their secondary conformations.

In contrast to the very poor binding shown towards CPSs from *K. pneumoniae*, the LPS bound with relatively high affinity. As with the pneumococcal CPSs, LPS was recognised both when surface bound and when in solution.

The O-antigenic sidechain of the O3 *K. pneumoniae* LPS serotype consists of a linear polymer of D-Man residues. As expected, this LPS type bound well to the MR. In addition, however, both the poly-galactan O-antigen serotypes (O1 and O2a) and the rough type LPS were recognised. The rough LPS and O2a (K3) LPS serotype in particular showed very strong binding to the MR comparable with yeast mannan.
The structures of the core oligosaccharide regions of *K. pneumoniae* LPS have only recently been determined (42,43) and are generally not as well defined as those from *Salmonella* or *Escherichia coli*. Analysis of the core regions from eight serotypes revealed that they all had similar structures variously containing terminal D-Glc and D-Hep residues as well as, unusually for LPS, D-GalA. LPS from a rough strain of *K. pneumoniae* expressed an unusual small oligosaccharide composed of D-Hep residues (44). The ring structure of D-Hep has the same configuration as D-Man and is therefore potentially a high affinity ligand for the MR, although this has not been tested directly. At this stage we have not determined which part of the LPS molecule is bound by the MR, that is, whether the O-antigenic sidechains are bound directly or whether all binding is directed towards the core oligosaccharide regions.

As well as recognition of LPSs from *K. pneumoniae*, some binding to the rough LPSs from *Salmonella* was observed, but at a much lower affinity. These LPSs variously contain terminal D-Hep residues as well as terminal D-Glc or D-Gal residues. The roughest LPS mutant, containing only terminal KDO was also recognised. Similarly to CPS therefore, the structural features of the LPS polysaccharides supporting MR binding are not clear, again indicating a conformational dependence.

Not all LPSs tested were found to bind. Those from *Ps. aeruginosa*, *H. influenzae* and *N. meningitidis* showed no binding at all. It may be of interest to note that *H. influenzae* and *N. meningitidis* are pathogens and *Ps. aeruginosa* causes serious infection in cystic fibrosis patients whereas *K. pneumoniae* is not pathogenic under normal circumstances.

Previous studies have reported that the binding and phagocytosis of whole cells of *K. pneumoniae* by alveolar MØs occurs through the recognition of CPS by the MR (16). In *in vitro* assays using purified CPS and the purified MR protein however we were unable to demonstrate any significant binding. *K. pneumoniae* CPS is released from cells as a complex of CPS, LPS and protein as well as being associated with the cell surface (45). The crude
CPS extract in all the strains analysed, except K26, showed very weak binding to the MR in vitro (data not shown). On further purification, however, all binding, in nearly all cases, was shown to be due to the LPS component. It is possible that LPS on the cell surface may contribute to binding to MØs or that other MØ receptors such as class A scavenger receptors (46), complement receptor type 3 (47,48) or the recently identified β-glucan receptor, dectin (49) are involved.

The Ca^{2+} dependent lectins of the collectin family, mannose binding protein (MBP) and the two surfactant proteins A and D, recognise a wide variety of different microorganisms (50-52). MBP recognises different monosaccharides with the same binding specificity as the MR, and the mechanism of sugar recognition by the CRD of MBP is analogous to that of CRD4 of the MR. The prediction would be for both lectins to recognise similar microbial polysaccharides. In this respect, MBP has been reported to recognise rough LPS structures from *Salmonella* but not to bind to whole cells of *S. pneumoniae* (53,54). In the case of MBP, binding to the purified bacterial component did not always reflect actual binding to the bacterial surface experimentally. This is perhaps not surprising as different surface components may interact in concert or one component may prevent binding of another. In the case of *S. pneumoniae*, for example, the pneumococcal surface protein A extends from the cell wall and protrudes outside the capsule. The exposed part of this protein has a highly electronegative charge which reduces complement activation on the bacterial surface (55). Surface protein A may also repel the binding of other molecules such as the MBP. With respect to *K. pneumoniae*, it is possible that the LPS is still accessible even in capsular strains (56).

Similar to the conclusions made during the present study for MR recognition, no direct correlation could be made between MBP binding to microbial polysaccharides and the
primary polysaccharide structure (53,54), again indicating an important conformational effect regarding recognition of complex polysaccharides.

The sugar binding specificities of the surfactant proteins A and D are not as clearly defined as, and differ from, those of the MR and MBP. They have both been reported to bind bacterial CPS and LPS and knock out mice have demonstrated an important role for surfactant protein A in pulmonary innate immunity (51,52).

Both purified bacterial polysaccharide and bacterial polysaccharide or oligosaccharide in the form of protein glycoconjugates are used in vaccines. The initial interaction of the CPS with the host is mediated by components of innate immunity such as natural IgM anti-carbohydrate antibodies, complement and lectins. The MR, either on MØs or as a soluble protein, forms one such constituent and its overall importance is not known. Factors such as the binding, uptake, transport, localisation and rate of degradation and clearance of polysaccharide antigens, however, are poorly understood and all may be modified by MR recognition. It should be noted that a physiological concentration of blood glucose of approximately 6 mM may be sufficient to reduce binding of weak ligands to the MR.

Some bacterial polysaccharides, including pneumococcal CPSs, activate the alternative complement pathway resulting in the covalent attachment of complement components such as C3d (57). Covalent attachment of complement results in targeting of the polysaccharide to splenic marginal zone B cells and to follicular DCs both of which express high levels of the complement receptors CD21 and CD35. The spleen and particularly marginal zone B cells are important in anti-carbohydrate immune responses. Complement activation and attachment to CPS may increase, or be critical to, the antibody response, similar to the effect observed with protein antigens (58). The MR is a heavily glycosylated molecule, most probably sialylated, which may serve to reduce CPS mediated complement activation. If bound at a sufficient density sMR may also affect recognition and cross-linking of B cell
receptors by the CPS. Overall, the effect would be to potentially reduce the anti-CPS immune response.

Another possibility is that the sMR targets antigen to cells expressing ligands for the CR domain of the MR ie metallophilic marginal zone MØs and DCs (11,12,59). The structures of the sulphated oligosaccharide ligands expressed by these cells are not known. In agreement with (10) we show SO₄-3-Gal to be a good ligand as well as SO₄-3-GalNAc and SO₄-4-GalNAc. Expression of SO₄-4-GalNAc occurs on pituitary glycoprotein hormones and is responsible for the rapid clearance of these glycoproteins from the bloodstream by hepatic endothelial cells mediated by MR recognition (60). Sulphated GalNAc however is an unusual modification, probably specific to pituitary hormones, and it is more likely that terminal SO₄-3-D-Gal is recognised on metallophilic marginal zone MØs and DCs.

We have shown that the sMR can bind at the same time CPS through its CRD domains and sulphated oligosaccharide through the CR domain. With a few rare exceptions among organisms living in extreme environments (61), bacteria do not incorporate sulphate into their polysaccharides so that CR lectin interactions can be thought of as host cell specific. It has been speculated that DCs expressing ligands for the CR domain may serve to transport antigens via surface bound sMR. Such DCs have been traced migrating from sites of immunisation to B cell areas in secondary lyphoid tissues (12,59). CPS may therefore be targeted to specific cells via sMR attachment. The potential effects of MR recognition of CPS and LPS on immune responses is currently under investigation.
References

1. Stahl, P. D., and Ezekowitz, A. B. (1998) *Curr. Opin. Immunol.* **10**, 50-55

2. Pontow, S.E., Kery, V., and Stahl, P.D. (1992) *Int. Rev. Cytol.* **137B**, 221-244

3. Linehan, S. A., Martinez-Pomares, L., Stahl, P. D., and Gordon, S. (1999) *J. Exp. Med.* **189**, 1961-1972

4. Sallusto, F.M., Cella, M., Daniella, C., and Lanzavecchia, A. (1995) *J. Exp. Med* **182**, 389-400

5. Engering, A. J., Cella, M., Fluitsma, D., Brockhaus, M., Hoefsmit, E.C.M., Lanzavecchia, A., and Pieters, J. (1997) *Eur. J. Immunol.* **27**, 2417-2425

6. Weis, W.I., Taylor, M.E., and Drickamer, K. (1998) *Immunol. Revs.* **163**, 19-34

7. Taylor, M.E., Bezouska, K., and Drickamer, K. (1992) *J. Biol. Chem.* **267**, 1719-1726

8. Taylor, M. E., and Drickamer, K. (1993) *J. Biol. Chem.* **268**, 399-404

9. Fiete, D.J., Beranek, M.C.,and Baenziger, J.U. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 2089-2093

10. Leteux, C., Chai, W., Loveless, R.W., Yuen, C-T., Uhlin-Hansen, L., Combarnous, Y., Jankovic, M., Marc, S.C., Misulovin, Z., Nussenzweig, M.C., and Feizi, T. (2000) *J. Exp. Med.* **191**, 1117-1126

11. Martinez-Pomares, L., Kosco-Vilbois, M., Darley, E., Tree, P., Herren, S., Bonnefoy, J- Y., and Gordon, S. (1996) *J. Exp. Med.* **184**, 1927-1937

12. Berney, C., Herren, S., Power, C.A., Gordon, S., Martínez-Pomares, L., and Kosco-Vilbois, M. H. (1999) *J. Exp. Med.* **190**, 851-860

13. Martínez-Pomares, L., Mahoney, J. A., Káposzta, R., Linehan, S. A., Stahl, P. D., and Gordon, S. (1998) *J. Biol. Chem.* **273**, 23376-23380
14. Jennings, H.J., and Pon, R.A. (1996) in *Polysaccharides in Medicinal Applications* (Dumitriu, S., ed) pp 443-479, Marcel Dekker Inc., New York

15. Orskov, I., and Orskov, F. (1984) *Methods in Microbiol.* 14, 143-164

16. Athamna, A., Ofek, I., Kaisari, Y., Markowitz, S., Dutton, G. G. S., and Sharon, N. (1991) *Infect. Immun.* 59, 1673-1682

17. Kenne, L., and Lindberg, B. (1983) in *The Polysaccharides, vol.2* (Aspinall, G.O., ed) pp 287-363, Academic Press, Inc., New York

18. Dutton, G.G., Parolis, H., Joseleau, J.P., and Marais, M. F. (1986) *Carb.Res.* 149, 411-423.

19. Parolis, L.A.S., and Parolis, H. (1988) *Carb.Res.* 179, 301-314

20. Cescutti, P., Toffanin, R., Kvam, B.J., Paoletti, S., and Dutton, G.G.S. (1993) *Eur.J.Biochem.* 213, 445-453

21. Stenutz, R., Erbing, B., Widmalm, G., Jansson, P.E., and Nimmich, W. (1997) *Carb.Res.* 302, 79-84

22. Ravenscroft, N., Stephen, A.M., and Merrifield, E.H. (1987) *Carb.Res.* 167, 257-267.

23. Galfre, G., Milstein, C., and Wright, B. (1979) *Nature* 277, 131-133

24. Linehan, S.A., Martinez-Pomares, L., da Silva, R., and Gordon, S. (2001) *Eur.J.Immunol.* 31, 1857-1866

25. Knutson, C.A., and Jeanes, A. (1968) *Analytical Biochem.* 24, 470-481

26. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. & Smith, F. (1956) *Anal. Biochem.* 28, 350-356

27. Laemml, U.K. (1970) *Nature, London, U.K.* 227, 680-685

28. Tsai, C-M., and Frasch, C.E. (1982) *Anal. Biochem.* 119, 115-119

29. Min, H., and Cowman, M.K. (1986) *Anal. Biochem.* 155, 275-285

30. Westphal, O., and Jann, K. (1965) *Methods in Carb. Chem.* 5, 453-54
31. Masoud, H., Moxon, E.R., Martin, A., Krajcarski, D., and Richards, J.C. (1997)
   *Biochemistry* **36**, 2091-2103

32. Raetz, C.R.H. (1990) *Annu. Rev. Biochem.* **59**, 129-170

33. Pavliak, V., Brisson, J.R., Michon, F., Uhrin, D., and Jennings, H.J. (1993) *J.Biol.Chem.*
   **268**, 14146-14152

34. Anderson, S.R., Bjune, G., Lyngby, J., Bryn, K., and Jantzen, E. (1995) *Microbial. Pathog.* **19**, 159-168

35. Hansen, D.S., Mestre, F., Alberti, S., Hernandez-Alles, S., Alvarez, D., Domenech-Sanchez, A., Gil, J., Merino, S., Tomas, J.M., and Benedi, V.J. (1999) *J.Clin. Microb.* **37**, 56-62

36. Mullin, N.P., Hitchen, P.G., and Taylor, M.E. (1997) *J.Biol.Chem.* **272**, 5668-5681

37. Rutherford, T.J., Jones, C., Davies, D.B., and Elliott, A.C. (1994) *Carb.Res.* **265**, 79-96

38. Jones, C., Currie,F., and Forster, M.J. (1991) *Carb.Res.* **221**, 95-121

39. Ciuffreda, P., Colombo, D., Ronchetti, F., and Toma, L. (1992) *Carb.Res.* **232**, 327-339

40. Wessels, M.R., and Kasper, D.L. (1989) *J.Exp.Med.* **169**, 2121-2131

41. Isaac, D.H. (1985) in *Polysaccharides: Topics in Structure and Morphology* (Atkins, E.D.T., ed) pp 141-184, Macmillon Publishing Ltd., U.K

42. Vinogradov, E., and Perry, M.B. (2001) *Carb. Res.* 335, 291-296

43. Severn, W.B., Kelly, R.F., Richards, J.C., and Whitfield, C. (1996) *J.Bacteriol.* **178**, 1731-1741

44. Susskind, M., Brade, I., Brade, H., and Otto, H. (1998) *J.Biol.Chem.* **273**, 7006-7017

45. Straus, D.C. (1987) *Infect. Immun.* **55**, 44-48

46. Peiser, L., Mukhopadhyay, S., and Gordon, S. (2002) *Curr. Opin. Immunol.* **14**, 123-128

47. Thornton, B.P., Vetvicka, V., Pitman, M., Goldman, R.C., and Ross, G.D. (1996) *J.Immunol.* **156**, 1235-1246
48. Albanyon, E.A. & Edwards, M.S. (2000) *Infect. Immun.* **68**, 5794-5802

49. Brown, G.D., and Gordon, S. (2001) *Nature* **413**, 36-37

50. Jack, D.L., Klein, N.J., and Turner, M.W. (2001) *Immunol. Reviews* **180**, 86-99

51. Crouch, E., Hartshorn, K, & Ofek, I. (2000) *Immunol. Revs.** 173**, 52-65

52. Lawson, P.R., and Reid,K.B.M. (2000) *Immunol. Revs.** 173**, 66-78

53. Devyatyarova-Johnson, M, Rees, I., Robertson, B, Turner, M., Klein, N., and Jack D. (2000) *Infect. Immun.** 68**, 3894-3899

54. Neth, O., Jack, D.L., Dodds, A.W., Holzel, H., Klein, N.J., and Turner, M.W. (2000) *Infect. Immun.** 68**, 688-693

55. Jedrzejas, M.J. (2001) *Microbiol. Mol. Biol. Revs.** 65**, 187-207

56. Tomas, J.M., Camprubi, S., Merino, S., Davey, M.R., and Williams, P. (1991) *Infect. Immun.* **59**, 2006-2011

57. Harms, G., Hardonk, M.J., and Timens, W. (1996) *Infect. Immun.* **64**, 4220-4225

58. Carroll, M.C. (1998) *Annu. Rev. Immunol.* **16**, 545-568

59. Martinez-Pomares, L., and Gordon, S. (1999) *The Immunologist* **7/4**, 119-123

60. Roseman, D.S., and Baenziger, J.U. (2000) *Proc. Natl. Acad. Sci. U S A** 18, 9949-9954

61. Messner, P (1997) *Glycoconj. J.* **14**, 3-11
Footnotes

1 The abbreviations used are: AATGal, 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose; CPS, capsular polysaccharide; 4,6-(S)-pyruvate, 4,6-cyclic pyruvate acetal; CRD, carbohydrate recognition domain; CRD(4-7)Fc, CRDs 4–7 of the mannose receptor fused to human IgG1 Fc; CR-Fc, the cysteine-rich domain of the mannose receptor fused to human IgG1 Fc; DC, dendritic cell; ELISA, enzyme linked immunosorbent assay; L-Fuc, L-fucose; L-FucNAc, N-acetyl-L-fucosamine; D-Gal, D-galactose; D-GalNAc, N-acetyl-D-galactosamine; D-GalA, D-galacturonic acid; D-Glc, D-glucose; D-GlcNAc, N-acetyl-D-glucosamine; D-GlcA, D-glucuronic acid; D-Hep, L-glycero-D-manno-heptose; HPLC, high performance liquid chromatography; KDO, 3-deoxy-D-manno-octulosonic acid; LAL, limulus amoebocyte lysate; LPS, lipopolysaccharide; D-Man, D-mannose; D-ManNAc, N-acetyl-mannosamine; MBP, mannose binding protein; MR, mannose receptor; PAA, polyacrylamide; PEA, phosphoethanolamine; L-Rha, L-rhamnose; Rib, ribose; sMR, soluble mannose receptor.

2 Martinez-Pomares, L., Reid, D.M., Taylor, P.R., Linehan, S.A., Zamze, S., Wong, S.Y.C. & Gordon, S (2002) Shedding controls surface expression of the murine mannose receptor in primary cells.

Acknowledgements:
We thank Dr Chris Jones for his help and supplying us with pneumococcal CPSs, Dr Harry Deneer for kindly sending us strains of *K. pneumoniae*, Dr Svein R. Anderson and Geoffrey Guile (Jenner Institute) for *N. meningitidis* LPS and for help with HPLC analysis, respectively.
Figure legends

Fig. 1

Purification of the sMR

sMR was affinity purified from culture supernatants of MR transfected NIH 3T3 cells using MR5D3 as described under Experimental procedures. The recovered protein was quantified by the BCA assay and analysed by (a) SDS-PAGE and (b) gel filtration chromatography.

(a) SDS-PAGE profile of affinity purified sMR

SDS-PAGE was carried out on a 10% (w/v) slab gel under reducing conditions and proteins stained with Coomassie blue. Lane 1, molecular weight markers, lanes 2 & 3, sMR.

(b) Gel filtration profile of affinity purified sMR

Gel filtration chromatography was carried out on a TSK G3000 SW (7.5 x 600 mm) column. The buffer used was 10 mM Tris-HCl, pH 7.4, 0.154 M NaCl and 10 mM Ca^{2+}. The flow rate was 0.25 ml/min and the absorbance of the eluant was monitored at 214 and 280 nm. Arrows indicate the elution positions of gel filtration molecular weight protein standards (Bio-Rad) of 158, 44, 17 and 1.3 kDa as indicated. v and t indicate the void and total volumes of the column, respectively.

Fig. 2

Purification of *K. pneumoniae* CPS

(a) Gel filtration profile of *K. pneumoniae* CPS

*K. pneumoniae* CPSs were extracted and purified by gel filtration chromatography as described under Experimental procedures. Chromatography was carried out on a TSK
G5000 xl column in PBS containing 0.25% (w/v) sodium deoxycholate at 60 °C. The flow rate was 0.25 ml/min. The refractive index (panel c), and the absorbance of the eluant at 205 nm (panel a) and 280 nm (panel b) was monitored. Peaks a and b were pooled, dialysed against distilled water, freeze dried and residual detergent removed by precipitation with 80% (v/v) ethanol. Total carbohydrate in pools a and b was quantified by the phenol-sulphuric acid reaction. Recovery was 80% or greater. The figure shows a representative profile of CPS from strain K31. All CPS extracts gave very similar results. v and t indicate the void and total volumes of the column, respectively, filtration range for dextrans 7,000 kDa to 50 kDa.

(b & c) SDS-PAGE analysis of pools a and b

The CPS and LPS content of pools a and b was analysed by SDS-PAGE on either 10 or 15% (w/v) gels. LPS was visualised by silver staining and CPS by staining with alcian blue followed by silver staining. *K. pneumoniae* CPS was not detectable without the use of alcian blue. Fig. 2b; lanes 1 & 2, LPS extracted from capsular serotype strain K55, 0.4 and 0.2 µg; lanes 3 & 5, K55 CPS extract, pool a, and lanes 4 & 5, K55 CPS extract, pool b. Separation was on a 15% (w/v) gel and detection by silver staining. LPS is clearly visible in pool 2, showing the characteristic O-antigen banding pattern, identical to that shown by LPS extracted with 45% (w/v) aqueous phenol. LPS is not detectable in pool a. Fig.2c; lanes 1 & 2, K3 CPS extract, pool b; lanes 3 & 4, K3 CPS extract, pool a. Separation was on a 10% (w/v) gel and detection with alcian blue followed by silver staining. Lanes 1 & 2 show the O-antigen banding pattern of K3 LPS (the low molecular weight core-LPS component has been eluted from the gel), lanes 4 & 5 show the high molecular weight CPS migrating as a broad smear at the top of the gel, but no lower molecular weight components or banding pattern indicative of LPS.
Fig. 3
Concentration of mannose receptor required for detection by ELISA

Different concentrations of CRD(4-7)Fc and sMR were tested to establish the concentrations required for detection of binding to polysaccharide substrates. Proteins were incubated for 2 h at room temperature in the wells of coated plates and bound CRD(4-7)Fc and sMR detected as described under Experimental procedures.

♦ and ■, binding of CRD(4-7)Fc to yeast mannan and α-D-Man-PAA, respectively. △ and □, binding of the sMR to yeast mannan and α-D-Man-PAA, respectively.

Fig 4
Inhibition of binding of the mannose receptor by monosaccharides

The inhibition of binding of CRD(4-7)Fc and sMR to α-D-Man-PAA coated plates by different monosaccharides was assayed. Different concentrations of monosaccharide were pre-incubated with 2 µg/ml of MR protein for 30 min followed by 2 h incubation in coated plates followed by detection of bound MR as described in Experimental procedures.

(a), inhibition of binding of the sMR, (b) inhibition of binding of CRD(4-7)Fc

◆ D-Man, ■ L-Fuc, ▲ L-Rha, ○ D-GlcNAc, □ D-Glc.

The $K_i$ values, where $K_i$ is the concentration of inhibitor resulting in 50% inhibition of MR binding, were between 0.28 and 0.32 mM for the inhibition of binding of sMR by D-Man and L-Fuc, and between 4 and 7 mM for inhibition by D-Glc, D-GlcNAc and L-Rha. The $K_i$ values for inhibition of binding of CRD(4-7)Fc by D-Man and L-Fuc were between 1 and 1.8 mM; those for inhibition by D-Glc, D-GlcNAc and L-Rha were in the range of 7-20 mM.
Fig 5

Recognition of *S. pneumoniae* CPSs by the mannose receptor

CPSs were coated onto the wells of ELISA plates and the direct binding of CRD\(_{4-7}\)Fc and sMR assayed by incubation with 2 µg/ml of protein and detection of bound protein as described in Experimental procedures. All CPSs coated the plates as shown by titration with anti-CPS specific antisera. Coating concentrations were optimal from 10 µg/ml and upwards for detection with antisera. For detection with the MR a concentration of 50 µg/ml was used for coating which resulted in maximal readings for each CPS.

The results are expressed as percentage binding relative to yeast mannan (100%). Results are ±10% taken as the average of 4-6 experiments.

Open bars, CRD\(_{4-7}\)Fc; shaded bars, sMR.

Fig 6

Inhibition of binding of CRD\(_{4-7}\)Fc to CPS by D-Man and D-Glc

(a) Inhibition with 0.1, 0.5, 1 and 5 mM D-Man; black, open, dark shaded and light shaded bars, respectively. YM., yeast mannan.

(b) Inhibition with 1, 5 and 10 mM D-Glc; black, open and shaded bars, respectively.

CRD\(_{4-7}\)Fc was incubated at 2 µg/ml with different concentrations of monosaccharide for 2 h in the wells of ELISA plates coated with CPS. Detection of CRD\(_{4-7}\)Fc was as described in Experimental procedures. Inhibition of binding to yeast mannan is shown for comparison.
Fig 7

Inhibition of MR binding by soluble *S. pneumoniae* CPS.

CRD\(_{(4-7)}\)Fc (2 µg/ml) was pre-incubated with different concentrations of CPS for 30 min followed by incubation in the wells of α-D-Man–PAA coated plates for 2h. Black, dark shaded, open and light shaded bars, incubation with CPS at 10, 50, 100 and 200 µg/ml, respectively. Bound CRD\(_{(4-7)}\)Fc was detected as described under Experimental procedures. The percentage inhibition of binding relative to controls with no inhibitor is shown.

Fig. 8

Inhibition of binding of CRD\(_{(4-7)}\)Fc to LPS by D-Man and D-Glc

(a) Inhibition with 0.5, 1 and 5 mM D-Man; black, open and shaded bars, respectively.

(b) Inhibition with 1, 5 and 10 mM D-Glc; black, open and shaded bars, respectively.

CRD\(_{(4-7)}\)Fc was incubated at 2µg/ml with different concentrations of monosaccharide for 2 h in the wells of ELISA plates coated with LPS. Detection of CRD\(_{(4-7)}\)Fc was as described in Experimental procedures. Inhibition of binding to yeast mannan is shown for comparison. YM., yeast mannan.

Fig 9

Dual recognition of microbial polysaccharide and sulphated ligand by the mannose receptor

a) Increase in detection of binding of sMR to SO\(_4\)-3-β-D-Gal-PAA following incubation with yeast mannan, CPS and LPS.

sMR (2 µg/ml) was pre-incubated with yeast mannan, CPS and LPS for 30 min followed by incubation in the wells of SO\(_4\)-3-β-D-Gal-PAA coated plates and detection of bound sMR as previously described. Yeast mannan co-incubation was at 10 and 1 µg/ml and CPS and LPS at 50 µg/ml.
The percentage increase in detection of bound sMR relative to control binding with no added polysaccharide is shown.

b) Detection of CPS bound to $SO_4$-3-β-D-Gal-PAA coated plates following incubation with sMR.

CPS and sMR were co-incubated as previously described. CPS bound to the plate was detected with rabbit anti-CPS specific antisera followed by anti-rabbit IgG alkaline phosphatase conjugate, species absorbed. Open bars, incubation of $SO_4$-3-β-D-Gal-PAA coated plates with CPS alone; filled bars co-incubation of CPS and sMR.
TABLE 1a

Structures of *S. pneumoniae* capsular polysaccharides

Structures are taken from reference 14.

All sugars are in the pyranose conformation.
| Type | Structure |
|------|-----------|
| 1    | \((\rightarrow3)\alpha\text{-AATGal}\rightarrow4\alpha\text{-D-GalA}\rightarrow3\alpha\text{-D-GalA}\rightarrow3\) |
| 3    | \((\rightarrow4)\beta\text{-D-Glc}\rightarrow3\beta\text{-D-Gla}\rightarrow3\) |
| 4    | \((\rightarrow3)\beta\text{-D-ManNAc}\rightarrow3\alpha\text{-L-FucNAc}\rightarrow4\alpha\text{-D-GalNAc}\rightarrow1\) |
| 6B   | \((\rightarrow2)\alpha\text{-D-Gal}\rightarrow3\alpha\text{-D-Glc}\rightarrow3\alpha\text{-L-Rha}\rightarrow4\) |
| 9N   | \((\rightarrow4)\alpha\text{-D-GlcA}\rightarrow3\alpha\text{-D-Glc}\rightarrow3\beta\text{-D-ManNAc}\rightarrow4\) |
| 9V   | \((\rightarrow4)\alpha\text{-D-GlcA}\rightarrow3\alpha\text{-D-Gal}\rightarrow3\beta\text{-D-ManNAc}\rightarrow4\) |
| 14   | \((\rightarrow4)\beta\text{-D-Glc}\rightarrow6\beta\text{-D-Gal}\rightarrow4\beta\text{-D-GlcNAc}\rightarrow3\beta\text{-D-Gal}\rightarrow4\) |
| 18C  | \((\rightarrow4)\beta\text{-D-Glc}\rightarrow4\alpha\text{-L-Glc}\rightarrow2\alpha\text{-D-Gal}\rightarrow4\alpha\text{-D-Glc}\rightarrow3\alpha\text{-L-Rha}\rightarrow1\) |
| 19A  | \((\rightarrow4)\beta\text{-D-ManNAc}\rightarrow4\alpha\text{-D-Glc}\rightarrow3\alpha\text{-L-Rha}\rightarrow1\) |
| 19F  | \((\rightarrow4)\beta\text{-D-ManNAc}\rightarrow4\alpha\text{-D-Glc}\rightarrow2\alpha\text{-L-Rha}\rightarrow1\) |
| 23F  | \((\rightarrow4)\beta\text{-D-Glc}\rightarrow4\alpha\text{-L-Rha}\rightarrow2\beta\text{-D-Gal}\rightarrow4\beta\text{-L-Rha}\rightarrow1\) |
| CW-PS | \((\rightarrow6)\beta\text{-D-Glc}\rightarrow3\alpha\text{-D-AATGal}\rightarrow4\alpha\text{-D-GalNAc}\rightarrow3\beta\text{-D-GalNAc}\rightarrow1\) |

TABLE 1(a)
Table 1b

Structures of *K. pneumoniae* capsular polysaccharides

Structures are taken from reference 17, except for the following: ¹(18)., ²(19)., ³(20)., ⁴(21)., ⁵(22).

? Anomeric linkage not known.
| Type | Structure |
|------|-----------|
| K1   | \[\rightarrow 4\)-[2,3-(S)-pyruvate]-\beta-D-GlcA-(1→4)-\alpha-L-Fuc-(1→3)-\beta-D-Glc-(1→) \]          |
| 1\(^{1}\)K3 | \[\rightarrow 2\]-[(4,6-(S)-pyruvate)-\alpha-D-Man-(1→4)]-\alpha-D-GalA-(1→3)-\alpha-D-Man-(1→2)-\alpha-D-Man-(1→3)-\beta-D-Gal-(1→) \] |
| K11  | \[\rightarrow 3\]-\beta-D-Glc-(1→3)-[(4,6-(S)-pyruvate)-\alpha-D-Gal-(1→4)]-\beta-D-GlcA-(1→3)-\alpha-D-Gal-(1→) \] |
| K17  | \[\rightarrow 3\]-\alpha-L-Rha-(1→4)-\beta-D-Glc-(1→2)-[(\alpha-L-Rha-(1→3)]-\alpha-L-Rha-(1→4)-\alpha-D-GlcA-(1→) \] |
| 2\(^{2}\)K22 | \[\rightarrow 4\]-\beta-D-Glc-(1→3)-[(4-O-lactate)]-\beta-D-GlcA-(1→6)-\alpha-D-Glc-(1→4)]-\beta-D-Gal-(1→) \] |
|       | \[\text{6}\] \[\text{1}\] \[OAc\] |
| K26  | \[\rightarrow 3\]-\beta-D-Gal-(1→2)-[(4,6-(S)-pyruvate)]-\beta-D-Glc-(1→4)-\beta-D-Glc-(1→6)-\alpha-D-Glc-(1→4)]-\alpha-D-GlcA-(1→3)-\alpha-D-Man-(1→2)-\alpha-D-Man-(1→) \] |
| K27  | \[\rightarrow 3\]-\beta-D-Glc-(1→3)-?Gal-(1→3)-[(\beta-D-Glc-(1→4)]-?Gal-(1→6)-\beta-D-Glc-(1→) \] |
|       | \[\text{6}\] \[\text{1}\] \[\beta-D-GlcA\] |
| K31  | \[\rightarrow 3\]-\beta-D-Gal-(1→3)-\beta-D-Glc-(1→3)-[(4,6-(S)-pyruvate)]-\beta-D-Glc-(1→2)-\alpha-D-Man-(1→4)]-\alpha-D-GlcA-(1→) \] |
| K36  | \[\rightarrow 2\]-\alpha-L-Rha-(1→3)-\beta-D-Gal-(1→3)-[(4,6-(S)-pyruvate)]-\beta-D-Glc-(1→4)-\beta-D-GlcA-(1→2)]-\alpha-L-Rha-(1→3)-\alpha-L-Rha-(1→) \] |
| 3\(^{3}\)K40 | \[\rightarrow 3\]-\alpha-L-Rha-(1→2)-\alpha-L-Rha-(1→4)-\alpha-D-Glc-(1→2)-\alpha-D-Man-(1→2)-\alpha-D-Man-(1→3)-\alpha-D-Gal-(1→) \] |
| K46  | \[\rightarrow 3\]-\alpha-D-Gal-(1→3)-\beta-D-Gal-(1→3)-[(\beta-D-Glc-(1→3)]-(4,6-(S)-pyruvate)]-\alpha-D-GlcA-(1→3)-\alpha-D-Man-(1→) \] |
| 4\(^{4}\)K52 | \[\rightarrow 3\]-[(\alpha-D-Gal-(1→2)]-\alpha-D-Gal-(1→4)-\alpha-L-Rha-(1→3)-\beta-D-Gal-(1→2)-\alpha-L-Rha-(1→4)-\beta-D-GlcA-(1→) \] |
| K55  | \[\rightarrow 3\]-\beta-D-Glc-(1→4)-[(\alpha-D-GlcA-(1→3)]-\alpha-D-Gal-(1→3)]-\alpha-L-Rha-(1→) \] |
|       | \[\text{2}\] \[\text{1}\] \[\text{OAc}\] |
| K60  | \[\rightarrow 3\]-[(\alpha-D-Glc-(1→4)]-\beta-D-GlcA-(1→3)-[(\beta-D-Glc-(1→2)]-\beta-D-Gal-(1→3)-[(\beta-D-Glc-(1→2)]-\alpha-D-Man-(1→3)-\beta-D-Glc-(1→) \] |
| 5\(^{5}\)K64 | \[\rightarrow 4\]-\alpha-D-GlcA-(1→3)-\alpha-D-Man-(1→3)-\beta-D-Glc-(1→4)-[(4,6-(S)-pyruvate)]-\beta-D-Glc-(1→2)]-\alpha-D-Man-(1→) \] |

\[\text{3}\] \[\text{1}\] \[\alpha-L-Rha\]
Table 2a

**Structures of the O-antigenic sidechains of *K. pneumoniae* LPS**

**O-serotype**

| 01 | Galactan I and Galactan II: \( \rightarrow 3 \)-\( \beta \)-Galp-(1→3)-\( \alpha \)-D-Gal-(1→) and \( \rightarrow 3 \)-\( \alpha \)-D-Galp-(1→3)-\( \beta \)-D-Galp-(1→), respectively. |
| 02a | Galactan I |
| 03 | Polymannose: \( \rightarrow 3 \)-\( \alpha \)-D-Man-(1→3)-\( \alpha \)-D-Man-(1→2)-\( \alpha \)-D-Man-(1→2)-\( \alpha \)-D-Man-(1→) |
| 04 | \( \rightarrow 2 \)-\( \beta \)-Ribf-(1→4)-D-Galp-(1→) |

Structures are taken from reference 15. The majority of LPSs expressed by isolates of *K. pneumoniae* are of the 01, 02 and 03 serotypes.
Table 2b

Structures of LPSs tested for binding to the MR

The LPS structures of (a) *H. influenzae* type b strain Eagen (31) (b) *S. typhimurium* (32) and (c) *N. meningitidis* immunotype L3 (33,34) are shown.

The mutant forms of *Salmonella* and *Neisseria* LPS types are indicated by the dotted lines.

The core structures of the different LPS types extracted from strains of *K. pneumoniae* in this study are not known.
Table 3

**Direct binding of the MR to authentic standards**

| Substrate                  | CRD4-7Fc | sMR | CR-Fc |
|----------------------------|----------|-----|-------|
| Yeast mannan               | 1.1 (100%) | 1.2 (100%) | 0.00 |
| α-L-Fuc-PAA                | 0.84 (76%) | 0.90 (75%) | 0.00 |
| α-D-Man-PAA                | 0.70 (64%) | 0.66 (55%) | 0.00 |
| α-D-Man_{(3)}-PAA          | 0.81 (74%) | 0.84 (73%) | 0.00 |
| β-D-Gal-PAA                | 0.00     | 0.00 | 0.00  |
| S04-3-β-D-Gal-PAA          | 0.00     | 0.59 | 0.60  |
| S04-3-β-D-GalNAc-PAA       | 0.00     | 1.6  | 1.6   |
| S04-4-β-D-GalNAc-PAA       | 0.00     | 0.24 | 0.3   |
| S04-6-β-D-Gal-PAA          | 0.00     | 0.05 | 0.06  |
| S04-6-β-D-GlcNAc-PAA       | 0.00     | 0.05 | 0.06  |

Soluble sugar-polyacrylamide substrates were coated onto the wells of ELISA plates and direct binding of MR fusion proteins or sMR assayed by incubation with 2 µg/ml of protein for 2 h at room temperature followed by detection of bound proteins as described under Experimental procedures.

Results are the average of three assays ± 10% and are shown as absorbance readings at 405 nm taken after 30 min incubation with substrate and in brackets as percentage binding relative to yeast mannan.
Table 4

Recognition of LPS by the mannose receptor

The different LPS serotypes of *K. pneumoniae* were extracted from the following capsular serotype strains: O1, K1; O2a, K3; O3, K55; O4, K36 and rough type, K17 (15,35).

The presence or absence of O-antigen and the banding patterns indicative of poly-galactose or poly-mannose O-antigen were confirmed by SDS-PAGE and silver staining (data not shown). It was noted that LPSs from capsular strains K3 and K55 contained a relatively high proportion of LPS molecules with O-antigen whereas LPS from K1 and K36 contained less O-antigenic material.

The structures of the *K. pneumoniae* LPS O-antigens and of the other LPS types used in this assay are shown in Table 2a&b.

LPSs were coated onto the wells of ELISA plates at 50 µg/ml and the direct binding of CRD_{4-7}Fc and sMR assayed as described under Experimental procedures. The results shown are percentage binding relative to yeast mannan.
Table 4

| LPS                          | CRD\textsubscript{(4,7)Fe} | sMR |
|------------------------------|-----------------------------|-----|
| **K. pneumoniae**            |                             |     |
| O1 (galactan I and II)       | 30                          | 19  |
| O2a (galactan I)             | 94                          | 100 |
| O3 (polymannose)             | 76                          | 52  |
| 04 (ribose/galactose)        | 0                           | 0   |
| Rough type                   | 87                          | 89  |
| **Salmonella**               |                             |     |
| Rb\textsubscript{2}          | 12                          | 9   |
| Rc                           | 14                          | 12  |
| Rd\textsubscript{1}          | 12                          | 20  |
| Rd\textsubscript{2}          | 20                          | 19  |
| Re                           | 19                          | 18  |
| **Ps. aeruginosa**           |                             |     |
|                              | 0                           | 0   |
| **H. influenzae**            |                             |     |
|                              | 0                           | 0   |
| **N. meningitidis**          |                             |     |
| immunotype L3                | 0                           | 0   |
| Mu-4                         | 0                           | 0   |
Fig 2b/c

b  
LPS+O-antigen

core-LPS

1  2  3  4  5  6

c  
1  2  3  4
Fig 3
Fig. 4

(a) Percentage inhibition vs. mM concentration inhibitor

(b) Percentage inhibition vs. mM concentration inhibitor
Fig. 6

(a)

(b)
Fig. 7

The graph shows the percentage inhibition of different polysaccharides. The x-axis represents various polysaccharides (1-23F, CW-PS), and the y-axis represents the percentage inhibition ranging from 0 to 100.

Poly saccharide
Fig. 8

(a)

(b)
Fig. 9

(a)

(b)
Recognition of bacterial capsular polysaccharides and lipopolysaccharides by the macrophage mannose receptor

Susanne Zamze, Luisa Martinez-Pomares, Hannah Jones, Philip R. Taylor, Richard J. Stillion, Siamon Gordon and Simon Y.C. Wong

J. Biol. Chem. published online August 23, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M207057200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts