Macrophage Receptors for Influenza A Virus: Role of the Macrophage Galactose-Type Lectin and Mannose Receptor in Viral Entry

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Although sialic acid has long been recognized as the primary receptor determinant for attachment of influenza virus to host cells, the specific receptor molecules that mediate viral entry are not known for any cell type. For the infection of murine macrophages by influenza virus, our earlier study indicated involvement of a C-type lectin, the macrophage mannose receptor (MMR), in this process. Here, we have used direct binding techniques to confirm and characterize the interaction of influenza virus with the MMR and to seek additional macrophage surface molecules that may have potential as receptors for viral entry. We identified the macrophage galactose-type lectin (MGL) as a second macrophage membrane C-type lectin that binds influenza virus and is known to be endocytic. Binding of influenza virus to MMR and MGL occurred independently of sialic acid through Ca2+-dependent recognition of viral glycan by the carbohydrate recognition domains of the two lectins; influenza virus also bound to the sialic acid on the MMR. Multivalent ligands of the MMR and MGL inhibited influenza virus infection of macrophages in a manner that correlated with expression of these receptors on different macrophage populations. Influenza virus strain A/PR/8/34, which is poorly glycosylated and infects macrophages poorly, was not recognized by the C-type lectin activity of either the MMR or the MGL. We conclude that lectin-mediated interactions of influenza virus with the MMR or the MGL are required for the endocytic uptake of the virus into macrophages, and these lectins can thus be considered secondary or coreceptors with sialic acid for infection of this cell type.

Infection of host cells by influenza virus is initiated by attachment of virus to sialic acid residues on the host cell surface through the receptor-binding site at the distal tip of the viral hemagglutinin (HA) (43). After attachment, the virus is internalized by endocytosis, and acidification of the endosome triggers a conformational change in viral HA that results in fusion of the viral envelope and host cell membrane (34). At the cell surface, sialic acid residues are commonly found at the termini of oligosaccharide chains that are attached in O or N linkage to cell surface proteins; they are also an essential component of acidic glycosphingolipids (gangliosides) that are present in all mammalian cell membranes. Although the abundance of sialic acid on mammalian cells provides influenza virus with multiple potential receptors, virus attachment does not always lead to virus entry (5, 8, 46). Furthermore, sialic acid-independent infection of Madin-Darby canine kidney (MDCK) cells by influenza virus has been reported (35). The specific host cell molecules that serve as functional receptors (or coreceptors) for the infectious entry of influenza virus have yet to be defined.

We have studied the infectious entry of influenza virus into macrophages (Mφ), which represents an early event in recognition of the virus by the innate immune system (23, 44). After intranasal infection of mice, influenza virus replicates productively in cells of the respiratory epithelium. Mφ are also infected and viral proteins are produced, but replication is abortive and no live progeny are released (32); infection of Mφ is thus a dead-end for the virus leading to a reduction in viral load. In addition, influenza virus infection of Mφ stimulates production and release of proinflammatory cytokines and alpha/beta interferon (28), which may assist in further limiting viral replication and spread within the respiratory tract. Depletion of airway Mφ from mice prior to intranasal influenza virus infection leads to increased virus titers in the lung, testing to the important role of Mφ in early host defense against the virus (38, 44).

We observed in a previous study (30) that influenza A virus strains differed in their ability to infect murine Mφ, strains carrying a more highly glycosylated hemagglutinin (HA) molecule being more efficient at infecting Mφ than less glycosylated strains, although binding of viruses to the Mφ cell surface was equivalent. Our investigation of this phenomenon indicated involvement of the Mφ mannose receptor MMR (CD206), a C-type lectin, in infectious viral entry (29, 30). The involvement of other receptors was not excluded, and our subsequent observation that influenza virus can infect the RAW 264.7 Mφ cell line, which does not express the MMR, indeed points to the existence of other routes of infectious entry of the virus into Mφ.
In the present study we used direct binding methods to confirm and characterize the interaction of influenza virus with the MMR and to seek additional Mø surface molecules that may have potential as receptors for viral entry. We identify the Mø galactose-type lectin (MGL) as a second Mø membrane C-type lectin that binds influenza virus and investigate its involvement in the infectious process.

**Materials and Methods**

**Viruses.** The influenza A viruses used were A/Guangdong/25/93 (H1N2) (Guangdong/93), A/HKx31 (H2N2), and the M. Sinai strain of A/PRI/83/4 (H1N1) (PR8). HKx31 is a high growth reassortant of PR8 with A/Aichi/2/68 (H2N2) that bears the H3N2 surface glycoproteins (2). Viruses were grown in the allantoic cavity of 10-day embryonated hen's eggs by standard procedures and infectivity titers were determined by plaque assay on MDCK cells (1). For binding studies, viruses were concentrated from allantoic fluid by precipitation with 8% (w/v) polyethylene glycol 6000 (BDH Chemicals) and purified by rate zonal sedimentation on 25 to 70% (w/v) sucrose gradients. Purified Guangdong/93 virus was grown in the absence of trypsin and concentrated by ultracentrifugation. Viruses were grown in the essential medium (Gibco) supplemented with 2 mM glutamine, 2 mM pyruvate, 1 mM MgCl2 (PBS-Ca2+, EDTA), and 10% FCS (v/v) in 250 ml of culture flasks, rinsed three times with ice-cold PBS containing 1 mM CaCl2 and cultured for 30 min. Unreacted biotin was quenched with PBS-Ca2+. The reaction was terminated by adding an equal volume of 2 M glycine, the fixed virus was dialyzed extensively with Tris-buffered saline (TBS; 0.05 M Tris-HCl, 0.15 M NaCl [pH 7.2]), and aggregates were removed by centrifugation (10,000 x g, 5 min).

**Cell lines.** The Mø cell lines J774E (a mutant line of J774 Mø selected for enhanced expression of MR [11]) and RAW 264.7 were maintained in essential medium (Gibco) supplemented with 2 mM glutamine, 2 mM pyruvate, 10% fetal calf serum (FCS) (sMEM, Invitrogen), with 60 mM thiouguanine added to media for J774E Møs. MDCK cells and LA-4 cells, a murine airway epithelial cell line, were passaged in RPMI 1640 supplemented with 2 mM glutamine, 2 mM pyruvate, 10% FCS, and 10% ECS (ESR, Japan). Resident alveolar Møs from C57BL/6 mice were obtained and cultured as previously described (30).

**Preparation of Mø cell lysates.** Møs grown to semiconfluence in 8-cm² tissue culture flasks (1 x 10⁶ to 3 x 10⁷ cells/flask) were rinsed twice with ice-cold phosphate-buffered saline (PBS) and harvested with cell scrapers. The cells were washed three times by centrifugation, resuspended in 0.5 ml of PBS on ice, and then mixed with an equal volume of 2% lysis buffer (final concentration; 50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% Triton X-100, 1 mM CaCl2, 1 mM MgCl2) and protease inhibitor cocktail (EDTA-free complete inhibitor from Boehringer Mannheim). After 1 h on ice, the lysate was centrifuged for 3 min at 10,000 g. Final protein concentrations of the lysates ranged from 1–3 mg ml⁻¹.

**Sialidase treatment.** Mø cell lysates (100 µl) were treated with 1 µl (5 µL) of neuraminidase from Vibrio cholerae type III (Sigma) at 37°C for 4 to 6 h. The reaction was terminated by adding an equal volume of 2% nonreducing sodium dodecyl sulfate (SDS) sample buffer (final concentration = 2% SDS, 10% glycerol, 0.005% bromophenol blue, 62.5 mM Tris-HCl [pH 6.9]).

**Cell surface labeling.** Møs were grown to semiconfluence in 80-cm² tissue culture flask cells, rinsed three times with ice-cold PBS containing 1 mM CaCl2 and 1 mM MgCl2 (PBS-Ca2+, Mg2+), and surface labeled by incubation for 30 min on ice with 0.5 mg ml⁻¹ EZ-Link Sulfo-NHS-biotin (Pierce) in PBS-Ca2+, Mg2+; 5 ml per flask. Buffer was removed, and a second 5 ml of biotin solution was added for 30 min. Unreacted biotin was quenched with PBS-Ca2+, Mg2+ containing 0.1 M glycine (10 ml per flask), and cell lysates were prepared as described above.

**Primary antibodies.** Monoclonal antibodies (Mab) specific for the HA of HKx31 and PR8 influenza viruses were generated within the Department of Microbiology and Immunology, University of Melbourne. Hyperimmune serum to Guangdong/93 virus was prepared in BALB/c mice by two intraperitoneal inoculations, 3 weeks apart, of virus in allantoic fluid. Polyclonal antisera to the virus strain was added to the blot for 2 to 3 h, followed by multiple washes and incubation with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako). After washing, the membranes were moved to room temperature, and a peroxidase substrate was immediately added. To study the effect of EDTA or monocarboxylic acids on virus binding, these were included during the virus binding step and in the washes immediately prior to and subsequent to incubation of virus on the blot. Blots were developed by standard methods using chemiluminescence or dianibenzidine and scanned, and the images were viewed with Adobe Photoshop software.

**Immunoblot detection of biotinylated proteins on blots.** Filters were blocked for 1 h with 5% nonfat milk powder in PBS containing 0.1% Tween 20. After incubation, the membranes were washed and incubated with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako). After washing, the membranes were moved to room temperature, and a peroxidase substrate was immediately added. To study the effect of EDTA or monocarboxylic acids on virus binding, these were included during the virus binding step and in the washes immediately prior to and subsequent to incubation of virus on the blot. Blots were developed by standard methods using chemiluminescence or diaminobenzidine and scanned, and the images were viewed with Adobe Photoshop software.

**Immunoprecipitation and chromatography.** Immunoprecipitate the MMR, J774E cell lysate was first precleared by incubation with protein G-Sepharose (Amersham Pharmacia Biotech) for 2 h at 4°C, followed by removal of the Sepharose beads by centrifugation. Rabbit anti-MMR IgG (5 µg per 250 µl of cellular protein) and additional protein G-Sepharose were added to the lysate, and the mixture was incubated on a rotating platform at 4°C for 16 h. Immune complexes bound by protein G-Sepharose were collected by centrifugation, washed extensively with lysis buffer, boiled in 50 µl of nonreducing SDS-sample buffer at 95°C for 10 min, and analyzed by VOPBA.

To isolate MGL by affinity chromatography, asialofetuin (ASF; Sigma) was coupled to CNBr-Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer's instructions using 5 mg of asf per ml of packed beads. J774E Mø lysate was treated with 5 mM EDTA to release endogenous ligands from MGL before addition to a 1/3 volume of ASF-Sepharose and recalcification with 20 mM CaCl2. After incubation, the Sepharose was collected, washed, and suspended in 0.3 M β-galactose in lysis buffer to elute J774E proteins.

**Viral precipitation of Mø proteins.** Mø lysate was prepared from biotin surface-labeled J774E cells and 1 ml of lysate (1 to 2 mg of total protein) was diluted to 5 ml with chilled lysis buffer. The suspension was centrifuged at ultracentrifugation for 100,000 x g for 45 min at 4°C (Beckman Optima series, Beverly, MA) using a swinging-bucket rotor (SW30 Ti) at 2°C. After centrifugation, clarified lysate, 100 µg of glutaraldehyde-fixed influenza virus (Guangdong/93) was added, and the suspension was adjusted to contain 10 mM CaCl2 or 5 mM EDTA (pH 7). Tubes containing virus only or clarified lysate only in the presence of 10 mM CaCl2 served as negative controls. After incubation at 4°C for 2 h on a rotating platform to allow interaction of virus with cellular molecules, the contents were diluted to 5 ml with lysis buffer (with or without supplemental CaCl2 or EDTA, as used during the incubation period) and centrifuged at 100,000 x g for 45 min at 4°C to pellet the virus and any bound cellular proteins. The precipitated virus complexes were washed three times with lysis buffer by sequential resuspension and ultracentrifugation and then heated at 95°C for 5 min in 50 µl of nonreducing sample buffer and subjected to SDS-PAGE.

**Detection of cell surface MMR and MGL by flow cytometry.** Aliquots of 10⁵ cells were incubated on ice for 20 min with supernatant from hybridoma 2.402 to block Fc receptors and then stained for 30 min on ice with biotin-labeled Mabs to either MMR (MCA-223SB) or MGL (MCA-14) or with appropriate biotin-labeled isotype control MAb in TBS containing 5 mM EDTA and supplemented with 10% normal mouse serum. After a washing step, bound MAbs were detected with streptavidin conjugated to allophycocyanin, and the cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences) by using data on 20,000 cells. The data were analyzed by using the FlowJo program (Treestar).

**Infection of Mø and epithelial cells by influenza virus.** Eight-well chamber slides (Lab-Tek, Nunc) were seeded with Mø or epithelial cells at 2 x 10⁴ cells/ml in 250 µl, followed by incubation overnight. The resulting monolayers were washed and incubated for 1 h at 37°C with 10⁵ PFU of influenza virus in 300
RESULTS

Interaction of influenza virus with the MMR. Our previous studies had implicated the MMR, a C-type lectin, as a major endocytic receptor in the infectious entry of influenza virus into murine Møs (30). In seeking to confirm a direct interaction between virus and MMR, we carried out a VOPBA in which lysate from J774E Møs was resolved by SDS-PAGE and probed with influenza A virus HKx31 in the presence of Ca2+. Detection of bound virus revealed its interaction with numerous proteins, with particularly intense binding to a molecule that migrated with an apparent molecular mass of 175 kDa (Fig. 1A). The mobility of this protein correlated with that of MMR, as evidenced by comparing duplicate blots that had been probed with virus and with polyclonal anti-MMR IgG (Fig. 1B). The distinct 175-kDa virus-binding band was not observed in VOPBA of lysate from the RAW 264.7 Mø cell line, which does not express the MMR (12). Immunoprecipitation of MMR from the J774E lysate led to marked depletion of the 175-kDa virus-binding protein, and virus bound strongly to MMR recovered from the immunoprecipitate (Fig. 1C).

Sialic acid represents the primary receptor for influenza virus, and the MMR is known to be sialylated (20). To determine whether virus binding to MMR was dependent on sialic acid, J774E lysate was treated with V. cholerae neuraminidase (sialidase) prior to separation by SDS-PAGE and VOPBA. As expected, virus binding to the majority of Mø proteins was abolished after sialidase treatment (Fig. 2A, HKx31 virus; Fig. 2B, Guangdong/93 virus). In contrast, desialylation of the MMR, which was accompanied by a slight increase in its mobility on SDS-PAGE (Fig. 2C), did not eliminate virus binding (Fig. 2A and B). Binding of virus to the desialylated MMR was abrogated in the presence of EDTA (Fig. 2B, lane 4), a finding consistent with Ca2+-dependent recognition of viral carbohydrate by the C-type lectin domains of the MMR. Interestingly, EDTA reduced but did not eliminate binding of virus to the untreated MMR (Fig. 2B, lane 3). This suggests that the interaction of virus with native MMR occurs in two ways, through binding of the viral HA to sialic acid on MMR, as well as by recognition of oligosaccharide on the viral glycoproteins by the C-type lectin activity of the MMR. The strong binding of virus to immunoprecipitated MMR observed in Fig. 1C likely reflects binding to sialic acid on MMR, since boiling of the immunoprecipitate prior to VOPBA would be expected to destroy lectin activity of the MMR.

Influenza virus binds to MGL. To investigate virus-binding proteins of lower molecular mass, a higher concentration of acrylamide (10%) was used for SDS-PAGE and subsequent VOPBA of J774E lysate. This revealed Guangdong/93 virus binding to a 44-kDa protein band and, in desialylated lysate, to a protein species of slightly faster mobility (Fig. 3A). The possible involvement of another lectin was considered, a candidate being the Mø galactose-type lectin (MGL, known previously as galactose/N-acetylgalactosamine-specific C-type lectin), which is an integral membrane glycoprotein with a reported molecular mass of 42 kDa (17, 19). In a duplicate blot probed with antibody specific to MGL, the mobilities of these virus-binding bands in native and desialylated lysates were indeed shown to correspond to those of MGL (Fig. 3A).

Like the hepatic asialoglycoprotein receptor, MGL func-
tions as an endocytic receptor for unsialylated glycoproteins (17, 26). To isolate MGL, J774E MΦ lysate that had been sialidase-treated (+) or not treated (−) was resolved in duplicate by SDS–10% PAGE, and blots were probed with virus (Guangdong/93) in a VOPBA or immunoblotted with MAb LOM-8.7 to detect MGL. (B) MGL was isolated from J774E MΦ by affinity chromatography on ASF (MGL) and resolved by SDS–10% PAGE alongside J774E cell lysate (MΦ). The binding of Guangdong/93 virus was examined in VOPBA using virus binding buffer NiBB-Ca^{2+} (control) or the same buffer supplemented with 0.1 M galactose or 0.1 M mannose. The approximate molecular mass positions are indicated in kilodaltons.

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FIG. 3. Influenza virus binds to MGL. (A) Comparison of MΦ proteins recognized by influenza virus and by anti-MGL antibody. J774E MΦ lysate that had been sialidase-treated (+) or not treated (−) was resolved in duplicate by SDS–10% PAGE, and blots were probed with virus (Guangdong/93) in a VOPBA or immunoblotted with MAb LOM-8.7 to detect MGL. (B) MGL was isolated from J774E MΦ by affinity chromatography on ASF (MGL) and resolved by SDS–10% PAGE alongside J774E cell lysate (MΦ). The binding of Guangdong/93 virus was examined in VOPBA using virus binding buffer NiBB-Ca^{2+} (control) or the same buffer supplemented with 0.1 M galactose or 0.1 M mannose. The approximate molecular mass positions are indicated in kilodaltons.

In VOPBA, binding of influenza virus to MGL was abrogated in the presence of 0.1 M D-galactose but not D-mannose, as shown in Fig. 3B for both MGL in the whole J774E lysate and isolated MGL. Binding of virus to MGL thus appears to be mediated solely through the galactose-specific C-type lectin activity of MGL, there being no evidence for additional binding through sialic acid as had been shown to occur for the interaction of influenza virus with MMR.

Analysis of cell surface proteins precipitated by influenza virus from MΦ lysates. Being endocytic receptors, only a fraction of MMR and MGL molecules are expressed at the cell surface at any one time, the remainder being located intracellularly (22, 45). Since the VOPBA blots described above were conducted with whole-cell lysates, some of the observed binding of virus would have been to lectin molecules derived from inside the cell. To confirm that influenza virus could bind to cell surface-expressed MMR and MGL, J774E cells were surface labeled with biotin prior to lysis, the lysate was mixed with influenza virus and subjected to ultracentrifugation, and the precipitate was analyzed for biotinylated molecules that had coprecipitated with the virus. In order to stabilize the virus in the presence of detergent in the MΦ lysate, glutaraldehyde-fixed virus was used for this experiment. Among the biotinylated proteins precipitated by Guangdong/93 virus were two major species, of masses ~175 and ~44 kDa, whose precipitation was abrogated in the presence of EDTA (Fig. 4). By immunoblotting with specific antibodies, these proteins were confirmed to be MMR and MGL, respectively (data not shown).

Infection of MΦ by influenza virus is blocked by ligands of MGL and MMR. We had shown previously that infection of MΦ by influenza virus was blocked at a postbinding step by yeast mannan, a ligand of MMR, suggesting a role for MMR in viral entry (30). The RAW 264.7 MΦ cell line lacks MMR expression and yet is readily infected by influenza virus. To investigate a possible role for MGL in viral entry, we examined the effect of ASF, a multivalent ligand of MGL, together with mannan, on influenza virus infection of J774E MΦ (MMR⁺, MGL⁺); RAW 264.7 MΦ (MMR⁺, MGL⁺); and LA-4, a mouse respiratory epithelial cell line that lacks expression of either receptor (Fig. 5A).

Cells were incubated with Guangdong/93 influenza virus in chamber slides in the absence or presence of various concentrations of ASF or mannan, and infection was monitored by

FIG. 4. Coprecipitation of MΦ cell surface proteins with influenza virus after ultracentrifugation. J774E cells were surface labeled with biotin prior to cell lysis. Influenza virus (Guangdong/93) was incubated with the lysate in the presence of 10 mM Ca^{2+} or 5 mM EDTA and then precipitated by ultracentrifugation and washed. Final pellets were boiled and then resolved by SDS–12% PAGE and blotted with streptavidin. Samples of biotinylated lysate only (MΦ) and virus only (virus) were subjected to the same treatment and served as controls. The approximate molecular mass is indicated in kilodaltons.
subsequent detection of viral NP expression by immunofluorescence microscopy. As shown in Fig. 5B, ASF was found to inhibit infection of both J774E Mφ, RAW 264.7 Mφ (RAW) or LA-4 epithelial cells. Cells were stained with biotin-labeled MAb to MMR or MGL (shaded histograms) or with appropriate biotin-labeled isotype control MAb (open histograms). A minimum of 20,000 cells were collected for analysis. (B) Monolayers of J774E Mφ, RAW 264.7 Mφ or LA-4 epithelial cells were incubated for 30 min at 37°C in serum-free medium alone or supplemented with increasing concentrations of either mannan or ASF, as indicated, prior to the addition of 10^6 PFU of Guangdong/93. Mannan or ASF were included during subsequent culture of the cells. Cells were fixed 6 to 8 h postinfection and stained for the expression of influenza virus NP, and the percentage of virus-infected cells was determined via immunofluorescence. The data represent the mean percent infection ± 1 standard deviation of three independent experiments.

**FIG. 5.** Expression of MMR and MGL by Mφ correlated with ability of mannan and/or ASF to inhibit infection by influenza virus. (A) Expression of MMR and MGL on J774E Mφ, RAW 264.7 Mφ (RAW) or LA-4 epithelial cells. Cells were stained with biotin-labeled MAb to MMR or MGL (shaded histograms) or with appropriate biotin-labeled isotype control MAb (open histograms). A minimum of 20,000 cells were collected for analysis. (B) Monolayers of J774E Mφ, RAW 264.7 Mφ or LA-4 epithelial cells were incubated for 30 min at 37°C in serum-free medium alone or supplemented with increasing concentrations of either mannan or ASF, as indicated, prior to the addition of 10^6 PFU of Guangdong/93. Mannan or ASF were included during subsequent culture of the cells. Cells were fixed 6 to 8 h postinfection and stained for the expression of influenza virus NP, and the percentage of virus-infected cells was determined via immunofluorescence. The data represent the mean percent infection ± 1 standard deviation of three independent experiments.

**FIG. 6.** The poor ability of strain PR8 to infect Mφ correlates with weak interactions with MMR and MGL. (A) Susceptibility of Mφ and epithelial cell lines to infection by Guangdong/93 ( ), HKx31 ( ), and PR8 ( ) influenza viruses. Alv, resident alveolar Mφ. Monolayers of cells were infected in chamber slides with 10^6 PFU of influenza virus as described in Materials and Methods. The percentage of infected cells was determined in five independent fields per chamber by fluorescence microscopy. The data are expressed as mean percent infected cells ± 1 standard deviation and are representative of at least two independent experiments.

Interaction of PR8 influenza virus with Mφ lectins. The Mt. Sinai strain of A/PR8/34 influenza virus infects murine Mφ very poorly (30). This phenomenon is illustrated in Fig. 6A, which compares the Guangdong/93, HKx31, and PR8 viruses for their ability to infect different Mφ populations and the

epithelial cell lines LA-4 and MDCK cells. HKx31 (H3N2) is a reassortant of PR8 (H1N1) with A/Aichi/68 (H3N2) virus and differs from PR8 only in its surface glycoproteins (2). The HA of PR8 (Mt. Sinai) is poorly glycosylated, bearing glycans on the fibrous stalk of the HA molecule but none on the globular head (6). In contrast, HKx31 carries two (39), and Guangdong
carries four (14) potential glycosylation sites on the head of the HA molecule, in addition to the stalk glycans. If MMR and MGL function as receptors for viral entry into Mφ, the poor infectivity of PR8 for Mφ may reflect a poor interaction with these C-type lectins.

The binding of PR8 to MMR and MGL was examined in VOPBA, and the results are shown alongside the binding pattern obtained for Guandong/93 in the same experiment (Fig. 6Bi and ii). (Note that the blot shown for Guandong/93 in Fig. 6Bi is the same as that shown in Fig. 2B, left panels, PR8 binding having been tested in parallel with Guandong/93 binding as part of the larger experiment depicted in Fig. 2B and C). Like Guandong/93 virus, PR8 virus bound to numerous proteins from the untreated J774E lysate, including to a 175-kDa protein corresponding in size to the MMR (Fig. 6Bi); unlike Guandong/93, however, binding of PR8 to the desialylated form of MMR was undetectable. This indicates that while PR8 may recognize sialic acid on MMR, the lectin domains of the MMR do not bind the carbohydrate on PR8 virus.

In virus blots to examine binding to MGL in J774E lysate and to isolated MGL (Fig. 6BiBii), Guandong/93 bound to MGL, as shown previously; in contrast, no binding of PR8 to MGL was detected at all. Together, these results support the hypothesis that the inefficiency of PR8 virus infection of Mφ is related to poor recognition by the C-type lectins MMR and MGL, a finding consistent with an important role for these lectins as routes of entry for influenza virus into Mφ.

**DISCUSSION**

Although sialic acid has long been recognized as the primary receptor for attachment of influenza virus to the cell surface, the particular receptor or coreceptor molecules involved in mediating viral entry have not yet been elucidated for any cell type. The present study has used direct binding methods to investigate the cellular receptors involved in infection of Mφ by influenza virus and extends our previous study that implicated the MMR in this process (30). As shown here by the VOPBA technique, virus bound to sialic acid on numerous Mφ glycoproteins. Binding of influenza virus Guandong/93 to the MMR was confirmed, and binding to a second Mφ surface C-type lectin, MGL, was demonstrated, using VOPBA and virus-precipitation techniques. Binding to these latter receptors occurred independently of sialic acid, through Ca\(^{2+}\)-dependent recognition of viral glycans by the carbohydrate recognition domains of the two lectins, although, as discussed further below, binding of virus to sialic acid on MMR also occurred. Both MMR and MGL are known to function as endocytic receptors (20, 26). Mannan and ASF, multivalent ligands of MMR and MGL, respectively, were found to block infection of Mφ by Guandong/93 in a manner that correlated with expression of these receptors on different Mφ populations, while having no effect on infection of epithelial cells which lack these receptors. The PR8 (Mt. Sinai) strain of influenza virus, which is poorly glycosylated and infects Mφ very poorly, failed to show detectable binding in VOPBA to the lectin domains of either MGL or MMR. Together, these findings point to the importance of lectin-mediated interactions of virus with MMR and/or MGL in the infection process.

The mode of binding of Guandong/93 virus to MMR was examined in VOPBA using conditions of desialylation and/or Ca\(^{2+}\) depletion with EDTA. The results indicated binding occurred in two ways: (i) by recognition of viral carbohydrate by the C-type lectin domains of MMR and (ii) by binding of the virus to sialic acid on MMR. An additional mode of ligand recognition by MMR has been reported, namely, Ca\(^{2+}\)-independent recognition of sulfated oligosaccharides by the cysteine-rich domain of the molecule (13, 21). Although sulfation of HA has been described (9, 16), sulfated oligosaccharides have been associated with the stalk rather than the globular head of HA molecule (41, 42) and may not be accessible for MMR binding. We obtained no evidence for this mode of binding to influenza virus, given that desialylation of MMR and removal of Ca\(^{2+}\) abrogated all binding of Guandong/93.

In contrast to the dual mode of binding of influenza virus to MMR, interaction of MGL appeared to be mediated by the C-type lectin activity of MGL alone, since the binding of Guandong/93 was fully abrogated in the presence of 0.1 M galactose (Fig. 3B) and PR8 virus showed no binding at all to MGL in VOPBA (Fig. 6BiBii). Although MGL is sialylated, as evidenced by its enhanced mobility after sialidase treatment (Fig. 3A), the type of sialic acid, or the nature of its linkage to adjacent sugars, is apparently not recognized by influenza virus.

Sialidase treatment of Mφ greatly reduces susceptibility to influenza virus infection (30). Thus, although binding of Guandong/93 virus to desialylated MMR and MGL was clearly demonstrated in VOPBA (Fig. 2A and B and Fig. 3A), such interactions at the Mφ cell surface are not sufficient to mediate efficient infection. Efficient infection requires the contribution of both sialic acid and the lectin activity of MMR and/or MGL. A similar phenomenon has been reported previously where the enhanced entry of H5N1 pseudotype viral particles into stable cell lines expressing the C-type lectin DC-SIGN was abolished if cells were treated with bacterial sialidase prior to exposure to virus (40).

Our results support a model for infection of Mφ by influenza virus in which MMR and MGL act as coreceptors with sialic acid. After attachment of virus to multiple sialylated molecules (glycoproteins and glycolipids) at the cell surface, virus binds through its glycans to the lectin domains of MGL or MMR. These endocytic receptors subsequently mediate the uptake of virus into endosomes, along with adjacent sialylated molecules to which the virus remains bound, the HA-sialic acid interaction being required for efficient fusion of viral and endosomal membranes at acid pH in the late endosome (10). The dual nature of binding of MMR to influenza virus, through both sialic acid and C-type lectin interactions, may enhance the efficiency of this route of entry. Indeed, through mediating both binding and entry functions, the MMR may conceivably behave as a functional receptor in its own right. Interestingly, VOPBA of PR8 virus on Mφ lysate appeared to indicate binding to the sialic acid on MMR (Fig. 6BiBii). If so, such binding is clearly not sufficient to mediate efficient infection by PR8 virus, suggesting engagement of the lectin domains of MMR is essential for efficient entry of the virus to occur.

In RAW 264.7 Mφ, which express MGL but not MMR, infection by Guandong/93 virus was blocked by ASF but not by mannan, a finding consistent with viral entry being mediated by MGL in this cell type (Fig. 5). In J774E Mφ, which express both MMR and MGL, strong blocking of infection (>80%)
could be achieved by either ligand alone. This suggests that when both MMR and MGL are present, they may act cooperatively to facilitate viral entry and that in blocking experiments, engagement of one type of receptor with its multivalent ligand affects the expression or functioning of the other receptor. Such a situation could occur if MMR and MGL are physically associated or clustered in the M\(\phi\) membrane and the binding of mannann to MMR, or ASF to MGL, sterically hinders access of viral glycans to the other receptor or results in its down-modulation by coendocytosis within the same coated pit. Experiments to monitor the surface expression of MMR and MGL on J774E M\(\phi\) in the absence or presence of cognate and noncognate ligand may help to resolve this issue.

The binding of influenza viruses to the lectin domains of MMR and MGL will reflect the type, number, and accessibility of glycans carried on the virus. Glycosylation of influenza virus glycoproteins is N linked, and glycans may be high mannose, complex, or hybrid in type (3, 18). Complex glycans of influenza virus bear terminal galactose due to removal of sialic acid by the viral neuraminidase (4) and thus represent potential ligands for galactose-binding lectins such as MGL. High-mannose glycans represent a likely ligand for MMR, although glycans with exposed fucose or N-acetylglucosamine residues also represent potential targets (20, 33). There is considerable variation among influenza A viruses in the level of glycosylation of the HA molecule, particularly on its globular head. HKx31 and Guangdong/93 viruses carry two and four glycosylation sites, respectively, on the head of HA (14, 39). In contrast, PR8 virus carries no glycans on the head of its HA molecule (6) although, like all influenza A viruses, it carries glycosylation on the stalk region of HA at sites which are highly conserved between subtypes (24, 25, 31). The failure of PR8 virus to bind in VOPBA to the lectin domains of either MMR or MGL suggests that the stalk glycans on HA of intact virions are not readily accessible to binding by either receptor, and it is the glycans on the head of HA that are important in the interaction.

Two other recent studies have attempted to dissect the steps involved in binding and entry of influenza virus into cells. Stray et al. (35) found that influenza virus was able to infect sialidase-treated MDCK cells, albeit at lower efficiency, indicating the existence of sialic acid-independent functional receptors for influenza virus on this cell type. Chu and Whittaker (8) reported that influenza viruses could bind to but not infect Lec1 cells, a mutant line of CHO cells. Lec1 cells have a mutation in the N-acetylgalactosaminyltransferase 1 gene (GnT1) that results in deficient terminal N-linked glycosylation, although gangliosides are still produced and O-linked glycosylation should not be deficient. Influenza virus displayed efficient binding to these cells, but infection was arrested at the level of internalization, and the virus was not endocytosed. These studies support a model of influenza virus infection in which the receptors for viral entry are distinct from primary attachment receptors.

The functional receptors for influenza virus appear to differ in different cell types. In the present study, receptors for influenza virus entry into murine M\(\phi\) were identified as C-type lectins, the MMR and MGL, which are not expressed in MDCK cells or in LA-4 respiratory epithelial cells. Although the specific virus receptors on these latter cells have not yet been identified, other mannose- or galactose-specific lectins would not appear to be involved since mannan and ASF had no effect on infection of these cell types. Likewise, the functional receptors on MDCK and LA-4 cells would appear to be absent from murine M\(\phi\) since PR8 influenza virus, which is markedly inefficient at infecting M\(\phi\), is able to infect MDCK and LA-4 cells with high efficiency. PR8 is a highly mouse-adapted virus and its ability to evade both MMR and MGL receptors and subsequent uptake and destruction by mouse M\(\phi\), could be one factor contributing to its virulence for mice. Whereas mouse M\(\phi\) do not support productive replication of influenza virus (32), studies suggest that at least some human macrophage populations do (7, 27). Thus, the precise role of airway M\(\phi\) in host defense against influenza virus may differ in mice compared to humans.

Recently, mice have been shown to express two closely related MGL proteins, MGL1 and MGL2, with distinct carbohydrate specificities (37). MGL1 shows specificity for Lewis\(^x\) trisaccharides, whereas MGL2, like human MGL, preferentially binds to terminal N-acetylgalactosamine residues (36, 37). In the studies presented here we used MAbs LOM-8.7 (specific for MGL1) and LOM-14 (cross-reactive with MGL1 and MGL2) to detect expression of murine MGL. Subsequent studies have shown that J774 and RAW 264.7 M\(\phi\) cell lines express MGL1, but not MGL2, at the cell surface (K. Denda-Nagai and T. Irimura, unpublished results). Our data pointing to MGL as a receptor for influenza virus entry into murine M\(\phi\) can thus be interpreted as function of MGL1. Whether MGL2 can also function in this way requires further study.

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