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*J Immunol* 2002; 168:2872-2879; doi: 10.4049/jimmunol.168.6.2872

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Optimal T Cell Responses to Cryptococcus neoformans Mannoprotein Are Dependent on Recognition of Conjugated Carbohydrates by Mannose Receptors

Michael K. Mansour,* Larry S. Schlesinger, † and Stuart M. Levitz²*

Cryptococcosis is a leading cause of death among individuals with compromised T cell function. Soluble Cryptococcus neoformans mannoproteins (MP) have emerged as promising vaccine candidates due to their capacity to elicit delayed-type hypersensitivity and Th type 1-like cytokines, both critical to the clearance of this pathogenic yeast. In this study, the mechanisms responsible for the potent immunostimulatory properties of MP were explored. Using Chinese hamster ovary cells expressing human macrophage mannose receptor (MMR), we determined that MP is a MMR ligand. Functionally, competitive blockade of multilectin mannose receptors (MR) on APCs diminished MP-dependent stimulation of primary T cells from immunized mice and the MP-reactive CD4⁺ T cell hybridoma, P1D6, by 72 and 99%, respectively. Removal of O-linked saccharides from MP by β-elimination inhibited MP-dependent stimulation of P1D6 and primary T cells by 89 and 90%, respectively. In addition, MP-dependent stimulation of P1D6 was abrogated after digestion with proteinase K, suggesting the protein core of MP contributed the antigenic moiety presented by APC. Stimulation of P1D6 by MP also was abolished using APC obtained from invariant chain-deficient mice, demonstrating Ag presentation was MHC class II restricted. Our data suggest that MP is a ligand for the MMR and that T cell stimulation is functionally inhibited either by competitive blockade of MR or by removal of carbohydrate residues critical for recognition. The demonstration that efficient T cell responses to MP require recognition of terminal mannose groups by MMR provides both a molecular basis for the immunogenicity of cryptococcal MP and support for vaccination strategies that target MR. The Journal of Immunology, 2002, 168: 2872–2879.

The opportunistic yeast Cryptococcus neoformans is a significant cause of disease in patient populations with T cell dysfunction. Major risk factors include AIDS, lymphoma, and chronic receipt of immunosuppressive medication (1, 2). While cryptococcosis has multiorgan involvement, meningoen cephalitis is the most common clinical manifestation of cryptococcus (3). The annual prevalence of cryptococcosis among HIV-infected patients at risk in New York City in 1991 was estimated to be ~7% (4). Considerably higher prevalence rates are seen in parts of the developing world. Even with recent advances in therapy, morbidity and mortality are high. In patients with AIDS, indefinite maintenance therapy is required to prevent relapse (5). In light of these daunting statistics, identification of immunoprotective cryptococcal Ags that could serve as vaccine candidates has been declared a public health priority (6).

Through observation of immunocompromised patients and the development of infectious animal models, it has become clear that the cryptococcal immune response relies heavily on an intact cell-mediated immune response. Elimination of the CD4⁺ and/or CD8⁺ T cell populations results in a diminished capacity for clearance of C. neoformans (7–10). Other studies have also demonstrated a role for Ab and B cells (11, 12). Initial attempts using whole organism vaccination for protection were moderately successful (13); however, the use of eukaryotic cells as vaccines presents potential autoimmune and inflammatory complications. Therefore, investigators have fractionated cryptococcal culture supernatants to identify immunostimulatory components. Separation of supernatants by Con A affinity chromatography yields a highly mannosylated protein fraction termed mannoprotein (MP) (14). MP can elicit delayed-type hypersensitivity reactions and induces production of the cytokines TNF-α (15), IL-12, and IFN-γ (16), shown to be critical in decreasing fungal loads in murine models of cryptococcosis (17–20). Moreover, T cells from patients who have recovered from cryptococcosis proliferate and secrete cytokines in response to MP (21, 22). To further characterize the immunoreactive moieties present in culture supernatants, we previously generated CD4⁺ T cell hybridoma cell lines with specificity for MP (23). One such hybridoma, designated P1D6, has specificity for a serine/threonine-rich MP, MP98, with an apparent molecular mass of 98 kDa. While other laboratories have also identified immunoreactive cryptococcal Ags (24, 25), it is likely that a successful vaccine will consist of multiple components able to elicit both T and B cell-mediated immunity.

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Received for publication November 1, 2001. Accepted for publication January 4, 2002.

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1 This work was supported by National Institutes of Health Grants RO1 AI37532 and RO1 AI25780 (both to S.M.L.), and T32 AI07309 (to M.K.M.), as well as partial support from Veterans Affairs (to L.S.S.). S.M.L. is a recipient of a Burroughs Wellcome Fund Scholar Award in Pathogenic Mycology.

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0022-1767/02/$02.00

Abbreviations used in this paper: MP, mannoprotein; FT, flow-through; CR, crude cryptococcal supernatant; MR, multilectin mannose receptor; MMR, macrophage mannose receptor; PAS, periodic acid-Schiff; ManP, methyl α-D-mannopyranoside; GalP, methyl α-D-galactopyranoside; PK bead, protease K-conjugated agarose bead; PG bead, protein G-conjugated agarose bead; LN, lymph node; CHO, Chinese hamster ovary; Ii, invariant chain.
C-type lectins include a family of related proteins that have more than one putative lectin domain. These multilectin mannose receptors (MMR) include the dendritic cell receptor DEC-205, phospholipase A2 receptor, and the macrophage mannose receptor (MR), a well-characterized pattern-recognition receptor (reviewed in Ref. 26). Although these proteins are all on immune cells, their precise cellular distribution, ability to bind carbohydrates, and immune function appear to be quite different. Both the MMR and DEC-205 serve as links between innate and adaptive immunity by colocalizing acquired Ag with MHC class II-rich endosomes (27–29). The MMR can recognize patterns of exposed hexoses, including mannose and fucose, but not galactose (26). The MMR can interact with a range of mannose-decorated whole pathogens and Ags, including Borrelia burgdorferi (30) and lipoparabinomannans from pathogenic mycobacteria (31–33). We hypothesized that APCs, which express MMR and DEC-205, target cryptococcal MP for receptor-mediated uptake and subsequent efficient Ag presentation to T cells. To test this hypothesis, we assessed the capacity of MP to bind the MMR and be processed by APC to stimulate primary T cells from immunized mice as well as the MP-reactive T cell hybridoma P1D6. Moreover, we investigated the potential downstream consequences on T cell activation resulting from interference with MR-dependent MP uptake by APCs.

Materials and Methods

Materials

All materials were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. RPMI 1640, PBS, and geneticin were purchased from Life Technologies (Rockville, MD). PBS and HBSS were purchased from Bio-Whittaker (Walkersville, MD). Trinitiated thymidine was purchased from NEN Life Sciences (Boston, MA). Cell culture was performed in a humidified 37°C incubator supplemented with 5% CO2. RPMI-10 is defined as RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10 mM HEPES buffer.

Isolation of soluble C. neoformans MP fraction

MP was isolated from culture supernatants of C. neoformans acapsular strain Cap67 (no. 52817; American Type Culture Collection, Manassas, VA) with a previously described (34), with minor modifications. Briefly, fungi were grown for 5 days at 30°C in an orbital shaker in medium consisting of 6.7 g/L yeast nitrogen base, 27 mM dextrose, 64 μM L-histidine, 134 μM L-methionine, and 97 μM L-tryptophan. Cultures were allowed to settle overnight at 4°C and the supernatant was collected, filtered-stereilized (0.22-μm pore size), and concentrated 60- to 80-fold using a 10-KDa-cutoff regenerated cellulose tangential filtration cassette (Millipore, Bedford, MA). The concentrated crude cryptococcal supernatant (CR) was exchanged for PBS and subjected to Con A Sepharose chromatography using fast performance liquid chromatography (AKTA model; Amersham Pharmacia Biotech, Piscataway, NJ). The nonbinding flow-through (FT) fraction was collected followed by a MP fraction eluted using 0.2 M methyl α-D-mannopyranoside (ManP). The fractions were dialyzed against deionized water (Pierce, Rockford, IL) and subsequently lyophilized. After reconstitution with PBS, protein concentration was assessed using the bicinchoninic acid assay (Pierce) while total carbohydrate was measured using the phenol-sulfuric acid assay (35). Aliquots were stored at −80°C. All fractions were boiled for 5 min to inactivate any biologically active Con A leached from the affinity column (36).

SDS-PAGE analysis

Culture supernatant fractions were run under denaturing conditions using 12% SDS-PAGE. Silver staining, periodic acid-Schiff (PAS), and Western blotting were performed using standard protocols. Briefly, for silver staining of proteins, gels were fixed by sequential incubations for 30 min in 50% methanol/10% acetic acid, overnight in 5% methanol/7% acetic acid, and for 30 min in 10% glacial acetic acid. Following extensive washing in deionized water, the gel was stained for 15 min using 23.5 mM AgNO3/20 mM NaOH/2% NH4OH. After washing with deionized water, the bands were visualized using a solution of 240 μM citric acid/0.02% formaldehyde. The development of the bands was stopped by incubating the gel for 20 min in 10% acetic acid. To assess carbohydrate association by PAS staining, gels were fixed overnight in PAS buffer (40% ethanol/25 mM NaCl/25 mM acetic acid). Carbohydrates were oxidized for 1 h using 1% sodium periodate in PAS buffer. After washing unreacted m-periodate, Schiff’s reagent was added in the dark (10 min). The development of the bands was stopped with extensive washing. For Western blotting, following electrophoretic transfer (30 V, overnight, 4°C) polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) were blocked using a 0.1 mg/ml polyvinyl alcohol solution for 1 min followed by a BSA block reagent (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 1 h. After washing in TBS (25 mM Tris/0.15 M NaCl/0.05% Tween 20, pH 7.5), membranes were incubated sequentially for 1 h each in 0.1 μg/ml biotinylated Con A probe (Pierce) and a 1/40,000 dilution of streptavidin-HRP conjugate (Zymed Laboratories, San Francisco, CA). The blot was visualized using the Supersignal West Pico chemiluminescent substrate (Pierce).

Fractionation, β-elimination, and digestion of MP

After resolving the MP fraction using 12% SDS-PAGE, gels were sliced into three regions. Based on concurrently run, silver-stained molecular mass standards, region 1 contained bands with an apparent range of >60 KDa, region 2 contained bands with an apparent range of 60–30 KDa, and region 3 contained bands with an apparent range of <30 KDa. Gel slices were chopped into 2- to 3-mm cubes and placed in PBS overnight at 4°C to elute the glycoproteins. The gel slices were washed once with deionized water and the pooled buffer fractions were dialyzed against PBS using 3.5-KDa-cutoff dialyzer cassettes (Pierce).

For enzymatic O-linked deglycosylation, the MP fraction or OVA was incubated at a concentration of 0.65 mg/ml in PBS containing 0.1 M NaOH at 37°C. At the indicated time points, NaOH was neutralized using glacial acetic acid (37, 38).

For protein digestion, 0.4 mg/ml MP was incubated with 4% cross-linked agarose beads conjugated to proteinase K or protein G at a final concentration of 16 μg/ml. The mixtures were incubated rotating at 37°C for 4 h in RPMI 1640. The reaction was stopped by spinning the mixture through a 0.45-μm filter trap to remove the agarose beads.

Competitor HRP assay

The pVex expression vector containing human MMR cDNA (39) was used to generate a stably transfected MMR-expressing Chinese hamster ovary cell line (CHO-MMR) (40). Expression of MMR was confirmed by flow cytometry using a mAb specific for human MMR (BD Pharmingen, San Diego, CA). CHO-MMR and untransfected wild-type CHO cells (CHO-K1) were cultured in 24-well plates in RPMI-10 supplemented with 0.5 mg/ml active geneticin (for selection of transfected cells only). When a confluent monolayer was achieved, the cells were washed twice with HBSS containing 1 mg/ml BSA. Cryptococcal supernatant fractions or mannosylated inhibitors of the MMR were preincubated with the CHO cells in binding buffer (1% BSA in HBSS) at the indicated concentrations for 5 min at room temperature. Following the preincubation, 50 μg/ml HRP type II was added for 1 h at 37°C. HRP type II is a glycoprotein decorated with mannose, N-acetylgalcosamine residues, making it a natural ligand for the MMR (41). Following the incubation, the CHO cells were washed four times, lysed, and solubilized using 300 μl 0.1% Triton X-100. HRP uptake was measured as previously described (42), with minor modifications. Briefly, 25 μl of cell lysate was incubated with 225 μl of α-diaminodisulfate solution (0.008% α-diaminodisulfate/0.003% H2O2, pH 1.5 M sodium phosphate, pH 5). Color conversion was read at an absorbance wavelength of 405 nm. HRP activity was compared with a standard curve of known HRP concentrations. There was no conversion of color substrate in the presence of CHO cell lysate alone. Total protein from the cell lysate was assessed using the bicinchoninic acid assay. HRP uptake is expressed as a ratio of HRP activity per milligram of total protein.

Naïve splenocytes as source of APC

Spleens from naïve C57BL/6 mice, collected, were irradiated (3000 rad), and macerated through sterile wire mesh. Contaminating erythrocytes were lysed using 0.15 M ammonium chloride. The resulting cell suspension was purified over a Ficoll-Hypaque gradient and theuffy layer was collected and washed in RPMI-10. The spleen cell population contains B cells, dendritic cells, and macrophages as potential sources of Ag presentation.

Immunization of mice

Pathogen-free 6-wk-old male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were caged and housed under specific pathogen-free conditions at Boston University’s Laboratory Animal Science Center (Boston, MA). Immunizations were performed using...
the RiBi Adjuvant system. A total of 50 μg of CR were injected i.p. in 3-wk intervals for up to four immunizations. Seven days following the final boost, inguinal, para-aortic, axillary, and mesenteric lymph nodes (LN) were collected using asptic technique.

**Proliferation analysis**

Proliferation of T cells from LN of CR-immunized C57BL/6 mice or whole splenocytes from DO11.10-transgenic mice (a gift of A. Marshak-Rothstein, Boston University School of Medicine) was assessed in response to MP or OVA, respectively.

LNs were macerated to a single cell suspension using frosted glass slides. RBCs were lysed with 0.15 M ammonium chloride. For purified T cells, contaminating B cells, macrophages, and other APCs were depleted using standard complement-mediated lysis techniques (43), with minor modifications. Supernatants from two Ab-secreting B cell hybridomas specific for MHC class II (clone TIB 120, anti-I-A\(^{b,d}\), I-E\(^{a}\); and clone TIB 229, anti-I-A\(^{b}\); a gift of Dr. L. Wetzler, Boston University School of Medicine) were incubated with LN single cell suspensions for 45 min on ice at a concentration of 10\(^5\) cells per milliliter. After washing, cells were resuspended in rabbit serum (a gift of Dr. L. Wetzler) at a dilution of 1/20.

**T cell hybridoma**

The CD4\(^+\) T cell hybridoma cell line used in these studies, P1D6, is specific for an epitope found in cryptococcal MP, MP98 (23). Thus, in the presence of APC and MP98, P1D6 will secrete IL-2. Hybridoma cell lines were maintained in RPMI-10 supplemented with 1% hypoxanthine/thymidine, 0.35 μg/ml amphotericin B, 10 μg/ml ciprofloxacin, and 55 μM 2-ME. Hybridoma cells at 1 × 10\(^5\) cells per well were added to gamma-irradiated naive splenocytes at 2 × 10\(^5\) cells per well in the presence or absence of Ag and inhibitors. Gamma-irradiated C57BL/6 splenocytes, added at 5 × 10\(^5\) cells per well, served as a source of APC. Nonirradiated splenocytes from DO11.10 mice were prepared as described above and plated at 7 × 10\(^5\) cells per well.

Cells were incubated for 4 days and pulsed with tritiated thymidine during the final 18 h. Plates were subjected to a freeze-thaw cycle, then collected onto fiberglass filter strips using a cell harvester (Cambridge Technology, Cambridge, MA). Samples were counted by scintillation spectrometry (Delta 300; Searle Analytic, Des Plains, IL). Results are expressed as stimulation index, defined as stimulated cpm divided by unstimulated cpm.

**Statistical significance**

Student’s t test with a value of p ≤ 0.05 was used as a measure of statistical significance. Representative data are expressed as means ± SE.

**Results**

**Characterization of the MP fraction**

The mean protein concentrations in the CR and MP fraction were 5.5 and 3.3 mg/L, respectively. As calculated by mass, the MP and FT fractions had carbohydrate:protein ratios of 5.4:1 and 6.5:1, respectively. SDS-PAGE analysis of the MP fraction resolved at least 10 distinct bands ranging from <20 to >100 kDa (Fig. 1A, MP). Several bands exhibited negative staining on silver stain, suggesting the presence of heavy carbohydrate conjugation. The presence of polysaccharide was verified by PAS staining (Fig. 1B, MP) as well as an ultraviolet light-visible glycostain (Pro-Q Emerald 300 Glycoprotein Gel Stain kit; Molecular Probes, Eugene, OR) (data not shown). Western blotting with a biotinylated Con A probe showed five bands measuring at apparent molecular masses of 111, 96, 71, 36, and 29 kDa (Fig. 1C, MP). Staining with Con A suggests terminal mannosae conjugation. As expected, the FT fraction did not stain (Fig. 1C, FT).

**CHO cells heterologously expressing MMR bind C. neoformans MP**

Several members of the MR family have been described that exist on innate immune cells, including MMR and DEC-205 (26). We used a CHO cell line engineered to heterologously express functional MMR (CHO-MMR) (40) to determine whether MP binds specifically to the MMR. To detect binding of MP to the CHO-MMR cells, a known ligand of the MMR, HRP, was used in a competition uptake assay. To confirm the specificity of the HRP/CHO-MMR uptake assay, competitive inhibitors were used. A total of 50 mM ManP and mannans from *Saccharomyces cerevisiae* inhibited HRP uptake by 90 and 94%, respectively, as compared with 8% by methyl α-D-galactopyranoside (GalP) (Fig. 2). MP incubated at various concentrations demonstrated dose-dependent inhibition of HRP uptake, reaching a maximum of 82% at 50 μg/ml, FT, at 50 μg/ml, inhibited HRP uptake by 37%, suggesting that this fraction contains ligands that recognize the MMR but not Con A. CR, which contains significant amounts of MP, inhibited HRP uptake by 74%. The parent cell line, CHO-K1, demonstrated only a minor capacity for HRP acquisition. To test the possibility that individual components of the HRP uptake assay could have interfered with HRP enzymatic activity, 400 μg/ml MP, FT, and CR, as well as 100 mM ManP, 100 mM GalP, and 15 mg/ml mannan were coincubated with 50 pg/ml HRP at 37°C for 3 h. There was no inhibition of HRP-mediated substrate conversion with any of the coinubcations (data not shown).

**FIGURE 1.** Analysis of *C. neoformans* strain CAP67 supernatants by SDS-PAGE. CR, FT, and MP fractions were resolved by 12% SDS-PAGE and analyzed by silver stain (A), PAS (B), and Con A-biotin blot (C). Migration of commercial molecular mass standards, expressed in kilodaltons, is indicated to the left of the gels.
FIGURE 2. Competition of HRP uptake by cryptococcal MP. HRP type II, a natural ligand for the MMR, was incubated with CHO-MMR and control CHO-K1 cells for 1 h at 37°C in the presence of the indicated inhibitors or cryptococcal supernatant fractions. Cellular uptake of HRP was measured as described in Materials and Methods. Inhibitors and fractions include control (PBS), 1 mg/ml S. cerevisiae mannans, 50 mM ManP, 50 mM GaIP, C. neoformans MP at 1 (MP1), 10 (MP10), 25 (MP25), and 50 (MP50) μg/ml, FT (FT50) at 50 μg/ml, and CR (CR50) at 50 μg/ml. Data are representative of five independent experiments in duplicate. *, p < 0.03; **, p < 0.01 compared with control.

Effect of MR blockade on the activation of MP-specific T cell hybridomas

We next assessed the contribution of MR to the stimulation of the T cell hybridoma P1D6 by MP. Using gamma-irradiated naive splenocytes as sources of APC, MP with or without sugar analogs were incubated overnight in the presence of T cell hybridoma cells as described in Materials and Methods. Addition of the MMR competitors, ManP and mannans, inhibited IL-2 production by 99% at 40 μg/ml MP (Fig. 3). The osmotic control, GaIP, demonstrated nonsignificant decreases of IL-2 as compared with control (PBS). MP, mannan, or the sugar analogs did not nonspecifically affect individual components of the hybridoma/CTLL-2 bioassay, as MP-stimulated IL-2 production was seen only if both hybridoma cells and splenocytes were present in the wells (data not shown). In addition, neither mannan nor the sugar analogs had direct nonspecific effects on the proliferation of CTLL-2 cells (data not shown). Thus, these data suggest dependence on MR for hybridoma stimulation.

FIGURE 3. Effect of mannosylated inhibitors on activation of the MP-specific CD4+ T cell hybridoma, P1D6. Irradiated naive splenocytes and P1D6 cells were incubated for 24 h in the presence of the indicated concentration of MP plus 1 mg/ml S. cerevisiae mannans (Mannan), 50 mM ManP, 50 mM GaIP, or RPMI 1640 (Control). Activation of P1D6 was measured by assaying IL-2 production from tissue culture supernatant. Data are representative of four independent experiments in duplicate. *, p < 0.05 comparing control or GaIP vs ManP or mannan.

Dependence of O-linked carbohydrates for immunoreactivity of MP

Glycoprotein and proteoglycan carbohydrate residues may be conjugated to protein cores by O-, N-, or GPI-links (44). The amino acids serine and threonine serve as donors for O-links, whereas asparagine provides the means for N-links. Investigators have established the presence of abundant serines and threonines in MP fractions from C. neoformans (45). The MP98 sequence contains 103 serine/threonine residues to serve as potential sites for O-links, as well as 12 possible N-link sites (23). Removal of carbohydrates manifests as a shift in apparent molecular mass when glycoproteins are resolved on SDS-PAGE. Digestion of the MP fraction with enzymes specific for N-linked carbohydrates such as peptidase N-glyco idase F or EndoH (New England Biolabs, Beverly, MA) resulted in only slight decreases in the apparent molecular mass (data not shown). To assess the presence of O-links, chemical deglycosylation was used in the form of β-elimination. β-Elimination uses mild sodium hydroxide treatment to specifically cleave O-links via nucleophilic attack (37). Following β-elimination for 3 or 24 h, high molecular mass bands found in the MP fraction shifted to lower apparent molecular mass, as demonstrated by silver staining (Fig. 4A). Moreover, β-elimination resulted in loss of carbohydrate as demonstrated by PAS staining (Fig. 4B). This suggests O-links as the dominant form of carbohydrate conjugation used by C. neoformans strain CAP 67.

To assess the effect of deglycosylation on immune function, β-eliminated MP was incubated with primary T cells isolated from CR-immunized mice as well as with P1D6 (Fig. 4C). Gamma-irradiated splenocytes from naive mice served as APCs in both reactions. Compared with the 0-h β-elimination time point, which was immediately neutralized, progressively longer treatment resulted in maximal inhibitions of 90 and 89% as measured by incorporation of tritiated thymidine and IL-2 production, respectively. As a control for potential mitogens in the cryptococcal fractions, 50 μg/ml MP, FT, CR, and β-eliminated MP were co-incubated with naive C57BL/6 single splenocyte suspensions for 4 days and proliferation was measured by tritiated thymidine incorporation. The results demonstrate that there was no stimulatory activity attributable to the fractions alone (stimulation indices for MP, FT, CR, β-eliminated MP, and Con A (5 μg/ml as a positive control) were 1.07 ± 0.14, 2.68 ± 0.36, 0.56 ± 0.02, 0.81 ± 0.07, and 29.99 ± 1.21, respectively).

Extreme pH conditions can degrade disulfide bonds and hydrolyze the peptide backbone, altering primary and secondary protein...
structure (46). To control for possible cellular toxicities or protein degradation resulting from the process of β-elimination, we took advantage of the transgenic DO11.10 mouse strain, which has been genetically engineered to express an TCRαβ specific for a peptide sequence found in OVA (47). OVA was subjected to the β-elimination procedure by incubation with sodium hydroxide for 3 and 24 h. Isolated DO11.10 splenocytes were then incubated with various concentrations of OVA pre- and post-β-elimination, and proliferation was measured by tritiated thymidine incorporation. In three independent experiments, proliferation of the DO11.10 splenocytes was undiminished by progressively longer β-elimination treatments (stimulation indices using 100 μg/ml OVA β-eliminated for 0, 3, and 24 h were 57 ± 3, 83 ± 3.26, and 81 ± 2.73, respectively). These results suggest that the conditions used for O-linked deglycosylation leave the protein core intact.

Determination of the immunoreactive Ags

The next set of experiments was designed to determine which of the multiple bands in the MP fraction were immunodominant Ags. Separation of the cryptococcal MP fraction into three molecular mass ranges (60, 60-30, and <30 kDa) was performed by slicing 12% SDS-PAGE gels and eluting them into PBS. Approximately 90 μg of protein were collected per fraction per gel. The apparent molecular masses of the fractions and the efficiency of the elution procedure were confirmed by silver and PAS stains (data not shown). To determine where the immunodominant Ag(s) existed, T cells from LN of CR-immunized C57BL/6 mice were purified and coincubated with gamma-irradiated naive splenocytes. ManP and GalP were included to assess the contribution of MR to stimulation by competitive blockade. Only the Ags found within the high molecular mass range (>60 kDa) elicited T cell proliferation as measured by thymidine incorporation (Fig. 5). Blockade of mannose receptors using ManP resulted in a 72% inhibition of MR with 50 mM ManP compared with an osmotic control 50 mM GalP or no sugar (none). Data are representative of two independent experiments in triplicate. *p < 0.03; **p < 0.007 compared with 0-h time point.

MP core protein is required for hybridoma stimulation

The above experiments established the requirement for the carbohydrate portion of MP for optimal responses to adaptive immune cells. This final set of experiments was designed to determine 1) whether the protein core of MP contributes to T cell stimulation...
and 2) whether stimulation occurs via MHC. Neither can be assumed, as other MHC-like molecules, particularly CD1, have the capacity to stimulate T cells by presenting nonprotein Ags, such as lipids (48). To determine whether the T cell response to MP is dependent on the core protein, MP was digested by incubation with agarose beads coupled to proteinase K (PK beads). As a control for nonspecific adsorption of MP by the agarose beads themselves, agarose beads coupled to protein G (PG beads) were used. IL-2 production by P1D6 hybridoma cells was used to assess MP immunogenicity following protease treatment. A complete loss of reactivity to MP after treatment with PK beads, but not PG beads, was observed (Fig. 6), suggesting that the protein core of MP is necessary for its immunoreactivity.

**Invariant chain-deficient mice cannot respond to MP**

To determine whether the response to MP was MHC restricted, invariant chain (II)-deficient mice were used. APCs from II−/− mice cannot traffic functional or sufficient MHC class II molecules to the surface (49). Thus, II−/− APCs lack the ability to present Ag via the endocytic pathway. In contrast, presentation via CD1 molecules should remain intact, as CD1 trafficking to endocytic vesicles is independent of II (50). MP was incubated with P1D6 hybridoma cells in the presence of gamma-irradiated splenocytes isolated from naive wild-type C57BL/6 and II−/− mice. Deficiency of the MHC class II pathway resulted in an 88% decrease in IL-2 production in response to 40 μg/ml MP (Fig. 7). These data suggest MP-dependent stimulation is MHC class II restricted.

**Discussion**

In this report, we have begun to dissect the molecular mechanisms responsible for the potent immunostimulatory capacity of cryptococcal MP. The major findings are as follows: 1) MP is a ligand for the MMR; 2) inhibition of binding of MP to MR, either by chemical deglycosylation of MP or by competitive inhibition of MR, results in potent inhibition of T cell activation; 3) the bulk of the T cell stimulatory capacity of MP resides in a fraction with an apparent molecular mass of > 60 kDa; and 4) MP stimulation appears to require the protein core for stimulation and is MHC class II restricted.

**FIGURE 6.** Effect of proteinase K digestion on MP stimulation of P1D6. MP was treated with PK or PG beads, as a negative control. Treated MP was separated from beads by spin-trapping. The capacity of the indicated concentration of treated MP to induce IL-2 production from P1D6 in the presence of irradiated naive splenocytes as APC was assessed. Data are representative of three independent experiments in duplicate. *, p < 0.04 comparing PK vs PG beads.

**FIGURE 7.** Stimulation of P1D6 using APC from II−/− mice. The contribution of the endocytic Ag presentation pathway (MHC class II restricted) was assessed using II−/− mice. MP was added for 24 h in the presence of P1D6 cells and irradiated naive splenocytes from either II−/− (MHC class II-deficient) or C57BL/6 (wild-type) mice. Activation of P1D6 was assessed by assaying for IL-2 production. Data are representative of two independent experiments in duplicate. *, p < 0.04 comparing wild-type C57BL/6 vs II−/−.

Although their function is largely unknown, cryptococcal MPs account for a large component of the secreted material from C. neoformans. Therefore, it is likely that MPs are among the initial Ags that professional APCs engage at a cryptococcal focus of infection. In preliminary experiments using an i.v. model of cryptococcosis, compared with groups treated with adjuvant only, MP-vaccinated C57BL/6 mice demonstrated significantly increased survival and decreased organ fungal loads in kidneys and brain (M. K. Mansour and S. M. Levitz, unpublished data). MP can also be isolated from other fungi such as Candida albicans and Pencillium marneffei (51, 52). Consistent with cryptococcal MP, candidal MP has been shown to elicit anticalendal T cell responses (52). Thus, the development of MPs as antifungal T cell vaccines may have extended efficacy in the prevention of a variety of pathogenic fungi.

APCs can use a variety of pathways to acquire Ag, including macro- and micropinocytosis, as well as receptor-mediated endocytosis. In the case of the MMR, this receptor recognizes whole pathogens and microbial products decorated with terminal mannose groups, such as mycobacterial lipoarabinomannans (31–33). Unlike lower eukaryotes, such as yeast, extracellular mammalian glycoproteins rarely exhibit terminal mannosylation, but instead integrate mannose within the internal branching structure of complex oligosaccharides (44). In the case of DEC-205, the structural determinants of Ags for binding to the receptor are less clearly defined. It is uncertain whether DEC-205 binds carbohydrates (26). Based on competition HRP uptake studies, cryptococcal MP is a ligand for the MMR. Moreover, acquisition of MP through MR is essential for proper Ag presentation to T cells. Experimental evidence suggests that Ag acquired through multilectin receptors, such as MMR and DEC-205, is targeted for efficient processing and presentation (28, 29). Consistent with these observations, blocking MR on APC with mannose analogs and mannans resulted in a significant reduction in stimulation of primary T cells and CD4+ T cell hybridomas. Our data do not rule out that other multilectins on the surface of APCs may also participate with the MMR in the recognition of MP.

Terminal mannosylation on MP would be required to allow recognition by the MR. The lectin Con A, which recognizes terminal mannososes and glucose (53), was used to purify MP from CR. Not
surprisingly, MP, but not the FT fraction, resolved by SDS-PAGE stained with a biotinylated Con A probe. In addition, two components of the MP fraction, MP98 and MP88, have been sequenced and contain C-terminal serine/threonine-rich regions that presumably serve to form O-linked glycosylation domains (Ref. 23 and S. M. Levitz, unpublished data). Our deglycosylation experiments lend support to the importance of O-links for MP terminal mannosylation. Cleavage of these links by β-elimination dramatically decreased the apparent molecular mass of silver-stained bands and eliminated reactivity with the carbohydrate-specific PAS stain. In contrast, digestion of MP with glycosidases specific for N-links had little effect on the apparent molecular mass of MP. These O-linked carbohydrates appear to be critical for MP stimulation, as deglycosylation of MP by β-elimination profoundly decreased the capacity of APC to stimulate primary T cells and the MP-reactive T cell hybridoma P1D6. These results did not appear to be secondary to nonspecific effects of β-elimination on protein structure or cellular toxicity, as no decrease in Ag-specific responses to a control β-eliminated protein, OVA, was observed.

Ags acquired and processed through the endocytic pathway are normally presented on MHC class II (54). This appears to be the method used for MP presentation as well. Digesting MP with proteinase K, a nonspecific protease, dramatically diminished P1D6 reactivity, indicating a requirement for the protein core. However, the possibility that the protein was required to maintain proper carbohydrate conformation while being presented cannot be entirely ruled out, especially as crystallographic evidence supports the ability of MHC to accommodate monosaccharides (55). A requirement for an MHC class II-restricted pathway was suggested by our data demonstrating splenocytes from Ii– mice in the presence of MHC class II-restricted pathway was suggested. The protein was required to maintain protease, dramatically diminished P1D6 reactivity, indicating a requirement for the protein core. However, the possibility that the protein was required to maintain proper carbohydrate conformation while being presented cannot be entirely ruled out, especially as crystallographic evidence supports the ability of MHC to accommodate monosaccharides (55). A requirement for an MHC class II-restricted pathway was suggested by our data demonstrating splenocytes from Ii– mice in the presence of MHC class II-restricted pathway was suggested.

To date, a notable problem with vaccine technology has been the difficulty designing formulations that specifically elicit protective cell-mediated immune responses. The potential for carbohydrate patterning to increase antigenic potency has led to novel approaches to vaccine development. Examples include chemically conjugating mannos from S. cerevisiae to weak Ags (56), expressing recombinant proteins in S. cerevisiae (57), and targeting of DEC-205 with mAbs coupled to peptides (58). Thus, our data demonstrating that efficient T cell responses to MP require recognition of terminal mannosic monosaccharides by mannose receptor would provide both a molecular basis for the immunogenicity of cryptococcal MP and support for vaccination strategies that target MR members.

Acknowledgments

We thank Dr. Ann Marshak-Rothstein for the gift of D011,10-antigenic mice and Dr. Lee Wetzler for the gifts of T cell purification reagents. We also acknowledge Dr. Shu-hua Nong for his effort in the generation of the P1D6 cell line. For technical assistance and useful discussions we thank Liz Leadbetter, Elena Sabbagh, Dr. Michele Youd, Dr. Bruce Granger, and especially Dr. Zeina Dagher.

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