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Properties of a Rat Monoclonal Antibody Reactive with Both the Mannan of *Candida* Species and the O-Antigen 6,7 Polysaccharide of Serogroup C_1 Salmonellae

N. A. NNALUE, A. WEINTRAUB, AND A. A. LINDBERG*

Department of Clinical Bacteriology, Karolinska Institute, Huddinge University Hospital, S-141 86, Huddinge, Sweden

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Monoclonal antibody MASC1-MR9 of isotype immunoglobulin M was generated in LOU/C rats by immunization with heat-killed *Salmonella thompson* (serogroup C_1, O-antigen 6,7 lipopolysaccharide) bacteria which had been further enriched for O-antigen by coating with homologous lipopolysaccharide. Eight monoclonal antibodies were selected by screening against the lipopolysaccharide of *S. thompson*. In a subsequent test against *Candida* mannan, only one antibody, MASC1-MR9, was reactive. Immunofluorescence microscopy of various yeasts and bacteria with MASC1-MR9 showed that this antibody bound to the surfaces of 142 of 148 *Candida* strains of 10 different species tested but failed to bind to the surface of *Saccharomyces cerevisiae*. The *Candida* strains which failed to bind MASC1-MR9 were all five strains of *Candida krusei* and the single strain of *Candida utilis* tested. Several (11 of 33) *Salmonella* strains belonging to serogroup C_1 reacted with this antibody, as expected; however, 1 of 11 strains of *Morganella morganii* and 4 of 64 strains of *Escherichia coli* were also reactive. Serum-free supernatants of MASC1-MR9 agglutinated *S. thompson*, one strain of *Salmonella choleraesuis*, and 109 of 110 *Candida* strains tested. The immunochemical properties of MASC1-MR9 as studied by enzyme-linked immunosorbent inhibition assays show that it recognizes an epitope present in the mannan of *Candida* species as well as in the O-6,7 antigen of *Salmonella* species.

Candida species are opportunistic pathogens of humans which frequently cause severe systemic infections in patients with AIDS (14), cancer (3), and burns (28) as well as in those under immunosuppressive or radiation therapy (27). Immunocompetent individuals may become infected after suppression of indigenous bacterial flora with broad-spectrum antibiotics. Systemic *Candida* infection is associated with high mortality, as high as 90% in burn patients (26, 28) and 95% in cancer patients with deep organ infection (24). Diagnosis early in the course of infection is paramount for effective treatment but can be very difficult (15). The main problems in diagnosis are that most patients show few distinctive clinical signs (13), blood cultures are often negative (10), and cultural isolation of *Candida* species, which are a constituent of the normal human flora, is of questionable significance. Also, routine serological tests for anti-*Candida* antibodies do not give specific indication of invasive disease (25), because *Candida* agglutinins are common in the general population (15). In one study (20), similar levels of anti-*Candida* antibodies were found in sera from normal persons and patients with or without invasive disease.

Mannan is the major antigenic component of *Candida albicans* and is responsible for agglutination (29), precipitation (8), and immunofluorescence (32) reactions. Unlike the majority of mannans isolated from fungi, which have been α-linked polysaccharides (16), the mannans of *Candida* species (17, 30) contain both α- and β-linked mannose residues. Similar linkages have also been found in the serogroup C_1 O-antigen 6,7 polysaccharide of *Salmonella thompson* (22). Ekwall et al. (11) have described serologic cross-reactivity between *C. albicans* and *S. thompson* with a polyclonal antiserum specific for the O-6,7 antigen. On the basis of these facts, we thought it reasonable to exploit a standing set-up for the generation of monoclonal antibodies against *Salmonella* antigens in our laboratory to isolate clones that cross-react with mannan of *Candida* species. In this paper we report the isolation and properties of one such clone.

MATERIALS AND METHODS

Bacterial and fungal strains. Eleven *Salmonella* strains of serogroup C, one strain of serogroup E, and three strains of serogroup H were obtained from L. LeMinor of the World Health Organization Collaborating Centre for *Salmonella*, Paris, France. Other bacterial strains of various species were clinical isolates. *C. albicans* ATCC 2091, ATCC 29790, ATCC 10231, and ATCC 26790; *C. tropicalis* ATCC 750 and ATCC 780; *C. glabrata* ATCC 15126; and *C. parapsilosis* ATCC 7330 were from the American Type Culture Collection, Rockville, Md. Four strains of *C. albicans* of serotype A (2863/84, 1865/86, 3681/86, and BMK760224-1) and four strains of serotype B (2063/84, 2636/86, 4460/86, and BMK760224-2) as well as all the strains of *Candida krusei*, *Candida stellatoidea*, *Candida viswanathi*, *Candida guillermondii*, *Candida zeylanoides*, and *Saccharomyces cerevisiae* were from Kenneth Holmberg of Mycology Division, National Bacteriology Laboratory, Stockholm, Sweden. Other yeast strains were clinical isolates from this laboratory.

O-antigen 6,7 polysaccharide-specific antiserum. Rabbit antiserum specific for the O-6,7 antigen was produced by immunization of rabbits with a glycoconjugate consisting of a decasaccharide derived from the lipopolysaccharide (LPS) of *S. thompson* IS40 by hydrolysis with phage 14 (11) and

* Corresponding author.

then chemically coupled to bovine serum albumin (BSA) as described previously (19).

Cell culture methods. Rat myeloma line Y3.AG.1.2.3. (Y3M) (12) used as the fusion partner was maintained in RPMI 1640 (Gibco Limited, Paisley, Scotland) supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories Limited, Irvine, Calif.), 1 mM sodium pyruvate, 4 mM glutamine, and 100 U of a penicillin-streptomycin mixture (Gibco). The same medium additionally supplemented with hypoxanthine-aminopterin-thymidine (HAT) was used for hybridoma selection after cell fusion.

Antigen, rats, and immunization. LOU/C rats were purchased from ALAB (Stockholm, Sweden) and immunized with heat-killed, LPS-coated *Salmonella thompson* bacteria prepared as previously described (23). The first inoculation was administered intraperitoneally on day 0 and consisted of 10 μg of *S. thompson* antigen in 0.1 ml of phosphate-buffered saline (PBS) emulsified in an equal volume of complete Freund adjuvant (Sigma Chemical Co., St. Louis, Mo.). Booster doses containing the same amount of antigen in PBS only were administered intraperitoneally on day 24 and intravenously on day 36. The rats were killed on day 40, and their spleens were used for production of hybridomas.

Production of hybridomas. Cell fusions between rat spleen cells and Y3M cells were done by standard procedures (18). Briefly, approximately 2×10^7 spleen cells and 10^7 Y3M cells were mixed in a tube, washed with Hanks buffered saline (Gibco) and sedimented by centrifugation. After removal of the supernatant, the cell pellet was loosened by gentle tapping; the cells were then fused by adding 1 ml of 10% polyethylene glycol (E. Merck AG, Darmstadt, Federal Republic of Germany) drop by drop. Finally, the cells were resuspended in selective medium containing HAT and dispensed into 96-well flat-bottomed microtiter plates which had been incubated overnight with a feeder layer of 5×10^4 mouse spleen cells per well. Four fusions were performed, two per rat spleen, and plated in a total of 1,760 wells.

Screening, selection, and characterization of hybridomas. Putative hybrids were tested by an enzyme-linked immunosorbent assay (ELISA) with phenol-water-extracted LPS from *S. thompson* as the antigen. Positive clones (A_{405} at 100 min, >1.0) were further tested against an extract of *Candida* mannan (a gift from Susanne deBurg Wetterlund). One clone, MASC1-MR9, secreting antibody reactive with this antigen was subjected to two cycles of subcloning by limiting dilutions and further characterized. This consisted of isotyping by the gel diffusion procedure, using a commercial isotyping kit, RMT 01K (Serotec, Bicester, United Kingdom), and testing of reactivity with smooth LPS from various *Salmonella* strains of serogroup C_1. Finally, the ability of MASC1-MR9 to react with whole cells of various *Candida* and bacterial species was investigated by immunofluorescence microscopy.

ELISA. The ELISA used has been previously described in detail (6). Briefly, a 96-well microtiter plate (Nunc, Roskilde, Denmark) was coated overnight at 20°C with smooth (10 μg/ml) or rough (25 μg/ml) LPS in 0.05 M carbonate buffer (pH 9.6), overcoated with 1% BSA for 1 h at 37°C, and washed three times with washing buffer (0.15 M NaCl, 0.05% Tween 20). Hybridoma culture supernatant containing monoclonal antibody was diluted in PBS-T (PBS containing 0.05% Tween 20); 100-μl aliquots were added to wells, followed by a 4-h incubation at room temperature and washing as described before. Alkaline phosphatase-labeled rabbit antibody to rat immunoglobulin was diluted as recommended by the manufacturer (Sigma), and 100 μl was added to each well. Plates were incubated at 20°C and washed again. Substrate solution (100 μl of *p*-nitrophenyl phosphate at 1 mg/ml in 1 M diethanolamine–0.5 mM $MgCl_2$ buffer, pH 9.8) was added, and plates were incubated for 100 min at 37°C; the A_{405} was then read in a Titertek Multiscan photometer (Flow Laboratories, Irvine, Scotland).

IFL. Indirect immunofluorescence microscopy (IFL) was done as described previously (31). Bacteria were grown overnight in L agar at 37°C, and yeasts were grown for 2 days at room temperature in Sabouraud dextrose agar. Undiluted culture supernatant of MASC1-MR9 (or 1:20 dilution of rabbit antiserum) was used as the source of primary antibody and either rabbit anti-rat immunoglobulin-fluorescein isothiocyanate conjugate or similarly labeled goat anti-rabbit conjugate (Dakopatts AB, Hagersten, Sweden) was used as the secondary antibody at a working dilution of 1:100. Stained slides were examined in a Leitz Orthoplan fluorescence microscope with incident light and a mercury HBO-200 lamp as the light source.

Statistical analyses. The statistical significance of differences in the reactivity of MASC1-MR9 with the various antigens was tested point by point by paired, two-tailed Student's *t* tests.

RESULTS

Production and properties of hybridoma MASC1-MR9. Spleens from two immunized rats were used for four fusions, and the products were plated in a total of 20 96-well microtiter plates. Twenty hybridomas were obtained, of which eight reacted with *S. thompson* LPS in a screening test (data not shown). Only one of these clones, MASC1-MR9, was subsequently found to react also with an extract of *Candida* mannan by ELISA. MASC1-MR9 is of the IgM isotype and showed reactivity in ELISA with LPS from some members of *Salmonella* serogroup C_1 but not others. Among the LPSs tested, the MASC1-MR9 was most reactive with those from *Salmonella choleraesuis* SL2824 and *S. thompson* IS40. It, however, showed a stronger reactivity ($P < 0.005$) with *Candida* mannan than with these LPSs at all the dilutions of culture supernatant tested (Table 1). A glycoconjugate consisting of a decasaccharide derived from the O-antigen 6,7 polysaccharide of *S. thompson* IS40 by hydrolysis with phage and then chemically coupled to BSA was found to be at least as reactive with MASC1-MR9 as *Candida* mannan (data not shown). The three antigens, SL2824 LPS, IS40 LPS, and *Candida* mannan were effective as inhibitors of the interaction of MASC1-MR9 with each other in a dose-dependent manner (Table 2). It was consistently found that the interaction of MASC1-MR9 with mannan was somewhat more difficult to inhibit than that with LPSs and that mannan was also a better inhibitor than LPSs. These results show that the antibody recognized the same or closely related epitopes in these antigens and suggest that the epitope might be somewhat more abundant or more accessible in mannan than in LPS. The very low concentration of mannan (<0.005 μg/ml or less) or LPS (ca. 0.03 μg/ml) required for 50% inhibition indicates that MASC1-MR9 has a high affinity for these molecules.

Immunofluorescence and agglutination of *Candida* species and bacteria. The reactivity of a culture supernatant of MASC1-MR9 (ELISA endpoint titer = 10,000) with different *Candida* species and with *S. cerevisiae* was tested by IFL and by slide agglutination. The organisms examined included the following: *C. albicans* (123 strains including ATCC strains), *Candida tropicalis* (5 strains), *Candida glabrata* (5

TABLE 1. Titration by ELISA of serum-free culture supernatant of MASC1-MR9 against *Candida* mannan and *Salmonella* O-antigen 6,7 LPS[a]

Coating antigen	A_{405}[b] (mean ± SD) at the following culture supernatant dilution:						
	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400
Mannan	3.25 ± 0.08	2.48 ± 0.14	1.41 ± 0.23	0.73 ± 0.12	0.42 ± 0.13	0.21 ± 0.04	0.16 ± 0.05
S. thompson LPS	1.83 ± 0.29	1.52 ± 0.17	1.02 ± 0.23	0.59 ± 0.18	0.31 ± 0.19	0.12 ± 0.05	0.09 ± 0.07
S. choleraesuis LPS	1.87 ± 0.11	1.49 ± 0.27	0.98 ± 0.03	0.51 ± 0.22	0.24 ± 0.08	0.11 ± 0.04	0.08 ± 0.04

[a] ELISA plates were coated with mannan (5 μg/ml) or LPS (10 μg/ml).
[b] Data were pooled from two separate experiments and represent results from six wells.

strains), *C. parapsilosis* (4 strains), *C. kusei* (5 strains), *S. cerevisiae* (5 strains), and 1 strain each of *C. zeylanoides*, *C. stellatoidea*, *C. viswanathi*, *Candida guilliermondi*, and *Candida utilis*. All of the *C. albicans*, *C. tropicalis*, *C. glabrata*, and *Candida parapsilosis* strains reacted strongly with MASC1-MR9 in both the IFL and slide agglutination tests. The *C. zeylanoides*, *C. stellatoidea*, *C. viswanathi*, and *C. guilliermondi* strains were also strongly positive in IFL but were not tested for agglutination. The *C. albicans* strains tested included four strains known to belong to serotype A and four strains belonging to serotype B, and all reacted equally well with this monoclonal antibody. All the strains of *C. krusei* and *S. cerevisiae* and the lone strain of *C. utilis* were negative by IFL and were not tested for agglutination. MASC1-MR9 showed reactivity with some strains of several bacterial genera. These included 11 of 77 *Salmonella* strains (9 of 33 serogroup C_1 and 2 of 6 serogroup E strains tested), 4 of 64 *E. coli* strains, and one of 11 *Morganella* strains. The other bacteria tested, 12 *Pseudomonas* strains, 10 staphylococci, and 10 β-hemolytic streptococci, were negative. The sample of streptococci tested contained four group A strains, all of which actually gave positive IFL reactions after incubation with MASC1-MR9 and conjugate. However, they also reacted with the conjugate alone to the same extent that they did when treated with both MASC1-MR9 and conjugate and thus apparently did not interact specifically with MASC1-MR9. Evidently, these strains bind the conjugate directly. Both *S. thompson* IS40 (used as immunogen to generate MASC1-MR9) and *S. choleraesuis* SL2824 agglutinated in undiluted serum-free supernatant of MASC1-MR9, but other bacterial strains were not tested for agglutination. The reactivity of the rabbit anti-O-6,7 serum with some *Candida* species and bacterial strains was also examined by IFL. The four serogroup C_1 salmonella strains (IS40, SL2824, and two others) and 21 of 22 *C. albicans* strains of serotype A were all reactive. None of the *C. albicans* strains of serotype B reacted with this serum.

DISCUSSION

The incidence of systemic infections caused by *Candida* species is increasing despite greater awareness and attempts to prevent it in persons at risk. More *Candida* species are being recognized as agents of disease in immunocompromised persons, especially in cancer and AIDS patients (1, 2, 34). Of the various serological tests that have been developed for early detection of systemic candidiasis, the direct detection of *Candida* antigens is believed to be the most promising for widespread application in the clinical laboratory. Mannan seems a good target antigen for such an assay not only because it is at the cell surface but also because mannan antigenemia occurs during systemic candidiasis (9).

The results of this investigation clearly show that monoclonal antibody MASC1-MR9 is directed against *Candida* mannan and recognizes an epitope common to this component and the LPS of some serogroup C_1 salmonellae. This conclusion is based not only on the immunochemical properties of this antibody but also on solid structural evidence. MASC1-MR9 was isolated on the basis of reactivity with the *Salmonella* serogroup C_1 O-6,7 antigen polysaccharide which contains mainly mannose residues, some in linkages also present in *Candida* mannan. Other monoclonal antibod-

TABLE 2. Inhibition of the interaction of MASC1-MR9 with mannan and LPSs[a]

Coating antigen (concn) and inhibitor	% Inhibition (mean ± SD) at the following inhibitor concn (μg/ml):					
	0.00005	0.0005	0.005	0.05	0.5	5.0
Candida mannan (5 μg/ml)						
Mannan	9 ± 11	26 ± 5	65 ± 9	85 ± 5	92 ± 6	98 ± 2
S. thompson LPS	ND[b]	ND	28 ± 4	94 ± 1	96 ± 1	93 ± 0
S. choleraesuis LPS	ND	ND	9 ± 4	78 ± 1	92 ± 0	93 ± 0
S. thompson LPS[a] (10 μg/ml)						
Mannan	0	54 ± 8	89 ± 6	99 ± 1	99 ± 1	99 ± 1
S. thompson LPS	0	16 ± 2	41 ± 3	95 ± 6	98 ± 2	99 ± 1
S. choleraesuis LPS	0	0	13 ± 3	83 ± 17	95 ± 6	99 ± 2
S. choleraesuis LPS[c] (10 μg/ml)						
Mannan	0	50 ± 2	87 ± 9	99 ± 1	100	100
S. thompson LPS	0	12 ± 4	34 ± 5	94 ± 5	98 ± 2	99 ± 1
S. choleraesuis LPS	5.8 ± 1	2.7 ± 4	0	76 ± 14	94 ± 6	99 ± 1

[a] Data represent results from four or six wells.
[b] ND, Not determined.
[c] Data were pooled from two separate experiments.

ies that recognize cell surface components of *Candida* species have been described (4, 5, 7, 33), but none of these has been used to develop an assay for widespread use. The ELISA inhibition studies show that MASC1-MR9 has a high affinity for mannan, and we will investigate its suitability for clinical antigen detection.

MASC1-MR9 recognized 142 of 148 *Candida* strains of eight different species; the only strains it failed to recognize were all five strains of *C. krusei* and the only strain of *C. utilis* tested. This broad reactivity with *Candida* species sets it apart from other monoclonal antibodies against *Candida* antigens that have been described. The monoclonal antibody described by Cassone et al. (7) was reactive with *C. albicans* and three other species but not with *C. glabrata* and *C. parapsilosis*. One (H9) of two monoclonal antibodies described by Brawner and Cutler (4) did not react with *C. parapsilosis*, while the other (C6) failed to react with both this species and *C. glabrata*. Moreover, monoclonal antibody H9 had only marginal reactivity with *C. albicans* strains of serotype B (5), unlike MASC1-MR9 which reacted equally well with all defined serotype A or B strains tested. Tojo et al. (33) have characterized several monoclonal antibodies reactive with the phosphomannan-protein complex of *C. albicans*. Each antibody recognized at most all three strains of *C. albicans* and one strain of *C. tropicalis* tested but failed to agglutinate *C. guilliermondii*, *C. krusei*, *C. parapsilosis*, *C. stellatoidea*, and *C. glabrata*. With the exception of *C. krusei*, MASC1-MR9 agglutinated all of these strains. We are at present attempting to map the specific epitope recognized by MASC1-MR9 in *Candida* mannan. Its reactivity with the O-antigen polysaccharides of serogroups C_1 and E (E_1, E_2, and E_3) salmonellae would be helpful in this regard since the chemical structures of these antigens have been defined (21, 22). It is somewhat surprising that rabbit antiserum against the O-6,7 antigen of *S. thompson* was specific for serotype A and did not react with serotype B of *C. albicans*. The glycoconjugate used for immunization was at least as reactive with MASC1-MR9 in ELISA as was *Candida* mannan and thus contained the epitope recognized by this monoclonal antibody. There are at least two possibilities to explain the apparent absence of antibodies against this epitope in rabbit polyclonal antiserum. It might be that rabbits, unlike rats, did not respond immunologically to this epitope. On the other hand, given the low efficiency at which this hybridoma was generated, it is also possible that MASC1-MR9 derived from a B lymphocyte specific for the endogenous *Candida* species carried in the rat intestinal microflora. In either case, the data indicate that the epitopes mediating reactivity between the polyclonal antiserum and *C. albicans* strains of serotype A are distinct from that of MASC1-MR9 and we deduce that the O-6,7 antigen of *Salmonella* species has more than a single determinant in common with the mannan of *C. albicans*.

The structural and serological relationships between the various *Candida* species have not been well investigated. MASC1-MR9 defines an epitope present in the mannan of all the *Candida* species tested, except *C. krusei* and *C. utilis*. Monospecific reagents of this kind which recognize specific antigenic determinants will be of great value for characterization of mannan structure and of relationships between the different species.

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