Antibody Response That Protects against Disseminated Candidiasis†

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We previously showed that surface mannans of *Candida albicans* function as adhesins during yeast cell attachment to mouse splenic marginal zone macrophages. The mannan adhesin fraction was encapsulated into liposomes and used to vaccinate mice over a 5- to 6-week period. Circulating agglutinins specific for the fraction correlated with increased resistance to disseminated candidiasis. Antiserum from vaccinated animals protected naive BALB/cByJ mice against *C. albicans* serotype A and B strains and *Candida tropicalis*. Antiserum also protected SCID mice against disseminated disease. The serum protective ability was stable at 56°C, but this ability was adsorbed by *C. albicans* cells. The antiserum was divided into three fractions after separation by high-performance liquid chromatography. One fraction contained all of the agglutinin activity and transferred resistance to naive mice. A second fraction also transferred resistance. Two monoclonal antibodies (MAbs) specific for candidal surface determinants were obtained. MAb B6.1 is specific for a mannan epitope in the adhesin fraction, and MAb B6 is specific for a different epitope in the fraction. Both MAbs are immunoglobulin M, and both strongly agglutinate candidal cells, but only MAb B6.1 protected both normal and SCID mice against disseminated candidiasis. In one experiment, 10 normal mice were given MAb B6.1 and challenged with yeast cells. Six mice survived the 67-day observation period; 4 of the survivors were cured as evidenced by the lack of CFU in the kidney and spleen. Our studies show that antibodies against certain cell surface antigens of *C. albicans* help the host resist disseminated candidiasis.

Candida albicans is the most common cause of opportunistic fungal diseases in humans (45). In the immunocompromised patient, disseminated candidiasis is a serious disease which often results in death, even in patients who were treated with antifungal agents such as amphotericin B (1, 24). Difficulties associated with both the diagnosis of disseminated candidiasis (3, 44) and treatment of the disease by conventional means argue in favor of pursuing the development of preventive strategies and alternative forms of treatment. To these ends, we are exploring the efficacy of specific antibodies in aiding the host to resist disseminated candidiasis.

The role of *Candida*-specific antibodies in host defense against disseminated candidiasis is a contentious issue. Several lines of evidence militate against a protective function. Patients who develop disseminated disease often have *Candida*-specific antibodies in their serum (41). In one study, passive transfer of antibodies failed to protect mice (2). Specific antibodies may block phagocytosis of *C. albicans* (25, 48), suggesting that antibodies may even enhance the severity of disease.

Contrary to the observations presented above, Mourad and Friedman (35, 36) showed that specific antibodies may be protective in experimental disseminated candidiasis. Ten years later, Pearsall et al. (42) made similar observations, and others (16) have obtained indirect evidence for a protective effect of antibodies. Recently, patient recovery from disseminated candidiasis was found to be correlated with the presence of serum antibodies against human and candidal hsp 90 (30, 31), and these antibodies protected mice against disseminated disease. Although the presence of anti-hsp 90 is an autoimmune response with unknown consequences, these studies show that antibodies can be protective against disseminated candidiasis.

Our work focuses on cell wall determinants of *C. albicans*. We hypothesize that only certain kinds of surface antigens may induce protective antibody responses. There is indirect evidence to support this hypothesis. The surface antigen makeup of *C. albicans* is variable both in vitro (5, 6, 14, 22, 29, 34) and in vivo (7, 22). Only a few of the wall components account for the adherence of *C. albicans* to a variety of surfaces (10, 13, 26, 33, 47), including murine splenic marginal zone macrophages (21) and macrophages in specific areas of peripheral lymph nodes (17).

Mannan adhesins responsible for attachment of *C. albicans* to the splenic and lymph node macrophages have been isolated (17, 20) and appear to be part of the cell surface phosphomannan complex (PMC) described by others (23). An adhesin site in the PMC has been identified to structure (27), additional parts of the PMC participate in yeast cell adherence (21), and others have suggested that mannan epitopes are involved in a host protective response against candidiasis (15). In our studies, we show that a liposome-encapsulated yeast cell adhesin (L-adhesin) fraction induces in mice a protective response against disseminated candidiasis. Furthermore, antibodies specific for the adhesin fraction are at least partly responsible for the protection.

MATERIALS AND METHODS

Organisms, culture conditions, and isolation of the adhesin fraction. *C. albicans* serotypes A (strain 1) and B (strain A9) were used and have been previously characterized (8, 18, 20, 21, 49). *Candida tropicalis* CT-4 is from our stock collection, and species identification was confirmed with API 20C yeast identification strips (Analytab Products, Plainview, N.Y.). Stock cultures were stored and maintained as described previously (19, 20) and grown to stationary phase in GYEP broth (19, 20) at 37°C. The yeast cells were washed three times in sterile deionized water, suspended to the appropriate concentration in sterile Dulbecco's phosphate-buffered saline (DPBS) (Sigma Chemical Co., St. Louis, Mo.), and used to challenge mice.

The PMC (referred to as the adhesin extract) was obtained in crude form, as described before (19, 20), by a β -mercaptoethanol extraction of the serotype A isolate of *C. albicans*. Less than 1 μ g of this extract inhibited adherence of yeast cells to splenic and lymph node macrophages; hence, it contains the adhesins (17, 20). Chemically, the extract is primarily mannan with about 3.5% protein. After

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proteinase digestion, the protein content dropped to 0.47%, and yet all adhesin activity was retained (20).

Liposome encapsulation of the adhesin extract. The adhesin extract was encapsulated into multilamellar liposomes as described previously (11). Briefly, 200 μ l of phosphatidylcholine (100 mg of phosphatidylcholine per ml of chloroform) and 30 μ l of cholesterol (100 mg of cholesterol per ml of chloroform [molar ratio of phosphatidylcholine to cholesterol of approximately 3.2:1]) were combined into 10 ml of chloroform-methanol (1:1) in a 500-ml round-bottom flask. The solution was dried as a thin film by rotary evaporation at 37°C under reduced pressure. The film was dissolved in 10 ml of chloroform, evaporated again, dispersed at room temperature for 10 min in 5 ml of DPBS containing 10 mg of the adhesin extract, allowed to stand for 2 h, sonicated for 3 min, and held at room temperature for an additional 2 h. To separate non-liposome-associated antigen from liposome-encapsulated antigen, the preparation was sedimented by centrifugation at $1,000 \times g$ for 30 min. The pelleted liposomes were suspended in 5 ml DPBS and pelleted again, and this process was repeated two more times. The liposome-encapsulated adhesin extract (L-adhesin) was finally suspended in 4 ml of DPBS and stored at 4°C under nitrogen for up to 2 weeks. The amount of adhesin extract within the L-adhesin was 178 μ g/ml, as determined by the phenol-sulfuric acid reaction (12). Control liposomes were prepared exactly as described above, but buffer (DPBS) without adhesin extract was added during the preparation. This control liposome mixture is referred to as L-PBS.

Vaccination and challenge of mice. In all experiments, mice were used and housed in accordance with institutional regulations in an AAALAC-certified animal facility. BALB/cByJ (Jackson Labs, Bar Harbor, Maine) female mice, 6 to 7 weeks old, received the initial vaccine and weekly booster immunizations. Each injection consisted of 0.2 ml of L-adhesin administered intravenously (i.v.). Anti-adhesin titers in mouse sera were assessed by slide agglutination against latex beads coated with the adhesin extract. Adhesin coating was performed as described previously (19, 20, 27). When the agglutinin titers reached 40 or more (usually by the fourth booster immunization), the animals were challenged. Control mice received an equal volume and number of injections consisting of diluent (DPBS) only prior to challenge. The mice were challenged i.v. with viable yeast cells prepared to the appropriate concentration in 0.2 ml of DPBS.

Treatment of polyclonal antiserum. To characterize the nature of the protective factor(s) in antiserum, polyclonal antiserum samples were obtained from vaccinated mice and pooled. The serum fraction was either immediately stored at -20°C, heated at 56°C for 30 min prior to use, or adsorbed five times with formalin-killed washed *C. albicans* 1 cells at a ratio of 10 volumes of antiserum to 1 volume of DPBS-washed packed dead yeast cells.

The antiserum was also fractionated by passage through an ABx high-performance liquid chromatography (HPLC) column (J. T. Baker, Phillipsburg, N.J.), as described in reference 40, to obtain pools of various separated serum components, including a fraction which contained all of the agglutinin activity. Briefly, buffer A consisted of 25 mM MES [2-(N-morpholino)ethanesulfonic acid (Sigma)] at pH 5.2 to 5.8, and buffer B consisted of 1 M sodium acetate (pH 7.0). One part of polyclonal antiserum was mixed with 2 parts of buffer A, and the mixture was loaded onto the ABx HPLC column with buffer A at a flow rate of 1.5 ml/min; each fraction was 40 drops. At 10 min, the amount of buffer B was brought to 20% at 15 min the amount of buffer B was brought to 50%, at 20 min the amount was brought to 70%, at 25 min the amount was brought to 100%, and then the amount was retained at 100% until 55 min, at which time the run was terminated. Each of the peaks detected by A_{280} was collected and dialyzed against at least 100 volumes of DPBS at 4°C with a minimum of four changes of DPBS over a 36-h period, and then each pooled fraction was concentrated by ultrafiltration (PM30 Diaflo ultrafiltration membrane; Amicon Division, Beverly, Mass.). Each concentrated fraction was brought to approximately one-half of the original starting volume of antiserum applied to the column. Each was tested for the ability to agglutinate whole yeast cells and latex beads coated with the adhesin fraction.

Passive transfer experiments. Normal mouse serum (NMS [control]), polyclonal antiserum, antisera heated at 56°C, *C. albicans*-adsorbed antisera, and HPLC-fractionated polyclonal antisera were tested for their ability to transfer resistance against disseminated candidiasis to naive mice. For each condition, 7- to 8-week-old female or male BALB/cByJ mice (Jackson Laboratories) were given 0.5 ml of the test serum intraperitoneally, 4 h later they were given 0.2 ml of a suspension containing 2.5×10^6 yeast cells per ml of DPBS i.v., and 20 h later they were given another 0.2 ml of test serum intraperitoneally. Forty-eight hours after challenge, the numbers of candidal CFU per gram of kidney were determined as described below. In some experiments, passive transfer of immune serum and challenge with live yeast cells were done with 18- to 20-week-old male SCID mice (BALB/cByJSmn-scld/J; Jackson Laboratories).

Isolation and characterization of MAbs. Mice were immunized with whole yeast cells (4) or the L-adhesin (11), and two monoclonal antibodies (MAbs) specific for yeast surface epitopes were isolated as described before (4, 11). MAb B6 has the same specificity as MAb C6 (6), and MAb B6.1 is specific for an epitope in the PMC of *C. albicans* (19a). The epitope specificity of MAb B6 is unknown but differs from that of MAb B6.1, as evidenced by Ouchterlony lines of nonidentity against candidal cell wall extracts. Both of the MAbs agglutinate *C. albicans* cells, and both are immunoglobulin M (IgM), as indicated by reactions with commercial Ig heavy chain-specific antibodies (Sigma). The MAbs were produced in serum-free medium, concentrated by ammonium sulfate pre-

TABLE 1. Mice vaccinated against candidal adhesin extract have greater resistance to disseminated candidiasis than control animals^a

Challenge dose (CFU)	Vaccine preparation	Mean (SE) survival time (days) ^b	
		Expt 1	Expt 2
1×10^6	DPBS	12.5 (1.0)	Not done
	L-PBS	12.0 (0.0)	Not done
	L-adh	20.0 (2.2)	Not done
		$P < 0.05$	
5×10^5	DPBS	21.8 (4.2)	19.4 (7.9)
	L-PBS	17.3 (4.1)	20.6 (9.8)
	L-adh	31.8 (4.3)	46.0 (12.8)
		$P < 0.05$	$P < 0.05$
2.5×10^5	DPBS	25.0 (6.7)	38.2 (21.6)
	L-PBS	23.8 (8.3)	46.2 (25.5)
	L-adh	32.5 (3.8)	65.8 (13.9)
		$P < 0.1$	$P < 0.05$

^a Normal mice were given buffer (DPBS) alone, liposome-buffer (L-PBS), or the liposome-adhesin complex (L-adh) and challenged with various doses of *C. albicans* cells. Mean survival times for two separate experiments (Expt 1 and Expt 2) were determined, and results from the DPBS and L-adh groups were compared for statistical significance by the Kolmogorov-Smirnov one-sample test.

^b SE, standard error; Expt 1, four mice per group; Expt 2, five mice per group.

cipitation, and suspended and diluted in DPBS to give identical agglutinin titers. The same strategy described above for polyclonal antiserum was used to determine the ability of MAbs B6 and B6.1 to transfer protection. In these experiments, the agglutinin titers of each MAb were diluted to 20 (approximately 220 μ g/ml for MAb B6.1 and 290 μ g/ml for MAb B6) before administration to the BALB/cByJ mice. In one experiment, MAb B6 was obtained from ascites fluid, adjusted to an agglutinin titer of 320, and compared in terms of effect with MAb B6.1 at a titer of 20.

Assessment of resistance and susceptibility to disseminated candidiasis. To determine relative susceptibility or resistance to disseminated candidiasis, we used survival curves and/or counted the number of CFU per gram of kidney tissue in mice challenged i.v. with *C. albicans* cells. For survival curves, groups of test and control animals consisted of a minimum of five mice per group. Survival differences between the groups were calculated for statistical significance by the Kolmogorov-Smirnov one-sample test (9). The kidney is a target organ in experimental disseminated candidiasis; therefore, the number of *C. albicans* CFU in kidney tissue may be used as an indicator of disease severity (28, 43, 46). The CFU determinations were done by homogenizing the kidneys with glass tissue homogenizers as described previously (43), except that the kidneys were homogenized in 1 ml of DPBS and plated onto Mycosel agar (BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, Md.). The statistical significance of differences between test and control groups was determined with Student's *t* test.

RESULTS

Vaccinated mice have increased survival rates. Vaccinated mice showed more resistance to disseminated candidiasis than did control mice, as indicated by an increase in mean survival times after challenge (Table 1). To demonstrate a requirement for liposome delivery, some animals were given i.v. an equivalent amount of adhesin extract (178 μ g) in 0.2 ml of DPBS but without liposomes. The mean survival times of these animals did not differ from those of animals that received only DPBS (data not shown).

Serum from vaccinated mice transfers protection. Pooled polyclonal antiserum from vaccinated mice protected both naive normal BALB/cByJ mice and SCID mice from disseminated disease (Fig. 1). Whereas heat treatment (56°C for 30 min) had no effect on the protective ability of the antiserum, adsorption with *C. albicans* cells removed the activity (data not shown).

To determine if the vaccine induces protection against both serotypes of *C. albicans* and against other *Candida* species, mice were passively given, as described above, the anti-sero-

FIG. 1. Polyclonal antiserum (Ab) protects normal and SCID mice against disseminated candidiasis. Polyclonal antiserum from L-adhesin-vaccinated mice was administered i.v. with BALB/cByJ (A) and SCID (B) mice, the animals were challenged i.v. with *C. albicans*, and the resulting kidney candidal CFU per gram of tissue were determined. Bars, standard errors. Differences between the values obtained from mice that received polyclonal antiserum and control mice that received NMS were significant ($P < 0.01$).

type A polyclonal antiserum and were challenged i.v. with either a serotype B strain of *C. albicans* (5×10^5 cells) or a strain of *C. tropicalis* (10^6 cells). Kidneys were removed 48 h later for CFU determinations. Antiserum-treated mice challenged with the serotype B strain had $(11.3 \pm 2.7) \times 10^3$ CFU/g of kidney tissue, while NMS-treated mice (controls) had $(41.4 \pm 7.0) \times 10^3$ CFU/g ($P < 0.001$) (mean $\pm$ standard error). Likewise, antiserum-treated mice challenged with *C. tropicalis* developed $(145 \pm 16) \times 10^3$ CFU/g of kidney compared with $(267 \pm 34) \times 10^3$ CFU/g for NMS-treated controls ($P < 0.001$) (mean $\pm$ standard error).

Two fractions from antiserum transfer protection. Antiserum separated by use of an ABx HPLC column yielded three major fractions: Fr.I, Fr.II, and Fr.III (Fig. 2). All of the detectable agglutinin activity was associated with Fr.III. Fr.III gave the strongest evidence for ability to transfer protection, but protective activity was also associated with Fr.II. That is, mice given either NMS (negative control), unfractionated polyclonal antiserum (positive control), or Fr.I, Fr.II, or Fr.III and challenged with *C. albicans* i.v. resulted in (109 ± 33.3) , (40.9 ± 2.3) , (93.2 ± 9.5) , (59.2 ± 11.4) , and $(50.9 \pm 9.7) \times 10^3$ CFU/g of kidney, respectively (mean $\pm$ standard error). The differences were significant to $P < 0.05$ when the values for polyclonal antiserum, Fr.II, or Fr.III were compared with the number of CFU per gram of tissue for animals treated with NMS. In these experiments, the total amounts of protein received by each mouse were 22.4 mg of Fr.I, 3.8 mg of Fr.II, and 22.6 mg of Fr.III. The antibody activity (agglutination titer) of Fr.III (22.6 mg) was the same as the agglutinin activity of unfractionated polyclonal antiserum.

MAb B6.1 transfers protection, but MAb B6 does not. Although both MAbs are strong agglutinins and are of the same class, only MAb B6.1 transferred protection against disseminated candidiasis to naive BALB/cByJ mice. This result was demonstrated by both counts of CFU per gram of kidney (Fig. 3A) and by survival curve analysis (Fig. 3B). In these experiments, both MAbs were standardized to have the same agglutinin titers, as indicated in Materials and Methods. In one experiment, the titer of MAb B6 was increased to approximately 16 times that of MAb B6.1 and administered to mice at the volumes and schedules indicated in Materials and Methods. Even though the agglutinin titers at day 2 after adminis-

FIG. 2. Fractionation of polyclonal antiserum by HPLC column. Polyclonal antiserum obtained from vaccinated mice yielded three major fractions (Fr.I, Fr.II, and Fr.III) when passed through an ABx HPLC column. The various fractions were pooled, dialyzed against DPBS, concentrated by ultrafiltration, and reconstituted in DPBS to approximately one-half the volume of the amount of antiserum loaded onto the column. Agglutinin activity was found exclusively associated with Fr.III.

tration were 10 for animals that received MAb B6 and 2 for mice that received MAb B6.1, no protection due to MAb B6 was observed compared with mice given DPBS prior to yeast cell challenge (data not shown). In the survival experiments, six of 10 BALB/cByJ mice treated with MAb B6.1 survived the entire 67-day observation period, whereas all MAb B6-treated mice died by day 25, and all control (DPBS-treated) mice died by day 19 (Fig. 3B). Likewise, SCID mice treated with MAb B6.1 survived significantly ($P < 0.01$) longer than control mice (Fig. 4). In experiments with BALB/cByJ mice, the 67-day survivors were sacrificed, and their kidneys and spleens were plated for candidal CFU. No CFU in splenic tissues were detected in any of the animals. However, the kidneys from two of the mice showed CFU development (97.7×10^3 /g and 207.7×10^3 /g), whereas no CFU were detected in undiluted homogenates of kidneys from four of the mice.

DISCUSSION

This work provides strong evidence that antibodies specific for certain cell surface determinants on *C. albicans* aid the host in resistance against disseminated candidiasis. First, mice with enhanced resistance were those that were L-adhesin vaccinated and had agglutinin titers of 40 to 80. Second, mice vaccinated with only the adhesin extract developed low anti-adhesin titers (less than 5) and showed no enhanced resistance. Third, polyclonal antiserum from vaccinated mice protected naive normal BALB/cByJ mice and SCID mice from disseminated disease (Fig. 1). SCID mice, however, did not make antibodies or develop a protective response as a result of the vaccinations (data not shown). Fourth, heat treatment (56°C for 30 min) had no effect on the protective ability of the polyclonal antiserum, but adsorption with *C. albicans* removed the activity. Fifth, fractionation of the antiserum by an ABx HPLC column yielded a fraction that contained all of the agglutinin activity, and this fraction transferred protection to

FIG. 3. MAb specific for a phosphomannan fraction that contains candidal adhesins protects mice against disseminated candidiasis. BALB/cByJ mice were given polyclonal antiserum (pAb), MAbs specific for either the mannan adhesin fraction (MAb B6.1) or some other cell surface determinant (MAb B6), or buffer (DPBS) as a control. The animals were challenged i.v. with 5×10^5 viable yeast cells, and susceptibility to disseminated candidiasis was assessed by determining candidal CFU in the kidneys (A) or by survival curves (B). In both cases, significant differences ($P < 0.01$) between mice that received either polyclonal antiserum or MAb B6.1 and DPBS controls were found.

naive animals. Sixth, mice that received MAb B6.1, which is specific for the adhesin extract and is a strong agglutinin of whole yeast cells, developed fewer CFU in their kidneys after challenge, and both normal and SCID mice survived significantly longer than control animals (Fig. 3 and 4). In this experiment (Fig. 3B), 6 of 10 of the BALB/cByJ animals treated survived the entire 67-day observation period and 4 of 6 of the survivors appeared to be cured, as evidenced by the lack of CFU recoverable from their spleens and kidneys.

Our results also show that an antibody with specificity for a cell surface determinant of *C. albicans* may not necessarily protect animals against disseminated disease. These findings may well explain the variable results earlier workers have obtained regarding the role of antibodies in protection against disseminated candidiasis. In our work, animals that received the agglutinating IgM MAb B6 were just as susceptible as controls to disseminated candidiasis, even when MAb B6 was given at about 16 times the titer of MAb B6.1, resulting in in vivo titers higher than those in animals that received MAb B6.1. We have also found two additional MAbs specific for surface determinants that do not protect (unpublished data).

FIG. 4. MAb B6.1 protects SCID mice against disseminated candidiasis. BALB/cByJSmn-scid/J male mice were given MAb B6.1 intraperitoneally and challenged i.v. with 5×10^5 *C. albicans* cells. The resulting survival curves were plotted and found to significantly ($P < 0.01$) differ from those of mice given buffer (DPBS) instead of the MAb.

These results support our hypothesis that antibodies with only certain specificities against *C. albicans* are protective. Our findings are somewhat similar to those of Casadevall and coworkers (37 to 39), who have shown that antibodies protective against experimental cryptococcosis have specificity for the glucuronoxylomannan portion of the capsular material.

Strains of *C. albicans* are either serotype A or B, and both types can cause disseminated disease (41). In addition, there are an increasing number of candidiasis cases due to other candidal species such as *C. tropicalis* (26, 32). In our work, the vaccine induces in mice a response that also protects against disseminated disease due to a *C. albicans* serotype B strain and *C. tropicalis*. These data suggest that antiserum from vaccinated mice contains antibodies that are broadly protective. We have not attempted to determine if MAb B6.1 also protects mice against serotype B and *C. tropicalis* strains. The explanation for the broad protection of polyclonal antiserum may involve antibodies with various specificities or with specificity for the B6.1 epitope, or it may involve a yet undefined serum fraction in polyclonal antiserum that transfers protection.

Further experimentation is necessary to determine the mechanism(s) by which anti-adhesin extract antibodies protect the host against disseminated candidiasis. Since we found that MAb B6.1 also protects SCID mice, neither T nor B cells appear to be involved in the protection. One possible mechanism is that antibodies in the mouse cause simple agglutination of the yeast cells, which effectively reduces the number of independent infection units. This explanation does not seem likely, because MAb B6 does not protect, but it is a strong agglutinin. In fact, it causes larger agglutinates at a given titer than MAb B6.1 does (unpublished observations). The agglutinin titers in the serum of mice that received MAb B6 or MAb B6.1 were essentially the same. The lower number of CFU in MAb B6.1-treated animals, but not in MAb B6-treated mice, also militates against the argument that CFU are artificially reduced because of the presence of serum agglutinins. In addition, animals that passively received MAb B6.1 had enhanced survival compared with mice that received MAb B6.

Two other possibilities are that MAb B6.1 alters the adherence of yeast cells in vivo and/or enhances the phagocytosis of yeast cells by neutrophils and macrophages. The first possibility is under investigation. If the latter explanation is correct, the mechanism would not involve Fc receptors on phagocytic cells, because MAb B6.1 is an IgM. However, MAb B6.1 may

promote complement opsonization more efficiently than the nonprotective IgM agglutinin MAb B6. This intriguing possibility is under investigation.

There are possible applications of our investigations to human medicine. If antibodies with specificity as described in our work also protect people against disseminated candidiasis, individuals at high risk for disease may be either actively immunized by administration of the vaccine or passively immunized by transfer of preformed antibodies.

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