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Evidence that *Candida albicans* Binds via a Unique Adhesion System on Phagocytic Cells in the Marginal Zone of the Mouse Spleen

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We recently demonstrated by using an ex vivo adhesion assay that *Candida albicans* yeast cells exhibit a unique binding affinity for the marginal zone of the spleen. This binding event provides a working model for studying mechanisms of organ dissemination of the fungus from the blood. By using the ex vivo assays reported here, we showed by bright-field and electron microscopic techniques that mouse spleen marginal zone cells capable of ingesting India ink particles are also involved in yeast cell attachment. During splenic clearance of yeast cells from the circulation in vivo, *C. albicans* is also associated exclusively with marginal zone cells capable of ingesting India ink. The ability to ingest the ink particles is not necessarily related to yeast cell adherence, because the fungal cells did not bind to phagocytic cells in the splenic red pulp. In fact, the marginal zone phagocytic cells appear to have a unique binding system, because yeast cells also did not bind to phagocytes in other tissues, such as the thymus and peritoneum, or to seven different myeloid cell lines. In addition, antibodies to a number of well-characterized murine adhesion molecules, such as leukocyte integrins, LECAM-1, and CD44, had no effect on binding. On the basis of these results, we propose that splenic marginal zone phagocytes express a novel adhesion system that involves either a unique adhesion molecule or previously described adhesion molecules with unique binding activities.

Infectious disease organisms usually target specific host tissues rather than invading organs randomly. The identification of host ligands and parasite adhesion molecules involved in the tissue specificity of infectious agents will explain mechanisms by which these agents disseminate within the host and may lead to innovative diagnostic and therapeutic approaches against deep-seated infectious diseases.

Candida albicans is the most common cause of opportunistic fungal diseases and is one of the leading infectious disease agents overall as a cause of nosocomial infections (6). In immunocompromised patients, disseminated candidiasis is a serious disease, yet existing methods of diagnosing disseminated disease are inadequate, and treatments are often toxic for the patient. Of critical importance is understanding how *C. albicans* targets host organs following candidemia.

Over the past few years, four categories of adhesion molecules of *C. albicans* have been defined (2, 4): protein moieties on *C. albicans* that react with host cell surface proteins; lectinlike protein molecules on *C. albicans* that react with sugar residues of host cell surface glycoproteins; carbohydrate moieties on *C. albicans* that react with unidentified host cell surface molecules; and unidentified surface molecules of *C. albicans* that are responsible for hydrophobic interactions with host cells. Despite the array of adhesins identified, the specificity of *C. albicans* for particular host organs and tissues remains undefined.

Recently, we adapted an ex vivo adherence assay to study the mechanisms of *C. albicans* dissemination from the blood to internal organs (5). This same method was originally used to define lymphocyte homing receptors and mechanisms by which inflammatory cells accumulate at sites of tissue injury (1, 14, 19). Our investigations revealed the remarkable adherence specificity of *C. albicans* yeast cells for tissue within the marginal zone of the spleen and the trabecular sinus of lymph nodes rich in tissue macrophages. Furthermore, the ex vivo assay was found to accurately reflect tissue binding sites of *C. albicans* during dissemination of the fungus from the circulation to the spleen, lymph nodes, and other tissues (5).

The splenic binding pattern was especially intriguing because it provided a working model for defining a tissue-specific interaction between *C. albicans* and host tissue. The spleen is also an important organ for consideration because it participates in the vascular clearance of foreign particles, it actively takes up *C. albicans* from the circulation in experimental animals, and splenic lesions due to *C. albicans* are being recognized with increasing frequency and are associated with certain types of immune system defects (20). The yeast binding specificity in the spleen may serve as a model for understanding the mechanisms of dissemination of *C. albicans* from the blood and may lead to the identification of a new adhesion ligand specific for the splenic marginal zone.

Here we determined whether the tissue-specific adhesion of *C. albicans* to the marginal zone of the spleen involves a host cell type specific for the marginal zone.

MATERIALS AND METHODS

Organisms and culture conditions. *C. albicans* A9, described previously (12, 21), was used throughout the study. Yeast cells stored in 50% glycerol at −80°C were spread onto GYEP (2% glucose, 1% peptone, 0.3% yeast extract) agar plates or slants and allowed to grow for 48 to 72 h at 37°C. A loopful of yeast cells from the plate or slant was placed in 25

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ml of GYEP broth in a 50-ml Erlenmeyer flask, and the mixture was aerated by rotation at 180 rpm (Gyrotory shaker-incubator; New Brunswick Scientific Co., Inc., Edison, N.J.) for 24 h at 37°C. Three drops of the broth culture of yeast cells was used to inoculate 75 ml of GYEP broth in a 125-ml Erlenmeyer flask, and the mixture was incubated for 24 h at 37°C under aeration. The stationary-phase yeast cells were harvested by centrifugation, washed three times in cold sterile deionized water, and counted by use of a hemacytometer. Yeast cells were suspended to the desired concentration in phosphate-buffered saline (PBS, 20 mM, pH 7.2), and their hydrophobicity was determined as described previously (12) with latex microspheres (standard Dow latex [mean diameter, 0.815 μm] or Unisphere latex 24; Serva Fine Biochemicals, Westbury, N.Y.). In the experiments reported here, yeast cell suspensions were used only when they showed 100% hydrophilicity (i.e., 0% hydrophobicity). The analyzed yeast cell suspensions were stored in ice and used within 2 h.

Ex vivo adherence assay. The adherence assay was performed by the recently described stationary method (16). In brief, spleens were obtained from BALB/cByJ mice 5 min after intravenous (i.v.) inoculation of 0.1 ml of 40% luconyl blue (44 E 3172; BASF Aktiengesellschaft). All spleens, except those processed for electron microscopy (see below), were placed in Tissue Tek O.C.T. compound (Miles Inc., Elkhart, Ind.) immediately after removal from the animals and rapidly frozen on dry ice. The specimens were stored at −80°C until cryosectioned. Cryosections were cut 8 to 10 μm thick at −20°C (Frigocut 2800 N cryostat; Reichert-Jung, Leica, Inc., Deerfield, Ill.) and mounted onto Gold Seal Rite-On Micro Slides (Becton-Dickinson Labware, Lincoln Park, N.J.). The sections were air dried as described previously (16), and 100 μl of a yeast cell suspension (2×10^8 cells per ml) in Dulbecco modified Eagle medium (Sigma Chemical Co., St. Louis, Mo.) containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (GIBCO, Grand Island, N.Y.) and 5% fetal bovine serum (Sigma) and adjusted to pH 7.4 with 1 N NaOH was added to the sections and incubated without rotation for 15 min at 5 to 8°C. The sections were fixed, stained with either crystal violet (16) or periodic acid-Schiff's reagent and hematoxylin (PASH) (5), and mounted in Permount, and cells were counted manually as described previously (16).

In some experiments, antibody blocking studies were done to examine the potential role of known host adhesion molecules in the binding of *C. albicans* to the splenic marginal zone. Monoclonal antibodies specific for mouse leukocyte integrins LFA-1 (FD445.1 [17]), Mac-1 (M1/70 [18] and SK431 [13a]), and VLA4 (R1/2 [13]), L-selectin (previously called LECAM-1 or homing receptor) (MEL-14 [9]), and CD44 (MJ64 and IM7 [22]) were tested. All of the antibodies were of rat origin and were partially purified by ammonium sulfate precipitation. *C. albicans* yeast cells (2×10^8/ml) were treated with 50 μg of each antibody per ml in Dulbecco modified Eagle medium plus 5% fetal bovine serum and 10 mM HEPES for 20 min on ice prior to the adhesion assay. After the incubation period, the yeast cells were used directly in the binding assay, which was done as described above. In other experiments, the spleen sections were also treated with monoclonal antibodies for 20 min prior to the addition of antibody-treated yeast cells.

In vivo adherence of *C. albicans* to the splenic marginal zone. To examine the marginal zone cell type to which yeast cells attach, we injected 0.1 ml of India ink (waterproof drawing ink no. 4415; Faber-Castell Corp., Newark, N.J.) diluted 1:4 in PBS i.v. into mice. Thirty minutes later, some animals were given 0.1 ml of a suspension containing 1.5×10^8 *C. albicans* yeast cells i.v. Forty-five minutes later (i.e., 75 min after injection with India ink), all mice were sacrificed and their spleens were removed for cryosectioning as described above; the sections were stained with PASH or used in the ex vivo assay. Some splenic tissue was also processed for transmission electron microscopy (TEM) as described below.

SEM. For scanning electron microscopy (SEM), splenic cryosections mounted on glass coverslips instead of glass slides were used in the adherence assay as described above. Fifteen minutes after the interaction of the tissue with yeast cells, the sections were fixed by treatment with 3% glutaraldehyde in 20 mM PBS (pH 7.2) for 2 h at 5 to 8°C. The sections were gently washed five times in PBS, dehydrated in a graded ethanol series, critical point dried with liquid CO_2 (model 020 critical point dryer; Balzers, Arlington Heights, Ill.), gold sputter coated (Ted Pella, Inc., Redding, Calif.), and viewed in a JEOL-100 CX electron microscope with an ASID-4D resolution scanning system.

TEM. TEM was used to observe the *C. albicans*-marginal zone interaction during in vivo and ex vivo adherence. For in vivo adherence, splenic tissue from mice injected with India ink alone, *C. albicans* alone, or a combination of ink and yeast cells was cut into small pieces with a razor blade so that each piece contained white and red pulp, as discerned by inspection with a light microscope. The appropriate pieces were fixed in 3% glutaraldehyde in 20 mM PBS (pH 7.2) for 2 h at 5 to 8°C, washed four times in PBS, postfixed in 2% OsO_4 in PBS for 2 h at 22 to 25°C, washed in deionized water, dehydrated in a graded ethanol series and then in acetone, embedded in Epon 812, and polymerized for 24 h at 65°C.

For ex vivo adherence, spleens were removed from animals injected i.v. with India ink as described above, rapidly frozen in Tissue Tek O.C.T. compound, and cryosectioned at a thickness of 16 μm. The sections were collected on glass coverslips for ex vivo adherence as described above. Following yeast cell adherence, the sections were fixed in 3% glutaraldehyde for 2 h at 5 to 8°C, washed three times in PBS, postfixed in 2% OsO_4 for 2 h, and dehydrated in an ethanol series and then in acetone. Epon resin was added to the surface of each coverslip, and the sample was polymerized as described above. The coverslip was dissolved by treatment with hydrofluoric acid for 30 min at 22 to 25°C. The flat Epon blocks were oriented in a new Epon block to allow thin sections to be made at a right angle with respect to the tissue section. Thin sections were collected on copper mesh grids, stained with uranyl acetate and Reynolds' lead citrate, and viewed on a Zeiss EM10C/CA TEM at 65 kV.

RESULTS

Splenic cell type associated with ex vivo binding. At the light-microscopic level, the ex vivo adherence assay revealed a striking specificity of the stationary-phase hydrophilic yeast cells for the marginal zone areas of the spleen (Fig. 1A and B). Under the SEM, the association of *C. albicans* with the marginal zone was located at low power (Fig. 1C). At a higher magnification, evidence was found for two types of binding: tenacious (Fig. 1D) and tenuous (Fig. 1E). These observations suggested that the yeast cells interacted with the host cells, rather than intercellular material, such as extracellular matrix proteins, within the marginal zone.

To show more convincingly a cell-cell interaction, we did

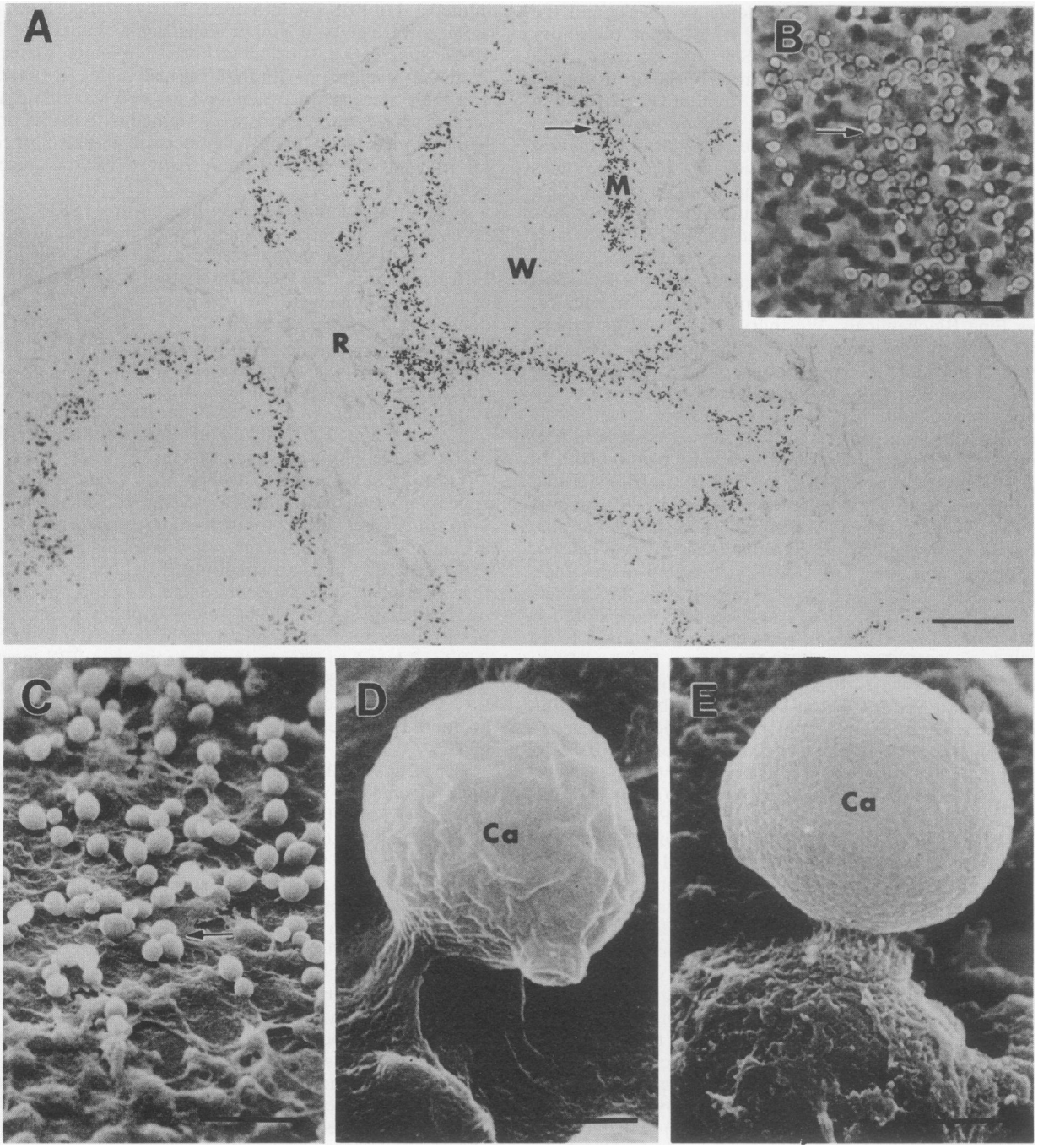

FIG. 1. Pattern of *C. albicans* binding to mouse splenic tissue. Yeast cells were allowed to interact with splenic tissue sections for 15 min, and the sections were fixed in glutaraldehyde, stained with 2% crystal violet, and examined with normal bright-field optics at low power (A; bar, 200 μm) or at a higher magnification with phase-contrast optics (B; bar, 10 μm). In both panels, *C. albicans* yeast cells were readily detected (arrows). M, marginal zone; W, white pulp; R, red pulp. Observations were also made by SEM. The marginal zone to which yeast cells attached was found with low power (C; bar, 10 μm) (arrow). At a higher magnification, it appeared that *C. albicans* (Ca) bound to splenic cells tenaciously (D; bar, 1 μm) or tenuously (E; bar, 1 μm).

studies to determine whether yeast cells attached specifically to marginal zone phagocytic cells, which are among the predominant cell types in the zone. Spleens from mice given India ink i.v. showed accumulation of the ink in the marginal zone in a pattern very similar to that of *C. albicans* (Fig. 2A). In addition, phagocytic cells in other areas of the spleen, such as the red pulp, also ingested the ink particles. The splenic tissue containing India ink was used in the ex vivo binding assay, and an association between areas of India ink and yeast cell binding was observed (Fig. 2B). To view the interaction at the level of electron microscopy, we allowed *C. albicans* to adhere to India ink-containing splenic sections

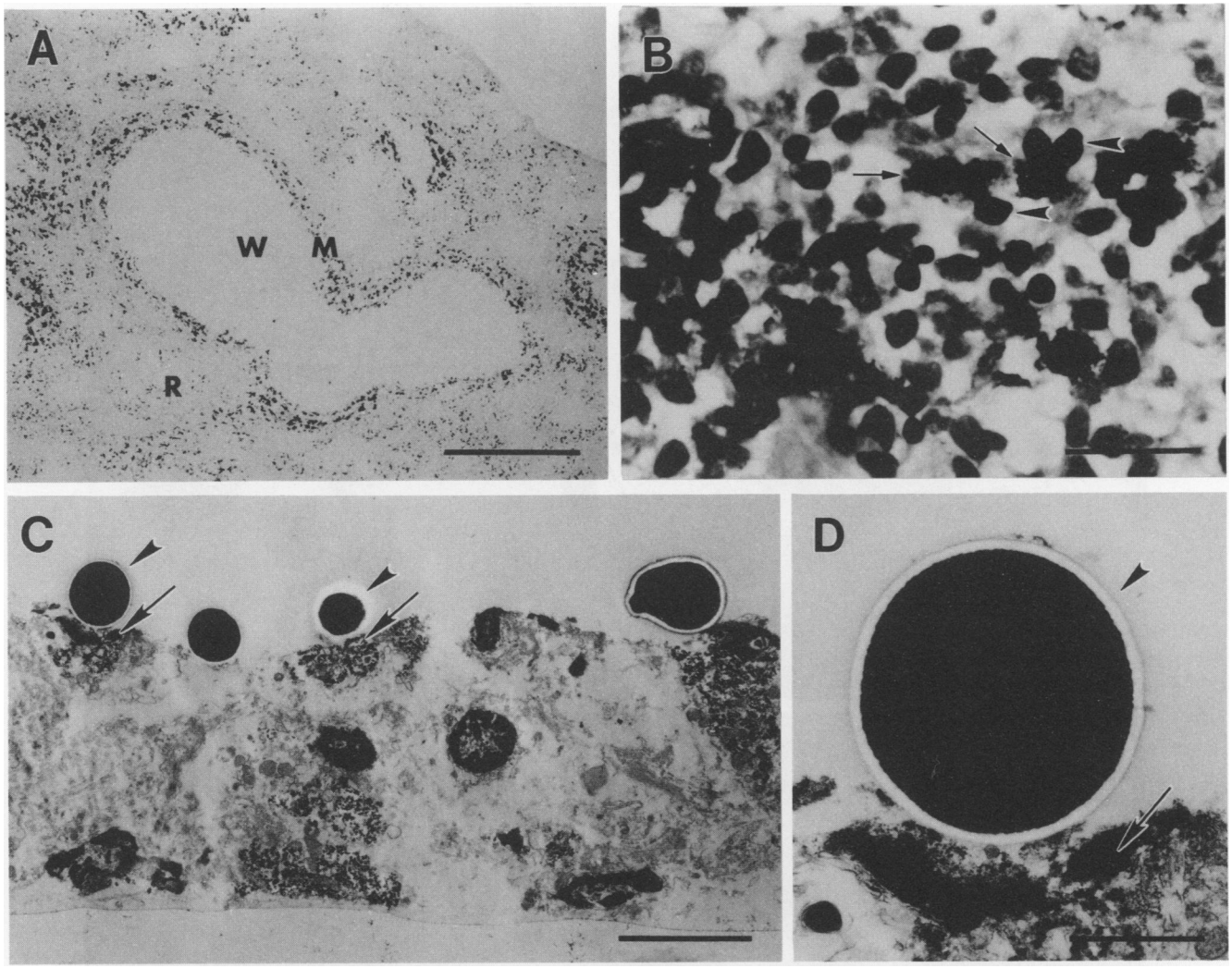

FIG. 2. Binding of *C. albicans* yeast cells to phagocytic cells in the splenic marginal zone in the ex vivo assay. Mice were injected i.v. with India ink prior to the removal of their spleens for use in the ex vivo assay. (A) Splenic sections examined prior to the addition of yeast cells showed India ink uptake in areas rich in phagocytic cells, such as the marginal zone (M) and red pulp (R) (bar, 200 μm). W, white pulp. (B) *C. albicans* yeast cells (arrowheads) became associated with marginal zone cells containing India ink particles (arrows) but not phagocytic cells in other areas of the spleen (bar, 10 μm). (C and D) Similar findings were obtained by TEM. Yeast cells (arrowheads) were found to be associated with host cells that had previously ingested India ink particles (arrows) (bars: C, 5 μm; D, 1 μm).

as before, but we oriented the sections in Epon resin to allow cutting of ultrathin cross-sections of the yeast-spleen interaction. The results of these studies clearly showed *C. albicans* attached to cells within the marginal zone. Furthermore, a correlation was noted between marginal zone cells that ingested India ink particles and host cells to which *C. albicans* yeast cells attached (Fig. 2C and D).

Splenic cell type associated with in vivo binding. Mice injected with India ink i.v. were given yeast cells i.v. and sacrificed as described in Materials and Methods. The spleens were removed from the animals and either cryosectioned, mounted on glass slides and stained with PASH for light microscopy or cut into small pieces with a razor blade, fixed in glutaraldehyde, and prepared for TEM. Viewed under bright-field microscopy, *C. albicans* was found associated with cells that had ingested India ink. At low power, India ink was ingested, as noted above, by phagocytes associated with the marginal zone and red pulp of the spleen, but yeast cells were found only in the marginal zone (Fig. 3A). At a higher magnification, yeast cells were found within vacuoles, indicating that they had been ingested by cells within the marginal zone. Furthermore, the same cells that ingested the India ink also ingested the *C. albicans* yeast cells (Fig. 3B to D).

By TEM, yeast cells were also found associated with marginal zone cells that were capable of ingesting India ink, but not with lymphocytic cells of the white pulp, which were devoid of India ink (Fig. 3E). In total, these observations indicate that *C. albicans* yeast cells attach to and then become ingested by phagocytic cells within the marginal zone of the spleen. Phagocytic cells that are located in other areas of the spleen and that are capable of ingesting India ink do not bind *C. albicans* yeast cells.

The role of known mouse leukocyte adhesion proteins in the binding of *C. albicans* to the splenic marginal zone was examined with specific monoclonal antibodies. Either spleen

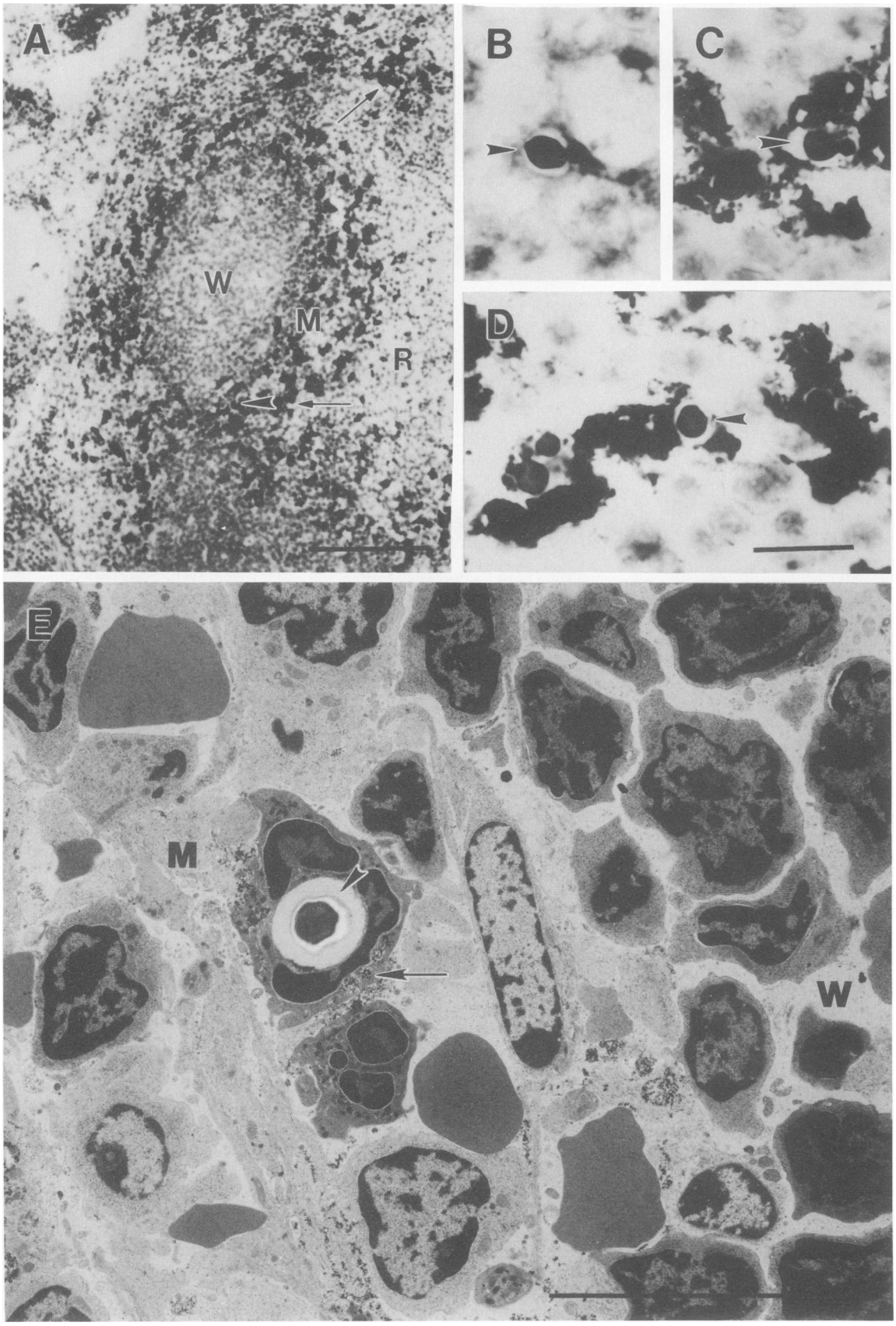
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FIG. 3. Binding of *C. albicans* yeast cells to phagocytic cells in the splenic marginal zone in vivo. Mice given India ink i.v. were given a suspension of viable stationary-phase yeast cells i.v. and sacrificed 45 min later. The spleens were removed and prepared for bright-field microscopy and for TEM. (A) With low-power bright-field microscopy, India ink (arrows) was found associated with marginal zone cells (M) and with cells located in the red pulp (R) (bar, 100 μm). Yeast cells (arrowhead) were barely discernible and appeared to be within vacuoles. W, white pulp. (B to D) At a higher magnification, yeast cells (arrowhead in B) were found enclosed within vacuoles of cells of spleens from animals not injected with India ink; in animals injected with India ink before *C. albicans*, yeast cells (arrowheads in C and D) were found enclosed within vacuoles of marginal zone cells that had ingested ink particles (bar, 10 μm). (E) The marginal zone (M) and adjacent white pulp (W) were located by TEM, and ingested yeast cells (arrowhead) were found associated with marginal zone cells that had ingested India ink particles (arrow) (bar, 10 μm).

sections were treated alone or sections and yeast cells were treated with antibodies against LFA-1, Mac-1, VLA4, CD44, and L-selectin as described in Materials and Methods. An antibody concentration which blocks the known adhesion activities of these proteins (50 μg/ml) had no effect on the binding of *C. albicans* to the splenic marginal zone (data not shown).

DISCUSSION

The spleen marginal zone cell type to which *C. albicans* yeast cells bind both in the ex vivo adherence assay and in vivo following experimental candidemia was identified. This was accomplished by use of bright-field, scanning, and transmission electron microscopic techniques.

Observations involving the ex vivo assay showed that yeast cells adhere to spleen marginal zone cells that are also capable of ingesting India ink. This association implies that these marginal zone cells are phagocytic cells. However, *C. albicans* yeast cells do not adhere to all phagocytic cells in the spleen, because red pulp phagocytes detectable by India ink uptake did not bind yeast cells. SEM observations of the yeast cell-marginal zone interaction showed that binding may be tenuous, involving relatively few host cell-yeast cell surface substances, or tenacious (Fig. 1D and E). These two types of interactions correlated with the observation under bright-field microscopy that some yeast cells appear to be rigidly fixed to splenic tissue, while others tend to waver when the slides are moved.

We previously determined that during experimental candidemia in mice, yeast cells from the circulation become attached to the splenic marginal zone in a pattern identical to that observed in the ex vivo assay (5). This observation was extended in this report to show that within 45 min following the induction of candidemia, the yeast cells become engulfed by marginal zone phagocytic cells. Using high-power bright-field microscopy and TEM, we obtained evidence that yeast cells are ingested by marginal zone host cells that also have the ability to ingest India ink particles. As in the ex vivo assay, *C. albicans* yeast cells are not associated with India ink-positive cells in the red pulp or with host cells that do not take up India ink, such as lymphocytes in the white pulp.

Since phagocytes appeared to be the cell population which supported *C. albicans* adhesion in the splenic marginal zone, we examined further whether binding might be unique to these cells. The ex vivo assay demonstrated that mononuclear phagocytes in the thymus, splenic red pulp, and lymph node paracortex did not bind *C. albicans* (5; this study). We further examined cells isolated from uninflamed peritoneum and peritoneum inflamed for 3 days with thioglycolate (sources rich in resident and inflammatory macrophages, respectively), bone marrow neutrophils (14), and six different myeloid cell lines (WEHI3, WEHI78/24, WEHI274, WEHI265, J774.1, and RAW264) for *C. albicans* adhesion under the conditions of the ex vivo assay at 4°C with no opsonization. None of these cell populations supported binding (data not shown).

Others have demonstrated that *C. albicans* produces integrinlike molecules that bind extracellular matrix proteins and the iC3b complement fragment (7, 8, 10, 15). However, it is unlikely that these molecules are involved in the adherence described here because the yeast cells do not adhere to thymus tissue (5) or to multiple sites within the spleen. Furthermore, the adherence appears to involve yeast cells and specialized phagocytic cells.

The marginal zone is believed to be the site of entry of lymphocytes into the spleen (3, 11); therefore, we tested whether the adhesive event between *C. albicans* and the splenic marginal zone involved known adhesion pathways in mice. This was done by examining the effects of monoclonal antibodies to a variety of known murine adhesion proteins. These included antibodies to integrins (LFA-1 [FD445.1], Mac-1 [M1/70 and SK431], and VLA4 [R1/2]), CD44 (MJ64 and IM7), and LECAM-1 (MEL-14). The antibodies were used to treat both the yeast cells and the tissue section in the ex vivo assay, but these antibodies had no effect on the binding of *C. albicans* yeast cells in the marginal zone.

Our studies indicate that the marginal zone of the spleen contains a unique adhesion system and is involved in the clearance of *C. albicans* from the circulation during fungemia. We propose that splenic marginal zone phagocytes express either a unique adhesion molecule or previously described adhesion molecules with unique binding activities. The adhesion system which binds *C. albicans* yeast cells potentially could be used by other cells, such as host lymphocytes, to gain entry into the spleen.

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