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Evasion of Host Defense by In Vivo-Produced Protoplast-Like Cells of the Insect Mycopathogen *Beauveria bassiana*†

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In vivo cells (hyphal bodies) of the hyphomycetous insect pathogen *Beauveria bassiana* collected from host *Spodoptera exigua* larval hemolymph were osmotically sensitive and lacked a well-defined cell wall. In light and electron microscope studies, a galactose-specific lectin purified from *S. exigua* hemolymph, concanavalin A (specific for α-mannose), and a polyclonal antibody to *B. bassiana* cell walls all bound to surfaces of in vitro-produced *B. bassiana* blastospores; however, none of these probes labelled the thin layer of extracellular material covering the plasma membranes of hyphal bodies. These cells were observed freely circulating in *S. exigua* hemolymph at 36 h postinfection, although immunocompetent hemocytes were known to be present. Additionally, association of hyphal bodies with hemocytes in monolayers was significantly less than for opsonized in vitro blastospores or submerged conidia. The absence of antigenically important galactomannan components on in vivo cells may therefore allow these cells to escape recognition and phagocytosis. Lack of structural components (e.g., chitin, as evidenced by the absence of binding of wheat germ agglutinin) may also be important with respect to evasion of host cellular defense mechanisms. Production of wall material resumed 48 to 60 h postinfection and therefore may coincide with loss of phagocytic capabilities of the hemocytes due to immunosuppressive effects of fungal metabolites. The protoplast-like cells may be formed by the action of hydrolytic enzymes in the hemocytes or by inhibition of fungal cell wall synthetases.

Fungal cell walls are generally composed of chitin and β-glucans as the major structural constituents (8); in addition, other polysaccharides [e.g., α(1→3)glucans] and glycoproteins (e.g., mannoproteins and galactomannoproteins) may form antigenic surface layers that are important in the regulation of the host response to fungal pathogens (23). For example, mannoproteins located in a fibrillar layer on walls of *Candida albicans* cells are apparently multifunctional and therefore interact in a variety of ways with vertebrate host cells (9). They may act as lectins binding to carbohydrate residues on host cell membranes, or, alternatively, carbohydrate (mannan) ligands on the fungal surface may interact with host cell receptors. Similarly, in insects, surface components of potentially pathogenic fungi may determine whether or not the invading cells are recognized by hemocytes (4). Hyphal bodies of the hyphomycete *Nomuraea rileyi* lack specific surface residues which are necessary for opsonization by a galactose-binding humoral lectin present in some lepidopteran hosts (e.g., *Spodoptera exigua*) and are not recognized by the insect hemocytes. Alternatively, blastospores of other hyphomycetes (*Paecilomyces farinosus* and *Beauveria bassiana*) which have the appropriate surface residues may become opsonized and subsequently recognized and phagocytosed by the hemocytes (21).

Regardless of the host system, changes in surface entities due to rearrangement (25) or loss (2, 6, 23) of fungal wall components may affect host-pathogen interaction. With respect to insect mycopathogens, wall-less forms of zygomycetes (*Entomophthorales*) are well known (15), and the absence of wall material appears to be important in relation to the pathogenicity of these fungi. Beauvais et al. (2) showed that hemocytes of *Galleria mellonella* (greater wax moth) larvae encapsulated walled hyphal bodies of *Entomophaga aulicae* but did not react to protoplasts of the fungus. They suggested that chitin and β(1→3) glucan on hyphal body walls mediated recognition of these cells and that protoplasts were able to evade encapsulation because of their lack of cell wall components. In our studies on *B. bassiana*, we have found that in vivo-produced cells (hyphal bodies) of the fungus were freely circulating and therefore appeared to evade recognition by host *S. exigua* hemocytes (12). Since hyphal bodies were also osmotically sensitive, we decided to compare the cell wall characteristics of these cells with those of in vitro-produced blastospores. Consequently, this is the first report of naturally occurring protoplast-like cells from a hyphomycetous insect pathogen.

MATERIALS AND METHODS

Maintenance of fungal cultures. *B. bassiana* UFL 5477 isolated from adult mole crickets, *Scapteriscus vicinus*, was maintained on Sabouraud maltose agar plus 2% yeast extract (SMY) at 25°C. Flasks containing Sabouraud dextrose broth (SDB) or SDB plus 2% yeast extract (SDY) were inoculated with conidia from the agar plates and placed on a shaker for 48 to 60 h. Resultant blastospores were separated from large mycelial fragments by filtering the broth medium through a layer of Miracloth (Calbiochem Co., San Diego, Calif.). Blastospores were washed twice by centrifugation (2,000 × *g*) in deionized water in preparation for various experimental procedures.

Growth and collection of in vivo-produced hyphal bodies. The life cycle of *B. bassiana* within host lepidopteran insects appears to be similar to that of other insect-pathogenic hyphomycetes (16). In our studies, the initial phases of the infection cycle (conidial attachment, germination, and penetration) were bypassed by injecting blastospores directly into hemocoels of *S. exigua* larvae. The larvae were reared

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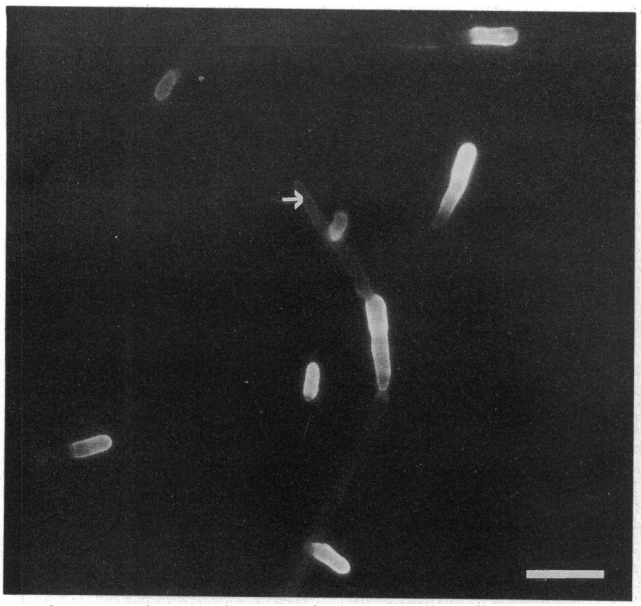

FIG. 1. In vitro-produced *B. bassiana* cells collected 48 h after inoculation of SDB. Fluorescent blastospores were indirectly labelled with galactose-specific lectin purified from *S. exigua* hemolymph. Germ tubes (mycelia) forming from blastospores were less intensely labelled (arrow). Bar, 10 μm.

to the fifth instar on an artificial diet at 26°C (10). Larvae to be treated were immobilized at 4°C for 1 h and then injected into the hemocoel through the proleg with 5×10^2 *B. bassiana* blastospores in 5 μl of 0.85% sterile saline. Injections were done with a 30-gauge needle attached to a 1-ml tuberculin syringe mounted on a microapplicator (ISCO Corp., Lincoln, Nebr.; see reference 12).

Hyphal bodies were collected from the larvae at 48 h postinjection. For electron microscope (EM) cytochemical studies, hemolymph was collected directly into cold 4% paraformaldehyde plus 0.4% glutaraldehyde in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–saline (HS) buffer (0.02 M, pH 7.0). The cells were fixed for 1 h on ice, washed three times by centrifugation (1,000 × *g*) in phosphate-buffered saline (PBS; pH 7.2), and dehydrated in a graded series of ethanol solutions. They were then embedded in LR White resin (Polysciences, Inc., Warrington, Pa.).

Cells collected at 60 h postinjection were fixed in 2% glutaraldehyde in PBS for 1 h and washed three times in PBS. The cells were embedded in 2% agar and postfixed in 1% buffered osmium tetroxide. They were then dehydrated and embedded in Spurr's resin (24).

For light microscope (LM) cytochemical studies, hemolymph was collected into 4% paraformaldehyde in HS from larvae at 48 h postinjection and cells were fixed for 1 h on ice. After being washed in PBS, cells were treated with various cytochemical probes (see below). In vitro blastospores taken from SDB medium at 48 or 60 h postinoculation were processed for EM or LM at the same time as the in vivo cells.

For monolayer experiments, 2 ml of hemolymph taken from larvae at 48 h postinjection were dripped directly into an anticoagulant (EDTA) buffer and hyphal bodies were separated from hemocytes with a Percoll (Sigma, St. Louis, Mo.) gradient (14). Cells were first washed free of sera by centrifugation (1,000 × *g*) for 3 min at 4°C. The cell pellet was suspended in anticoagulant buffer and applied to 10 ml of a preformed gradient (10 to 70%) prepared in 0.2 M sucrose in Tris-EDTA buffer, and the gradient solution was then centrifuged (250 × *g*) for 15 min. The band containing hyphal bodies was collected and washed in HS before being applied to monolayers. In some cases, in vivo cells for LM were also processed by this method.

Cytochemical methods. For LM, a concanavalin A (ConA)-fluorescein isothiocyanate (FITC) conjugate (Sigma) and a wheat germ agglutinin (WGA)-FITC (Sigma) conjugate were used to localize mannose and *N*-acetylglucosamine (chitin), respectively, as previously described (18). Briefly, in vivo hyphal bodies or in vitro blastospores prepared as outlined above were incubated in the conjugates (250 μg/ml of PBS) for 1 h at room temperature. Control solutions contained 0.25 M α-methylmannoside (ConA) or 1 mg of chitotriose per ml (WGA). After incubation, the cells were washed twice in PBS and examined with a Leitz Laborlux S microscope fitted with epifluorescence optics and a Wild MPS 46/52 photomicrographic system. Kodak T-MAX film (ASA 400) was used for photography.

Fungal cells were also indirectly labelled with a galactose-specific lectin purified from *S. exigua* larval hemolymph (5, 17). Briefly, the cells were incubated in the lectin (25 μg/ml of PBS) or a lectin solution containing 0.25 M galactose (control) for 1 h, washed, and incubated for 1 h with a monoclonal antibody (MAb 5H4-2H6) against the lectin diluted 1/10 in PBS. Cells were then labelled with anti-mouse immunoglobulin G-FITC (Sigma) diluted 1/30 in PBS con-

TABLE 1. Carbohydrate analysis of cell walls of in vitro- versus in vivo-produced cells of *B. bassiana* as revealed by various microscopic probes[a]

Probe	Carbohydrate specificity of cells	Labelling[b] of:	
		In vitro-produced cells	In vivo-produced cells
ConA-FITC	α-D-Glucose, mannose	++	+, apical region only
ConA→peroxidase-gold		++, outer wall layer	−
S. exigua lectin→anti-lectin MAb	Galactose	++	−
S. exigua lectin→anti-lectin MAb→anti-mouse-gold		++, outer wall layer	−
WGA-FITC	*N*-Acetylglucosamine	±, apical region	NT
WGA→ovalbumin-gold		++, inner wall region	+, septa only
Polyclonal antibody→anti-rabbit immunoglobulin G–gold	In vitro *B. bassiana* cell wall	++, outer wall layer	−

[a] Fungal cells were harvested at 48 h postinoculation of SDB or postinjection of *S. exigua* larvae (500 blastospores per larva). LM probes labelled the cell surfaces, whereas EM probes were used on thin-sectioned material.

[b] ++, strong labelling; +, moderate labelling; ±, weak labelling; −, no labelling; NT, not tested.

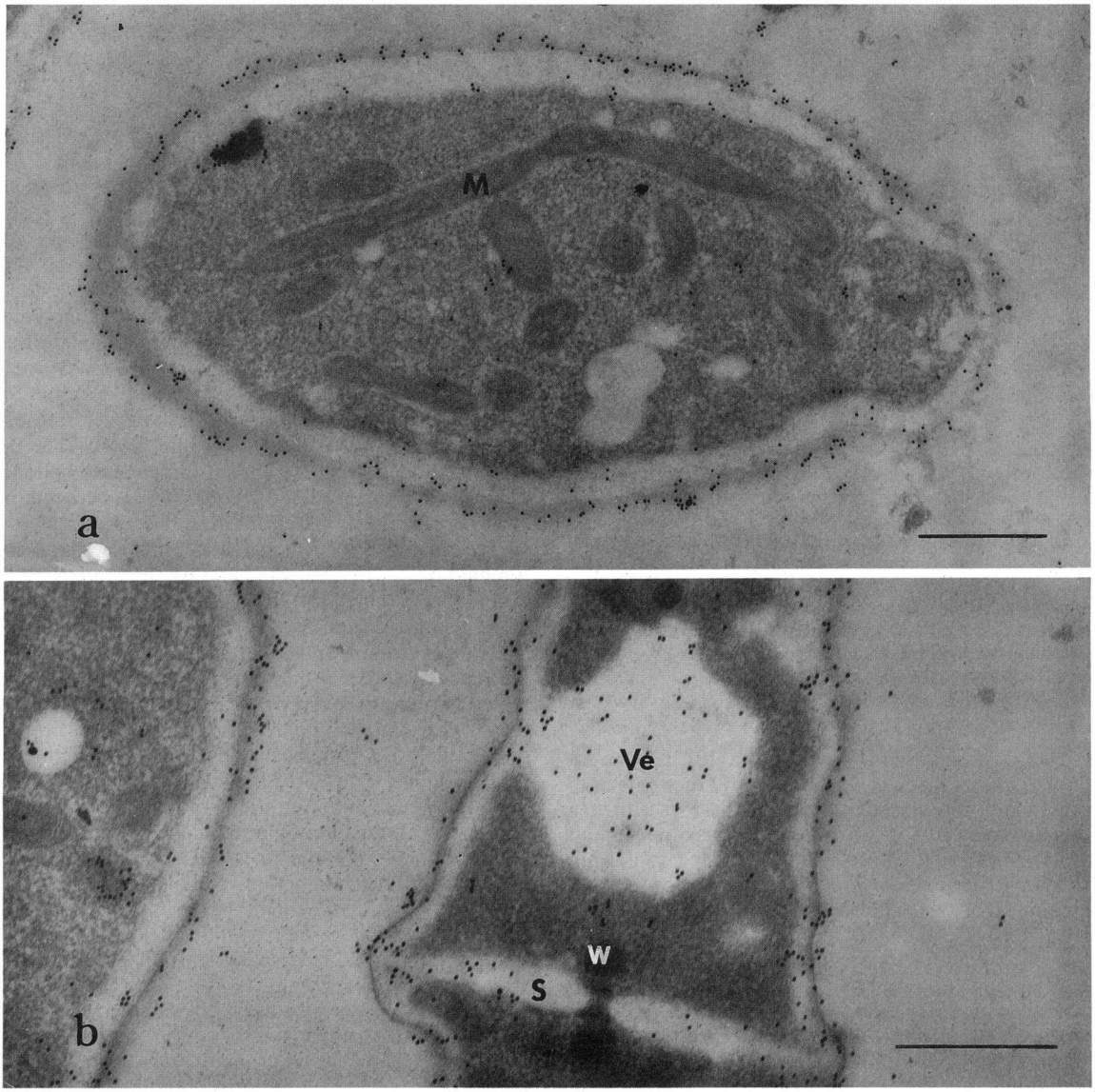

FIG. 2. Thin sections of in vitro-produced *B. bassiana* cells labelled with *S. exigua* lectin. In panel a, an outer galactose-containing layer on the blastospore is heavily labelled, while in panel b, labelling is apparent on the mycelial surface and in cytoplasmic vesicles. The vesicles (Ve) containing the lectin-binding component appear to be a source of the wall material. M, mitochondrion; S, septum; W, Woronin body. Bar, 0.5 μm.

taining 1% bovine serum albumin (BSA), washed, and examined. MAbs were prepared at the Hybridoma Core Laboratory, University of Florida, Gainesville, and screenings of hybridoma supernatants by enzyme-linked immunosorbent assay were also done at this facility. Positive supernatants were then further screened in our laboratory by fluorescence microscopy (19, 20).

For EM cytochemistry, labelling of the LR White-embedded material with *S. exigua* lectin and WGA was done as previously described (19). Thin sections on Formvar-coated nickel grids were preincubated in PBS-BSA. They were then incubated in purified *S. exigua* lectin (25 μg/ml of PBS-BSA) or in lectin solution containing 0.25 M D-galactose (control) for 1 h, washed in PBS-BSA, and incubated in hybridoma supernatant (MAb 5H4-2H6) diluted 1/100 in PBS. After 1 h, grids were washed several times in PBS-BSA and incubated for 30 min in an anti-mouse immunoglobulin G–gold conjugate (Sigma) diluted 1/20 in PBS-BSA. Grids were finally washed in PBS and water, poststained in uranyl acetate and lead citrate, and examined with a JEOL JEM 100CX EM. Additional controls included omission of the lectin or MAb.

For localization of chitin, preblocked grids were incubated for 1 h in unconjugated WGA (100 μg/ml of PBS-BSA), washed in PBS, and transferred to an ovomucoid-gold conjugate (Sigma) diluted 1/20 in PBS containing 0.2 mg of polyethylene glycol (molecular weight, 15,000 to 20,000) per ml. After 30 min, grids were rinsed in PBS and water and poststained. Control WGA solutions contained 1 mg of chitotriose per ml; for other control grids, WGA was omitted from the labelling sequence.

ConA labelling was accomplished by incubating preblocked grids in 100 μg of ConA per ml of PBS-BSA or in a

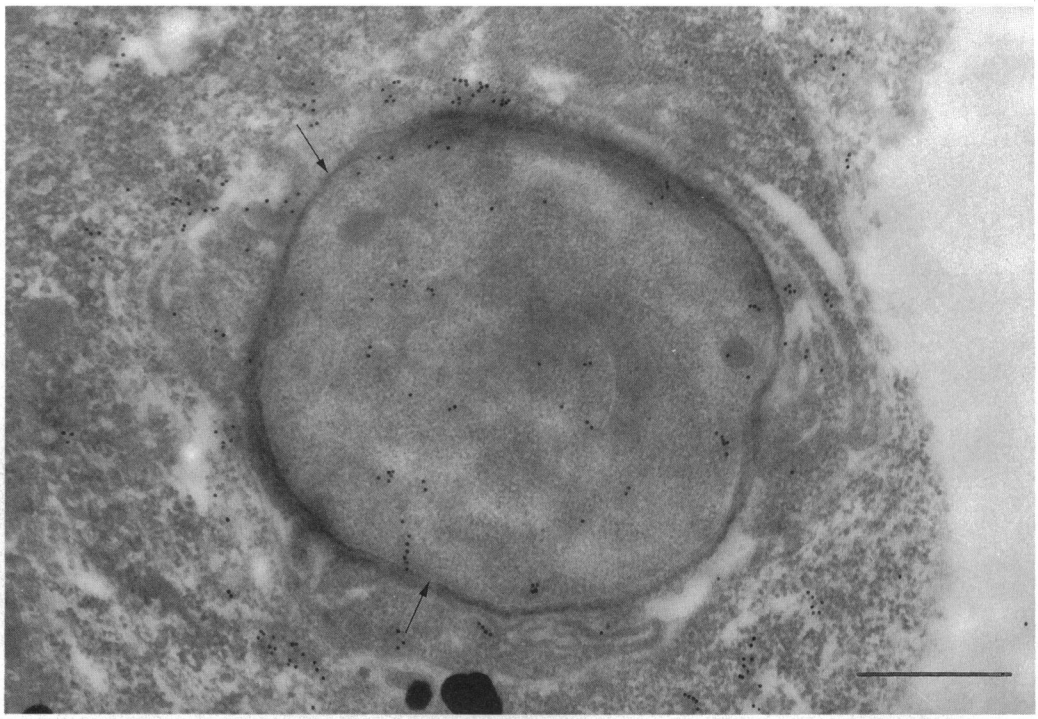

FIG. 3. Thin section of an in vivo-produced *B. bassiana* hyphal body located in an *S. exigua* larval hemocyte at 48 h postinjection. There is no well-defined fungal cell wall, and only a few scattered gold particles are evident after labelling with the *S. exigua* lectin. The arrows indicate the fungal cell plasma membrane. Bar, 0.5 μm.

ConA solution containing 0.25 M α-methylmannoside (control) for 1 h and then incubating them in a peroxidase-gold conjugate (Sigma) diluted 1/20 in PBS-BSA plus 0.05% Tween 20 (Sigma). After 30 min, grids were washed and poststained as described above.

Thin sections were also labelled with a polyclonal antibody against walls from in vitro-produced *B. bassiana* cells. The antibody was produced in rabbits (Kel-Farms, Alachua, Fla.), and serum was screened at our facility by enzyme-linked immunosorbent assay. Thin sections were labelled for 1 h with serum from a challenged rabbit or in preimmune rabbit serum (control) diluted 1/400 in PBS-BSA. After being washed in PBS-BSA, grids were incubated for 30 min in anti-rabbit immunoglobulin G–gold (Sigma) diluted 1/20, washed, and poststained.

Preparation and treatment of hemocyte monolayers. Hemolymph was collected directly into HS and further diluted with this buffer to a concentration of 1.2×10^5 granulocytes per ml. Granulocytes constituted the predominant type of hemocyte forming the monolayer. Twenty microliters of this suspension was applied to a glass slide, the hemocytes were allowed to attach for 10 min, and the resultant monolayers were washed with HS. Fungal cells (see Results) to be tested on monolayers were harvested from SDB (submerged conidia; see reference 11) or SDY (blastospores) medium at 48 h postinoculation, filtered, washed, and suspended in HS. Some of the blastospores (10^7/ml) were opsonized in 100 μg of *S. exigua* lectin per ml of HS for 1 h, washed, and suspended in HS. Preparation of in vivo hyphal bodies for monolayers has been outlined above. Approximately 10^4 fungal cells in HS were applied to each monolayer and allowed to incubate for 30 min. Monolayers were then washed with HS and examined. The percentage of granulocytes with one or more surface-adherent or endocytosed fungal cells (percent association) was determined after counting 500 to 600 granulocytes per monolayer; three to five monolayers were made for each fungal type.

RESULTS

LM and EM cytochemistry. Binding of galactose-specific *S. exigua* humoral lectin to surfaces of in vitro-produced *B. bassiana* cells is shown in Fig. 1. Fluorescence was especially intense on blastospore surfaces; in comparison, mycelia forming from these cells were weakly labelled (arrow) although stronger fluorescence was often observed at apical regions. As previously reported, ConA-FITC bound to in vitro surfaces in a similar pattern and labelling by WGA-FITC usually occurred only at apical regions (18; see Table 1). With the exception of occasional fluorescence at apical regions of ConA-treated cells, no labelling was observed when in vivo-produced hyphal bodies were tested with these probes (Table 1). Such results indicated either that a galactomannan surface layer was not being produced in vivo or that host-derived components were blocking the ability of the lectins to bind to the fungal surface; EM cytochemistry of thin sections was therefore used to further define cell wall characteristics.

As shown in Fig. 2, in vitro-produced cells were labelled in specific areas when thin sections were probed with *S. exigua* lectin. Labelling was restricted to the cell wall surface layer and to vesicles in the cytoplasm (Fig. 2b); such vesicles containing the lectin-binding component appeared to be a source of wall material. ConA and a polyclonal antibody against *B. bassiana* cell wall fragments stained the in vitro

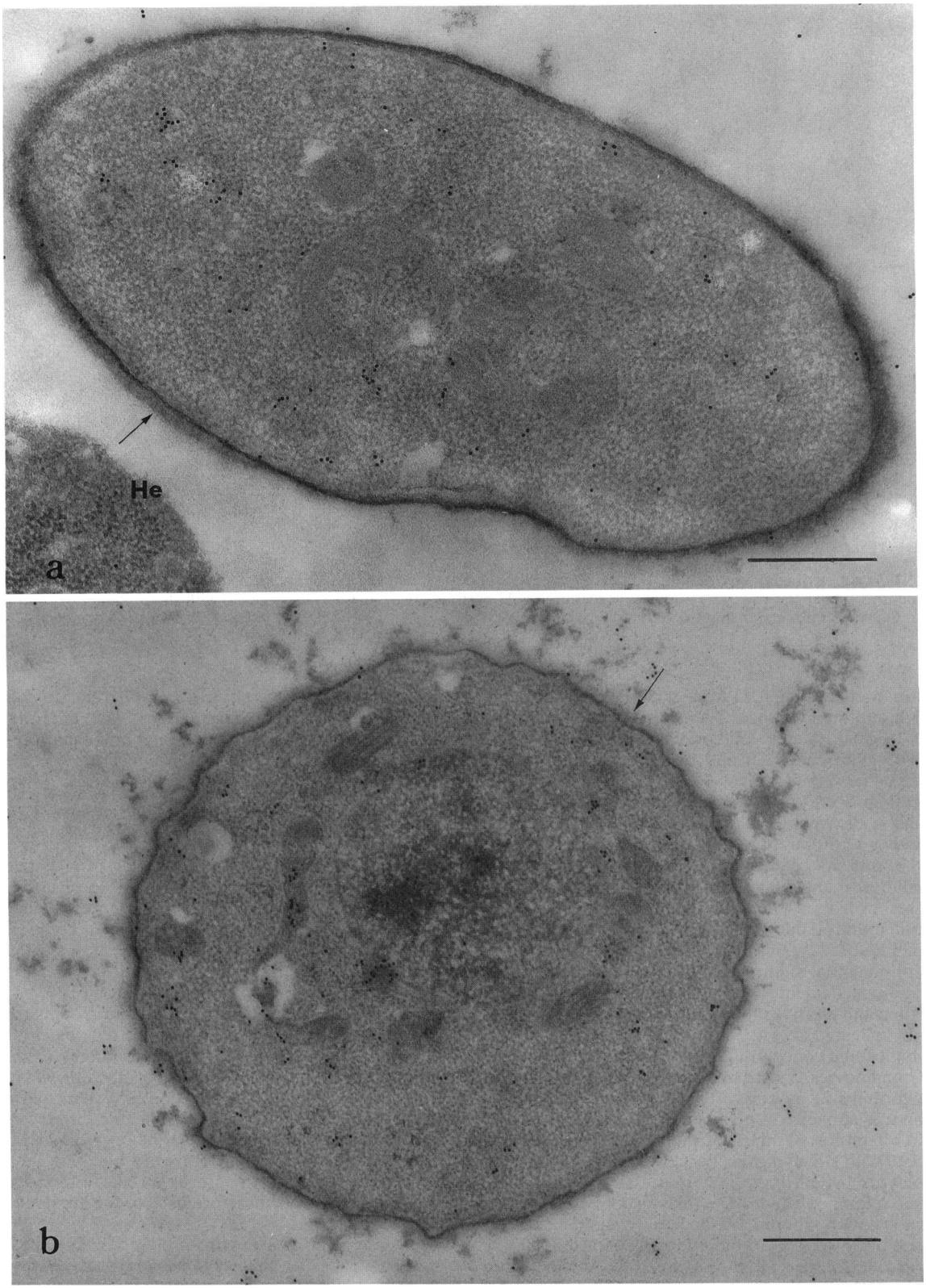

FIG. 4. Freely circulating *B. bassiana* hyphal bodies collected from *S. exigua* hemolymph at 48 h postinjection. Only a thin layer of fibrillar material (arrows) covers the plasma membrane of each cell, and there is no specific labelling of this material with the *S. exigua* lectin. In panel a, the typical oblong shape of a hyphal body is shown in longitudinal section. He, hemocyte. A cross-section of a hyphal body is shown in panel b. Note the convoluted appearance of the plasma membrane. Bars, 0.5 μm.

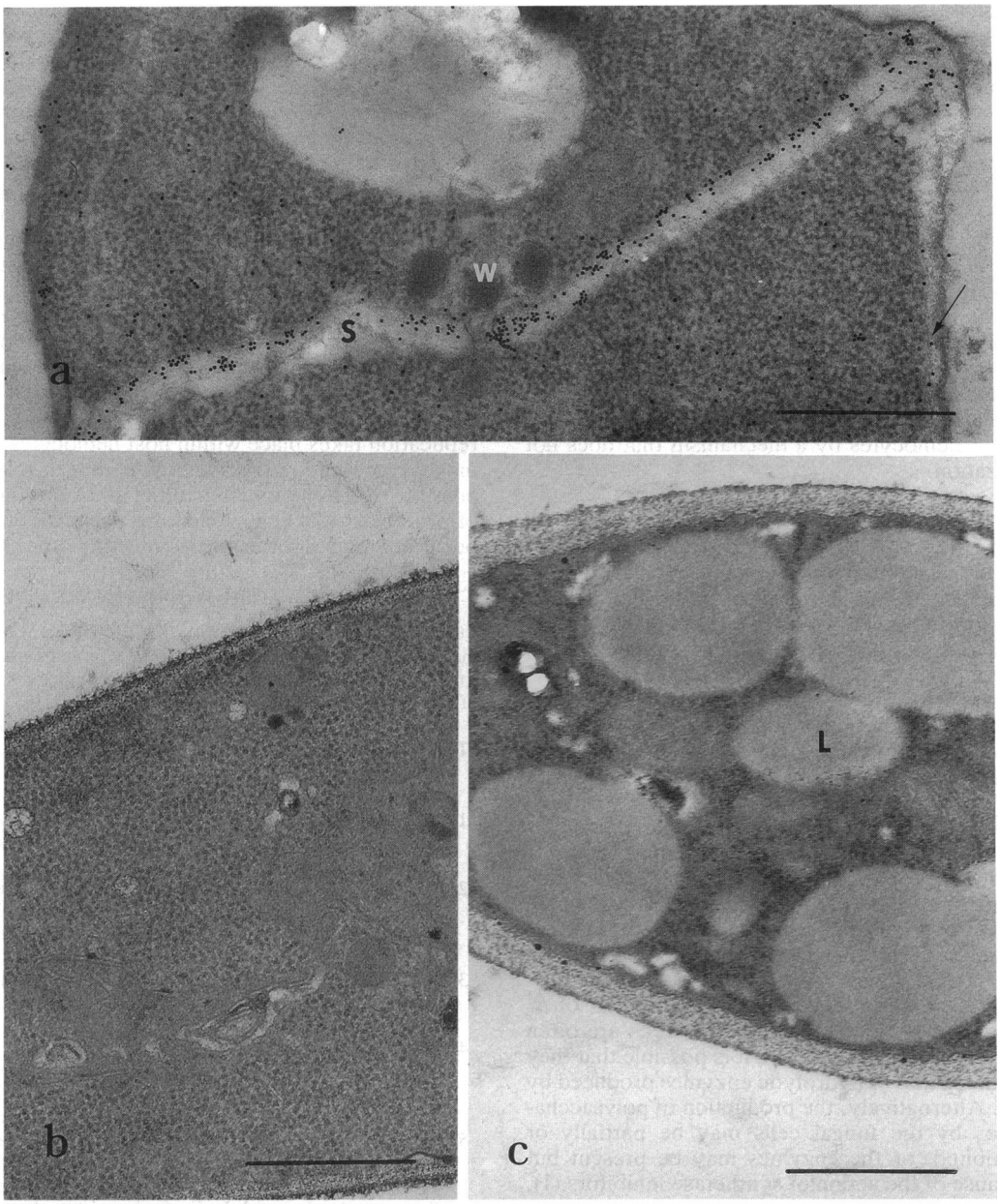

FIG. 5. (a) *B. bassiana* in vivo-produced hyphal body at 48 h postinjection. The septum (S) is heavily labelled with WGA, but there is only sparse labelling along the lateral wall (arrow). W, Woronin body. (b) In vivo-produced hyphal body at 60 h postinjection. The cell wall is only 28 nm thick. (c) In vitro-produced cell at 60 h postinoculation of SDY broth. The cell wall is 75 nm thick. L, lipid. Bars, 0.5 μm.

cells in a similar pattern, whereas WGA bound to an inner region of the cell wall near the plasma membrane (Table 1).

Surprisingly, thin sections of in vivo-derived hyphal bodies revealed that only a sparse layer of extracellular material covered the plasma membrane (Fig. 3 and 4). In Fig. 3, the hyphal body was located within a hemocyte and, as can be seen, the *S. exigua* lectin did not bind to the fungal cell, even in the region outside the plasma membrane. Other hyphal bodies, which were freely circulating (Fig. 4), showed a similar lack of labelling with the *S. exigua* lectin, ConA, and the polyclonal antibody to *B. bassiana* cell wall fragments (Table 1). Protoplast-like hyphal bodies were typically oblong in shape as seen in longitudinal section (Fig. 4a). The plasma membrane of cells in cross-section sometimes had a ruffled appearance (e.g., Fig. 4b), further indicating the absence of structurally rigid wall material.

Although most of the in vivo cells collected at 48 h postinjection lacked a distinct cell wall, some wall material was visible on cells which were forming hyphae; however, there was no labelling of these walls with any of the probes tested except WGA, which bound only to septa and not to lateral wall material (Fig. 5a). Wall regeneration became more evident at 60 h postinjection, when a thin (28-nm) layer of wall material was visible on the hyphal bodies (Fig. 5b). Walls on cells from culture media were, in comparison, thicker (75 nm) and better defined (Fig. 5c).

Hemocyte monolayer experiments. Results of in vitro hemocyte monolayer experiments confirmed that the lack of phagocytosis of hyphal bodies observed in hemolymph at 36 to 48 h postinjection was not just due to possible immunosuppressive effects of fungal toxins (12). In an experiment in which association of *B. bassiana* cells with granulocytes in *S. exigua* hemocyte monolayers was tested, the percentages of granulocytes with one or more surface-adherent or endocytosed fungal cells were as follows: submerged conidia, 83.3%; nonopsonized blastospores, 5.3%; opsonized blastospores, 42%; in vivo-derived hyphal bodies, 11.8%. Association of in vivo-derived hyphal bodies with phagocytic granulocytes was therefore significantly lower than that for submerged conidia or opsonized blastospores, and usually occurred at apical regions of the fungal cells, where some ConA-binding material was detected (Table 1). The presence of such material (e.g., mannoproteins) may allow hyphal bodies to bind to hemocytes by a mechanism that does not require opsonization.

DISCUSSION

Evasion of the host immune response by changing or shedding of components on surfaces of pathogenic organisms has been reported to be an effective strategy in a variety of parasitic systems, e.g., nematodes (3) and protozoans (22) that infect vertebrate hosts. The existence of wall-less hyphal bodies may therefore be an important factor in the pathogenicity of *Beauveria bassiana* for beet armyworms. Shedding of the galactomannan coat is in itself significant, since this layer bears receptors for the opsonic hemolymph lectin, as well as other molecules (e.g., mannoproteins) which, depending upon the morphological state of the fungus (e.g., submerged conidia; see reference 11), may allow for recognition and phagocytosis regardless of opsonization (see Results). The additional loss of underlying wall material may further enhance the ability of fungal cells to escape insect defense mechanisms, since such components [chitin and β(1→3) glucans] could also serve as recognition sites (2).

The process by which in vivo protoplast-like cells of *B. bassiana* are formed is unknown. Since the cells are often observed within granulocytes (Fig. 3), it is possible that they are formed by the action of hydrolytic enzymes produced by the hemocytes. Alternatively, the production of polysaccharide synthetases by the fungal cells may be partially or completely inhibited, or the enzymes may be present but ineffective because of the action of synthetase inhibitors (1). Whatever the mechanism, the existence of the wall-less state appears to be to the advantage of the fungus.

To summarize the in vivo life cycle of the fungus, it is probable that injected in vitro blastospores become opsonized and phagocytosed and that loss of the cell wall is a postphagocytic event. The wall-less cells may then replicate within the hemocytes (e.g., see references 7 and 13), as indicated by limited EM observations of budding hyphal bodies within granulocytes. The confinement of fungal elements within host cells (26) may be the reason why no freely circulating hyphal bodies are observed until approximately 36 h postinfection. Hyphal bodies released at this time are not recognized in vivo by hemocytes, which have been shown to be immunocompetent at this stage of infection (12). In addition, the hyphal bodies attach to granulocytes in monolayers at a much lower rate than opsonized in vitro blastospores or submerged conidia, and when they do attach it is at apical regions where, as indicated by fluorescence studies, ConA-binding components (mannans) may be present (Table 1). At the later stages of infection (48 to 60 h postinjection), hemocytes are no longer capable of a defensive response, probably because of the immunosuppressive effects of fungal metabolites (12). Fungal cells, even those in which extensive wall regeneration has occurred, are therefore not phagocytosed and are able to replicate and complete the infection cycle (16).

It is unclear whether the fibrillar material outside the plasma membrane of hyphal bodies is of fungal or host origin. Polyclonal antibodies to *S. exigua* hemolymph and hemocyte lysates bind to the hyphal body surface, as observed in fluorescence studies. However, these antibodies also bind to surfaces of in vitro-produced *Beauveria* blastospores, and further studies (cross-absorptions and cross-blotting) are necessary before a conclusion can be made. We will also continue microscopic studies aimed at elucidating the in vivo life cycle of the fungus and confirming that replication takes place within host hemocytes. Although our model represents a specific host-pathogen relationship, it is possible that loss of wall material and subsequent formation of protoplast-like cells which are not recognized as nonself occur among fungi capable of infecting other types of hosts.

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