

The Role of Surface Components of the Entomopathogenic Fungus *Entomophaga aulicae* in the Cellular Immune Response of *Galleria mellonella* (Lepidoptera)

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Larvae of *Galleria mellonella* (Lepidoptera) displayed a strong cellular reaction to the walled-stage hyphal bodies of the entomopathogenic fungus *Entomophaga aulicae* (Entomophthorales). In contrast, no reaction was observed against the wall-less protoplasts. This phenomenon was not due to an inhibition of the cellular response by secretion of a toxin by protoplasts. Furthermore, no molecular mimicry was observed between host and protoplast antigens. Host macromolecules which could protect them from being recognized as foreign did not attach to the protoplasts. Ultrastructural and chemical studies of hyphal body and protoplast surfaces of *E. aulicae* demonstrated the presence of 1,3- β -glucan and chitin on the hyphal wall, whereas these components were absent on the protoplast surface. Since purified 1,3- β -glucan and chitin isolated from *E. aulicae* and from another entomophthoralean species, *Conidiobolus obscurus*, induced their haemocytic encapsulation *in vitro*, it is suggested that these wall components are responsible for the recognition of hyphal body elements, and that their absence accounts for the ability of protoplasts to escape encapsulation.

INTRODUCTION

The immune system of insects responds to the invasion of fungal pathogens in a multicellular rather than humoral fashion (Rowley & Ratcliffe, 1981; Vey & Götz, 1986). Infection by species of Entomophthorales, following the attachment of adhesive conidia to the insect cuticle, involves penetration into the haemocoel during germ tube formation and the subsequent development of walled hyphal bodies or naked protoplasts (Brobyn & Wilding, 1977; Nolan, 1985). The latter escape encapsulation by host haemocytes (Dunphy & Nolan, 1980).

The main defence reaction by the lepidopteran *Galleria mellonella* to fungal pathogens present in the haemocoel is the formation of multicellular granulomata, which engulf foreign elements upon recognition by haemocytes (Ratcliffe & Walters, 1983; Rowley & Ratcliffe, 1979; Vey, 1969). This involves prophenoloxidase cascade activation, resulting in melanin deposition, at the centre of the nodule enrobing the invader (Pye, 1978). Because the immune reaction of *G. mellonella* has been relatively well studied, and *Entomophaga aulicae* (Entomophthorales) produces both hyphal bodies and protoplasts *in vitro* (Beauvais & Latgé, 1988) this system was

Abbreviations: Anti-Gm, antiserum directed against *G. mellonella* haemolymph; anti-P, antiserum directed against *E. aulicae* protoplasts; Con A, concanavalin A; FCS, foetal calf serum; FITC, fluorescein isothiocyanate; PBS, 0.05 M-sodium phosphate buffer containing 0.15 M-NaCl; PNA, pea agglutinin; RCA₁₂₀, *Ricinus communis* agglutinin; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TRITC, tetra-rhodamine isothiocyanate; WGA, wheatgerm agglutinin.

chosen to investigate the role of fungal surface components in the elicitation or the inhibition of the multicellular insect defence reaction.

METHODS

Fungal material. *E. aulicae* (isolate 818) was isolated from *Choristoneura fumiferana* (Centre de Foresterie des Laurentides, Québec, Canada).

Protoplasts of *E. aulicae* were initially obtained after inoculation of a hyphal body suspension in liquid GLEN medium, containing 0.4% (w/v) glucose, 0.5% (w/v) yeast extract, 0.65% (w/v) lactalbumin hydrolysate, 0.77% (w/v) NaCl and supplemented with 10% (v/v) FCS (Serva) (Beauvais & Latgé, 1988). Successive transfers were maintained in this medium in unshaken flasks at 20 °C. Protoplasts were collected and washed with PBS by centrifugation at 3000 g for 5 min.

Hyphal bodies of *E. aulicae* were produced at 20 °C in liquid culture in shaken flasks containing 6% (w/v) glucose and 2% (w/v) yeast extract. They were collected and washed with PBS by centrifugation (8000 g, 5 min).

Hyphal walls of *E. aulicae* were prepared as described by Latgé *et al.* (1984). 1,3- β -Glucan and chitin were extracted from the walls of *E. aulicae* and of another entomophthorean species, *Conidiobolus obscurus*, by successive base and acid treatments (Latgé *et al.*, 1984). The purity of the fractions was ascertained by incubating 1,3- β -glucan (2 mg ml⁻¹) extract with a mixture of chitinase (2 mg ml⁻¹; Serva) and papain (2 mg ml⁻¹; Sigma) or chitin (2 mg ml⁻¹) with a mixture of exo-1,3- β -glucanase (2 mg ml⁻¹; laminarinase from mollusk, Sigma) and papain (2 mg ml⁻¹) in sodium acetate buffer, pH 5.3. Polysaccharide fractions were washed with 0.15 M-NaCl and stored freeze-dried.

Insects. The insects used were fifth-instar larvae of *Galleria mellonella* (Lepidoptera) from the insectarium of the Station de Recherches de Pathologie comparée, INRA, Saint-Christol-les-Alès, France.

Antisera. Antisera directed against the protoplasts of *E. aulicae* (anti-P) and against the haemolymph of *G. mellonella* (anti-Gm) were prepared as follows.

Anti-P was obtained by injecting a rabbit subcutaneously at several points along the spine and through the footpad with 25 mg lyophilized *E. aulicae* protoplasts suspended in 0.15 M-NaCl/Freund's complete adjuvant (1:1, v/v). Three weeks later, the animal was boosted with 15 mg lyophilized *E. aulicae* protoplasts suspended in 0.15 M-NaCl/Freund's incomplete adjuvant (1:1). Beginning one month later, intravenous inoculations of 10 μ g protoplast protein in 0.15 M-NaCl were performed for one month at a rate of two injections per week. The presence of specific antibodies in the antiserum was verified by fused rocket immunoelectrophoresis (Laurell, 1965).

For preparation of anti-Gm, BALB/c mice were injected subcutaneously with 50 μ g protein of larval haemolymph (obtained as described below) diluted in 0.1 ml PBS/Freund's complete adjuvant (1:1) once a week for three weeks. The presence of specific antibodies was verified by ELISA. Plates were coated with *G. mellonella* proteins at a concentration of 0.33 mg ml⁻¹ in 0.05 M-sodium carbonate buffer, pH 9.6. Anti-Gm was added at 0.1% dilution and antibody binding was detected using an anti-mouse-IgG-peroxidase conjugate.

Fungus-insect confrontation. (a) *In vivo injection experiments.* The following fungal materials, suspended in 0.15 M-NaCl, were injected into the haemocoel of 10 larvae per experiment: (i) protoplasts of *E. aulicae* (minimum 10 per insect); (ii) protoplasts (30–100 per insect) killed by fixation for 1 h in PBS containing 2.5% (w/v) paraformaldehyde, or by irradiation (90 Gy min⁻¹); (iii) protoplasts preincubated for 1 h in PBS/1% BSA containing 1/200 anti-P, and washed with PBS/0.1% BSA, then 0.15 M-NaCl; (iv) hyphal bodies of *E. aulicae* (10–40 per insect); (v) wall extract; (vi) culture filtrate from *E. aulicae* protoplasts after 1, 3, 5 and 7 d growth. Control larvae were injected with 0.15 M-NaCl.

(b) *In vitro experiments.* To collect haemolymph and haemocytes from the general cavity of the larvae, an incision was made with a scalpel on one proleg after anaesthetization at 4 °C for 1 h. Haemolymph was taken up with a capillary tube and directly added to an equal amount of the fungal material suspended in 0.15 M-NaCl on a microscope slide. The following fungal samples were tested. (i) protoplasts (haemocyte: protoplast ratio 10:20:1); (ii) hyphal bodies (haemocyte: hyphal body ratio 20:1); (iii) 1,3- β -glucan or chitin; (iv) anti-P-treated protoplasts as described above.

The preparation was gently mixed for 5 min, and observed under a light microscope. Controls comprised a drop of haemolymph mixed with an equal drop of 0.15 M-NaCl; for anti-P-treated protoplasts, a drop of hyphal bodies in 0.15 M-NaCl containing 1/200 anti-P was added to a drop of haemolymph. Experiments were done before melanization began.

Surface labelling of protoplasts and hyphal bodies. (a) *Lectin labelling experiments.* Localization of α -D-mannose or α -D-glucose, D-galactose and N-acetyl-D-glucosamine residues on the surface of protoplasts and hyphal bodies grown *in vitro* was done with FITC conjugates of Con A (50 μ g ml⁻¹; target α -D-mannose, α -D-glucose; Sigma), WGA (50 μ g ml⁻¹; target N-acetyl-D-glucosamine; Sigma) or RCA₁₂₀ (50–200 μ g ml⁻¹; target β -D-galactose; Sigma), and TRITC-conjugated pea agglutinin (PNA) (50 μ g ml⁻¹; target D-galactose; Sigma). Fungal cells were incubated for 1 h in the lectin solutions in PBS, then washed with PBS and observed under a fluorescent microscope. Binding of the lectins to protoplast surfaces was also tested using transmission electron microscopy

(TEM). Protoplasts were fixed for 1 h in a solution of PBS containing 2.5% (v/v) glutaraldehyde. After several washes in PBS, they were incubated for 1 h in ConA or WGA (each $50 \mu\text{g ml}^{-1}$), and then incubated for 1 hour with the specific ferritin-conjugated sugar (mannosyl-ferritin or chitobiosyl-ferritin, both from IBF) at the concentration of $250 \mu\text{g ml}^{-1}$ in PBS. After washing with PBS, the protoplasts were post-fixed in 0.1 M-sodium cacodylate containing 1% (v/v) osmium tetroxide and prepared for TEM as outlined below. Specific sugar inhibitor (Sigma), methyl α -D-mannopyranoside (0.3 M), di-N-acetylchitobiose (0.1 M), D-galactose (0.3 M) and lactose (0.3 M) were preincubated with the respective lectins and the solutions were used as control.

(b) *Localization of 1,3- β -glucan.* The presence of 1,3- β -glucan on the surface of the protoplasts and hyphal bodies was investigated using a rabbit anti-laminaribiosyl serum prepared by M. Horisberger and M. Rouvet-Vauthey (Horisberger & Rouvet-Vauthey, 1985) and aniline blue as a specific fluorochrome for 1,3-linked glucan (Currier & Stigger, 1956). For the aniline blue experiments, protoplasts and hyphal bodies were incubated for 1 h in 0.066 M- K_2HPO_4 containing 0.15 M-NaCl and 0.1% aniline blue. After washing with PBS containing 25% (v/v) glycerol, they were examined with a fluorescent microscope, with excitation at 300–400 nm. Cellulose was used as a negative control. For experiments with the anti-laminaribiosyl serum, protoplasts and hyphal bodies were: (i) fixed in a solution of PBS containing 2.5% paraformaldehyde and 0.1% glutaraldehyde for 1 h; (ii) washed with PBS containing 10 mM- NH_4Cl and incubated for 1 h in the same solution; (iii) washed with PBS and incubated for 1 h in the anti-laminaribiosyl antiserum diluted 1/10 in PBS/1% BSA; (iv) washed with PBS/0.1% BSA and incubated for 1 h in a solution of anti-rabbit IgG coupled to 40 nm colloidal gold (GAR 40, Jansen) and diluted to 2.5% in PBS/1% BSA. After washing with PBS/0.1% BSA and then PBS, the material was fixed, and prepared for scanning electron microscopy (SEM) as described below. Anti-laminaribiosyl serum at a 1/10 dilution previously incubated for 24 h at 37 °C in the presence of 50 mg laminarin ml^{-1} was used as control.

(c) *Surface charge.* The presence of anionic sites on the surface of protoplasts and hyphal bodies was detected by incubating the fungal cells in PBS containing cationized ferritin (250 – $500 \mu\text{g ml}^{-1}$). After 1 h incubation, cells were washed with PBS and subsequently prepared for TEM as outlined below.

(d) *Immunocytochemical labelling.* Twenty-four hours after infection with protoplasts, protoplasts and haemocytes were recovered from the haemolymph by centrifugation at 3000 g. After fixation for 1 h in PBS containing 2.5% paraformaldehyde and 0.1% glutaraldehyde and washing with PBS containing 10 mM- NH_4Cl and then PBS, protoplasts and haemocytes were incubated for 1 h in a solution of PBS/1% BSA containing anti-P or anti-Gm (1/200) and subsequently incubated for 45 min at 25 °C in a solution of PBS/1% BSA containing an FITC-conjugated anti-rabbit or anti-mouse IgG (1/400) (Biosis). Protoplasts and haemocytes were washed and observed under a fluorescent microscope. Non-immunized rabbit or mouse antisera were used as controls.

A similar experiment was performed on protoplasts incubated *in vitro* for 10 min with haemolymph from an uninfected larva.

Histology and electron microscopy. For light microscopy, inoculated larvae were fixed in Carnoy's fixative after 30 min to 8 d infection, and embedded in Paraplast. Sections (4–7 μm) were stained by the periodic acid/Schiff reaction, haemalun-picro-indigo carmine and modified Heidenheim's Azan technique (Ganter & Jollès, 1970).

All samples for electron microscopy were fixed in 2.5% glutaraldehyde for 1 h at room temperature or overnight at 4 °C, and post-fixed in 1% osmium tetroxide for 1 h at room temperature in 0.1 M sodium cacodylate pH 7.2. Dehydration and embedding in Epon 812 was performed as previously described (Latgé *et al.*, 1982). Infected larvae for TEM observation were fixed by an injection into the haemocoel of 0.15 M-NaCl containing 2.5% glutaraldehyde and immersed for 1 h in 0.1 M-sodium cacodylate containing 25% glutaraldehyde. Fixed larvae were cut into 2–3 mm pieces and washed with 0.1 M-sodium cacodylate as indicated above.

Material used for SEM was dehydrated (Samson *et al.*, 1979), deposited on glass coverslips coated with 0.1% poly-L-lysine, then critical-point dried, and examined (JEOL) using a back-scattering electron device.

RESULTS

Morphology of hyphal bodies and protoplasts

E. aulicae hyphal bodies were similar to those of other species of Entomophthorales: in liquid culture, unicellular multinucleate elements developed, being characterized by a one-layered wall of fibrillar structure, many glyoxysome-like bodies with a crystalline electron-dense inclusion, and nuclei containing a nucleolus and patches of heterochromatin (Figs 1, 2 and 3).

Protoplasts of *E. aulicae* in GLEN medium developed as spindle-shaped protoplasts giving rise to spherical structures containing one vacuole (Fig. 4a). The size of these spheres increased, with a voluminous central vacuole and a compartmental cytoplasm at the periphery (Fig. 4b). Later a chain of spindle-shaped protoplasts formed (Fig. 4c), and dissociated into individual spindle-shaped protoplasts. TEM observations confirmed the absence of wall in all protoplast stages, which also contained fewer lipid inclusions and glyoxysome-like bodies than hyphal

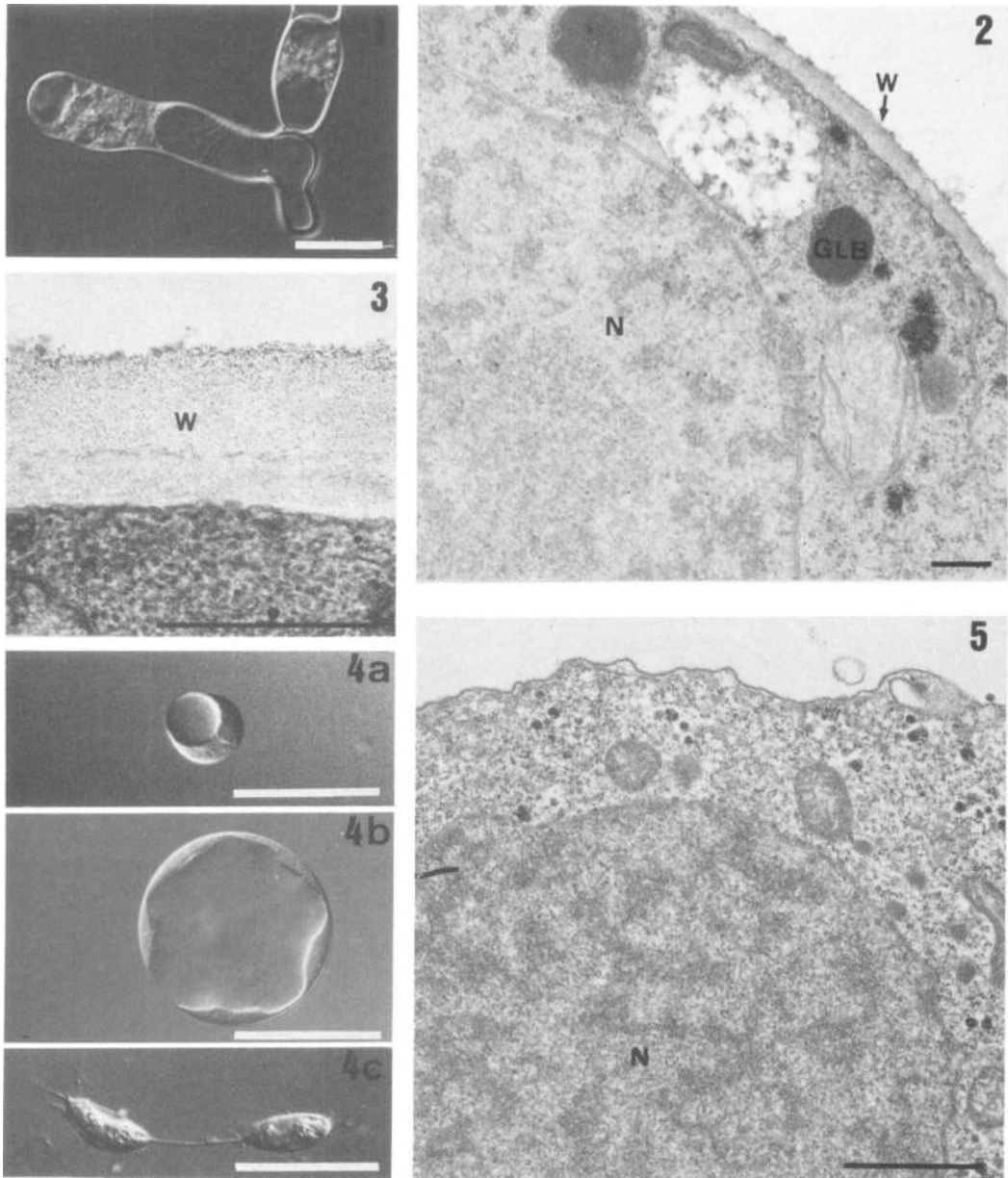


Fig. 1. Hyphal bodies of *E. aulicae* in liquid culture medium. Bar, 10 μm .

Fig. 2. Ultrastructural organization of the hyphal body. Note the nucleus (N) with abundant heterochromatin and one nucleolus, the glyoxysome-like bodies (GLB) and the one-layered wall (W). Bar, 1 μm .

Fig. 3. Transmission electron micrograph showing the fibrillar structure of the wall (W). Bar, 0.5 μm .

Fig. 4. (a-c). Successive stages of development of the protoplasts in liquid GLEN medium: spherical protoplast (a), protoplasmic sphere with a voluminous central vacuole and compartmental cytoplasm at the periphery (b), and chain of spindle-shaped protoplasts (c). Bars, 10 μm .

Fig. 5. Ultrastructural organization of a protoplast. Note the nucleus (N) with abundant heterochromatin and the absence of wall. Bar, 1 μm .

Table 1. Reactions of hyphal bodies and protoplasts to different markers, showing the chemical composition of their surface

Marker	Target	Reaction of:	
		Hyphal bodies	Protoplasts
Anti-P	Protoplasts	—	+
Con A (50 µg ml ⁻¹)	α-D-Mannose, α-D-glucose	+++	+
WGA (50 µg ml ⁻¹)	N-Acetyl-D-glucosamine	++	—
PNA (50 µg ml ⁻¹)	β-Galactose	—	—
RCA (50–200 µg ml ⁻¹)	β-D-Galactose	—	—
Aniline blue	1,3-Glucan	++	—
Anti-laminaribiosyl serum	1,3-β-Glucan	++	—
Cationized ferritin (250–500 µg ml ⁻¹)	Anionic sites	—	—

bodies; nuclei were also characterized by abundant heterochromatin and the absence of a visible nucleolus (Fig. 5).

Cellular immune response of G. mellonella to hyphal bodies and protoplasts

After injection of 10 to 40 hyphal bodies per larva, no disease appeared. The larvae continued their growth until the adult stage.

Histological observations demonstrated that as early as 5 min after the confrontation of hyphal bodies and haemocytes *in vitro*, or after injection of the hyphal bodies into the insect body, the fungus was encapsulated and melanized by the haemocytes (Fig. 6). The granuloma increased in size and the fungus eventually died (Fig. 7). Ultrastructural observations of the *in vitro*-formed granuloma revealed a total degranulation of the haemocytes after 5 min and a dense material surrounding the hyphal bodies (Fig. 8). A similar encapsulation reaction was observed when purified wall was used, demonstrating that the wall alone will trigger the cellular reaction.

Protoplasts were not encapsulated by haemocytes, either *in vivo* or *in vitro* (Figs 9 and 10). *In vivo*, the protoplasts invaded the haemolymph, killing the insect. The time from injection to death was dependent on the protoplast dose: 250 protoplasts caused 100% mortality in 5 d, whereas larvae died 15 d after 10 protoplasts were injected. Histological sections occasionally showed loose aggregates of haemocytes and protoplasts, different from the typical granuloma usually found after injection of foreign material, as no concentric haemocyte lines were seen in these aggregates and no melanization was observed (Fig. 11). The haemocytes did not degranulate (Fig. 12). Protoplasts *in vivo* were generally larger than the haemocytes, multinucleate and pleomorphic with numerous lipid inclusions (Fig. 12). In contrast to *in vitro* experiments, they were never seen spherical with a voluminous central vacuole (Fig. 10). Protoplasts killed by paraformaldehyde or irradiation to preserve the surface antigenic composition, and protoplasts preincubated with anti-P serum to mask their surface antigens, were not recognized as foreign particles. Anti-P itself had no influence, since hyphal bodies were normally encapsulated in the presence of anti-P. The injection of protoplast culture filtrates had no influence on the larvae.

Chemical composition of the surface of the protoplasts and hyphal bodies

The surface of the protoplasts produced *in vitro* was labelled with anti-P. The same labelling, with an identical dilution of anti-P, was obtained on protoplasts incubated in the plasma *in vitro* or recovered from injected insects (Fig. 13a, b). Moreover, protoplasts produced *in vivo* did not react with anti-Gm, whereas haemocytes did react (Fig. 14a, b). These results demonstrate that surface antigens of the protoplasts and haemocytes are different and that no host components were deposited on the surface of the protoplasts during the infection process.

Anti-P did not label hyphal bodies (Table 1), demonstrating that the surface components differed from those of protoplasts. Since these components may play an important role in

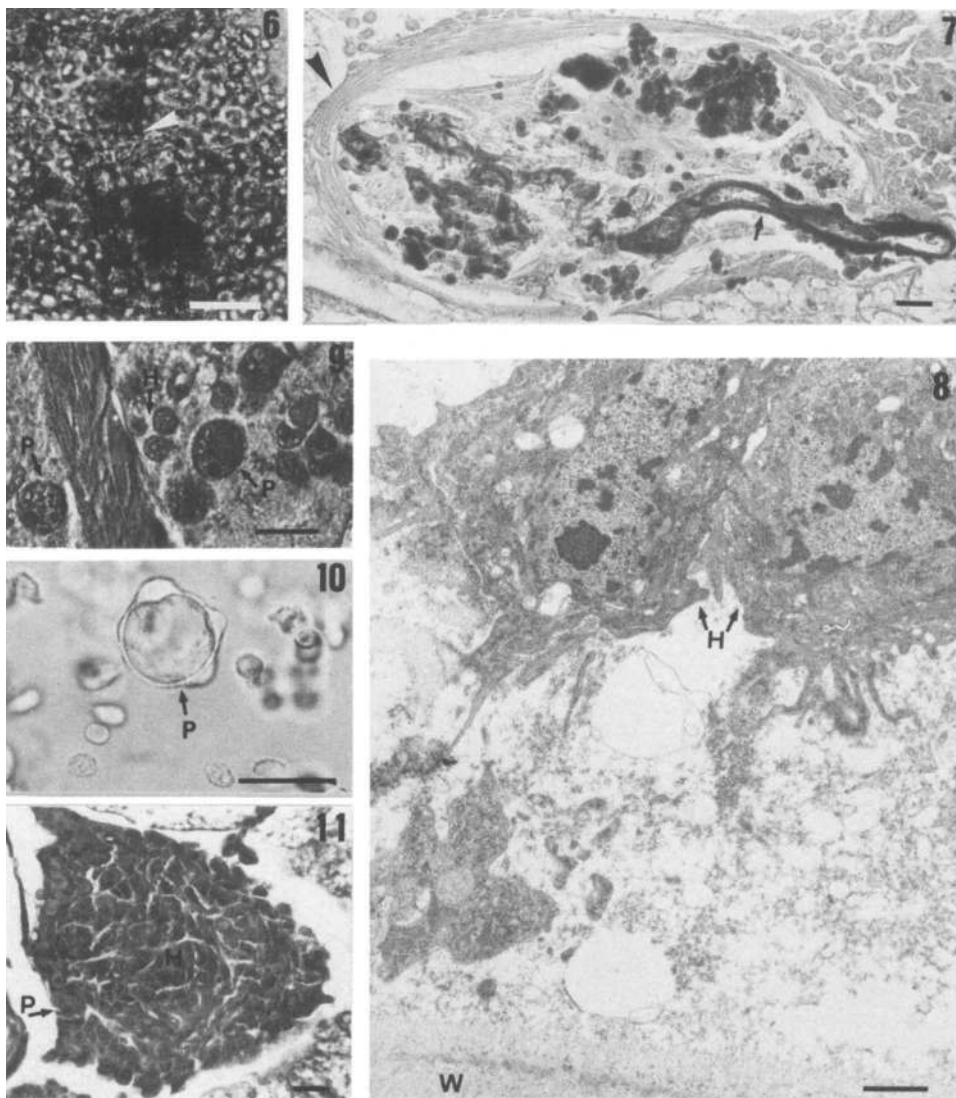


Fig. 6. *In vitro* encapsulation of the hyphal bodies of *E. aulicae* (arrowhead) by haemocytes of *G. mellonella*. Bar, 20 μ m.

Fig. 7. *In vivo* encapsulation of the hyphal bodies of *E. aulicae* 8 d after injection. Note the concentric lines of clumped haemocytes (arrowhead) and the melanized dead hyphal bodies (arrow). Bar, 20 μ m.

Fig. 8. *In vitro* encapsulation of the hyphal bodies under TEM, showing the degranulation of the haemocytes (H) and the dense material surrounding the wall (W) of the hyphal body. Bar, 1 μ m.

Fig. 9. Protoplasts of *E. aulicae* *in vivo* 4 d after injection into *G. mellonella*. Note the absence of recognition of the protoplasts (P) by haemocytes (H). Bar, 20 μ m.

Fig. 10. *In vitro* confrontation of protoplasts (P) and haemocytes.

Fig. 11. *In vivo* aggregation between protoplasts (P) and haemocytes (H) 24 h after injection. Note the absence of both classical granuloma structure and melanization. Azan coloration shows that protoplasts are present on the left of the aggregates (protoplasts show an orange-yellow cytoplasm and red nucleus whereas haemocytes show a blue cytoplasm and red nucleus). Bar, 20 μ m.

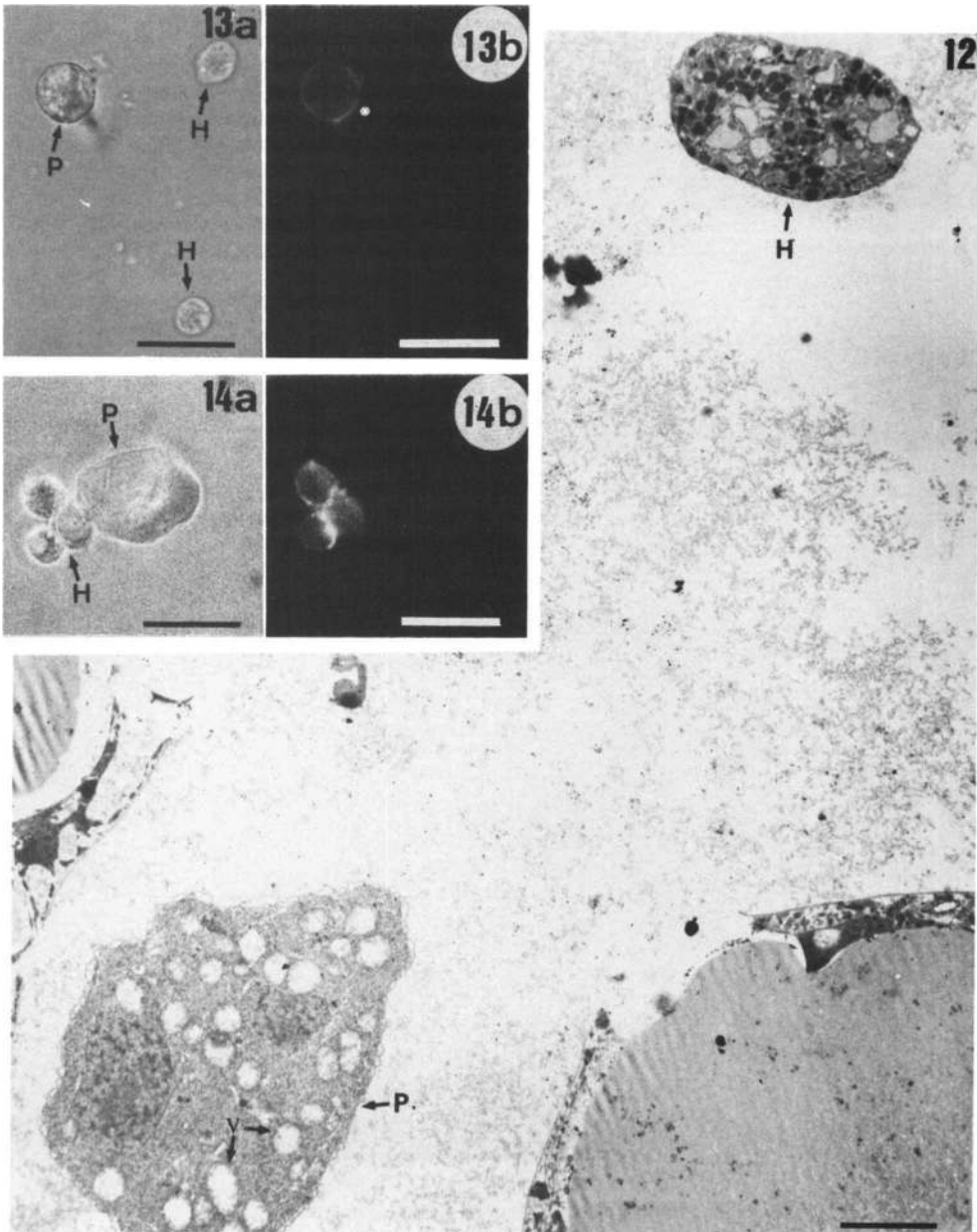


Fig. 12. Protoplasts of *E. aulicae* *in vivo* under TEM, showing the pleomorphic multinucleate protoplast (P) with vacuoles (V) and an undegranulated haemocyte (H). Bar, 1 μ m.

Fig. 13 (a, b). Positive antiserum labelling of protoplasts (P) recovered from infected larvae. Note the absence of labelling in haemocytes (H). (a) Transmitted light; (b) epifluorescence. Bars, 10 μ m.

Fig. 14. (a, b). Absence of labelling by an antiserum against *G. mellonella* hemolymph of a protoplast (P) recovered from infected larvae. Note the positive labelling of the surface of the haemocytes (H). (a) Transmitted light; (b) epifluorescence. Bars, 10 μ m.

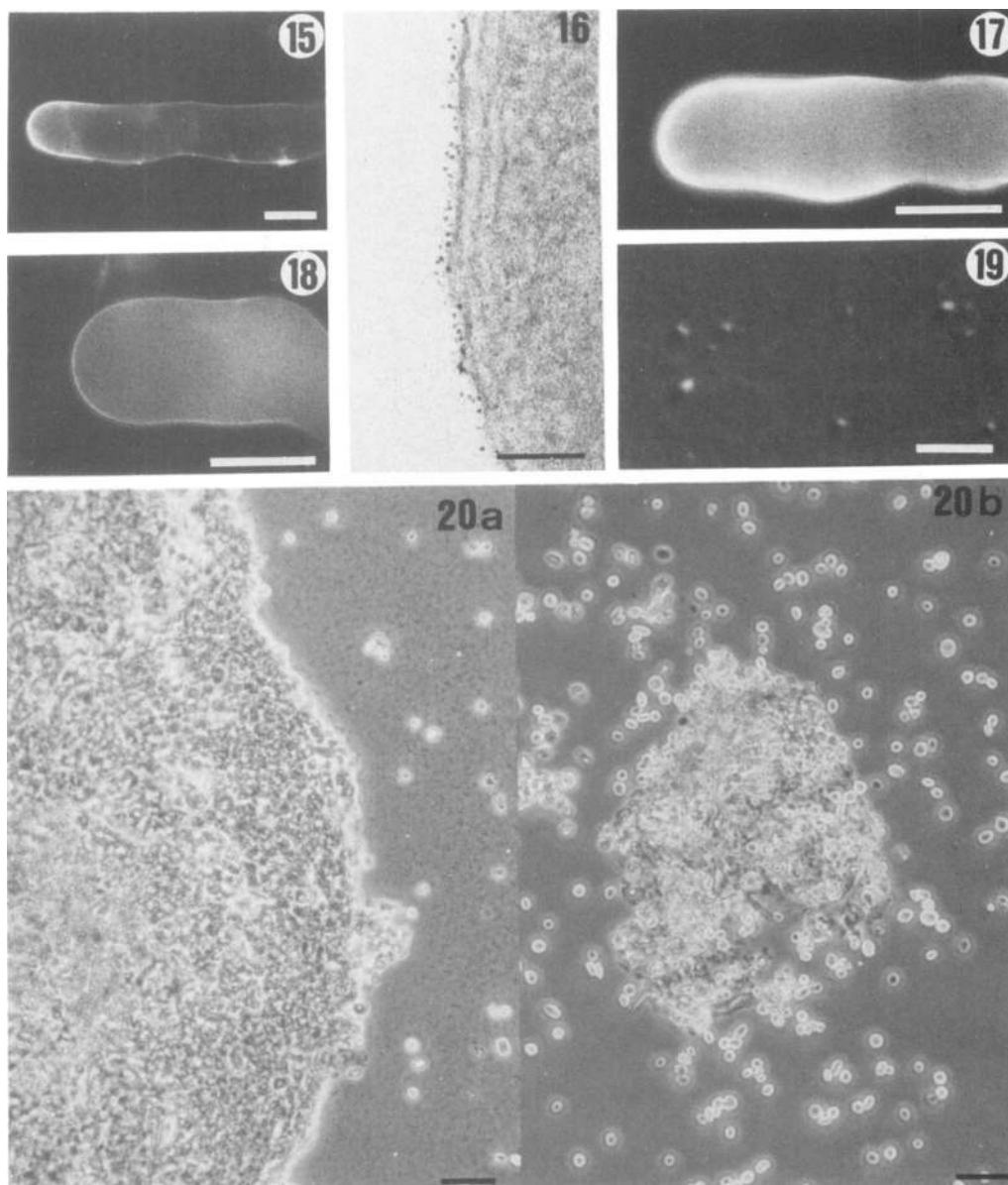


Fig. 15. Con A positive labelling of hyphal body wall using FITC-Con A. Bar, 20 μ m.

Fig. 16. Con A positive labelling of protoplast surface using Con-A-mannosyl-ferritin. Bar, 0.1 μ m.

Fig. 17. WGA positive labelling of hyphal body wall using FITC-WGA. Bar, 10 μ m.

Fig. 18. Hyphal body wall labelled with aniline blue fluorochrome. Bar, 10 μ m.

Fig. 19. Scanning electron micrograph of hyphal body wall showing positive labelling with anti-laminaribiosyl antiserum. Bar, 0.1 μ m.

Fig. 20 (a, b). *In vitro* granuloma provoked by addition of 1,3- β -glucan (a) and chitin (b). Bar, 20 μ m.

inducing the defence reaction, a comparative cytochemical study of the surface determinants of protoplasts and hyphal bodies was undertaken (Table 1).

The hyphal body and protoplast surfaces reacted to Con A (Figs 15 and 16). However, hyphal bodies were more strongly labelled than protoplasts. This indicated the presence of glycoprotein

on the surface of both stages of *E. aulicae*, probably more abundant on hyphal bodies. Neither protoplasts nor hyphal bodies demonstrated binding to PNA and RCA, even at high concentrations, suggesting the absence of galactose residues on the surface of both propagules. Protoplast and hyphal body surfaces did not bind to cationized ferritin at concentrations of 250–500 $\mu\text{g ml}^{-1}$. This shows that the outer layer of the hyphal body and protoplasts of *E. aulicae* is not negatively charged. However, material from the culture medium sometimes stuck to the surface of the hyphal bodies or protoplasts, giving a positive reaction to cationized ferritin. The biggest difference concerned chitin and 1,3- β -glucan. Hyphal bodies gave a strongly positive reaction to WGA, to the fluorochrome dye aniline blue and to the anti-laminaribiosyl serum (Figs 17, 18 and 19). The SEM observations demonstrated a uniform labelling of the wall surface of the hyphal body by the anti-laminaribiosyl serum (Fig. 19). Protoplasts did not react to the 1,3- β -glucan or chitin probes. These results indicate the presence of 1,3- β -glucan and chitin on the hyphal body surface only. *In vitro*, haemocytes demonstrated an encapsulation reaction which was stronger with 1,3- β -glucan than with chitin (Fig. 20a, b).

DISCUSSION

E. aulicae protoplasts are able to evade encapsulation due to the lack of 1,3- β -glucan and chitin on their surface. Our results established that the presence of these components on the hyphal body wall leads to recognition by the host and subsequent granuloma formation in *G. mellonella*. The absence of recognition of protoplasts of *Entomophaga grylli* (Macleod *et al.*, 1980) and *Entomophthora muscae* (unpublished), even in non-natural hosts, could be due to similar reasons.

Although it has been previously suggested that recognition of *E. aulicae* protoplasts can be induced by treatment with β -galactosidase, histological or chemical confirmation of the presence of galactose residues on the protoplast surface was not provided (Dunphy & Chadwick, 1985); in the present study no galactose residues were detected by PNA or RCA. Since we established that foreign proteins can easily stick to the cell surface of protoplasts and hyphal bodies, we suggest that the destruction of the protoplasts may be due to a non-specific adhesion of the enzyme to the protoplasts, inducing a non-specific defence reaction.

The absence of cell reaction to protoplasts was not due to the secretion of an inhibitor of the encapsulation, since it also occurred with dead protoplasts. Moreover, protoplast culture filtrate did not have any toxic effect on the insect host. The labelling by anti-P of protoplasts of *E. aulicae* produced *in vivo* contrasts with trypanosoma infection in tsetse flies (Vickerman, 1978) and indicates that protoplasts did not change the antigenic nature of their surface during infection. Unlike the cercariae of the schistosome *Trichobilharzia ocellata* in its host *Lymnaea stagnalis* (Van der Knaap *et al.*, 1985), *E. aulicae* protoplasts did not have their antigens masked by host substances.

However, the data presented here suggest that the protoplasts are able to evade the defence reaction due to the lack of 1,3- β -glucan and chitin on their surface. 1,3- β -Glucan and chitin were the only components which triggered *in vitro* a defence reaction by the haemocytes of *G. mellonella*. The importance of chitin and 1,3- β -glucan in the defence reaction of invertebrates has been previously demonstrated. The main metabolic pathway involved in the arthropod defence reaction is the prophenoloxidase system, which has been proposed to constitute a primitive form of the vertebrate complement system (Söderhäll & Smith, 1983). The activation of the vertebrate complement is classically induced by 1,3- β -glucan through the alternative pathway (Hamuro *et al.*, 1981). The prophenoloxidase system, like complement, can be activated by 1,3- β -glucan (Söderhäll & Unestam, 1979). Yoshida *et al.* (1987) demonstrated recently the presence of 1,3- β -glucan receptor in the plasma of *Bombix mori* larvae. The role of *N*-acetylglucosamine in the defence reaction has been poorly studied until now. Chitin does not activate the prophenoloxidase system (Söderhäll & Unestam, 1979). However, addition of *N*-acetylglucosamine *in vitro* to granulocytes of spruce budworm larvae enhanced their adhesion to *Absidia repens* spores (Dunphy & Nolan, 1982).

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