Journal of Invertebrate Pathology **78,** 201–209 (2001)
doi:10.1006/jipa.2001.5069, available online at http://www.idealibrary.com on IDEAL®

Survival and Differential Development of *Entomophaga maimaiga* and *Entomophaga aulicae* (Zygomycetes: Entomophthorales) in *Lymantria dispar* Hemolymph

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Received February 23, 2001; accepted August 20, 2001

The closely related entomophthoralean fungi *Entomophaga aulicae* and *E. maimaiga* are both host-specific pathogens of lepidopteran larvae. However, these fungi do not have the same host range. The first objective of this study was to compare the fate of *E. aulicae* in the nonpermissive host *Lymantria dispar* with the fate of the successful pathogen *E. maimaiga* over the same time period. In the hemolymph of *L. dispar* injected with *E. maimaiga* protoplasts, the number of hemocytes demonstrated a decreasing trend after the first day postinjection and hemocytes completely disappeared by day 5, with the majority of larvae dying in 5.6 ± 0.1 days. In *L. dispar* larvae, *E. maimaiga* infections developed successfully, evidenced by increasing numbers of protoplasts and hyphal bodies prior to host mortality. In contrast, at day 5 hemocytes were readily visible in hemolymph of *E. aulicae*-injected larvae, but *E. aulicae* cells did not increase in numbers, although persisting in the hemolymph for at least 16 days postinjection. For both fungal species, when hemolymph samples from injected insects were introduced to culture media viable fungal cultures were always produced. Both *E. aulicae* and *E. maimaiga* occurred in hemolymph initially after injection as protoplasts. For *E. maimaiga*, after day 3, <50% of fungal cells were hyphal bodies until insect death when most cells regenerated cell walls. For *E. aulicae*, from day 2 equal numbers of fungal cells in the hemolymph occurred as protoplasts and hyphal bodies. To investigate the cause of fungistasis in *E. aulicae*-injected larvae, *E. aulicae* cell cultures exposed to partially purified protein fractions from hemolymph of larvae infected with either fungus displayed increased lysis and decreased viability at lower concentrations of protein fractions compared with *E. maimaiga* cell cultures. These studies demonstrate that *E. aulicae* does not increase in *L. dispar* hemolymph, although it persists and results suggest that proteinaceous factors induced within the hemolymph may limit the capacity of *E. aulicae* to develop successful infections. © 2001 Elsevier Science (USA)

***Key Words:* *Entomophaga aulicae; Entomophaga maimaiga*; entomopathogens; fungi; insects; fungistatic activity; immunity; *Lymantria dispar*; Entomophthorales.**

INTRODUCTION

The insect immune response is one of the principal factors determining whether an entomopathogenic fungus can successfully develop within an insect (Boucias and Pendland, 1998). One of the distinguishing characteristics of entomopathogenic fungi is that their cell walls are rich in β-1,3 glucans. These sugars are often readily detected by the insect immune system and, in response to their presence, cellular immune defenses are mounted. Some entomophthoralean fungi occur in host hemolymph as protoplasts lacking the sugar-rich cell walls; it has been shown that occurrence as protoplasts helps in avoiding the cellular immune response (Beauvais *et al.,* 1989, Butt *et al.,* 1996).

Entomophthoralean fungi that occur *in vivo* as protoplasts are still usually highly host specific (Hajek and Butler, 2000). The closely related entomophthoralean fungi *Entomophaga aulicae* and *Entomophaga maimaiga* are both pathogens of lepidopteran larvae. Both fungi grow as protoplasts within the hemocoels of hosts but these fungi do not have the same host range. For example, *E. aulicae* cannot infect the lymantriid *Lymantria dispar* (gypsy moth) and *E. maimaiga* cannot infect the geometrid *Lambdina fiscellaria* (hemlock looper), although the opposite relationships result in successful infections (Bidochka and Hajek, 1996). For

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0022-2011/01 $35.00
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E. maimaiga, this host specificity occurs after injection of fungal cells into the hemocoel and thus is independent of differential cuticular penetration by conidia of these two fungal species (Hajek *et al.,* 1995). A recent study has shown that *E. maimaiga* and *E. aulicae* differ in glycoprotein composition of the surfaces of protoplasts (Bidochka and Hajek, 1996); it was suggested that these cell surface components could be used in nonpermissive hosts to recognize these cells as nonself. Based on this hypothesis, protoplasts of some species could still be recognized in nonhosts and the immune system could then respond.

It is still unclear what mechanisms are responsible for blocking development of *E. maimaiga* or *E. aulicae* in nonpermissive hosts. After injection into *L. dispar* hemolymph, *E. aulicae* cells evoked a minimal cellular response; they were never found to be phagocytosed, were only rarely encapsulated, and were assumed to have been lysed (Butt *et al.,* 1996). In addition to the cellular immune responses, insects display humoral immune responses. Antibacterial peptides are known to occur in many insect species to assist with protection against bacterial infections. More recently, antifungal peptides have also been identified in insect hemolymph (Gillespie *et al.,* 2000a), e.g., drosomycin (Fehlbaum *et al.,* 1994) and metchnikowin (Levashina *et al.,* 1995) from *Drosophila*, AFP from *Sarcophaga peregrina* (Iijima *et al.,* 1993), and an unnamed peptide from *Heliothis virescens* (Lamberty *et al.,* 1999).

The objective of this study was to further investigate the fate of *E. aulicae* cells in the nonpermissive host *L. dispar,* drawing comparisons with successful infections by *E. maimaiga*. Our goals were twofold: to count the numbers of hemocytes and fungal cells in hemolymph through time and to determine the types of fungal cells present during the infection cycle. Studies were subsequently conducted to identify potential mechanisms that prevented successful proliferation of *E. aulicae* within *L. dispar,* with primary emphasis on possible occurrence of fungistatic factors within the hemolymph.

MATERIAL AND METHODS

Fungal Cultures

Cultures of *E. aulicae* (FPMI 893; Forest Pest Management Institute, Saulte Ste. Marie, Ontario, Canada) and *E. maimaiga* (ARSEF 6174; USDA, Agricultural Research Service Collection of Entomopathogenic Fungal Cultures, Ithaca, NY) were grown as protoplasts in Grace's insect tissue culture medium (GIBCO-BRL, Gaithersburg, MD) supplemented with 5% (v/v) fetal bovine serum (GIBCO-BRL) and incubated at 20°C in complete darkness. Cultures were transferred to fresh media every 2 days to maintain growth as protoplasts. After transfer number eight, a new aliquot from the same original culture was thawed for use.

Infection of Insects

L. dispar were obtained and maintained on artificial diet as previously described (Hajek *et al.,* 1995). Protoplast concentrations of either *E. aulicae* or *E. maimaiga* were adjusted to 1×10^5 protoplasts/ml with Grace's insect tissue culture medium. Ten microliters of this suspension (10^3 protoplasts) were injected into 4th instar larvae of *L. dispar* through the ventral surface using a microinjector fitted with a 3-ml syringe and a 23-gauge needle. Control larvae were injected only with Grace's medium. After injection, larvae were incubated on artificial diet, at 20°C with a photoperiod of 15 h light and 9 h dark.

In Vivo Studies

L. dispar larvae were injected with protoplast suspensions of either *E. maimaiga or E. aulicae*, or with Grace's medium. At 24-h intervals after injection, larvae were surface-sterilized by dipping in 1% sodium hypochlorite for ca. 2 s, followed by rinsing in sterile deionized water for 5–10 s, and drying on sterile paper towels. A larval proleg was cut with fine scissors and 20 μl of hemolymph was collected with sterile pipette tips and then mixed with 0.85% NaCl, with 50 μg/ml phenylthiourea (PTU) added as a melanization inhibitor. We used the stain Calcofluor to distinguish protoplasts from hyphal bodies because this stain is only taken up by the cell walls of hyphal bodies and not by the plasma membrane surrounding protoplasts. For microscopic observation, 20 μl of hemolymph was mixed with 20 μl of 0.1% Calcofluor (Sigma Chemical) (Butt, 1997), 5 μl of 0.85% NaCl, and 5 μl of 50 μg/ml PTU using five replicates for each treatment. Drops of this mixture were observed either on hemocytometers or on glass slides with cover slips (see below). Cells were observed using epifluorescence at 400× with a Zeiss Axioskop (exciter 340–370, dichroic 395, barrier 420–530).

No distinction was made among types of hemocytes (Fig. 1A). For *E. maimaiga* hemolymph samples, fungal cells were easily differentiated from hemocytes (Fig. 1B), so a hemacytometer was used for quantification of fungal cells. Cell counts were made daily until day 5 postchallenge because larvae died in 5.6 ± 0.1 days. Fluorescence microscopy to differentiate cell types was conducted on days 1–4 after injection.

For hemolymph from *E. aulicae*-infected larvae, total hemocyte and fungal cell counts were performed from 2 days postinjection for both treated and control insects, continuing at 2-day intervals until day 16 posttreatment. Cell counts were made using a grid (0.00784 mm^2) in the microscope eyepiece. It was not possible to differentiate protoplasts of *E. aulicae* from hemocytes of *L. dispar* using a hemacytometer since on this sur-

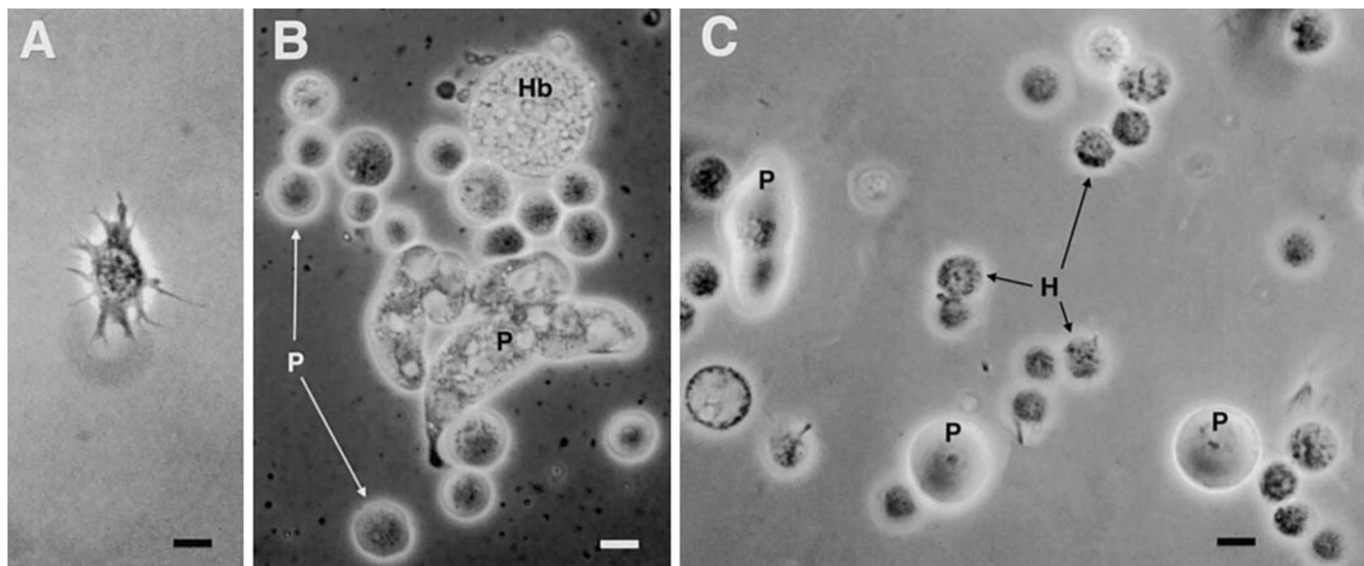

FIG. 1. Light micrographs of fungal and insect cells in samples of *Lymantria dispar* larval hemolymph demonstrate visual distinctions among cell types. (A) Phase contrast micrograph of a control hemocyte at 5 days postinjection with Grace's tissue culture medium. Bar, 83 μm. (B) Phase contrast micrograph of *Entomophaga maimaiga*-treated hemolymph at 5 days postinjection with fungal cells. Bar, 83 μm. (C) Fluorescence micrograph of *E. aulicae*-treated hemolymph at 5 days postinjection with fungal cells. Bar, 94 μm. H, hemocytes; P, protoplasts; Hb, hyphal bodies.

face, both appear very similar in shape and size. Therefore, we used the differential attachment of protoplasts versus hemocytes to surfaces of glass slides to distinguish cell types; using phase contrast microscopy, on glass slides a halo could be seen around the protoplasts while hemocytes lacked the halo (Fig. 1C). For each of five replicates observed every second day, cells in 10 randomly chosen grids were counted. Fluorescence microscopy was used to differentiate hemocytes from protoplasts on days 1–5 postchallenge.

In Vitro Studies

For these studies, hemolymph samples were placed into 24-well plates (Falcon, Franklin Lakes, NJ) using 5 replicate wells for each treatment. Hemolymph from *E. aulicae*- and *E. maimaiga*-injected *L. dispar* larvae was collected (20 μl per insect) and inoculated into 1 ml Grace's insect tissue culture medium supplemented with 5% fetal bovine serum plus 10 μl/ml gentamycin (Gentocin; Schering-Plough Animal Health Corp., Kenilworth, NJ) and 50 μg/ml PTU. Cells were incubated at 20°C in complete darkness. For *E. maimaiga,* observations were made on days 2 and 4 after initiation of cultures. For *E. aulicae,* observations and cell counts were performed every 48 h beginning at 2 days, through a total of 16 days. To detect cell walls of hyphal bodies, the same staining procedures with Calcofluor were used for each observation as with *in vivo* studies. For all samples, cells within the ocular grid were quantified for 10 randomly selected microscopic fields.

Extraction of Antifungal Peptides from Lymantria dispar Hemolymph

Fourth instar *L. dispar* larvae were injected with *E. aulicae* or *E. maimaiga* protoplasts or with Grace's insect tissue culture medium using the procedures above. At 3 days postchallenge, hemolymph was collected directly into sterile chilled microfuge tubes containing 20 μl each of the protease inhibitor aprotinin (50 μg/ml) (Sigma Chemicals) and 50 μg/ml PTU. Hemolymph was centrifuged at 14,000*g* for 1 min at 4°C to pellet cells; the supernatant was kept at −20°C until use.

Partial Purification of Antifungal Factors

Partial fractionation was carried out similarly to methods described in Lamberty *et al.* (1999). Cell-free hemolymph was acidified with trifluoroacetic acid (25 μl/ml) to pH 3 and then left on ice for 30 min. After centrifugation at 10,000*g* for 30 min at 4°C, the supernatant was loaded onto a solid-phase extraction (SPE) C18 column prewashed with acetonitrile and then conditioned with 0.05% trifluoroacetic acid (TFA) in deionized water. Sequential elutions were performed with 0.05% TFA in water, followed by 10, 40, and 100% acetonitrile with 0.05% TFA. The bulk fractions were dried in a centrifugal concentrator (Savant) and then weighed, dissolved in sterile deionized water, and sonicated to aid in dissolution. When tested on an equivalent weight basis, preliminary separations indicated that the bulk of fungistatic and lytic activity resided in

the fraction eluted with 40% acetonitrile in 0.05% TFA, typically constituting 2 mg of total protein from 3 ml of starting hemolymph (approximately 5% of dry weight). For assays comparing hemolymph samples from control and infected larvae, samples were prepared on an equivalent weight basis (2 mg/ml in water) and then sterilized by passage through a 0.2-μm nylon filter, prior to testing at 50–150 μg weight basis. For later assays comparing hemolymph from larvae infected with *E. maimaiga* or *E. aulicae,* dissolved samples were centrifuged to remove debris, then analyzed for protein using a Bradford dye microassay with bovine serum albumin as a standard (Pierce Chemical), and adjusted to equivalent protein concentrations with deionized water. Samples were then filtered through a 0.2-μm nylon filter into a sterile tube for bioassay analysis.

Bioassays

E. maimaiga and *E. aulicae* were cultured in standard Grace's insect tissue culture medium. For bioassay, 80 μl of a culture and 20 μl (40 μg/well) of either fraction or sterile deionized water were added to wells of 24-cell-well plates, which were then incubated at 20°C in darkness. All samples were preadjusted with concentrated Grace's medium prior to addition of cells to maintain osmolarity. Cell counts of *E. maimaiga* and *E. aulicae* were taken over a 72-h period. Later assays were performed by counting fungal cells using a hemacytometer at 48 h; viability of cells was determined by fluorescence microscopy using fluorescein diacetate and propidium iodide to indicate whether cells were living or dead, respectively (Firstencel *et al.,* 1990). For comparison of hemolymph taken 1–3 days postinfection, cell counts were taken after 48 h of incubation.

Statistical Analysis

To compare cellular densities and types in hemolymph, data were subjected to analyses of variance using two-way ANOVA followed by post hoc multiple comparisons, dividing the α of 0.05 by the number of comparisons.

RESULTS

Cellular Distribution in Hemolymph of E. maimaiga-Infected L. dispar

The number of hemocytes in the hemolymph of *L. dispar* infected with *E. maimaiga* significantly decreased relative to control hemolymph on days 4 (t = 5.56; P = 0.001) and 5 (t = 5.85; P = 0.0043) after injection; hemocytes completely disappeared by day 5 (Fig. 2). Counts of hemocytes on days 2 and 3 were numerically greater for controls than treated insects but were not significantly different (day 2: t = 2.67; P = 0.0292; day 3: t = 0.87; P = 0.4134) due to variability among individual insects sampled. The number of fungal cells increased from day 1, and fungal cells were the predominant cells found in hemolymph by day 5, with up to 153,400 cells/μl of hemolymph in one individual very close to death (average = 43,390 ± 26,525; mean ± SE). No cellular responses to the presence of *E. maimaiga* cells within insects were observed.

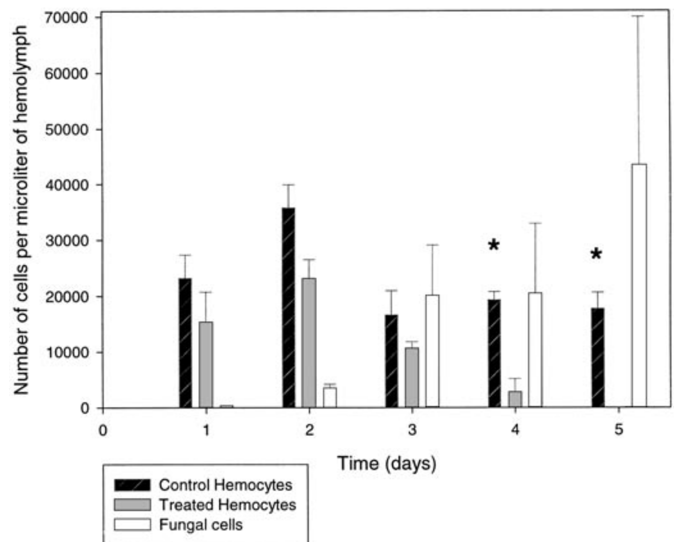

FIG. 2. Concentrations (mean ± SE) of *Entomophaga maimaiga* cells and *Lymantria dispar* hemocytes in hemolymph after injection of fungal cells compared with hemocyte concentration in controls. *Significantly different values for hemocyte concentrations in control and treated insects.

E. maimaiga protoplasts were readily visible in *L. dispar* hemolymph following injection and were easily observed through day 5. Protoplasts were the predominant form of fungal cells present, comprising between 60 and 100% of fungal cells (Fig. 3). Hyphal bodies were observed by day 3 when they constituted 38% of the total fungal cells. The insects sampled at day 4 were almost certainly at different stages of disease progression due to the variability in cell type; hyphal bodies were evident but there were still more protoplasts. As an abrupt change, in insects approximately 2–3 h before death (barely able to move on day 5 postchallenge), we observed that almost all of the fungal cells in the hemolymph had regenerated cell walls to become hyphal bodies.

Cellular Distribution in Hemolymph of E. aulicae-Infected L. dispar

Hemocytes were easily visible as a major cell type in the hemolymph of *E. aulicae*-infected insects under phase contrast microscopy; fungal cells were evenly distributed throughout the sample, but at low numbers. Hemocytes in *E. aulicae*-treated hemolymph typ-

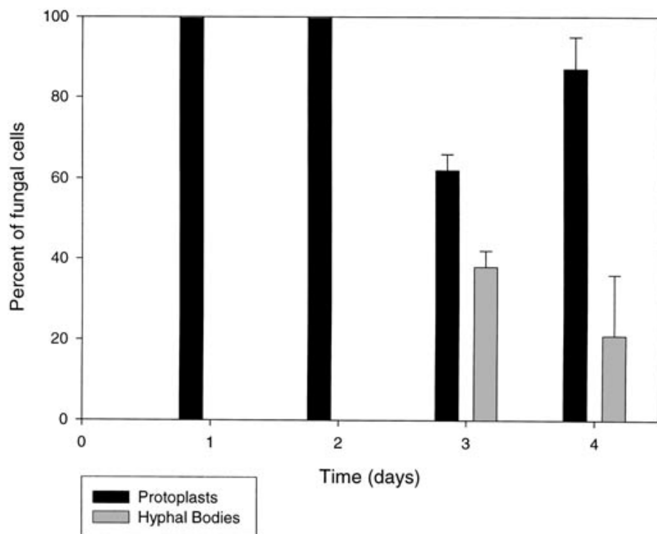

FIG. 3. Percentages of *Entomophaga maimaiga* cells (mean ± SE) as protoplasts or hyphal bodies within *Lymantria dispar* hemolymph after injection of fungal cells.

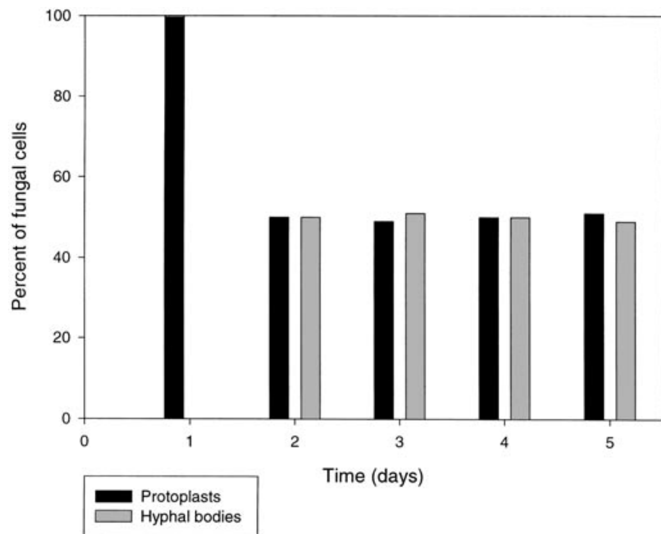

FIG. 5. Percentages of *Entomophaga aulicae* cells (mean ± SE) as protoplasts or hyphal bodies within *Lymantria dispar* hemolymph after injection of fungal cells.

ically maintained densities similar to those in control hemolymph. No significant differences in the number of hemocytes in treatments vs control were detectable except for day 16 ($F = 9.95$; $P = 0.0025$) when there were more hemocytes in treated than in control larvae (Fig. 4). No cellular response to infection was observed during this experiment except for a few cellular aggregations around fungal cells at days 12, 14, and 16 posttreatment. The densities of *E. aulicae* cells declined between days 2 and 16 (testing slope = 0; $t = -3.74$; $P = 0.0006$). However, between days 6 and 16, the decrease in the titer of *E. aulicae* cells over time was no longer evident (testing slope = 0; $t = -0.98$; $P = 0.3361$). By day 16, only 6% of treated larvae had died, although not apparently due to *E. aulicae* infections, and the remainder of treated larvae survived as prepupae or pupae.

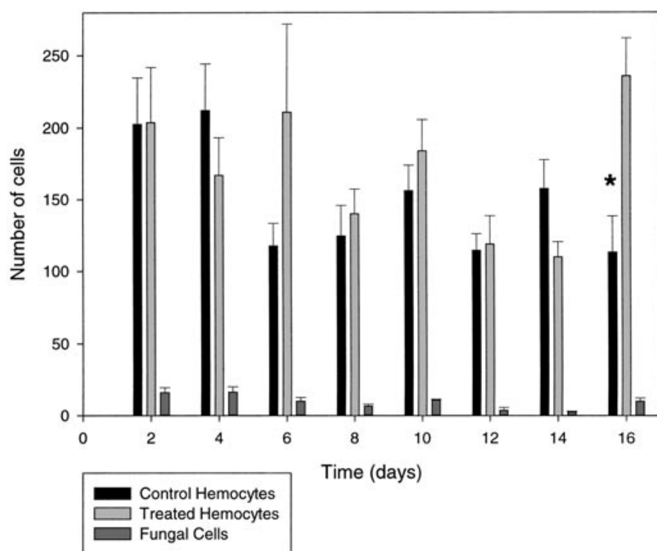

FIG. 4. Concentrations (mean ± SE) of *Entomophaga aulicae* cells and *Lymantria dispar* hemocytes in hemolymph after injection of fungal cells compared with hemocyte concentration in controls. Concentrations quantified as numbers of cells in 10 0.00784-mm^2 grids in the microscope eyepiece. *Significantly different values for hemocyte concentrations in control and treated insects.

The types of fungal cells in the hemolymph of *E. aulicae*-infected insects differed from those observed with *E. maimaiga* infections (Fig. 5). Equivalent levels of protoplasts and hyphal bodies were observed from day 2 to day 5, following the initial injection with protoplasts (day 0). Although not quantified after day 5, both protoplasts and hyphal bodies of *E. aulicae* could be observed up to day 16 postinfection and could still be cultured *in vitro* when recovered from hemolymph samples.

Variation in Development of E. maimaiga and E. aulicae in Vitro

These studies demonstrated that the cells of both *E. maimaiga* and *E. aulicae,* when removed from hemolymph, were viable, although *E. aulicae* cells were not increasing in number within insects. Protoplasts of both *E. maimaiga* and *E. aulicae* were the sole fungal cell type 2 days after hemolymph samples were transferred to cell well plates. For insects bled on day 4 postinjection, 2 days after *in vitro* cultures were initiated, protoplasts and hyphal bodies were present at equivalent numbers (*E. aulicae*: $\chi^2 = 0.83$; *E. maimaiga*: $\chi^2 = 2.13$; both $P > 0.05$) with no difference by

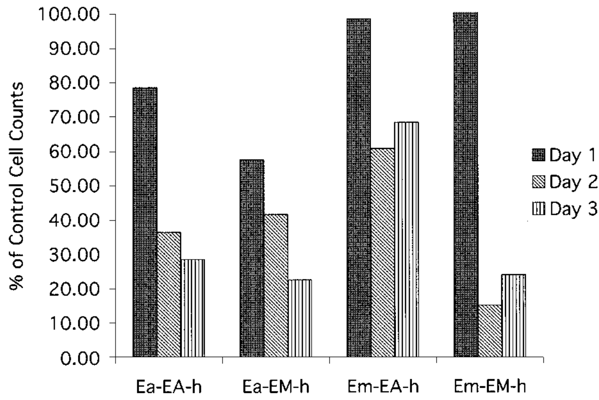

FIG. 6. Effect of hemolymph fractions on cell counts of *E. aulicae* and *E. maimaiga* in culture. Hemolymph fractions were removed from *L. dispar* at days 1, 2, and 3 postinjection with either *E. aulicae* or *E. maimaiga*, fractionated, and then added at an equivalent weight basis (50 μg) to cell cultures. Cell counts were taken after 48 h of incubation; control cell counts were 25.4×10^4 *E. maimaiga* cells/ml and 7.9×10^4 *E. aulicae* cells/ml. Ea-EAh, *E. aulicae* fraction against *E. aulicae* protoplasts in culture; Ea-EMh, *E. aulicae* fraction against *E. maimaiga* protoplasts in culture; Em-EAh, *E. maimaiga* fraction against *E. aulicae* protoplasts in culture; Em-EMh, *E. maimaiga* fraction against *E. maimaiga* protoplasts in culture.

fungal species ($\chi^2 = 0.238$; $P > 0.05$). For the following samples taken at 2-day intervals, 2-day-old cultures from hemolymph samples of *E. aulicae*-challenged insects contained a mixture of protoplasts and hyphal bodies, but fewer hyphal bodies than protoplasts were present in each sample.

Fungistatic Activity Present in Hemolymph

To determine whether fungistatic factors might be present in insect hemolymph to regulate fungal development, we partially fractionated hemolymph on SPE (Lamberty *et al.,* 1999) to test for antifungal activity against *E. maimaiga* and *E. aulicae* cell cultures on an equivalence basis. Little reduction in fungal cell growth was observed in the initial rinse or in 10% or 100% acetonitrile-eluted fractions, while reduced cell growth and cell lysis were detectable in the fraction eluted with 40% acetonitrile with 0.05% TFA, similar to that reported for hemolymph fractions of *H. virescens* (Lamberty *et al.,* 1999). The active fraction represented less than 10% of the dry weight of the starting material, constituting less than 1 mg/ml of protein. Thus, the 40% fraction was used to compare activities from hemolymph obtained from larvae inoculated with Grace's medium, *E. aulicae,* or *E. maimaiga.*

Hemolymph samples from fungal-challenged insects inhibited growth of both *E. aulicae* and *E. maimaiga* cells, and there was a two- to threefold greater activity found in samples from fungal-challenged larvae added at equivalent weights than in controls. Hemolymph collected at 1 day postinfection had little activity relative to water controls, while hemolymph collected at 2 and 3 days postinfection contained considerably greater activity (Fig. 6). For comparison, control cell counts were 25.4×10^4 *E. maimaiga* cells/ml and 7.9×10^4 *E. aulicae* cells/ml.

Hemolymph, collected at 3 days postinjection with *E. maimaiga* and *E. aulicae* and adjusted to equivalent protein concentrations, was used to determine activity against both fungal cell cultures *in vitro* (Fig. 7). Loss of cell viability over the range of protein concentrations was consistent with the observed loss of fluorescence and a decline in cell counts relative to controls. Both *E. aulicae*- and *E. maimaiga*-infected hemolymph fractions reduced cell counts in both *E. aulicae* and *E. maimaiga* cultures relative to the control. The *E. auli-*

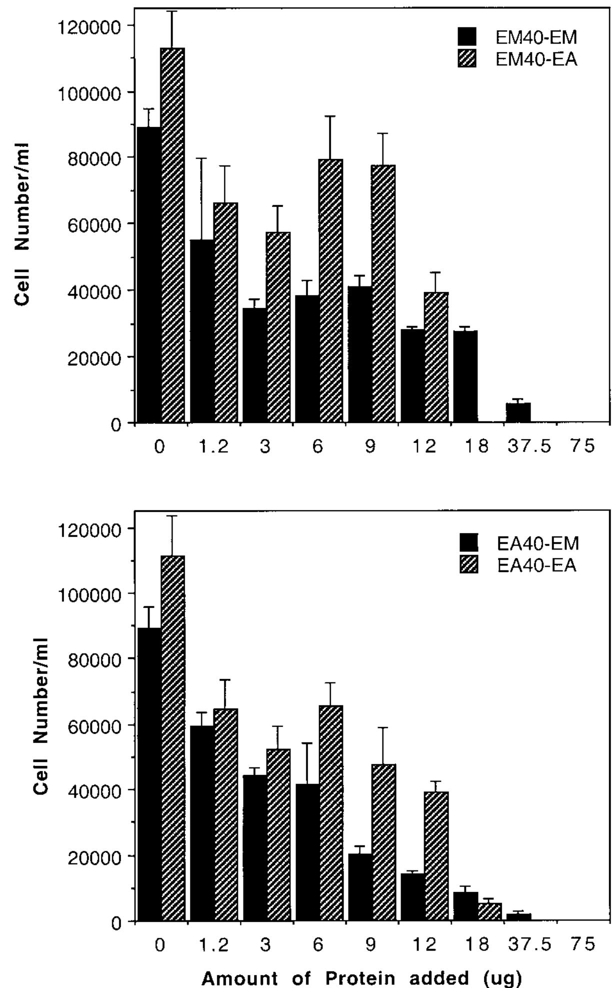

FIG. 7. Effect of *E. aulicae*- and *E. maimaiga*-extracted fractions from *Lymantria dispar* hemolymph, against *E. aulicae* and *E. maimaiga* cells in *in vitro* cultures (mean ± SE). The 40% acetonitrile SPE fractions from hemolymph samples were compared on an equivalent protein basis. EM40-EM, *E. maimaiga* hemolymph fraction against *E. maimaiga* cells; EM40-EA, *E. maimaiga* hemolymph fraction against *E. aulicae* cells; EA40-EM, *E. aulicae* hemolymph fraction against *E. maimaiga* cells; EA40-EA, *E. aulicae* hemolymph fraction against *E. aulicae* cells.

cae-infected hemolymph fraction appeared to have greater activity, as evidenced by a more pronounced reduction of cell counts in both cultures over the range of protein concentrations tested. However, both *E. aulicae* and *E. maimaiga* cells were still viable at up to 18 μg of protein in *E. aulicae* hemolymph.

E. aulicae cell cultures, however, were more sensitive than *E. maimaiga* cell cultures to both hemolymph fractions. No viable cells of *E. aulicae* remained in the presence of 18 μg of the *E. maimaiga* hemolymph fraction while *E. maimaiga* cell cultures were still viable at up to 37.5 μg of either hemolymph fraction. At a concentration of 75 μg of either hemolymph fraction, both culture cell counts decreased to zero.

DISCUSSION

Two days after injection of *L. dispar* larvae with protoplasts, *E. aulicae* and *E. maimaiga* cells present in hemolymph included only protoplasts but, by day 4, walled hyphal bodies were also present. Although walled cells were present, we detected only a minimal cellular immune response for *E. aulicae* at 12 days postinoculation. Previous studies in which hyphal bodies from cultures were injected into insects suggested that sugars in fungal cell walls are used for recognition of nonself with a resulting response by the cellular immune system (Beauvais *et al.,* 1989). In our study, the presence of walled hyphal bodies that developed from protoplasts injected into insects was associated with only a minimal, lagged response with *E. aulicae* only. Nonresponse to hyphal bodies by circulating hemocytes in *Spodoptera exigua* larvae has also been reported with the entomopathogen *Nomuraea rileyi* (Lopez Lastra and Boucias, 1994). Five days after injection of protoplasts, *E. maimaiga* had mounted a successful infection, overwhelming the host with no cellular immune response, although walled hyphal bodies had developed. In contrast, *E. aulicae* cells remained in the hemolymph throughout 16 days in significant numbers, the number of cells decreasing little, and insects did not die. We hypothesized that an inducible fungistatic factor in larval hemolymph could be preventing cells of *E. aulicae* from proliferating, although not killing the cells.

We found indications of antifungal factors present in hemolymph of *L. dispar* larvae challenged with *E. maimaiga* or *E. aulicae,* and low levels of activity were present in hemolymph of mock-challenged insects. In this study, partial fractionation of infected hemolymph and testing of this material in the presence of *E. aulicae* and *E. maimaiga* cells affected fungal cell viability and caused cell lysis. *E. aulicae* cell cultures, however, were more sensitive than *E. maimaiga* cell cultures to fractions purified from either *E. aulicae*- or *E. maimaiga*-infected hemolymph. In contrast, *E. maimaiga* cells were affected only at higher concentrations of the added fractions. Differences in the responsiveness of *E. aulicae* and *E. maimaiga* cells to this partially purified material correlate with their ability to mount successful infections, but further studies will be needed to assess the composition of this fungistatic agent(s) and its relative activity toward these two entomopathogens.

Numerous immune proteins, either directly or indirectly inhibiting microorganisms, are known from insects, including putatively nonspecific proteins such as lysozyme produced in the fat body (Gillespie *et al.,* 2000a). Protease inhibitors in the hemolymph are thought to possibly inhibit enzymes produced by fungi to obtain nutrients. Much of the work on inducible antimicrobial proteins in insects has been conducted by introduction of bacteria into hosts and subsequent testing using nonpathogenic microbes (Meister *et al.,* 1997; Gillespie *et al.,* 2000a). Several reports (Fehlbaum *et al.,* 1994; Levashina *et al.,* 1995; Lamberty *et al.,* 1999), however, have demonstrated the presence of antifungal activity by isolated insect hemolymph proteins, although these studies have tested the purified antifungal proteins against fungi that are nonpathogenic to the hosts tested. It is still unclear whether these antifungal proteins have any effects against fungal pathogens of insects and whether they play a role in determining host range.

Butt *et al.* (1996) reported that injected *E. aulicae* protoplasts were mostly lysed in the hemolymph of *L. dispar* larvae. We did not observe lysis in the present study, where *E. aulicae* fungal cells (protoplasts and hyphal bodies) were detectable in the nonpermissive host hemolymph up to 16 days after initial injection. However, we also did not observe increases in numbers of fungal cells. Although we observed the presence of a few aggregations of hemocytes around fungal cells at 12, 14, and 16 days posttreatment (in treated hemolymph), we did not observe hemocytic melanization, as previously described by Butt *et al.* (1996).

In previous studies, Hung and Boucias (1992) reported that infection of *S. exigua* larvae by *Beauveria bassiana* resulted in gradual suppression of the phagocytic competence of circulating hemocytes and, at 24 h postchallenge, a marked decrease in the number of hemocytes was seen. The number of hemocytes in controls was always higher than that in treated larvae, and at 48 hours hemocyte counts in controls were twice as high as those in treated insects (Hung and Boucias, 1992). In this study, we found that the number of hemocytes in *L. dispar* following challenge with *E. maimaiga* protoplasts decreased over 5 days, over which time the number of fungal cells increased. At 5 days postinjection, we could detect no circulating hemocytes, with complete replacement in the hemolymph by *E. maimaiga* fungal cells. When *Choristoneura fumiferana* was challenged with two isolates of *E. aulicae* (= *Entomophthora egressa*), titers of hemocytes

declined as titers of fungal cells increased, with this effect being more rapid with the isolate originating from this host than with a strain isolated from a different host (Dunphy and Chadwick, 1985). A decrease in total hemocyte counts has also been observed late in the infection cycle for *Schistocerca gregaria* infected with *Metarhizium flavoviride* (Gillespie *et al.,* 2000b). In contrast, for *E. aulicae*-treated *L. dispar* hemolymph, the number of hemocytes in treated vs control insects was not significantly different for up to day 16 postchallenge.

When hemolymph from challenged *L. dispar* was cultured under *in vitro* conditions, no significant differences in the number or type of fungal cells were apparent for *E. aulicae* or *E. maimaiga*. If inhibitory factors were present in the hemolymph, the dilution of such factors would have occurred with the addition of medium to support fungal growth. The *in vitro* results suggest that *E. aulicae* can persist in significant numbers under what appears to be a fungistatic environment within the hemolymph *in vivo* for a prolonged period but, when transferred to tissue culture medium, cells were still competent to proliferate.

In studies with the tortricid *C. fumiferana,* Murrin and Nolan (1987) reported that initially *E. aulicae* fungal cells in the hemolymph of infected insects predominantly grew as protoplasts but soon thereafter some of the fungal cells in the hemolymph of infected insects were present as hyphal bodies. In contrast, Soper *et al.* (1988) reported that *E. maimaiga* within *L. dispar* hemolymph remained as protoplasts until late in the infection cycle. We used fluorescence staining to aid in the evaluation of changes in fungal cell type during the infection cycle. We were able to document that, although initially *E. maimaiga* occurred as wall-less protoplasts, there was an increase in hyphal bodies, which constituted about 40% of total fungal cells 3 days after challenge. This pattern persisted until 5 days, when shortly before death the majority of cells regenerated cell walls. Such a pattern with growth as cells lacking sugar-rich walls early in infection has also been reported for the hyphomycetes *Metarhizium anisopliae* (Gillespie *et al.,* 2000a) and *B. bassiana* (Pendland *et al.,* 1993). Interestingly, although *E. aulicae* cells remained within larvae for 16 days, except for the initial 2-day sample, hyphal bodies were always present, yet response by hemocytes was very limited and was only seen on days 12, 14, and 16 postchallenge. It seems possible that after the initial protoplastic growth of the fungus, the phagocytic activity of hemocytes against fungal cells is inhibited, as seen with *B. bassiana* (Hung and Boucias, 1992) and *M. anisopliae* (Vilcinskas *et al.,* 1997). However, for these hyphomycetes, it has been suggested that toxic fungal secondary metabolites mediate cellular defences. With species of Entomophthorales, presence of such secondary metabolites midway through an infection cycle is not known.

ACKNOWLEDGMENTS

The authors thank the National Research Council of Argentina (C.O.N.I.C.E.T.) for a postdoctoral fellowship provided to C.C.L.L., NYC 139417 for providing financial support, and the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) (R. Humber and K. Hansen) for kindly providing fungal cultures. P. Kogan assisted with preliminary experiments leading to these studies. We also thank A. Encalada and F. Vermeylen for assistance with statistical analysis and J. D. Vandenberg for comments on the manuscript.

REFERENCES

Beauvais, A., Latgé, J. P., Vey, A., and Prevost, M. C. 1989. The role of surface components of the entomopathogenic fungus *Entomophaga aulicae* in the cellular immune response of *Galleria mellonella* (Lepidoptera). *J. Gen. Microbiol.* **135,** 489–498.

Bidochka, M. J., and Hajek, A. E. 1996. Protoplast plasma membrane glycoproteins in two species of entomophthoralean fungi. *Mycol. Res.* **100,** 1094–1098.

Bidochka, M. J., and Hajek, A. E. 1998. A non-permissive entomophthoralean fungal infection increases activation of insect prophenoloxidase. *J. Invertebr. Pathol.* **72,** 231–238.

Boucias, D. G., and Pendland, J. C. 1998. "Principles of Insect Pathology." Kluwer Academic, Dordrecht.

Butt, T. M., Hajek, A. E., and Humber, R. A. 1996. Gypsy moth immune defenses in response to hyphal bodies and natural protoplasts of entomophthoralean fungi. *J. Invertebr. Pathol.* **68,** 278–285.

Butt, T. M. 1997. Complementary techniques: Fluorescence microscopy. *In* "Manual of Techniques in Insect Pathology" (L. A. Lacey, Ed.), pp. 355–364. Academic Press, San Diego.

Dunphy, G. B., and Chadwick, J. M. 1985. Strains of protoplasts of *Entomophthora egressa* in spruce budworm larvae. *J. Invertebr. Pathol.* **45,** 255–259.

Fehlbaum, P., Bulet, P., Michaut, L., Lagueux, M., Broekaert W. F., Hetru, C., and Hoffman, J. A. 1994. Septic injury of *Drosophila* induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides. *J. Biol. Chem.* **269,** 33159–33163.

Firstencel, H., Butt, T. M., and Carruthers, R. I. 1990. A fluorescence microscopy method for determining the viability of entomophthoralean fungal spores. *J. Invertebr. Pathol.* **55,** 258–264.

Gillespie, J. P., Bailey, A. M., Cobb, B., and Vicinskas, A. 2000a. Fungi as elicitors of insect immune responses. *Arch. Insect. Biochem. Physiol.* **44,** 49–68.

Gillespie, J. P., Burnett, C., and Charnley, A. K. 2000b. The immune reponse of the desert locust *Schistocerca gregaria* during mycosis of the entomopathogenic fungus *Metarhizium anisopliae* var. *acridum. J. Insect Physiol.* **46,** 429–437.

Hajek, A. E., and Butler, L. 2000. Predicting the host range of entomopathogenic fungi. *In* "Nontarget Effects of Biological Control" (P. A. Follett and J. J. Duan, Eds.), pp. 263–276. Kluwer Academic, Dordrecht.

Hajek, A. E., Butler, L., and Wheeler, M. M. 1995. Laboratory bioassays testing the host range of the gypsy moth fungal pathogen *Entomophaga maimaiga. Biol. Contr.* **5,** 530–544.

Hung, S. Y., and Boucias, D. G. 1992. Influence of *Beauveria bassiana* on the cellular recognition response of the beet armyworm, *Spodoptera exigua. J. Invertebr. Pathol.* **60,** 152–158.

Iijima, R., Kurata, S., and Natori, S. 1993. Purification, characterization and cDNA cloning of an antifungal protein from the hemolymph of *Sarcophaga peregrina* (flesh fly) larvae. *J. Biol. Chem.* **268,** 12055–12061.

Lamberty, M., Ades, S., Uttenweiler-Joseph, S., Brookhart, G., Bushey, D., Hoffman, J. A., and Bulet, P. 1999. Isolation from the lepidopteran *Heliothis virescens* of a novel insect defensin with potent antifungal activity. *J. Biol. Chem.* **274,** 9320–9326.

Levashina, E., Ohresser, S., Bulet, P., Reichart, J., Hetru, C., and Hoffmann, J. 1995. Metchnikowin, a novel immune-inducible proline-rich peptide from *Drosophila* with antibacterial and antifungal properties. *Eur. J. Biochem.* **233,** 694–700.

Lopez Lastra, C. C., and Boucias, D. G. 1994. Studies on the cellular reactions of *Spodoptera exigua* larvae infected with the fungus *Nomuraea rileyi. J. Invertebr. Pathol.* **63,** 101–102.

Meister, M., Lemaitre, B., and Hoffman, J. A. 1997. Antimicrobial peptide defense in *Drosophila* with antibacterial and antifungal properties. *Bioessays* **19,** 1019–1706.

Murrin, F., and Nolan, R. A. 1987. Ultrastructure of the infection of spruce budworm larvae by the fungus *Entomophaga aulicae. Can. J. Bot.* **65,** 1694–1706.

Pendland, J., Hung, S. Y., and Boucias, D. G. 1993. Evasion of host defence by *in vivo*-produced protoplast-like cells of the insect mycopathogen *Beauveria bassiana. J. Bacteriol.* **175,** 5962–5969.

Soper, R. S., Shimazu, M., Humber, R. A., Ramos, M. E., and Hajek, A. E. 1988. Isolation and characterization of *Entomophaga maimaiga* sp. nov., a fungal pathogen of gypsy moth, *Lymantria dispar,* from Japan. *J. Invertebr. Pathol.* **51,** 229–241.

Vilcinskas, A., Matha, V., and Götz, P. 1997. Inhibition of phagocytic activity of plasmatocytes isolated from *Galleria mellonella* by entomogenous fungi and their secondary metabolites. *J. Insect Physiol.* **43,** 475–483.