A Monoclonal Antibody to Pink Bollworm (Lepidoptera: Gelechiidae) Egg Antigen: A Tool for Predator Gut Analysis

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ABSTRACT We describe the development, selection, and application of a monoclonal antibody (MAb) to eggs of pink bollworm, *Pectinophora gossypiella* (Saunders). We tested this MAb against all pink bollworm life stages and the egg stage of 10 other insect species using an enzyme-linked immunosorbent assay (ELISA). In all cases, the MAb was highly specific to pink bollworm egg and adult female antigens. A Western blot analysis showed that the MAb reacted with two egg polypeptides with molecular weights between 46 and 60 kDa. Predation studies were conducted in the laboratory to test the usefulness of this MAb for studying predator-prey interactions. Most predators fed either one or two pink bollworm eggs responded positively to the MAb in a serological analysis of gut contents. These data suggest that this MAb can be used as a diagnostic probe for gut content analysis of potential predators of pink bollworm eggs under field conditions.

KEY WORDS *Pectinophora gossypiella*, monoclonal antibodies, predation

THE PINK BOLLWORM, *Pectinophora gossypiella* (Saunders), a key cotton pest in the southwestern United States, has been responsible for continual economic loss in Arizona and California since 1967. Mainly because of pink bollworm pressure, cotton production in California's Imperial Valley has dropped from >40,000 ha to <6,000 ha in the last decade (Henneberry 1989). Estimates from Arizona in 1990 indicate that >90% of the cotton acreage was infested by pink bollworm and that an average of seven insecticide applications were made against this pest during the growing season (Head 1991). Insecticides remain the primary method of suppressing pink bollworm populations; however, the overreliance on pesticides has resulted in increased insecticide resistance, secondary pest outbreaks, destruction of the natural enemy complex, and environmental pollution. Researchers and cotton growers, as well as the general public, now seek more cost-effective and environmentally benign methods of control. One such method receiving renewed attention is biological control via the introduction of exotic natural enemies and the exploitation of indigenous arthropod predators that inhabit cotton in the southwestern desert (Naranjo et al. 1993).

Despite their potential, indigenous predators have not been consciously used in present management systems for pink bollworm. Most of the common arthropod predator species found in desert cotton fields feed on one or more stages of pink bollworm. To date, 25 species have been identified as potential predators based on laboratory feeding trials and field cage studies (Orphanides et al. 1971, Irwin et al. 1974, Fye 1979, Shields & Watson 1980, Henneberry & Clayton 1985). Although limited work suggests that predation of artificially placed pink bollworm eggs may reach 95% over a 3-d period (Henneberry & Clayton 1982), the effect of these predators under natural conditions in the field is poorly understood. The major obstacle is the difficulty of studying predators in their natural environment. Direct observations of predation are difficult because of the small size, cryptic nature, and nocturnal activity of predators and pests. Furthermore, many predators preorally digest their foodstuffs, making direct gut examinations impossible (Cohen 1990).

One way to circumvent the problem of direct observation for identifying the more important predator species and for measuring their effect on pest population dynamics is to develop pest-specific monoclonal antibodies (MAbs) (Lenz & Greenstone 1988, Hagler et al. 1991). With highly specific MAbs, predator gut contents can be analyzed for the presence of pest-specific antigen by an enzyme-linked immunosorbent assay

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Table 1. Species and life stages examined for cross-reactivity to a species- and stage-specific monoclonal antibody to pink bollworm egg antigen

Species	Order: family	Stage
Pectinophora gossypiella[a]	Lepidoptera: Gelechiidae	Egg-Adult
Spodoptera exigua	Lepidoptera: Noctuidae	Egg
Heliothis virescens	Lepidoptera: Noctuidae	Egg
Tricoplusia ni	Lepidoptera: Noctuidae	Egg
Geocoris punctipes	Heteroptera: Lygaeidae	Egg
Lygus hesperus	Heteroptera: Miridae	Egg
Chlorochroa uhleri	Heteroptera: Pentatomidae	Egg
Euschistus inflatus	Heteroptera: Pentatomidae	Egg
Thyanta custator accerra	Heteroptera: Pentatomidae	Egg
Leptoglossus zonatus	Heteroptera: Coreidae	Egg
Bemisia tabaci	Homoptera: Aleyrodidae	Egg

[a] Pink bollworm eggs examined were collected from both a laboratory culture maintained at the Western Cotton Research Laboratory, Phoenix, AZ, and from cotton fields located near Maricopa, AZ.

(ELISA). This study reports the development, mass-screening, and application of a species- and stage-specific MAb to pink bollworm. We targeted the pink bollworm egg stage for identification because it is the most vulnerable stage to predator attack. Uses of this MAb for studying pink bollworm predation in the field are discussed.

Materials and Methods

Antibody Production. Pink bollworm eggs were analyzed for protein content by the Bradford method (1976) using a Bio-Rad protein assay kit. Three BALB/cByJ female mice (10–12 wk old) were then immunized by intraperitoneal injection of 100 μl of a 1:1 emulsion of Ribi's RAS (Ribi Immunochem Research, Hamilton, MT) adjuvant and 20 μg of crude egg protein from pink bollworm in phosphate-buffered saline (PBS). The mice received three booster injections of egg protein, the first and second with 20 μg protein in PBS in 100 μl Ribi's RAS adjuvant (1:1), and the third in PBS alone (20 μg protein) at 3-wk intervals. Mouse serum was collected 3 d after the final booster and the concentration of anti-pink bollworm egg antibodies were estimated by ELISA (Voller et al. 1976). The serum of the mouse with the strongest positive response at a 1:1,600 dilution determined which mouse was selected for fusion. The fusion of anti-pink bollworm egg antibody producing cells with SP2/O myeloma cells was identical to that described by Hagler et al. (1991).

Hybridoma Supernatant Screening. Supernatant screening of fused hybrid cells was performed using ELISA. Fifty μl of crude pink bollworm egg antigen (5 μg/ml) in PBS (pH 7.4) were placed in each well of a 96-well assay plate (Falcon Pro-Bind 3915) and incubated at 4°C overnight. After removal of the unbound antigen from the assay plate, 350 μl of 1.0% nonfat dry milk in distilled H_2O was added for 1 h at 37°C to block any unoccupied sites in the wells. Wells were briefly rinsed three times with PBS-Tween 20 (0.05%) and twice with PBS. Duplicate samples from individual supernatants (96 total) from each hybridoma culture were dispensed (50 μl) into wells of the pretreated 96-well assay plates. Each plate included a PBS blank, a positive control (polyclonal antiserum from the immunized mice diluted 1:800 in PBS), and a negative control (preimmune normal mouse serum diluted 1:800 in PBS). Plates were incubated for 1 h at 37°C, then rinsed as above. Aliquots (50 μl) of goat antimouse IgG/IgM conjugated to alkaline phosphatase (TAGO Inc., Burlingame, CA) diluted 1:500 in 1% nonfat milk were added to the wells and incubated for 1 h at 37°C. Plates were again rinsed as above, and 50 μl of 1.0 mg/ml p-nitrophenyl phosphate substrate (Sigma Chemical, St. Louis, MO) in 1M diethanolamine and 0.5mM $MgCl_2$ (pH 9.8) was added to each well. After 15 min, the absorbance of each well was measured using a Dynatec MR 700 (Chantilly, VA) microplate reader set at 405 nm. Of the 292 hybridomas and cloned cell lines we examined, the hybridoma designated S3D6-H2-H11 was selected for more extensive cross-reactivity tests because of its specificity and sensitivity toward pink bollworm egg antigen.

Monoclonal Antibody Cross-Reactivity Testing. The MAb S3D6–H2–H11 was mass-screened by ELISA for recognition of molecules within other insect species. The ELISA procedure was carried out exactly as described above, except that individual wells were coated with the insect homogenates presented in Table 1. Individual samples (≈20 per treatment) were prepared by grinding one egg or one pink bollworm life stage in 250 μl PBS. Individual wells of the 96-well assay plate were coated separately with a 50-μl aliquot of each sample.

Monoclonal Antibody Characterization. SDS-PAGE analysis of pink bollworm egg protein extract (10 μg total protein) was performed using the method of Laemmli & Favre (1973). A 4–15% gradient gel was run at 30 mA constant current for 4 h at room temperature. Proteins were stained with Coomassie blue R-250 (6 g in 500 ml

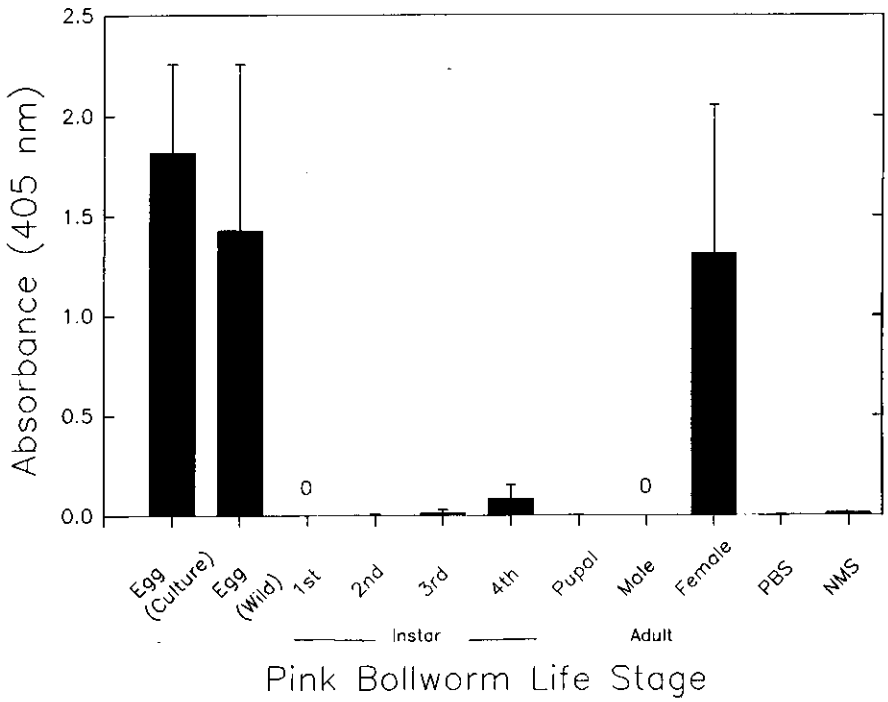

Fig. 1. Reactivity of pink bollworm egg-specific monoclonal antibody to different pink bollworm life stages, phosphate-buffered saline (PBS) blanks, and normal mouse serum (NMS) negative controls (mean ± SD, $n = 20$) as determined by ELISA.

methanol, 400 ml distilled H_2O, and 100 ml acetic acid) and destained in 10% (vol/vol) acetic acid plus 30% (vol/vol) methanol. Standards used to estimate the molecular weights of the egg proteins were obtained from Bio-Rad Laboratories (Richmond, CA); Rainbow protein molecular weight markers were obtained from Amersham Corp. (Arlington Heights, IL).

Polypeptides from the SDS-PAGE gel were electroblotted at 0.72 amps for 1 h onto a 0.45 μm nitrocellulose membrane (Schleicher & Schuell, Keene, NH) using a Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell (Richmond, CA). The transfer buffer consisted of 25mM Tris, 192mM glycine, and 20% vol/vol methanol (Towbin et al. 1979).

The protein-transferred nitrocellulose membrane was assayed against the MAb by rinsing the nitrocellulose in PBS-Tween (0.05% Tween-20) and PBS, then soaking for 30 min in 1.0% nonfat milk, rinsing three times with PBS-Tween, and incubating in MAb for 2 h. The nitrocellulose membrane was removed from the supernatant and rinsed as described above, then incubated in a 1:500 (vol/vol) dilution of goat antimouse IgG/IgM alkaline phosphatase-labelled secondary antibody (TAGO) for 1 h. The nitrocellulose membrane was developed using a Bio-Rad alkaline phosphatase conjugate substrate kit. The specific binding of the MAb was revealed by purple bands on the nitrocellulose. Antibody class and subclass were characterized by ELISA using reagents supplied in an ImmunoSelect (Gibco BRL, Grand Island, NY) isotype kit.

Predator Feeding Trial. We conducted a laboratory feeding trial to determine if pink bollworm egg antigen remains detectable in a predator's gut after consumption. The predators tested included adult *Collops vittatus* (Say) (Coleoptera: Melyridae), *Orius tristicolor* (White) (Heteroptera: Anthocoridae), *Geocoris punctipes* (Say) (Heteroptera: Lygaeidae), and third-instar *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae). Predators were collected from laboratory cultures, from sunflower fields located at the University of Arizona's Campbell Avenue Farm (Tucson, AZ), or from commercial alfalfa fields located 25 km NW of Tucson. In all instances, precautions were taken to guarantee that none of the predators collected was exposed to pink bollworm eggs before the laboratory feeding trial. Each predator was isolated in an individual petri dish and fed larvae of cabbage looper, *Tricoplusia ni* (Hubner), and water ad libitum for a minimum of 72 h. This is an adequate amount of time for a predator to digest any foodstuffs it might have contained at the time of collection (Hagler & Cohen 1990).

Before the feeding trials, 20 predators from each of the four species were placed individually in 9.0-cm petri dishes and isolated from any food overnight. Ten predators from each species were fed one or two pink bollworm eggs. After each

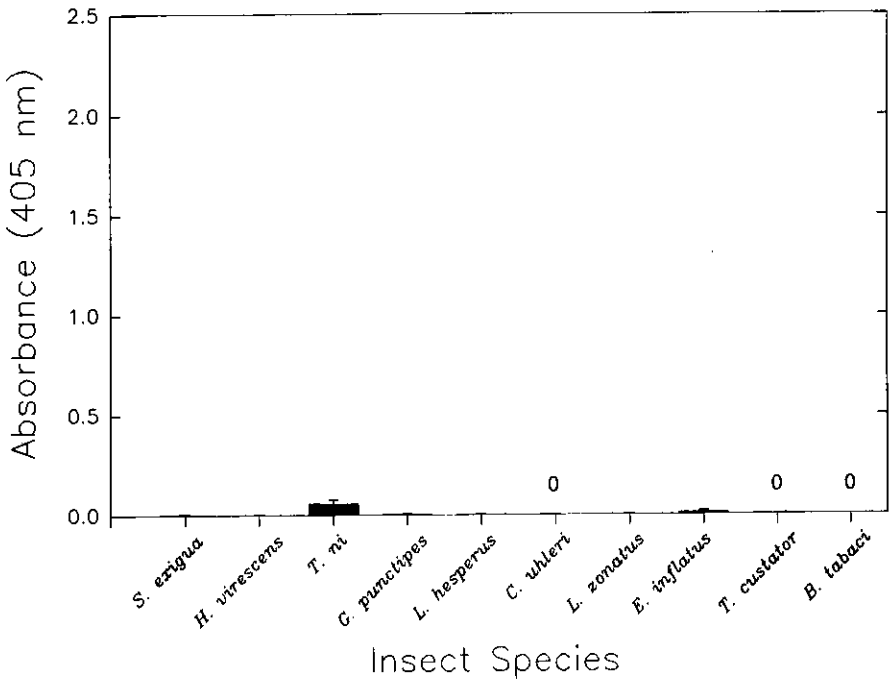

Fig. 2. Reactivity of pink bollworm egg-specific monoclonal antibody to eggs of several insect species (mean ± SD, $n = 20$) as determined by ELISA.

individual was observed feeding, the predator was isolated from any food source and held for 1 h at 27°C. Individuals were then macerated in 250 μl PBS and assayed by ELISA. The ELISA was exactly as described above, except that substrate incubation was for 1 h. The remaining 10 starved predators from each species (negative controls) were also assayed by ELISA.

Individual predators were scored positive for the presence of pink bollworm egg antigen if the absorbance value was three standard deviations above that of the respective negative control mean (Sutula et al. 1986). Significant differences between the mean ELISA absorbance values obtained from the negative controls and those fed pink bollworm eggs were determined using *t* tests.

Results

Hybridoma Supernatant Screening. After two clonings, a single hybridoma cell line designated S3D6–H2–H11 was selected for mass production. This particular MAb-producing cell line was selected for its specificity and sensitivity to pink bollworm egg antigen, its rapid growth rate, and its stability.

Monoclonal Antibody Cross-Reactivity Testing. This MAb was highly species- and stage-specific against the insects we tested. The pink bollworm stage specificity of S3D6–H2–H11 is presented in Fig. 1. The only positive responses were associated with the pink bollworm egg and adult female treatments. The positive ELISA response associated with the adult females was expected, because a gravid female carries egg proteins. When S3D6–H2–H11 was tested for species specificity against other insects, no positive responses were observed (Fig. 2). All other insect eggs tested yielded absorbance values near or below the value of the PBS blank and normal mouse serum (NMS) negative controls (Fig. 1).

Monoclonal Antibody Characterization. SDS-PAGE analysis revealed many pink bollworm egg polypeptides (gel not shown). When S3D6–H2–H11 was characterized for its affinity to these

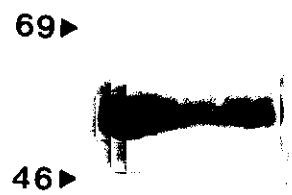

Fig. 3. Immunoblot of a 4–15% gradient (SDS-PAGE) gel containing 10 μg of pink bollworm egg protein. The blot was assayed against pink bollworm egg-specific monoclonal antibody.

Table 2. ELISA results testing for the presence of pink bollworm (PBW) egg antigen in the guts of predators using PBW egg-specific monoclonal antibody

Predator species	Negative Control Predators			Predators fed 1-2 PBW Eggs		
	Absorbance at 405 nm, mean ± SD (range)	Critical value[a]	No. positive reactions[b]	Absorbance at 405 nm, mean ± SD (range)	No. positive reactions	Significance[c]
Collops vittatus	−0.032 ± 0.016 (−0.048–−0.004)	0.016	0	0.571 + 0.350 (0.004–1.004)	9	***
Orius tristicolor	−0.009 ± 0.021 (−0.045– 0.007)	0.054	0	0.644 ± 0.591 (0.146–2.030)	10	**
Geocoris punctipes	−0.013 ± 0.009 (−0.027– 0)	0.014	0	0.061 ± 0.106 (−0.022–0.316)	6	*
Chrysoperla carnea	−0.035 ± 0.004 (−0.029–−0.043)	−0.022	0	0.041 ± 0.127 (−0.052–0.302)	5	NS

[a] Mean + 3 SD of the negative controls (Sutula et al. 1986).
[b] Based on the critical value of the negative control predators.
[c] Significant differences (*t* test) between negative control predators and their counterparts fed PBW eggs: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS, not significant ($n = 10$ for each treatment).

polypeptides by immunoblotting, it bound specifically to two sites with approximate molecular weights of 46 and 60 kDa (Fig. 3). Immunoglobulin subclass identification of S3D6–H2–H11 showed it to be IgG_{2A} with light kappa chains.

Predator Feeding Trial. All of the negative control predators we assayed yielded negative absorbance values (Table 2), indicating a lack of pink bollworm egg antigen in the gut. Of the predator species fed pink bollworm eggs, individuals representing *C. vittatus* and *O. tristicolor* yielded consistently high (positive) absorbance values. Conversely, individuals representing *G. punctipes* and *C. carnea* yielded inconsistent absorbance values. Only about half of the individual *G. punctipes* and *C. carnea* fed pink bollworm eggs scored positive.

No significant difference was found between the mean absorbance values of *C. carnea* fed pink bollworm eggs and their negative control counterparts (Table 2). In contrast, we found significantly higher absorbance values for *C. vittatus*, *O. tristicolor*, and *G. punctipes* that were fed pink bollworm eggs than those not fed any eggs. Although there was no significant difference between the two *C. carnea* treatments, 3 of the 10 individuals assayed yielded absorbance values well above the critical threshold value (i.e., 0.154, 0.191, and 0.302). The other two *C. carnea* that scored positive were closer to the critical value, with absorbances of −0.012 and 0.01, respectively.

Discussion

We developed a MAb that exclusively recognizes the pink bollworm egg and adult female stages. Such high specificity has rarely been achieved for predation studies. Lenz & Greenstone (1988) developed a stage-specific MAb to measure predation of fifth instar *Heliocoverpa zea* (Boddie) in the laboratory (Greenstone & Morgan 1989). Hagler et al. (1991, 1993) have developed MAbs to the egg stage of *Lygus hesperus* Knight and *Bemisia tabaci* (Gennadius) for measurement of predation in the field. We report here the fourth such highly species- and stage-specific MAb for use in predation studies.

Preliminary results of our laboratory feeding trials indicate that not all predators can be assayed equally effectively for the presence of pink bollworm egg antigen. For instance, 90% of the *C. vittatus* and 100% of the *O. tristicolor* that were fed pink bollworm eggs yielded a positive response. However, only 60% of the *G. punctipes* and 50% of the *C. carnea* fed pink bollworm eggs scored positive. Before testing for potential predators of pink bollworm in the field, we recommend further testing of the efficacy of this gut content assay under laboratory conditions.

Species- and stage-specific MAbs used in an ELISA are invaluable tools for studying predation in the field (Hagler et al. 1992). This technique eliminates many problems inherent with macroscopic, microscopic, and physiological evaluations of predation (Sunderland 1987). The use of MAbs as molecular probes for examining predator gut contents can significantly enhance the way we study insect predation. MAbs offer investigators a precise, easy, rapid, and economical method to evaluate predators in their natural environment (Hagler et al. 1992). The fact that this MAb reacted with pink bollworm adult females does not reduce its usefulness for predation studies and may in fact increase its utility when used in conjunction with knowledge of pest and predator biology. Pink bollworm adults are too large and evasive for many of the smaller predators to attack and consume. Therefore, a positive ELISA response by one of these types of predators will most likely be caused by egg predation. Likewise, a positive response in a larger predator that seeks mobile prey (i.e., assassin bugs, many spiders, etc.) would be more indicative of predation of an adult female moth.

Predation studies using MAbs will provide a better understanding of the key predators of important pests and allow a more realistic assessment of their feeding behavior in the field. Once key predators are identified, we can begin to study their habitat requirements more thor-

oughly and manipulate their populations more effectively for improved biological pest control. Moreover, these key predators could be mass-reared for use in augmentative biological control programs. Our pink bollworm egg MAb is currently being used in mass-screenings to identify key indigenous predators in pursuance of applying these biological control approaches.

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