

Stem Cells From Midguts of Lepidopteran Larvae: Clues to the Regulation of Stem Cell Fate

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Previously, we showed that isolated stem cells from midguts of *Heliothis virescens* can be induced to multiply in response to a multiplication protein (MP) isolated from pupal fat body, or to differentiate to larval types of mature midgut cells in response to either of 4 differentiation factors (MDFs) isolated from larval midgut cell-conditioned medium or pupal hemolymph. In this work, we show that the responses to MDF-2 and MP in *H. virescens* stem cells decayed at different time intervals, implying that the receptors or response cascades for stem cell differentiation and multiplication may be different. However, the processes appeared to be linked, since conditioned medium and MDF-2 prevented the action of MP on stem cells; MP by itself appeared to repress stem cell differentiation. Epidermal growth factor, retinoic acid, and platelet-derived growth factor induced isolated midgut stem cells of *H. virescens* and *Lymantria dispar* to multiply and to differentiate to mature midgut cells characteristic of prepupal, pupal, and adult lepidopteran midgut epithelium, and to squamous-like cells and scales not characteristic of midgut tissue instead of the larval types of mature midgut epithelium induced by the MDFs. Midgut stem cells appear to be multipotent and their various differentiated fates can be influenced by several growth factors. Arch. Insect Biochem. Physiol. 53:186–198, 2003. Published 2003 Wiley-Liss, Inc.[†]

KEYWORDS: midgut; stem cells; growth factors; differentiation; proliferation

INTRODUCTION

The midgut is the largest organ in insect larvae, save for the external epithelium. It is a processor of foodstuffs as well as an entry point for ingested toxins and viruses (Billingsley and Lehane, 1996; Keddie et al., 1989). Midgut cells from larvae of the Lepidoptera *Choristoneura fumiferana*, *Lymantria dispar*, *Bombyx mori* (Baines et al., 1994), *Manduca sexta* (Sadrud-Din et al., 1994), and *Heliothis virescens* (Loeb and Hakim, 1999) have been suc-

cessfully grown in vitro as primary and secondary cultures. The cells in these cultures are mixtures of typical larval midgut epithelial cell types, i.e., columnar, goblet, secretory, and stem cells. However, only the stem cells undergo mitosis and subsequent metamorphosis to the other cell types in vivo (Spies and Spence, 1985; Baldwin and Hakim, 1991; Billingsley and Lehane, 1996) and in vitro (Sadrud-Din et al., 1996; Loeb and Hakim, 1996) during molting and to repair injured midgut tissue (Spies and Spence, 1985; Loeb et al., 2001).

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Commercial products used in this study are not endorsed by the US Department of Agriculture.

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Received 13 September 2002; Accepted 14 May 2003

Thus, the developmental biology of the insect midgut epithelium is similar to that of mammals, where all mature gut epithelial cells are derived from gut stem cells (Watt and Hogan, 2000).

The midgut epithelium of larval Lepidoptera consists of a monolayer of mature goblet and columnar cells, with relatively few secretory cells, tightly connected to each other by pleated septate junctions during the intermolt period (Baldwin and Hakim, 1991; Billingsley and Lehane, 1996). The stem cells lie loosely associated among the bases of the epithelial cells, and insert themselves between existing mature cells as they differentiate during each larval molt (Baldwin and Hakim, 1991; Billingsley and Lehane, 1996). When whole lepidopteran midguts are incubated in culture medium, the more mobile stem cells tend to migrate away from the tissue mass before most of the other cell types; by collecting these loosely bound cells, it has been possible to prepare cultures highly enriched in midgut stem cells (Sadrud-Din et al., 1996; Loeb and Hakim, 1996; Elsen et al., 2002).

Proliferation, differentiation, and development of mammalian stem cells is controlled by numerous endogenous and circulating growth factors (Slack, 2000). Cell-free conditioned medium (CONMED) from cultured larval midgut induced stem cells isolated from prepupal midgut to differentiate to larval type columnar cells, while CONMED from prepupal midgut cultures induced stem cells from larval midgut to differentiate to prepupal types of cells, indicating that insect midgut differentiation is similarly chemically regulated (Loeb and Hakim, 1996). The number of well-characterized insect growth factors is still limited (Homma et al., 1996; Clark et al., 1997; Hatt et al., 1997; Hayakawa and Ohnishi, 1998; Mitsuhashi, 1998; Kawamura et al., 1999; Strand et al., 2000). We have characterized and synthesized four peptidic growth factors from cell-free larval midgut culture medium and from pupal hemolymph that stimulate the differentiation of lepidopteran midgut stem cells in culture (Loeb et al., 1999; Loeb and Jaffe, 2002). Pupal fat body, or an aqueous extract of that tissue (FBX), has been used to stimulate proliferation of midgut stem cells in vitro

(Sadrud-Din et al., 1994; Loeb and Hakim, 1999); the active protein from lepidopteran fat body extract has recently been isolated (Blackburn et al., unpublished observation). Use of these factors in this work has facilitated the study of the relationship between proliferation and differentiation in midgut stem cells from *H. virescens* and *L. dispar* in response to insect midgut growth factors. Although previous studies have indicated that vertebrate growth factors had little effect on insect cell lines in vitro (Nishino and Mitsuhashi, 1995), a number of commercially available mammalian factors were also incubated with midgut stem cells from *H. virescens*, with some success in changing the differentiation of stem cells from larval to putative prepupal and pupal types, and in inducing non-midgut fates.

MATERIALS AND METHODS

Experimental Animals

Eggs of the tobacco budworm, *Heliothis virescens* were provided by the Western Cotton Insects Laboratory, USDA (Phoenix, AZ). The larvae were raised on artificial diet (Stonefly Industries, Inc., Bryan, TX), at 30°C under LD 16:8 as described by Loeb and Hakim (1996). Gypsy moth eggs were obtained from the rearing facility at Otis Air Force Base, MA and reared on artificial diet under LD 12:12 at 25°C, as described in Bell et al. (1981).

Approximately 24 h prior to molting, larvae become swollen due to the release of molting fluid into the intercutaneous space (the puffy stage). A few hours prior to ecdysis, the existing head capsule is displaced forward, exposing the newly formed neck membrane (slipped-head stage). The slipped-head stage of 4th instar larvae of *H. virescens* and 4th and 5th instar *L. dispar* were used for most midgut cell cultures, as described in Loeb et al. (1999). Mixed cell cultures were also prepared from wandering, prepupal, pupal, and adult stages of *H. virescens*.

Stem Cell Cultures

Standard medium consisted of modified Grace's medium (Sadrud-Din et al., 1994, 1996) contain-

ing 25 $\mu\text{l/ml}$ fat body extract (FBX) and, in this work, $6 \times 10^{-8}\text{M}$ 20 hydroxyecdysone (20E) (Sigma Corp, St Louis, MO). Initial midgut stem cell preparations were made by incubating opened, washed midguts in standard culture medium for no longer than 1.5 h. Preparations were strained through 70- μm nylon sieves (Beckton Dickinson Corp, Franklin Lakes, NJ). Each sieve contained 50–75% stem cells. The cells were centrifuged at 600g; supernatant was removed and replaced by 1 ml fresh medium. The medium containing the stem and other cells were then layered on 3 ml of a commercial ficoll preparation (Ficoll Paque, Pharmacia, Uppsalla, Sweden), and centrifuged for 15 min at 600g; a majority of the stem cells remained in the first milliliter of the column, while most columnar and goblet cells moved to the lowest 250 μl of the column (Loeb and Jaffe, 2002). In Loeb and Jaffe (2002), we described washing the stem cell layer free of Ficoll Paque using sterile Insect Ringer solution. Since some of the stem cells tended to break apart during and after Ringer centrifugation, we used protein-rich sterile culture medium (Loeb et al., 1999) in this work instead of Ringer solution to more gently cleanse the cells of ficoll. The highly purified stem cells (80–95% stem cells) were maintained at 4°C for up to 25 days in standard medium in order to maintain their viability. Experimental procedures entailed use of conditioned medium, various growth factors, and hormones incubated with the stem cell preparations at 26°C.

Conditioned Medium

We maintained mixed secondary cultures containing all stages of *H. virescens* larval midgut cell types in 3 ml of standard medium in each well of 6-well plates (Falcon). Each week, cultures were fed by removing 1 ml of medium from each well and replacing it with 1 ml of fresh standard medium. Five hundred milliliters of medium taken from cultures 2 to 12 weeks old was collected, combined, and centrifuged at 800g for 10 min. The resulting cell-free supernatant was frozen in 1-ml aliquots. In order to provide uniformity, thawed aliquots of

this preparation were used as conditioned medium (CONMED throughout these studies

Experimental Methods

We used freshly prepared and ficoll-purified stem cell suspensions, as well as preparations that were stored for various amounts of time at 4°C in standard medium or at 4°C in 25% conditioned medium: 75% standard medium.

To set up an experiment, the number of stem cells in a 5- μl aliquot of a 1-ml suspension was counted, enabling accurate dispersion of 500 stem cells to each well of a 24-well plate (Falcon, Beckton Dickinson Corp). The total fluid volume in each well was adjusted to 400 μl . Experimental and control wells were prepared in duplicate or quadruplicate. Plates were sealed with tape, gently rotated to mix contents and then transferred to 26°C in the dark.

Hormones and Growth Factors

Midgut differentiation factors. The 4 synthetic midgut differentiation factors (MDFs) have statistically the same differentiation-inducing effect (approximately 40% differentiation) when administered separately (Loeb and Jaffe, 2002). Therefore, only one of the factors (synthetic MDF-2) was used at 10^{-8}M in all experiments in this series (Loeb and Jaffe, 1999).

Multiplication protein. The Multiplication Protein (MP) was isolated from FBX by use of cation exchange high pressure liquid chromatography and was available as a pure protein solution (verified by further HPLC and presence of a single band on SDS PAGE) containing 17 ng protein/ μl in bovine serum albumin equivalents (Blackburn et al., unpublished). The maximum effective dose of MP for stem cells from larval midguts of *H. virescens* was 50 ng protein (measured in BSA equivalents) in 3 μl fluid (Blackburn et al., unpublished), the dose used in all experiments herein described.

Peptidic growth factors. Stem cells were incubated in standard medium with the following commercially available factors: adipokinetic hormone, epi-

dermal growth factor (EGF), FMRamide, insulin, melatonin, nerve growth factor, β -Factor fragment 742–758 of platelet-derived growth factor (PDGF), proctolin, all trans retinoic acid (RA), transforming growth factor β (TGF β) (all at 10^{-6} M). Lutetotropic hormone and thyrotropic hormone were tested at 1 unit per ml. All of the factors were obtained from Sigma, St. Louis, MO.

Bioassay methods. In order to assay effects, cultures incubated with MDF-2 were maintained at 26°C for 6–7 days; cultures incubated with MP required 4 days; vertebrate growth factors required 2–3 weeks to show effects. Cultures were “fed” weekly by removing 100 μ l of fluid from each well, replacing it with an equal volume of fresh standard culture medium plus the factor under study. Identical numbers of cells in control wells were incubated in standard medium without added factors and were fed with standard medium only.

At the end of each observation period, the number of each cell type in each well was counted. The sum of the differentiated (non-stem) cells was divided by the total number of cells in each well to give the percent differentiated cells. Response to MP is shown as the mean number of cells in each well minus the mean number of cells in control untreated wells after 4 days of incubation at 26°C. In studies designed to ascertain the length of time that stored, refrigerated, stem cells would respond to MDF-2 and MP, identical experiments using the same batch of stored cells were set up at progressive time intervals.

Histology. Cultures were centrifuged at 600g through sterile Ringer solution 3 times to remove proteins. Drops of approximately 35 μ l were placed onto poly-L-lysine treated microscope slides (Goto et al., 2001) and dried in air overnight at room temperature. Cells on slides were hydrated in tris buffered saline (TBS) for 30 min and then stained using Carazzi’s hematoxylin method (Carazzi, 1911). After staining, cells were dehydrated by taking the slides through an ascending alcohol series and xylene, and mounted to cover slips (Poly-Mount Xylene, Polysciences, Inc., Warrington, PA).

Statistics. Statistics were calculated with the aid of Graph Pad (La Jolla, CA).

RESULTS

Response to Midgut Differentiation Factor-2

Freshly isolated *H. virescens* stem cells lost the ability to respond to MDF-2 after two days of storage at 26°C in standard culture medium (Loeb and Hakim, 1996) (data not shown); storage at 4°C lengthened MDF-2 responsiveness, although the cellular response continually decreased over 5 days (squares, dashed line in Fig. 1).

Since stem cells multiply readily in mixed culture, cell-free conditioned medium (CONMED) from mixed cultures was used to maintain stem cells at 4°C. Thirty percent conditioned medium: 70% standard medium at 4°C seemed to be opti-

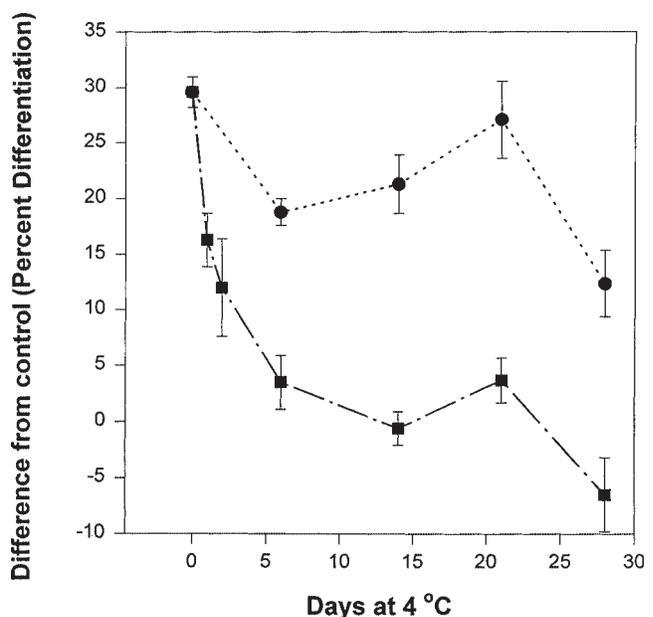


Fig. 1. Response of Ficoll-purified midgut stem cells from *Heliothis virescens* to MDF-2 (10^{-8} M) after storage at 4°C in standard culture medium (dashed line, squares) or in 25% conditioned medium: 75% standard culture medium (dotted line, circles). Cells were moved to 26°C just after addition of MDF-2 and incubated for 6–7 days before counting the differentiated (columnar and goblet) cells. The y axis indicates the difference between percent differentiation in untreated control cultures (no MDF-2) and those containing MDF-2. The x axis indicates the number of days that the same batch of stem cells was stored at 4°C. Error bars indicate SEMs. $n = 4$.

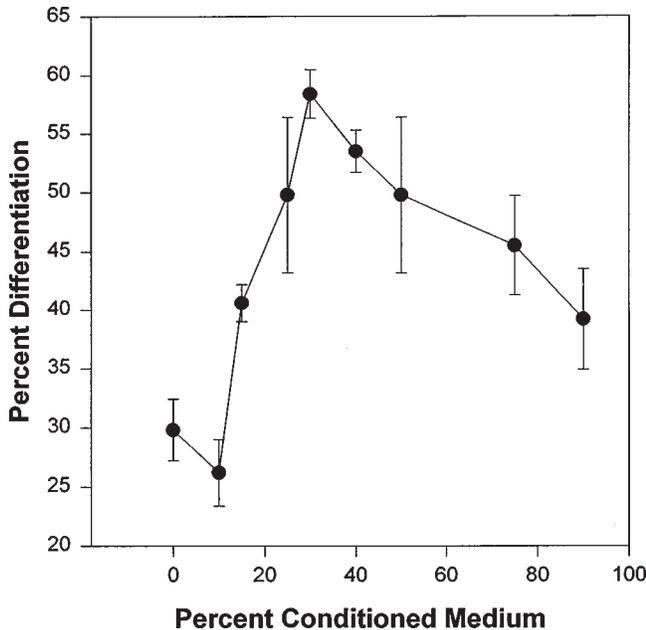


Fig. 2. Effect of increasing concentrations of conditioned medium (CONMED) on the effect of MDF-2 (10^{-8} M) in inducing differentiation of Ficoll-purified midgut stem cells from *Heliothis virescens* larvae. Cells were kept at 4°C for 5 days in various concentrations of CONMED, then incubated at 26°C in 10^{-8} M MDF-2. Cells were counted 7 days thereafter, and the % differentiated cells were calculated and plotted. Error bars indicate SEMs. $n = 4-8$ for each point.

imum for maintenance of midgut stem cells to respond to MDF-2 (Fig. 2), although we chose to use 25% conditioned medium: 75% standard medium for all experiments since it was high in the ascending portion of the response curve, and drop off in response occurred over 30% CONMED. It seemed safer to do this, since cell responses vary from batch to batch of cells (Fig. 2). Aliquots of stem cells maintained at 4°C in 25% CONMED remained responsive to MDF2 challenge at 26°C for at least 28 days, although a decline in activity was apparent after 21 days at 4°C (circles, dotted line in Fig. 1).

Exposure of stem cells to 25% CONMED at 4°C was not without serious effect. Newly prepared stem cell preparations that were not subjected to further Ficoll purification initially contained 50 to 75% stem cells. When such preparations were

maintained at 4°C in the presence of 25% CONMED for 1 to 20 days, washed, and then incubated in standard medium at 26°C , degenerating cells and cell fragments were observed within 24 h; approximately 40% of the original number of cells were lost. Counts of cell types revealed that most of the loss was due to degeneration of large mature cells. However, remaining stem cells continued to divide and differentiate, as indicated by the appearance of dividing pairs of stem cells and small differentiated cells (as seen in Fig. 5). Ten days after return to standard medium, a total of 3,265 columnar cells was counted in 3 separate lots of approximately 1,085 columnar cells each; $58 \pm 2.3\%$ of the new "columnar" cells in the medium were cuboidal in shape, each with a central nucleus and a microvillar fringe on one side, in contrast to larval-type columnar cells that were elongate hat or tongue-shaped cells with nuclei and a microvillar fringe in the apical portion of each cell (Fig. 5). A total of 2,405 columnar cells maintained in standard medium was counted in 2 batches of approximately 1,200 cells each; only $1.8 \pm 0.3\%$ of the control cells were cuboidal in shape and showed microvillar fringes. The rest of the cells with microvilli (98.2%) were elongate cells characteristic of larval columnar midgut.

Response to the Multiplication Protein by Stem Cells Maintained in Standard Medium at 4°C

Aliquots of *H. virescens* stem cells kept at 4°C in standard medium were moved to 26°C at various times and challenged with 50 ng of purified MP. In contrast to the limited lifetime of MDF-2 responses, stem cells were able to respond to MP even after 15 days at 4°C . A decline in responsiveness ensued thereafter (Fig. 3).

Response to the Multiplication Protein by Stem Cells Maintained in 25% CONMED at 4°C

H. virescens stem cells were maintained at 4°C with or without CONMED; at the end of 4 days, cells were washed twice, counted, distributed in groups of 500 to well plates, and incubated at 26°C

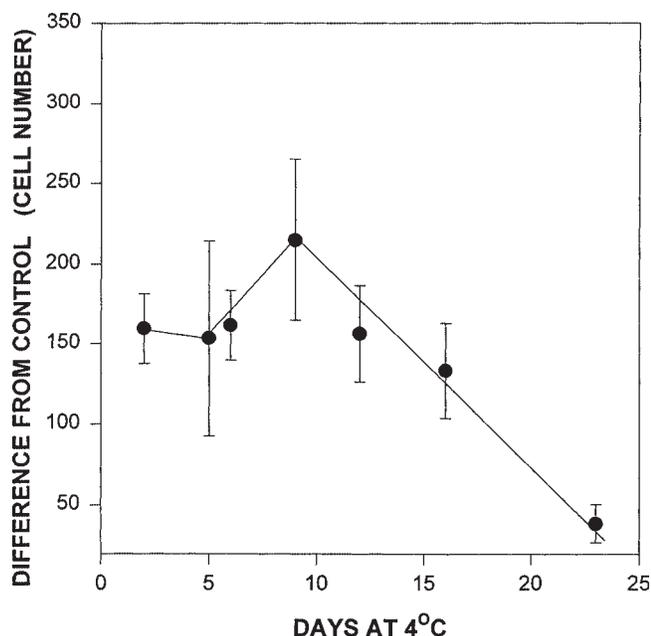


Fig. 3. Midgut stem cells, Ficoll purified, from *Heliothis virescens* larvae were kept at 4°C in standard medium for varying lengths of time (x axis) before moving to 26°C when MP (50 ng) was added. Cells were incubated at 26°C for 4 days prior to counting. The same procedures were followed in control cultures except for the addition of MP. Lines in this figure were drawn to approximate trends shown by the data. Error bars indicate SEMs. n = 6–8 for each point.

in standard medium with or without MP or MDF-2. Stem cells maintained at 4°C in standard medium responded to MP by increasing in number 2.6-fold after 4 days at 26°C. Although these MP-treated cells multiplied, only 4% had differenti-

ated at the end of 11 days, indicating that more proliferation than differentiation had occurred. In contrast, stem cells maintained in 25% CONMED at 4°C did not multiply in response to MP at 26°C (Table 1). Even though cells pre-treated with CONMED did not multiply when incubated with MP, they remained responsive to MDF-2 when it was added 4 days later; MDF-2 induced approximately 28% differentiation 7 days after its addition (Table 1).

Addition of MDF-2 to Stem Cells Prior to Addition of MP

Four cultures, each containing 500 freshly prepared *H. virescens* stem cells, were set up at 26°C in standard medium and 4 cultures were set up in medium containing 10⁻⁸M MDF-2. On the 6th day, the cultures were fed with either standard medium or standard medium containing MDF-2 (10⁻⁸M per well), respectively. At that time, MP was added to a pair of cultures in standard medium and a pair of cultures in standard medium plus MDF-2. Cell numbers and cell types were counted 10 days after the start of the experiment. The experiment was repeated. As shown in Figure 4, the total number of cells in the cultures containing standard medium or standard medium plus MDF-2 was approximately the same, although the percent of differentiated cells was 1.7 times greater in the cultures containing MDF-2. As expected, the total number of cells increased 1.5 times in cultures where MP was added on the 6th day. However, the percent

TABLE 1. Response in Number of Ficoll Enriched Midgut Cells of *Heliothis virescens* to Multiplication Protein, 25% Conditioned Medium (CONMED), and Differentiation in Response to MDF2 (10⁻⁸M) In Vitro*

Conditions	n	4th day (no. cells ± SEM)	Fold increase	n	11th day (% diffn ± SEM)
No CONMED, no MP	4	581 ± 48.0	1.2	4	4.3 ± 1.1
No CONMED (50 ng MP)	4	1285 ± 75.7	2.6	4	6.6 ± 1.0
CONMED (50 ng MP)	4	583 ± 44.3	1.2	—	—
CONMED + MDF-2	10	595 ± 34.8	1.2	6	28.1 ± 1.6
CONMED, MDF-2, + 50 ng MP	10	492 ± 53.2	0.99	6	27.4 ± 3.5

*Cells were stored at 4°C in either standard medium or CONMED as noted under Conditions, washed in standard medium; 500 cells were added to each well containing standard medium at 26°C. MP was added to some of the cultures, as noted. The number of cells in each well was counted after 4 days; MDF-2 was added at the 4th day, and cell number and types were noted on the 11th day; percent differentiation was calculated for the 11th day of culture.

CONMED = 25% conditioned medium; MP = multiplication protein; diffn = % differentiation; n = number of repeats; + implies addition of factor after the start of the experiment.

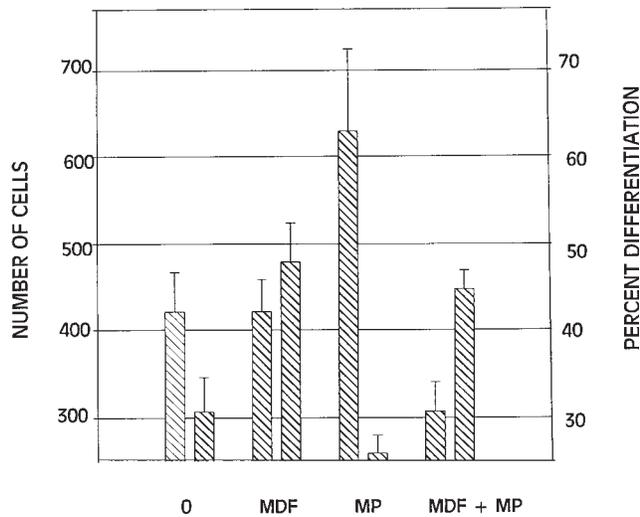


Fig. 4. Sixteen wells containing 500 freshly prepared (not Ficoll purified, and therefore with approximately 30% differentiated cells in the mixture) stem cells in standard medium were incubated at 26°C; an equal number of cultures in standard medium containing synthetic MDF-2 (10^{-8} M) was also prepared. MP was added to half of the control (without MDF) and half of the experimental wells (with MDF) 6 days after the start of the experiment. Total cell number and the number of differentiated cells were counted in each well on the 10th day of the experiment. 0 = untreated cells; MDF = synthetic MDF-2, administered only at the start of the experiment; MP = multiplication protein (50 ng in 3 μ l) added to untreated stem cells after 6 days in standard medium; MDF + MP = synthetic MDF-2, administered at the start of the experiment and MP administered on the 6th day. The left bar of each pair of bars represents the mean total number of cells, corresponding to the left y axis; the right bar of each pair, corresponding to the right y axis, represents the percent of differentiated cells under each condition. Error bars indicate SEMs. n = counts of cells in 4 cultures.

of differentiation in wells containing MP was 0.8 of the non-treated control. In contrast, when MDF-2 preceded MP, the total number of cells decreased to 0.7 that of the non-treated controls by the 10th day, even though the percent of cell differentiation was comparable to that in cultures receiving MDF-2 alone (Fig. 4). In all cases where differentiation increased in the presence of MDF-2, the resulting mature cells were all larval midgut epidermal forms.

Stem Cells Incubated With Growth Factors or Neuropeptides

Newly prepared stem cells were not further concentrated by Ficoll separation. Cells were maintained at 4°C in the presence of 25% CONMED for 1 to 20 days before the addition of the factors. However, cells were washed twice prior to addition of the factors, essentially removing non-bound CONMED.

Platelet-Derived Growth Factor (PDGF)

Mature *H. virescens* cells degenerated initially, although stem cells multiplied in PDGF, as evidenced by approximate doubling of the number of stem cells and the presence of stem cell doublets in each culture well by 10–14 days. After 15 days in 10^{-6} M PDGF, elongate granular cells, each with a prominent vacuole and no visible microvilli, were detected (Fig. 6) (Table 2).

Thyrotropic Hormone

In the presence of thyrotropic hormone (1 U/ml) for 7 days, *H. virescens* cells appeared to form clumps (Fig. 7). However, typical unclumped larval midgut cells were observed thereafter, despite the continuing presence of thyrotropic hormone. This effect was not detected in the presence of the 20E-containing standard medium nor in 20E at 10^{-6} M.

Epidermal Growth Factor (EGF) and Retinoic Acid (RA)

Newly prepared stem cells of *H. virescens* and *L. dispar* (not Ficoll purified), were incubated in standard medium containing EGF (10^{-6} M) or RA (10^{-6} M) for 36 and 48 h, respectively. These treatments caused both *H. virescens* and *L. dispar* midgut stem cells to multiply. Data for *L. dispar* midgut stem cells are shown in Table 3.

Exposure to EGF, RA, and EGF plus RA for 7 to 10 days elicited *H. virescens* stem cell daughters that were roughly twice as large as their stem cell mates

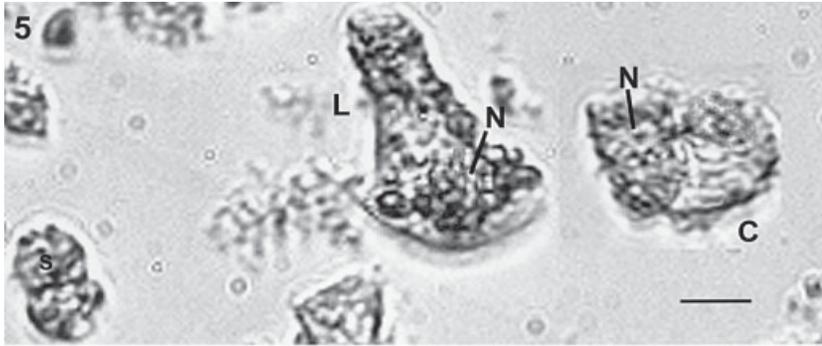


Fig. 5. Newly isolated *Heliothis virescens* stem cells were incubated in 25% CON-MED 4°C, and then returned to standard medium at 26°C for 10 days. Stem cells are dividing and differentiating. C = cuboidal columnar cell; L = larval columnar cell; N = nucleus; S = dividing midgut stem cells. Bar = 10 μ m.

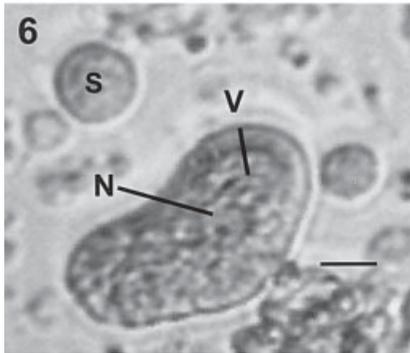


Fig. 6. Midgut cell from *Heliothis virescens* that has differentiated to an oval, vacuolated cell on exposure to PDGF (10^{-6} M). Vacuole (V) is marked. S = stem cell; Bar = 10 μ m.

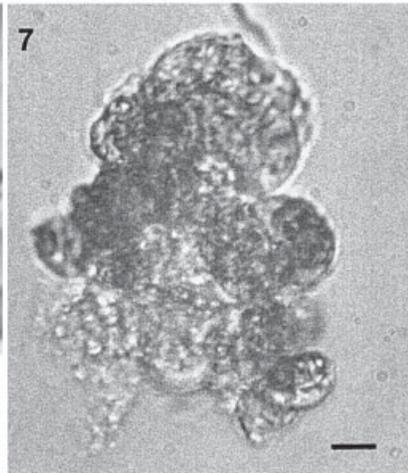


Fig. 7. Clumped stem cells from *Heliothis virescens* larval midgut stem cells treated with 1 U/ml thyrotropin for 7 days in vitro. Bar = 10 μ m.

(Fig. 8). After telophase, these partially differentiated daughter cells matured to large (60 μ m diameter), irregular, flattened cells with central nuclei that looked like typical squamous cells (Fig. 9). In some of the wells containing RA, we occasionally observed scales characteristic of those seen on adult wing in addition to the squamous-like cells (Fig. 10). The percent of squamous-like cells resulting from treatment with either RA or EGF, or EGF plus RA, was approximately 25% in each case. In contrast, untreated control cultures contained only 2% of squamous-like cell types; no scale-like types were observed in control cultures. (Fig. 11). It is pos-

sible that, given enough time, transformation of *L. dispar* stem cells might have also occurred after the 2–3-week incubation periods afforded to the *H. virescens* stem cells.

Other Factors and Hormones

H. virescens stem cells incubated for several weeks with insect factors adipokinetic hormone (Mordue and Stone, 1981), FMRFamide (Zitnan et al., 1990; 1993; Kingan et al., 1993), insulin (Smith et al., 1997), or proctolin (Brown and Starratt, 1975), or with mammalian factors melatonin, nerve growth

TABLE 2. Effects of PDGF (10^{-6} M) on Partially Purified Stem Cells of *Heliothis virescens* After 15 Days in Culture

No. cells counted	n	% stem	% vacuolated	% columnar types
Control ^a				
3651	4	80.8 \pm 2.7	0.07 \pm 0.05	20.5 \pm 1.9
PDGF				
2781	3	85.0 \pm 3.6	11.2 \pm 1.4	4.3 \pm 1.6

^aThe control consists of stem cells incubated in standard medium without PDGF.

TABLE 3. Effect of EGF and RA on Total Cell Number in Cultured *Lymantria dispar* Stem Cells, Counted after 48-H Exposure

	n	Cell no. mean \pm SEM*	Fold increase over control
Control (0)	6	417.6 \pm 1.1	
EGF	6	627.0 \pm 92.0	1.5
RA	6	587.3 \pm 53.8	1.4

* $P = 0.0664$, difference among the group means is significant.

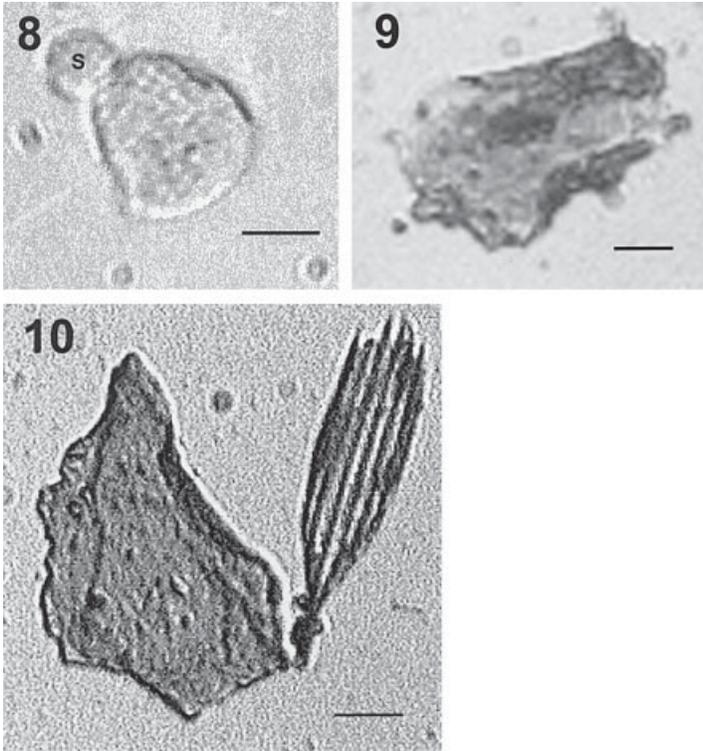


Fig. 8. Dividing midgut cell from *Heliiothis virescens* exposed to RA (10^{-6} M) for 10 days; one daughter cell is of normal size (S), while the other is enlarged and is probably differentiating to a squamous-like cell. Bar = approximately 20 μ m.

Fig. 9. Squamous-like cell derived from a *Heliiothis virescens* midgut stem cell exposed to EGF (10^{-6} M) for 14 days, stained using Carrazzi's hematoxylin method. Note its flatness and irregular shape. Central nucleus stained blue, cytoplasm stained pink. Bar = 10 μ m.

Fig. 10. Squamous-like cell (left) and scale-like cell (right) derived from midgut stem cells of *Heliiothis virescens* exposed to RA (10^{-6} M) for 14 days in vitro. Bar = 10 μ m.

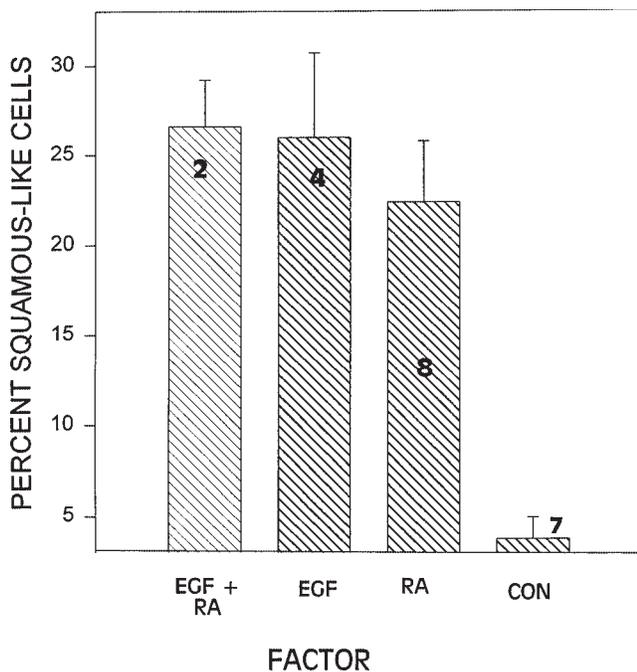


Fig. 11. Percent of squamous-like cells in the total population of midgut cells from *Heliiothis virescens* larvae treated with EGF or RA or EGF + RA. CON: The percent of squamous-like cells in control untreated populations. Data are presented as means \pm SEMs. The number placed in each column represents the number of repeats for each treatment.

factor, TGF β , or luteotrophic hormone, remained visibly unchanged (photographs not shown).

Cell Types Observed in Mixed Midgut Cultures Prepared From *H. virescens* in Wandering, Prepupal, Pupal, and Adult Stages

Mixed cultures containing all cell types were made in standard medium from whole midguts of wandering, prepupal, pupal, and adults of *H. virescens* and the cell types that differentiated in the cultures were noted 2 to 8 weeks after preparation. Cultures made from pupal midgut consisted predominantly of stem-like cells. However, cultures prepared from wandering, prepupal, and adult midguts were rich in cuboidal cells. Adult midgut cultures also contained vacuolated cells as well as a few squamous-like cells resembling those seen in Figures 6 and 9.

DISCUSSION

In vivo, premolt lepidopteran larval midgut stem cells multiply rapidly and then differentiate

to columnar and goblet cells as they intercalate among existing mature larval epithelium (Baldwin and Hakim, 1991), implying that different and precise signals control these separate functions. The predominant types of mature cells in the midgut that develop from the stem cells change as the role of the midgut changes from an organ of digestion and transport of nutrients in feeding larvae to one of storage and metabolic support in non-feeding wandering, prepupal, and pupal stages (Waku and Sumimoto, 1971; Baldwin et al., 1996).

Synthetic midgut differentiation factors 1 to 4 (MDFs) induce differentiation to larval types of midgut cells (Loeb et al., 1999; Loeb and Jaffe, 2002). However, exposure of larval midgut stem cells of *H. virescens* to medium containing CONMED induced a trend toward differentiation to cuboidal columnar cells characteristic of midguts of prepupal *M. sexta* (Loeb and Hakim, 1996; Baldwin et al., 1996), adult *B. mori* (Waku and Sumimoto, 1971), and in cells in mixed cultures observed in this work obtained from wandering, pupal, and adult stages of *H. virescens*. The CONMED probably contained differentiation factors in addition to known MDFs. Interestingly, 1–2% of each of the non-predominant columnar cell types were detected in all the large mixed midgut cultures maintained in the laboratory, indicating that, although a few stem cells could biochemically escape, the existing hormone and peptide milieu directed the majority of the stem cells to specific differentiated fates. We also showed that “mammalian” factors EGF, RA, and PDGF directed proliferation and differentiation of midgut stem cells from *H. virescens* larval midgut in vitro, although they appeared to act more slowly than the known midgut differentiation factors.

Freshly isolated larval midgut cells exposed to midgut differentiation factors (MDFs) differentiate to columnar or goblet cells within 6–8 days in culture (Loeb et al., 1999; Loeb and Jaffe, 2002). However, in this work we observed that after 2 days of storage at 26°C in standard medium, stem cells no longer differentiated in response to MDF-2 (data not shown) and that newly isolated stem cells from *H. virescens* larval midgut lost the ability to

respond to the midgut differentiation-inducing growth factor, MDF-2, after 5 days when stored at 4°C in standard medium. However, stem cell response to MP lasted 10–15 days under the same conditions. The response to MDF-2 could be prolonged for up to 20 days at 4°C by adding CONMED to the storage medium. If the differences in responsiveness are due to differences in receptor populations, these results may indicate that MDF-2 and MP may have different receptors. On the other hand, the receptors may be intrinsically linked to allow one response (differentiation) but not the other (e.g., multiplication). In vivo, rapid multiplication of stem cells usually precedes differentiation prior to molting in Lepidoptera (Baldwin and Hakim, 1991). Treatment of midgut stem cells with CONMED or MDF-2 appeared to block the response to MP and thus potentiate the action of growth factors that promote differentiation. Families of activated integrin, PDGF, Fibroblast Growth factor (FGF), and EGF receptors in mammalian cells can bind to one or several growth factors at once. Depending on the receptor kinases activated and interactions between the pathways generated, different downstream cascades direct proliferation, differentiation, death by apoptosis, or cell spreading (Malarkey et al., 1995; Sastry and Horowitz, 1996; Giancotti and Ruoslahti, 1999; Clatworthy and Subramanian, 2001). Integrin has been detected on the surfaces of cultured midgut cells derived from *H. virescens* larvae (Loeb and Hakim, 1999), *Manduca sexta* prothoracic gland (Chen et al., 1997), and in *Drosophila* cell lines (Miller et al., 2000), and may be a good candidate for study in this regard.

Although MDF-2 was isolated from a digest of the protein fetuin, MDF-1 was isolated from conditioned medium (Loeb et al., 1999). The minimum dose of CONMED needed to induce differentiation in stem cells of *M. sexta* was 50% (Sadrud-Din et al., 1996), but titers of conditioned medium higher than 30% were inhibitory to differentiation of *H. virescens* midgut stem cells in response to MDF-2. Perhaps low titers of MDF in CONMED preserved MDF receptors, as low titers of growth factors are known to do in mammalian systems (Enver et al.,

1998). However, hundreds of peptides are present in conditioned medium (Loeb et al., 1999), so that it is not now possible to define which of its constituents protected the receptors for MDF-2.

Thyrotrophic hormone is thought to have some growth hormone activity, and is related to the family of peptides that elicits steroidal synthesis (Thorngren and Hansson, 1977). Although thyrotrophic hormone did not elicit proliferation or specific differentiation, temporary cell clumping was observed. Similar clumping was exhibited by insect cells in response to the insect steroid, ecdysone (Courgeon, 1972; Berger et al., 1978), and thus it was thought relevant to include this reaction even though high titers (10^{-6} M) of 20E did not induce this effect.

PDGF generates signals for mammalian stem cell growth (Herbst et al., 1995) and movement (Woodard et al., 1998), modulates the action of other peptide hormones (Risbridger 1993), and can prevent apoptosis in an insect fat body cell line (Ottaviani et al., 2000). In the presence of PDGF, midgut stem cells from larval *H. virescens* differentiated to oval, vacuolated cells similar to those described in the prepupal and pupal stage midguts of *M. sexta* (Baldwin et al., 1996; Loeb and Hakim, 1996), in cells that fit the description of calcium-accumulating midgut cells in prepupae of *B. mori* (Waku and Sumimoto, 1971), as well as in mixed adult *H. virescens* midgut cultures (this work). Thus, PDGF induced differentiation of larval midgut stem cells to mature cells characteristic of post-larval stages of midgut development.

EGF stimulates mammalian cells to proliferate and differentiate. Adamson (1990) and Dominguez et al., (1998) have demonstrated that continually dividing tissues contain more EGF than static tissues. In insects, EGF and its receptor play vital roles in the proliferation and development of cells in eye imaginal discs of *Drosophila* (Dominguez et al., 1998). Retinoic acid is a morphogen that induces EGF receptor formation, and causes proliferation of mammalian cells (Adamson, 1990). EGF and RA induced *L. dispar* and *H. virescens* midgut stem cells to multiply, and *H. virescens* midgut stem cells to differentiate to cells resembling squamous epi-

thelium as well as scales, neither of which are accepted as characteristic of midgut epithelium (Waku and Sumimoto, 1971; Baldwin et al., 1996). However, small numbers of squamous-like cells were occasionally observed in cultures of adult *H. virescens* midgut in this work, suggesting that this development program may normally be present in midgut stem cells. Since combinations of RA and EGF induced the same amount and type of differentiation, it is possible that they affected the same receptor, possibly that for EGF.

This work demonstrates that lepidopteran midgut stem cells are multipotent, and can be prompted to develop to different cell types by exogenous or endogenous (Goto et al., 2001) growth factors, as are stem cells of vertebrates (Slack, 2000; Watt and Hogan, 2000; Schuldiner et al., 2000; Mansergh et al., 2000).

ACKNOWLEDGMENTS

The authors thank C. Goodman and J. Mitsuhashi for cogent comments on the manuscript. We also thank M. Shapiro for donations of gypsy moth larvae, D. Gelman for ecdysteroid radioimmunoassay, H. Do and N. Coronel for technical assistance.

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