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CONTROL OF LIFE, DEATH, AND DIFFERENTIATION IN CULTURED MIDGUT CELLS OF THE LEPIDOPTERAN, *HELIOTHIS VIRESCENS*[1]

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SUMMARY

Differentiated cells in the insect midgut depend on stem cells for renewal. We have immunologically identified Integrin β_1, a promotor of cell–cell adhesion that also induces signals mediating proliferation, differentiation, and apoptosis on the surfaces of cultured *Heliothis virescens* midgut cells; clusters of immunostained integrin β_1–like material, indicative of activated integrin, were detected on aggregating midgut columnar cells. Growth factor–like peptides (midgut differentiation factors 1 and 2 [MDF1 and MDF2]), isolated from conditioned medium containing *Manduca sexta* midgut cells, may be representative of endogenous midgut signaling molecules. Exposing the cultured midgut cells to *Bacillus thuringiensis* (Bt) toxin caused large numbers of mature differentiated cells to die, but the massive cell death simultaneously induced a 150–200% increase in the numbers of midgut stem and differentiating cells. However, after the toxin was washed out, the proportions of cell types returned to near-control levels within 2 d, indicating endogenous control of cell-population dynamics. MDF1 was detected immunologically in larger numbers of Bt-treated columnar cells than controls, confirming its role in inducing the differentiation of rapidly produced stem cells. However, other insect midgut factors regulating increased proliferation, differentiation, as well as inhibition of proliferation and adjustment of the ratio of cell types, remain to be discovered.

Key words: insect; midgut; cell signaling; stem cells; differentiation.

Lepidopteran midgut consists of a simple monolayer of epithelium sandwiched between the peritrophic membrane on the lumen side and a muscle and tracheal framework on the hemocoel side. Stem cells lie loosely attached to each other and to the bases of mature goblet and columnar cells that form most of the epithelium during intermolt (Baldwin et al., 1993). Figure 1 shows a normal population of *Heliothis virescens* midgut cells cultured in vitro. Only the stem cells divide and differentiate, providing new tissue to expand the original gut at molting, and replacing the mature cells that die by apoptosis as a result of age, or by other means from biological insult (Baldwin et al., 1993; Loeb and Hakim, 1996). This process also occurs in stem cell–dependent vertebrate tissues such as skin, intestine, or blood (Jones et al., 1995). Apoptosis was detected using the TUNEL method for immunostaining the 3′-ended strands of deoxyribonucleic acid (DNA) (Negoescu et al., 1998) (In Situ Cell Death Detection Kit; Roche Molecular Biochemical, Indianapolis, IN). Approximately 7% of normal cultured *H. virescens* midgut cells appeared to be apoptotic (Loeb et al., 2000) (Fig. 2). In vertebrate systems, regulation of the rate of stem cell mitosis, transition to the differentiating state, development to mature forms, and ultimate cell death depend on cell signaling; signaling can be directed by environmental cues, hormones, cytokines, cell-surface receptors, genes, and other intermediary molecules that interact in complex ways (Watt and Hogan, 2000).

A family of heterodimeric protein receptors, the integrins, is vital to cell signaling. They mediate cell–cell adhesion by binding to proteins such as collagen, fibronectin, or laminin, that may exist in extracellular matrices or on cell surfaces, but can also originate cascades of phosphorylations, ultimately resulting in the activation of the small signaling molecules that direct proliferation, differentiation, and apoptosis (Martin-Bermudo and Brown, 1996). Activated integrins tend to form clusters. Cytokines, such as transforming growth factor β (TGFβ), regulate the number and clustering patterns of integrin receptors on the surfaces of vertebrate cells, and influence the ultimate message delivered (Sastry and Horwitz, 1996; Kim and Yamada, 1997).

Cultured *H. virescens* midgut cells were stained with antibody to human β_1 integrin and, separately, antibody to bovine fibronectin, one of the proteins to which β_1 integrin binds (Loeb and Hakim, 1999). Figure 3 shows a monolayer of newly forming midgut epithelium in vitro, where the columnar cells contain clusters of integrin β_1–like immunopositive material that resemble the aggregating integrin receptors (Sastry and Horwitz, 1996). They surround a faintly immunopositive goblet cell. Goblet cells have almost never been observed as attached pairs in vivo, perhaps as a result of their dearth of B_1 integrin receptors, although columnar cells are frequently seen attached to each other. However, many small stem

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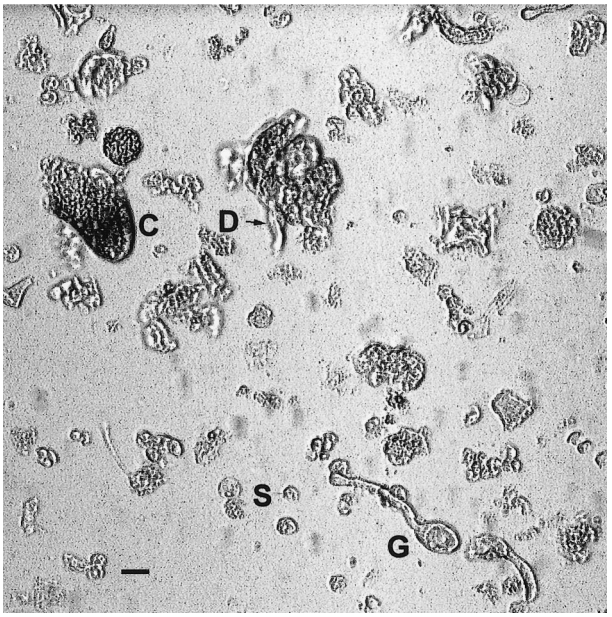

FIG. 1. A population of *H. virescens* midgut cells cultured in vitro for more than 30 d. *C:* columnar cell; *D:* differentiating cell, beginning to insert into a newly forming strand of midgut epithelium composed primarily of columnar cells; *G:* goblet cell; *S:* small round cells that are stem cells. Magnification: ×200. *Bar,* 10 nm.

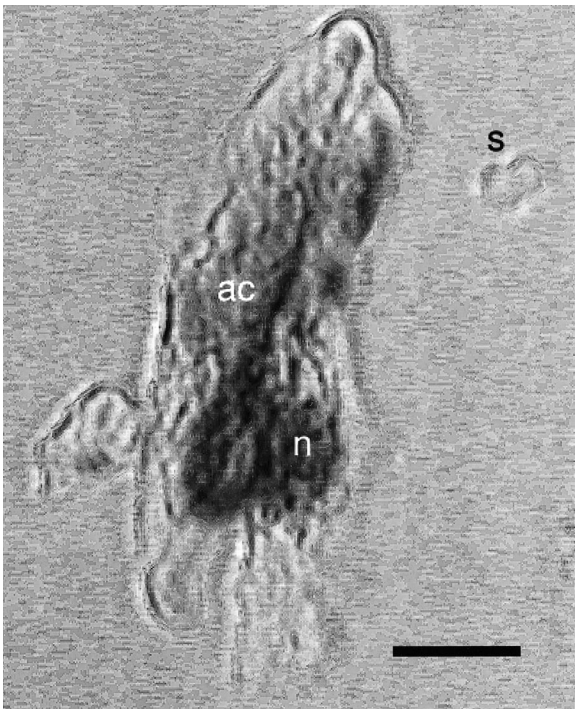

FIG. 2. Two attached columnar cells (*ac,* label on *left* columnar cell) stained by the TUNEL nuclear stain method for apoptosis. *Note* the diffuse nuclei (*n,* label on *right* columnar cell). Nonstaining, dividing stem cell at *right* (*s*). Absence of stain indicates nonapoptotic nuclei. *Bar,* 10 nm.

cells, which can be found in clusters at the bases of mature midgut cells, were stained with antibody to integrin β_1 (not shown) (Loeb and Hakim, 1999). Therefore, the data suggest that an integrin β_1–like molecule appears to be involved in adhesion and signaling in *H. virescens* midgut cells cultured in vitro.

Exposure to toxin from *Bacillus thuringiensis* (Bt) in vitro revealed another aspect of midgut cell signaling. Bt toxin generally attacks mature columnar cells by binding to specific receptors on microvillar membranes. Ion channel pores quickly penetrate the membranes at the sites of attachment, causing leakage of cellular contents, swelling, and cell lysis due to increased internal osmotic pressure (reviewed in Pietrantonio and Gill, 1996). However, 40–50% of cultured cells exposed to Bt toxin were observed in the cultures despite large doses of toxin (Baines et al., 1997; Loeb et al., 2000). It has been reported that sublethal doses of Bt toxin do not kill the intact larvae, since the damaged midgut cells appear to be replaced (Spies and Spence, 1985). Cultured cells from *H. virescens* midgut were treated with picogram quantities of Bt toxin from either Bt strains AA 1–9 or HD-73 for 2 d. The toxin was extracted from Bt spores by the use of sodium hydroxide solution at pH 10, and may have been a mixture of Cry toxins (Loeb et al., 2001). The doses described here were 1000 times lower than that previously used in vitro as a lethal dose (Baines et al., 1997), and can be represented as sublethal. A large number of ghosts of exploded columnar and goblet cells were observed, and the numbers of cells of normal appearance fell to approximately 50% of the untreated controls. The mature columnar and goblet cells that were observed in these cultures were about half the size of normal mature cells, indicating that they were newly differentiated and had not yet grown to mature size. In contrast, the numbers of stem and differentiating cells increased to approximately 200% of the controls (Fig. 4). The number of differentiating cells was higher than those of stem cells, indicating rapid conversion of stem cells to the differentiating state. Thus, it appeared that the rapidly produced mature cells replaced those destroyed by the toxin, confirming the assumptions of Spies and Spence (1985).

After 2 d of exposure to the Bt toxins, the cells were washed twice in fresh medium to remove the toxins, and maintained in the fresh medium for 2 d before scoring. As a result, the percentage of columnar and goblet cells in each population rose to approximately the control levels, while the percentage of stem and differentiating cells dropped to approach the control levels (Loeb et al., 2001) (Fig. 5).

Wounded vertebrate tissues, such as liver, blood, and intestine, are similarly repaired as a result of rapid production and differentiation of stem cells (Alison et al., 1998; Slack, 2000). As the wound is healed, the proliferation of stem cells ceases, and the excess numbers of stem and intermediary cells are removed by apoptosis (Slack, 2000). Similar processes were occurring in the cultured insect midgut cells.

In vertebrate tissues, the rate of stem cell proliferation and differentiation is orchestrated by multiple feedback controls involving hormones, growth factors, cytokines and other proteinaceous factors, smaller regulatory molecules, transcription factors, and, ultimately, gene expression (Slack, 2000; Watt and Hogan, 2000). However, many of the growth factors that influence the vertebrate systems do not appear to affect the cultured insect cells (Nishino and Mitsuhashi, 1995), despite the gene-sequences coding for the TGFβ family, epidermal growth factor, and others, described in

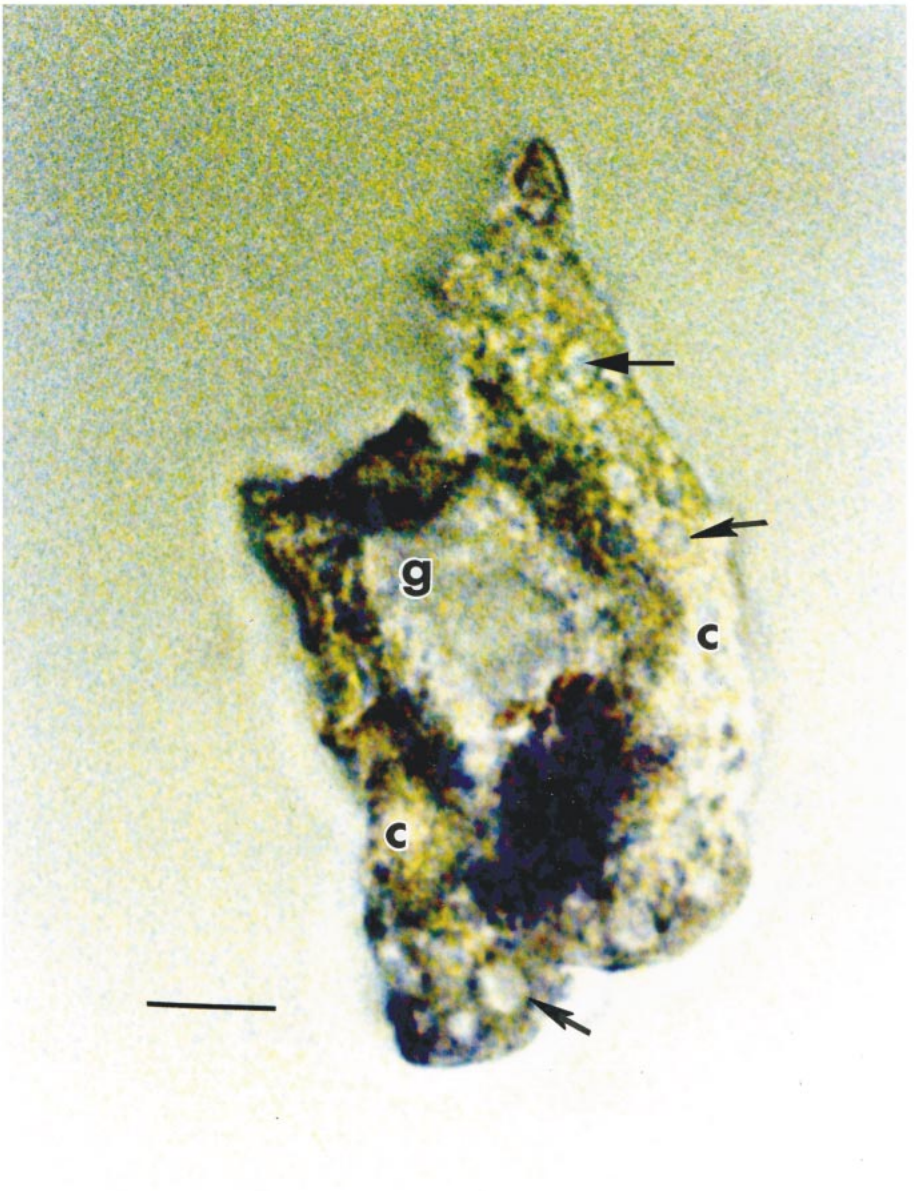

FIG. 3. A newly forming strand of midgut epithelium stained with polyclonal antibody to human β_1 integrin. A poorly staining goblet cell (*g*) is surrounded by two columnar cells (*c*). *Arrows* point to a cluster of immunostainable integrin-like material. *Note* the intense immunostain surrounding the goblet cell as it is attached to the two columnar cells. *Bar,* 10 nm.

Drosophila complementary DNA libraries (Padgett et al., 1987; Fleming et al., 1990). Although several insect growth factor–like factors have been described or partially purified (reviewed by Ferkovich et al., 1995), few have been characterized completely. A 24.4-kDa insulin-like factor (Ebberink et al., 1989) and a novel 96-kDa factor from conditioned medium from an embryonic cell line of *Sarcophaga peregrina* (Homma et al., 1996) have been sequenced. Several cytokines regulating muscle and plasmatocyte movement, as well as a growth inhibitor, have been described (Nachman et al., 1993; Strand et al., 2000). We have characterized two small peptidic factors, midgut differentiation factors 1 and 2 (MDF1 and MDF2), 2.6 and 0.68 kDa, respectively, from medium 'conditioned' by cultures of the *Manduca sexta* midgut. The factors are identical to sequences close to the terminus of bovine fetuin, and induce differentiation of cultured stem cells isolated from *M. sexta* as well as *H. virescens* midguts. They are maximally effective at physiological titers 10^{-8} and 10^{-7} *M,* respectively (Loeb et al., 1999). A polyclonal antibody to MDF1 stained the untreated cultured midgut cells; immunoreactivity was observed in 27.6 ± 0.3% of the control cells (Goto et al., 2000). MDF1 may regulate normal stem cell differentiation in a dynamic population. However, after exposure to Bt toxin (82 pg/μl) from Bt strain AA 1–9 for 24 h (4000 cells counted), immunoreactivity was observed in 41.6 ± 1.6% of the cells (Loeb et al., 2001). Therefore, the synthesis of MDF1 was greater in cells exposed to the toxin (Loeb et al., 2001). Many of the mature cells in the Bt-treated population

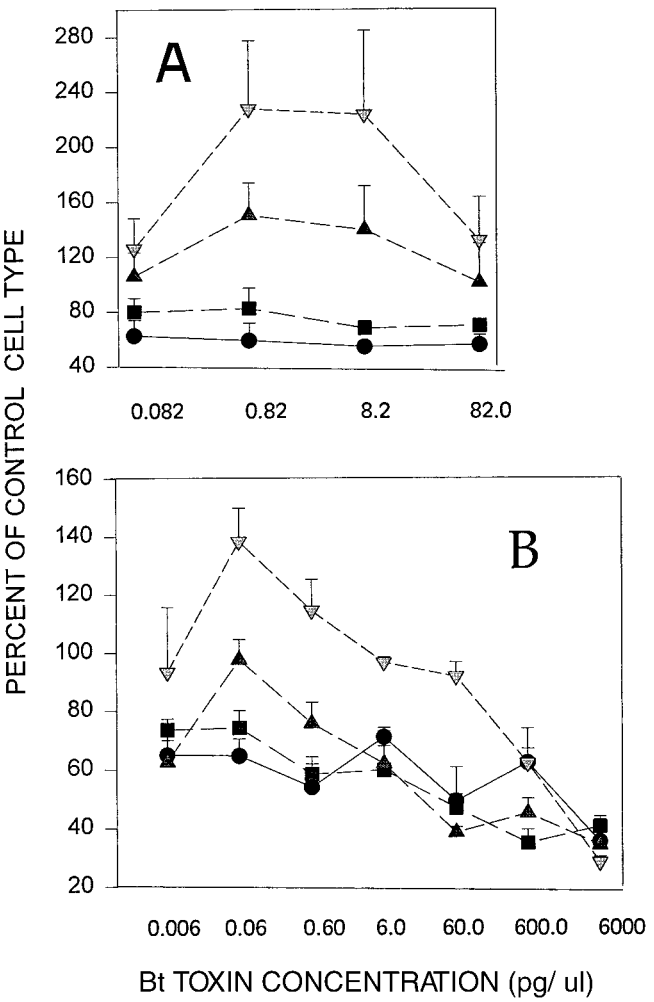

FIG. 4. Cell types observed in *H. virescens* midgut cultures after exposure to different concentrations of Bt toxin for 2 d. Data are expressed as the percentage of that type of cell found in control cultures. *Circles:* goblet cells; *squares:* columnar cells; *upright triangles:* stem cells; *inverted (gray) triangles:* differentiating cells. *A:* toxin from nonspecific Bt strain AA 1–9; *B:* toxin from Bt strain HD-73, specific for *H. virescens.* N = 4 experiments. *Bars* indicate SEM.

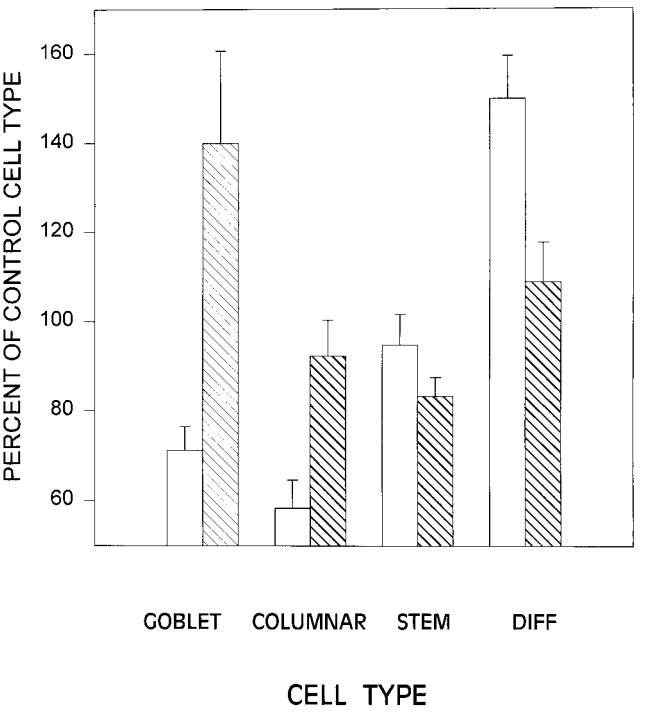

FIG. 5. Comparison of cell types observed after 2 d of exposure to Bt toxin from strain HD-73 (*open bars*) with cell types in the same cultures 2 d after the Bt toxin was washed out (*hatched bars*). Data is expressed as the percentage of that type of cell found in control cultures. Control cultures were washed with medium at the same time as experimental cultures, and also cultured for 2 d before scoring. N = 7–8 experiments; cell-count data from all concentrations of Bt HD-73 toxin (*see* Fig. 4*B*) were combined in this figure. *Bars* indicate SEM.

were younger than those in untreated populations because many existing mature cells were killed by the effect of the Bt toxin. Nonetheless, the data strongly implies that an MDF1-like molecule is produced in a rapidly differentiating midgut stem cell population. Columnar cells formed the great majority of the cells that were positively immunostained with anti-MDF1, implicating them as the major source of the factor in cultured cells (Goto et al., 2000). Since a plethora of interacting regulatory factors are known to control stem cell proliferation, differentiation, and shutdown after wounding in vertebrates, it is reasonable to suspect that many more insect growth factors and cytokines remain to be discovered in comparable insect systems.

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