Ecdysteroids in vitro promote differentiation in the accessory glands of male mealworm beetles[1]

K. A. Grimnes and G. M. Happ

Department of Zoology, University of Vermont, Burlington (Vermont 05405, USA), 21 October 1986

Summary. Physiological peak doses of 20-hydroxyecdysone were added to organ cultures of young pupal accessory glands of male *Tenebrio molitor*. During subsequent culture in vitro or in vivo, the glands accumulated adult-specific antigens. Control organ cultures showed no such antigen accumulation. In this system, ecdysteroid controls not only cell cycles but also differentiation.

Key words. 20-Hydroxyecdysone; ecdysone; cell cycle; cyto-differentiation; accessory gland; mealworm beetle; *Tenebrio molitor*.

The male accessory glands of *Tenebrio molitor* grow and differentiate during the 9-day pupal stage and the first week after adult ecdysis [2–5]. Cell division continues from pupal ecdysis (day 0) through the mid-pupal ecdysteroid peak (days 3–5) and then stops in the late pupa (days 6–7)[6,7]. Unlike its affect on Kc cells of *Drosophila*[8], 20-hydroxyecdysone accelerates mitotic rates when added in vitro to organ cultures of the accessory glands from young or midstage pupae[9].

Terminal differentiation of the male accessory glands begins in the late pupa (days 8–9) and is complete by 6–7 days after adult ecdysis[4,5]. Post-ecdysial increases of adult-specific antigens in the tubular accessory glands (TAGs) have been followed by bidimensional immunoelectrophoresis with polyclonal antibodies[10] and in the bean-shaped accessory glands (BAGs) by western blotting with monoclonal antibodies[11,12]. Mature BAGs contain 8 types of secretory cells[13]. Three monoclonal antibodies, designated PL3.4, PL6.3, and PL21.1, are specific for cell types 3, 7, and 4 respectively[11,12,14]. During development, these antigens are not detectable immunocytochemically in the mid-pupa but they are seen at the apices of the secretory cells on the penultimate pupal day. With dot blots, they are seen to increase sharply during post-ecdysial adult differentiation, as shown in figure 1 for antigens recognized by PL6.3.

After implantation for 8 days in male or female adult hosts, glands from 0-day or 1-day pupae do not show adult patterns of leucine incorporation on fluorographs of two-dimensional SDS-pI gels[14]. However, when the implanted accessory glands have been dissected from the mid-pupa, at the time of the large peak of ecdysteroids[15], they are competent to make adult proteins[14]. In this note, we report that administration of ecdysteroid in vitro to young pupal organs will bring about a change in competence for young pupal accessory glands.

In the first series of experiments, we looked for differentiation after application of 20-hydroxyecdysone to young pupal BAGs cultured in Landureau's S-20 medium[9,16]. In vivo, the major ecdysteroid peak begins at day 3 and the hormone titer reaches its apex at pupal day 5[15]. We compared explants from 2-day pupae (before the pupal ecdysteroid peak[15]) with those of 5-day pupae (previously exposed to the ecdysteroid peak in situ[15]). Glands for the experimental group were dissected from pupae and exposed to physiological peak doses of 20-hydroxyecdysone (10^{-5} M) in vitro. Control glands were cultured without hormone. Following 8 days in vitro, BAGs were fixed in alcoholic Bouin's and paraffin sections were prepared. After allowing primary monoclonal antibody (PL6.3) to bind to the tissue, we visualized the sites of binding with peroxidase-conjugated secondary antibody and diaminobenzidine[12]. BAGs from 6-day or younger pupae (before culture) did not stain while those from 0-day adults showed staining at the location corresponding to cell type 7.

Glands explanted from 5-day pupae were antigen-positive after 8 days of culture in basal Landureau's medium. There was no immunocytochemical staining after 2-day explants had been cultured for 8 days in vitro without hormone. However, the explants from 2-day pupae cultured with 10^{-5}M ecdysteroid were immunocytochemically-positive for PL 6.3 antigen, indicating the onset of adult differentiation. With this in vitro culture system, we never observed full quantitative peak differentiation such as that found when BAGs matured in situ in the intact post-ecdysial adult.

In the second series of experiments, we applied hormone in vitro and then implanted the pupal glands in abdomens of female adults. Female hosts were used to avoid any contamination from male host tissues, especially accessory glands or proteins which might be released from them. First, we cultured 0-day pupal accessory glands in Landureau's medium for 24 h. During that interval in vitro, glands were exposed to various concentrations of 20-hydroxyecdysone. Next, we transplanted the glands from the in vitro culture to the abdomens of female beetles which had just undergone adult ecdysis. After 10 days of culture in vivo, the hosts were killed and the implants recovered. In those implants which had been exposed to hormone in concentrations from 10^{-7}–10^{-5} M, there was greater total protein content than in controls cultured without hormone (fig. 2).

Differentiation was monitored by gross morphology and immunochemistry. The secretory cell types of the BAGs are readily recognized in the adult but are indistinguishable in the young and mid-pupa[13]. Our morphological indices of differentiation were the appearance of a milky patch in the secretory epithelium (characteristic of cell type 3), and of a semisolid secretory plug in the lumen of the gland (composed

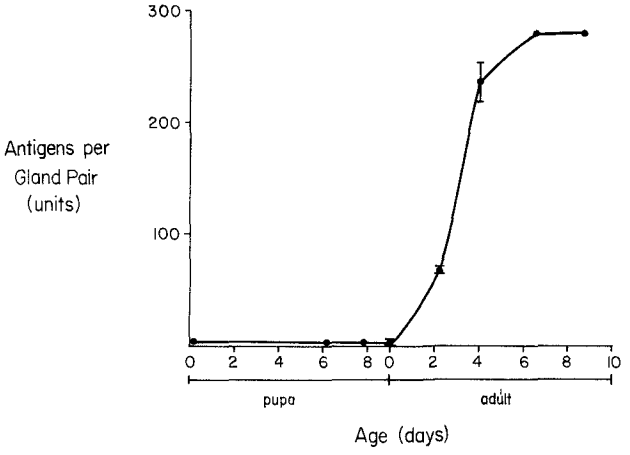

Figure 1. PL6.3 antigen in BAGs of increasing age. Aliquots of gland homogenates were serially diluted and spotted onto nitrocellulose. After successive exposure to PL6.3 antibody and secondary antibody conjugated with horseradish peroxidase, the blot was developed with *o*-diansidine. Reaction product intensity was scored (0–8) by comparison with standards processed in parallel. The results were normalized for dilution. Eight glands at each age were scored. No reaction was obtained from pupal gland homogenates. At 6 and 8 adult days, reaction was at the highest level for all glands tested. Error bars show standard errors.

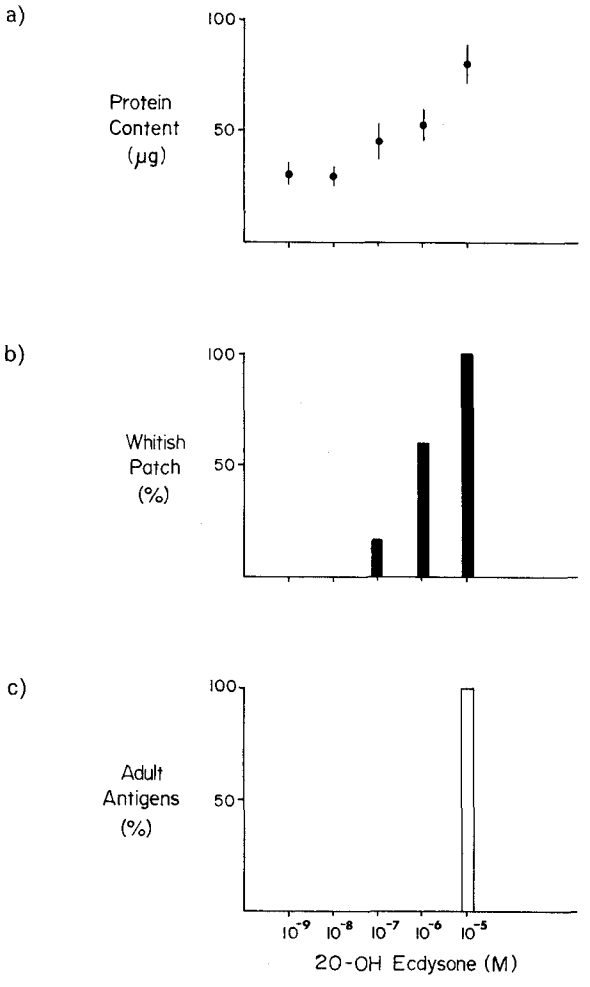

Figure 2. Growth and differentation of 0-day pupal BAGs after hormone administration in vitro followed by implantation into adult female hosts. 20-Hydroxyecdysone was supplied at the concentrations shown in Landureau's medium. Glands in medium with no hormone were indistinguishable from those in 10^{-9} M ecdysteroid. Experimental groups were 12–18 glands. After ten days of culture in vivo, the implants were recovered by dissection. *a* Protein content was determined by Lowry method, with bovine serum albumin as the standard. *b* The percentage of glands with a whitish patch was scored by inspection. *c* The percentage of glands containing adult-specific antigens recognized by PL3.4, PL6.3, and PL21.1 was determined by dot blot, using a 1:1:1 mixture of the three monoclonal antibodies as the primary antibody. In the latter case, adult-specific antigens were detected only in glands previously exposed to 10^{-5} M 20-hydroxyecdysone but not to lower doses.

of the secretions from all cell types). In the intact animal, the milky patch is visible at ecdysis and the plug appears within two days thereafter. Appearance of adult-specific antigens was monitored by dot blots with the monoclonal antibodies PL3.4, PL6.3, and PL21.1.

Hormone exposure promoted not only growth but also differentiation (fig. 2). All 17 glands exposed to 10^{-5} M ecdysteroid had a well-defined whitish patch and three of these also contained a secretory plug. This same concentration of ecdysteroid produced maximum acceleration of the cell cycle in young pupal BAGs after 24 h in vitro[9]. After culture in 10^{-6} M hormone in the present study, only one-third had a well-defined milky patch, another third showed traces of the patch, and none had a secretory plug. At lower doses the response declined, until at 10^{-8} M the hormone-treated glands were indistinguishable from the controls cultured in basal Landureau's medium.

Glands treated with 10^{-5} M ecdysteroid showed a positive reaction in dot blots with monoclonal antibodies PL3.4, PL6.3, and PL21.1, whether the antibodies were tested individually or as a mixture (fig. 2c). None of these three antigens was found in glands cultured in basal medium or in glands exposed to hormone concentrations of 10^{-6} M, 10^{-7} M, or 10^{-8} M. It is likely that intermediate hormone concentrations between 10^{-6} M and 10^{-5} M would have led to the appearance of the of antigens in an intermediate number of glands. Pooled gland homogenates from the 10^{-5} M ecdysteroid group were applied to SDS polyacrylamide gels for nondenaturing electrophoresis. Gels were electroblotted onto nitrocellulose, and probed with a mixture of the three antibodies[11, 12, 14]. Characteristic staining at 14 kDa, 23 kDa, and 28–29 kDa, (corresponding to the antigens for PL6.3, PL21.1, and PL3.4, respectively), confirmed the presence of these adult-specific antigens in the glands exposed to 10^{-5} M 20-hydroxyecdysone.

For both vertebrate and insect systems, it has been suggested that changes in the pattern of gene expression require a turn of the cell cycle[17–19]. But for certain cases, 'quantal mitoses' do not seem to be required for such changes[20–22]. On the basis of the data available at present for the BAG, we cannot determine whether ecdysteroid-driven effects on cell cycling and on cell differentiation are independent responses to the same hormone or whether division and differentiation are successive consequences of a single hormone-triggered developmental transition.

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2 Poels, A., Ann. Soc. R. Zool. Belg. *102* (1972) 199.
3 Gerber, G.H., Can. J. Zool. *54* (1976) 990.
4 Happ, G.M., Yuncker, C., and Dailey, P.J., J. exp. Zool. *220* (1982) 81.
5 Happ, G.M., Yuncker, C., and Huffmire, S., J. exp. Zool. *200* (1977) 223.
6 Grimnes, M.J., and Happ, G.M., Int. J. Insect Morph. Embryol. *9* (1980) 281.
7 Happ, G.M., and Happ, C.M., J. Morph. *172* (1982) 97.
8 Stevens, B., Alvarez, C.M., Bohman, R., and O'Connor, J.D., Cell *22* (1980) 675.
9 Szopa, T.M., Lenoir Rousseaux, J.-J., Yuncker, C., and Happ, G.M., Devl Biol. *107* (1985) 325.
10 Black, P.N., Landers, M.H., and Happ, G.M., Devl Biol. *94* (1982) 106.
11 Grimnes, K.A., and Happ, G.M., Insect Biochem. *16* (1986) 635.
12 Grimnes, K.A., Bricker, C.S., and Happ, G.M., J. exp. Zool. (1987) in press.
13 Dailey, P.J., and Happ, G.M., J. Morph. *179* (1983) 139.
14 Happ, G.M., in: Molecular Entomology. Ed. J. Law. UCLA Press, 1986, in press.
15 Delbecque, J.-P., Hirn, M., Delachambre, J., and DeReggi, M., Devl Biol. *64* (1978) 11.
16 Landureau, J.-C., and Grellet, P., C.r. Acad. Sci. Paris *274* (1972) 1372.
17 Holtzer, H., Weintraub, H., Mayne, R., and Mochan, B., Curr. Top. devl Biol. *7* (1972) 229.
18 Schneiderman, H.A., in: Insect Juvenile Hormones, Chemistry and Action. Ed. M. Beroza. Academic Press, New York.
19 Willis, J.H., A. Rev. Ent. *19* (1974) 97.
20 Kumaran, A.K., Differentiation *12* (1981) 121.
21 Nadal-Ginard, B., Cell *15* (1978) 855.
22 Levenson, R., Kernen, J., Mitrani, A., and Housman, D., Devl Biol. *74* (1980) 224.

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