Ecdysteroid Production by a Continuous Insect Cell Line

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The spent medium from ten established cell lines was extracted and tested for ecdysteroids by radioimmunoassay. Of the seven lepidopteran lines tested, only IAL-TNDI and MRRL-CH showed evidence of ecdysteroid production. However, the results were erratic and difficult to evaluate and these lines were dropped from further consideration. However, of the three cockroach cell lines tested, one, UMBGE 4, produces ecdysteroid and consistently releases virtually all of it into the medium. The main ecdysteroid was identified as ecdysone and the increase was logarithmic during the first 11 days of the subculture, with a decrease from day 11 to day 14.

UMBGE 4 is a vesicle cell line which also tested positive for chitin synthesis. When the pH of the medium was lowered from pH 7.4 to pH 6.3, both the chitin synthesis and the ecdysone synthesis dropped by roughly 50%.

Key words: ecdysone, radioimmunoassay, cockroach, tissue culture

INTRODUCTION

Selected cell lines are capable of synthesizing chitin or chitin proteins. Marks et al. [1] showed that a cell line from the tobacco hornworm (Manduca sexta) synthesized chitin and Kramerov et al. [2] showed that a cell line from Drosophila produced what they termed "chitin proteins." More recently we have found that a cell line from the German cockroach (Blattella germanica) consistently synthesizes chitin [3].

In vivo, the molting process is triggered by a surge of ecdysone which is converted to 20-hydroxyecdysone by the adjacent tissues. In vitro, epidermal...
tissues not previously exposed to molting hormone require 20-hydroxyecdysone priming in order to initiate chitin synthesis [4,5]. Since cell lines do not require such priming in order to produce chitin, we decided to test the hypothesis that these cell lines might be producing the hormone.

**MATERIALS AND METHODS**

**Cell Lines**

Ten established cell lines were tested for the secretion of ecdysteroids into the medium. These included three lines from *Blattella germanica* (UMBGE 1, 2, and 4) [6] and seven lepidopteran lines including IPRI-CF-124 [7], UMN-PIE [8], IAL-PID2 [9], IAL-TNDI [10], UMN-MDH [11], and MRRL-CH and its clone CHE [12].

**Culture Methods**

The lepidopteran cells were cultured in Yunker's modified Grace's medium with 15% fetal bovine serum and 0.5% gentamycin from Grand Island Biological Co. (Grand Island, NY). The cockroach cells were cultured in UMN-B1 medium [6] purchased from Kansas City Biological Inc. (Lenexa, KS) and supplemented with 10% fetal bovine serum and 0.5% gentamycin. Cells were seeded into 25-cm² Corning tissue culture flasks containing 5 ml of medium and maintained at 27 ± 1°C. The pH of the medium was adjusted to either 6.3 or 7.4 with KOH.

**Harvesting and Processing of Medium and Cells**

Cells were harvested with gentle pipetting and were separated from the medium by centrifugation at 125g for 5 min. The remaining medium was lyophilized and extracted with twice its volume of 80% methanol (HPLC* grade) and stored overnight at −30°C. The extract was warmed, vortexed, filtered (Whatman No. 2), and washed once with another volume of 80% methanol, and approximately 75% of the filtrate was evaporated under nitrogen at room temperature until the remaining liquid could freeze at −30°C. Frozen samples were then lyophilized and stored for later analysis. The cell pellet was washed twice in Puck’s saline and a portion of the suspended pellet was removed for protein assay. Those pellets extracted for ecdysteroid analysis were homogenized in a volume of 80% methanol equal to twice the volume of medium in which the cells were grown. The homogenate was stored overnight at −30°C, filtered, and dried as above. Cell lines that grew as single cells were quantitated with a Coulter cell counter. Vesicle lines or lines that grow as clumps were quantitated with the Pierce BCA protein assay kit (Pierce Chemical Co, Rockford, IL) with bovine serum albumin as a standard.

*Abbreviations used: EM = Electron microscopy; HPLC = high-performance liquid chromatography; PTTH = prothoracicotropic hormone; RIA = radioimmunoassay.
Ecdysteroid Production by a Continuous Cell Line

Ecdysteroid Extraction and Analysis

For initial analysis of the various cell lines, processed media were resolubilized in 500 μl of 75% methanol by vortexing and sonication and stored overnight at -20°C. The insoluble material was removed by centrifugation (3,000g, 20 min, 4°C). Aliquots of 100 μl were dried and analyzed by RIA according to Kelly et al. [13]. For comparison, some of the samples were processed by Sep-Pak extraction (see below) and the fractions analyzed by RIA.

For chromatography of ecdysteroids present in the UMN-B1 medium, UMBGE 4 cells were grown in 300 ml of medium for 7 days. The cells and medium were processed as described and the dry extract was taken up in 30 ml of 75% methanol and analyzed for ecdysteroids by RIA. An 800-μl aliquot of the medium extract containing 8,000–10,000 pg 20-hydroxyecdysone equivalents was dried and then redissolved with sonication and vortexing in 400 μl of HPLC-grade water. This solution was added to a methanol primed Sep-Pak (Waters, Milford, MA) along with a 400-μl rinse of the tube. The water eluate was collected and the Sep-Pak was sequentially eluted with 4 ml of 20% methanol followed by 4 ml of 100% methanol. A small portion of each fraction was analyzed for ecdysteroid activity and approximately 90% of the total activity was in the 100% methanol. The eluant was dried overnight and resolubilized in 40% methanol. This solution was then chromatographed on a Supelco-LC-18DB C18 column (Supelco, Bellefonte, PA; 4.6 mm id × 25 cm, 5-μm particle size) and eluted with 40% methanol at 2 ml/min, and 600-μl fractions were collected. Fractions were divided and one-half of each was assayed for ecdysteroid by RIA. The adjacent peaks showing high levels of ecdysteroid activity were pooled and chromatographed on a Shandon Hypersil-silica gel column (Shandon Southern Institute, Sewickley, PA; 3.9 mm id × 30 cm; 3 μm particle size), eluted with a mixture of methylene chloride: methanol: isopropyl alcohol: water (250:25:20:1), and assayed for RIA-positive ecdysteroids. The antiserum used for the RIA was 2.8 times more sensitive for ecdysone than for 20-hydroxyecdysone and showed a high level of cross-reactivity to some ecdysteroids that were conjugated at the C-22 position [14]. All ecdysteroid titers were reported as pg equivalents of 20-hydroxyecdysone and compared to each other by using cell number or total protein content.

RESULTS

Ecdysteroid Levels in Culture Medium

For the initial screening of RIA-detectable ecdysteroids we cultured cells for 7 days and compared the ecdysteroid levels in the expended medium with that in fresh medium. Among the lepidopteran cell lines (Table 1), only two (IAL-TND1 and MRRL-CH) showed levels well above the controls. In IAL-TND1 this was due to a single high reading of 356 pg-eq/ml/mg protein of 20-hydroxyecdysone, which could not be replicated. In the MRRL-CH the readings were inconsistent and varied widely between replicates. Among the cockroach cell lines (Table 2), only UMBGE 4 consistently showed ecdysteroid levels above the controls. Because of this consistency, the relatively high
TABLE 1. Ecdysteroid RIA Analysis of Medium From Lepidopteran Cell Lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Replicates</th>
<th>pg-eq/ml medium/ 10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grace’s medium</td>
<td>6</td>
<td>25.5 ± 4.4 eq/ml^a</td>
</tr>
<tr>
<td>(Yunker’s modification)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IPRI-CF-124</td>
<td>5</td>
<td>13.9 ± 6.5</td>
</tr>
<tr>
<td>UMN-PIE</td>
<td>6</td>
<td>23.9 ± 12.8</td>
</tr>
<tr>
<td>IAL-PID2</td>
<td>4</td>
<td>10.9 ± 2.2</td>
</tr>
<tr>
<td>UMN-MDH</td>
<td>6</td>
<td>23.4 ± 4.0</td>
</tr>
<tr>
<td>MRRL-CH</td>
<td>6</td>
<td>78.8 ± 14.9</td>
</tr>
<tr>
<td>MRRL-CH(CHE clone)</td>
<td>4</td>
<td>17.0 ± 5.4</td>
</tr>
<tr>
<td>IAL-TND1</td>
<td>6</td>
<td>103.7 ± 56.2 eq/ml/mg^b</td>
</tr>
</tbody>
</table>

^a Fresh medium without cells.

^b Cells were not easily dissociated and were quantitated by protein assay.

levels of ecdysteroid production, and the fact that this line also consistently produced chitin [3], this cell line was used for the remaining experiments.

Ecdysteroid Secretion in Line UMBGE 4

Preliminary analysis showed that more than 95% of the secreted ecdysteroid was in the medium, very little remaining in the cells. When cultured for 14 days, the level of ecdysteroid in the cells did not change, while in the medium there was a logarithmic increase from day 1 to day 11 (190–4,200 pg-eq/ml/mg protein) after which the level dropped to 2,900 pg-eq/ml/mg protein (Fig. 1).

Analysis of ecdysteroid production at different pH levels showed that at pH 6.3 the ecdysteroid level was 64.8±18.4 pg-eq while at pH 6.6 the level was 147.5±32.1 pg-eq and at pH 7.4 it was 150.5±9.9 pg-eq. This sharp increase in ecdysteroid production between pH 6.3 and 6.6 is accompanied by an increase in chitin production of roughly 50%.

Identification of the Secreted Ecdysteroid

The ecdysteroid secreted by the UMBGE 4 cells was identified by column chromatography. Of the material originally applied to the Sep-Pak cartridge, approximately 90% of the RIA-active material eluted with 100% methanol. This was then applied to a C_{18} reverse-phase column and analyzed by RIA. The major peak of the RIA-active material had the same retention time as ecdysone (Fig. 2A). A portion of the material from the ecdysone region was then rechromatographed on a normal-phase column and again eluted with
the same retention time as ecdysone (Fig. 2B). Thus the main RIA-active ecdysteroid released by the cells is identified as ecdysone.

DISCUSSION

A number of insect cell lines are known to be sensitive to ecdysteroids applied at physiological levels. The responses range from morphological changes [15], to induction of unique enzymes [16], changes in cell-surface glycoproteins [17], and changes in membrane transport systems [18, 19]. Although organ cultures have been shown to secrete and to respond to ecdysteroids, the secretion of ecdysteroids by established cell lines is only now being demonstrated. We have demonstrated that the established cell line UMBGE 4 synthesizes ecdysone and releases it into the medium. Furthermore, we have shown that this cell line synthesizes chitin [3]. Chitin synthesis is substantially increased by the addition of 20-hydroxyecdysone. Of the three cockroach cell lines tested, only the UMBGE 4 line secreted ecdysone and synthesized chitin.
The results obtained with the lepidopteran cell lines are not as clear. The cell lines IAL-TND1 and MRRL-CH are erratic in their production of both chitin and ecdysteroids and neither responds with increased chitin production when treated with $10^{-6}$ M 20-hydroxyecdysone. Our examination of these two cell lines was restricted to ecdysteroids released into the medium. We did not look at ecdysteroids which might be retained by the cells. Recently Lynn and Feldlaufer [20] reported that IAL-TND1 from *Trichoplusia ni* imaginal disks synthesized 20-hydroxyecdysone, which was retained within the cells. The positive results we obtained with IAL-TND1 probably were due to breakage of some cells during centrifugation. On the other hand, UMBGE 4, an embryonic cell line, secreted its newly synthesized ecdysone. It is possible that this difference between the cell lines results from a differ-
ence in the metabolism or transport of the ecdysteroids. If so, it may also account for the differences in chitin synthesis that we found between the UMBGE 4 and MRRL-CH cell lines.

Since the UMBGE 4 cell line acts more like the cultured prothoracic gland [21], producing ecdysone and releasing it into the medium, it would be interesting to determine whether PTTH stimulates hormone production. It is also possible that the cells that are producing both ecdysone and chitin are also producing PTTH. This possibility should be investigated.

The UMBGE 4 cell line was derived from eggs containing germ-band-stage embryos. These cells grow as vesicles in culture. It has not been possible to dissociate and clone these cells, and thus we do not know whether the same cells are producing both ecdysone and chitin. However, the cells do produce small particles that incorporate radiolabeled N-acetylglucosamine. When the pH of the culture medium is reduced from 7.4 to 6.3, the production of ecdysone, chitin, and particles is decreased, even though the cells still grow well. The level of both chitin and particle production can be increased by adding 20-hydroxyecdysone. This pattern of events is highly suggestive of that which has been reported from organ cultures [4].

The similarity of these findings to those obtained with organ cultures and indeed with whole insects indicates that this and perhaps other similar cell lines can be used as experimental material for studies on the biosynthesis and release of ecdysteroids at the cellular level. In addition to this it may have potential for use as an assay for the presence of the PTTH.

LITERATURE CITED