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INITIATION AND CHARACTERIZATION OF FIVE EMBRYONIC CELL LINES FROM THE COTTON BOLL WEEVIL *ANTHONOMUS GRANDIS* IN A COMMERCIAL SERUM-FREE MEDIUM

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SUMMARY

Five continuous cell lines were initiated from embryonic tissue of the cotton boll weevil *Anthonomus grandis* Boheman in a commercially available, serum-free medium (Excell 401) and have undergone in excess of 60 passages. Isoenzyme analysis confirmed that the lines originated from boll weevil tissue. Four of the lines grew as single attached cells of either epithelioid or fibroblastoid morphology. The fifth line, BRL-AG-2, grew primarily as cell aggregates and was found to release ecdysteroids (primarily ecdysone) into the culture medium. Evidence was also obtained suggesting that line BRL-AG-2 synthesizes chitin. Three lines, BRL-AG-1, BRL-AG-3A, and BRL-AG-3C, could be induced to produce an antibacterial factor(s) which was released into the culture medium.

Key words: isozymes; ecdysone; 20-hydroxyecdysone; antibacterial factors; serum-free cell culture; cellular chitin synthesis; insect immune factors; Coleoptera; Curculionidae.

INTRODUCTION

The cotton boll weevil is a major pest of cotton, and its control currently relies heavily on the use of chemical pesticides. Much effort is being directed toward the search for alternative control strategies, including the study in our laboratory of methods for disrupting exoskeletal cuticle formation. Several continuous insect cell lines have been reported to produce at least one component of the insect cuticle, the polysaccharide chitin (23,27,33,34,47). In addition, cell lines from the German cockroach *Blattella germanica*, the midge *Chironomus tentans*, and the cabbage looper *Trichoplusia ni* have demonstrated the ability to produce low levels of ecdysteroids (28,34,35,46,49) which are involved in the insect cuticle molting process.

We were interested in the possibility of using a coleopteran cell line for studying hormonal regulation of the synthesis of cuticle components, especially the cuticle proteins of the cotton boll weevil (48). There were, however, relatively few coleopteran cell lines available at that time (2,10,11,31). With the recent improvements in insect media formulations, cell lines have been established from previously difficult insect species, and some of these have subsequently been passaged (or serially subcultured) without the costly and sometimes inhibitory supplementation with commercial vertebrate serum (14,15,39). One such commercially available serum-free medium, Excell 401 (JRH Biosciences, Lenexa, KS) was chosen for initiating several cell lines from *A. grandis* embryonic tissue. We present here data concerning the general characterization of these apparently continuous cell lines as well as information concerning some specialized features of these lines. These cell lines are apparently the first invertebrate cells to have been both isolated and continuously subcultured in a serum-free tissue culture medium (39).

MATERIALS AND METHODS

Establishment of continuous cell lines. Between 300 to 400 eggs were obtained from a colony of Ebony strain cotton boll weevils (12,41). The eggs were of mixed age ranging from 4 to 48 h old. They were surface sterilized according to Goodwin (14) and washed 3 times in sterile water and once in Excell 401 medium.

The eggs were suspended in a small volume of Excell 401, transferred to 1.5 ml microcentrifuge tubes, and homogenized with gentle pressure using a sterile plastic pestle. The mixture was transferred to a 15-ml centrifuge tube, diluted to 5 ml with Excell 401, centrifuged at 500 *g* for 5 min, and the supernatant discarded. The cell pellet was suspended in 5 ml of medium and gently drawn up into a pipette several times to break up large clumps of cells. The cell suspension was transferred to a 25-cm^2 tissue culture flask and incubated at 28° C for 1 h. After this time the cell suspension was carefully poured off into a new flask, and fresh medium was added to the attached cells remaining in the first flask. This process was repeated several times with increasingly longer times allowed for cell attachment. The medium was poured off all the primary culture flasks after 12 to 24 h to remove dead cells and any traces of melanin. Up to this point the Excell 401 medium had been supplemented with 50 μg/ml of gentamicin, but this was discontinued from this time onward. After 1 wk of incubation at 28° C, and each week thereafter, the flasks were fed by removing 1 to 2 ml of medium and adding an equal volume of fresh Excell. After 3 to 5 wk the cells in several of the flasks had reached a sufficient density to allow subculture (initially at a ratio of 1 part cell suspension to 1 part fresh medium at 2-wk intervals). Cells from cultures at Passages 11 through 16 were frozen (at a rate between 1° and 2° C per minute) in Excell 401 plus 10% dimethyl sulfoxide (DMSO) and stored at −135° C.

Karyology. Log phase cell cultures were treated with 100 μg/ml 5-bromo-deoxyuridine for 16 h, washed 3 times with medium, and treated for 6 h with 2.5 μg/ml thymidine. Colcemid (0.06 μg/ml) was added for the

final 30 min (4). The cells were shaken loose, centrifuged at 500 *g* for 5 min, and all but 1 ml of the medium was removed. An equal volume of distilled water was added initially (and again at 10 and 15 min giving a final 1/8 dilution) and the cells were incubated at 34° C. After a total of 20 min of hypotonic solution-induced swelling, 10 drops of fresh fixative (1 part acetic acid, 3 parts methanol) were added and the cells gently mixed. The cells were centrifuged at 500 *g* for 5 min, the supernatant discarded, and 0.1 ml of fixative was added to the pellet. After 20 min an additional 2.9 ml of fixative was added and the cells were suspended and then allowed to sit for 10 min before being centrifuged. The cells were resuspended in a small volume of fixative and dropped onto clean slides. The slides were stained with Giemsa and at least 50 chromosome spreads from each cell line were counted at ×600 magnification.

Mycoplasma testing. All five cell lines were tested for mycoplasma contamination using the fluorescent dye Hoechst 33258 (9).

Isozyme patterns. Cells from confluent cultures were shaken loose, pelleted at 500 *g* (5 min), washed 3 times with sterile 1% NaCl, and finally suspended in a small volume of sterile 0.01 *M* tris (pH 7.6). The cell suspension was subjected to three rounds of freezing and thawing and then centrifuged at 12 000 *g* for 30 min at 4° C. The supernatant was removed, aliquoted, and stored at −135° C.

Cell lysates were separated by polyacrylamide gel electrophoresis on 6.5% gels run at 35 mA constant current and 4° C (38). After electrophoresis, the gels were stained at 37° C for the following six enzyme systems: glucose-6-phosphate dehydrogenase (G6pdh), glutamate oxaloacetate transaminase (Got), hexokinase (Hk), isocitrate dehydrogenase (Idh), malate dehydrogenase (Mdh), and phosphoglucomutase (Pgm) (6,18–20,37).

Antibacterial product testing. Nearly confluent cultures were treated with 0.02 mg/ml of an acetone extracted *Escherichia coli* TB-1 powder (17) for 48 h. The medium was then collected from treated and untreated control cultures and tested for antibacterial products using an agar diffusion test (22) with live *E. coli* TB-1 as the test organism. A clear zone around the wells after 24 h incubation at 37° C was an indication of the presence of antibacterial activity.

Ecdysteroid assay. Medium from 1-wk-old confluent cell cultures was removed and stored frozen at −20° C until processed. Samples from 4 different passages from each of the cell lines were evaluated for the presence of ecdysteroids. A 5-ml sample of the medium was partitioned against chloroform (1:1) and the aqueous portion was placed on a conditioned SEP-PAK C_{18} cartridge and then rinsed successively with 5 ml each of 25 and 60% methanol in water, respectively. The polar ecdysteroids eluted with the 25% methanol and the free ecdysteroids came off with the 60% methanol (25). The 60% methanol eluate was taken to dryness by vacuum centrifugation and then subjected to radioimmunoassay (RIA) for ecdysteroids.

The cell line with the highest RIA activity was analyzed further to determine what ecdysteroids were contributing to this activity. Samples of tissue culture medium were partitioned against chloroform to remove lipids, and the aqueous phase was then loaded onto a SEP-PAK C_{18} cartridge and eluted with 25 and 60% methanol (25). The 60% methanol fraction was taken to dryness, picked up in 1 ml of 40% methanol in water. Two 200-μl aliquots were taken for determination of total ecdysteroid content, and the remaining 600 μl were separated with a Beckman 126 System Gold high performance liquid chromatograph (HPLC) using a Waters Nova-Pak C_{18} Radial Pak column. The system was monitored at 243 nm. The column was eluted with 40% methanol at a flow rate of 2.0 ml/min. Twenty five 2-ml fractions were collected. Each fraction was divided into 3 aliquots, dried by vacuum centrifugation, and submitted to RIA. Background RIA activity was assumed to be the average of the values obtained for fractions 18 to 25. Ecdysteroids were identified by correlating the amount of RIA-positive material in each fraction that co-migrated with known standards. The standards consisted of ecdysone, 20-hydroxyecdysone, makisterone A (Sigma, St. Louis, MO) and 20,26-dihydroxyecdysone (provided by Dr. R. Lafont, Paris, France).

Ecdysteroid titers were determined by RIA (8). Antiserum, raised in rabbits against an ecdysone-22-hemisuccinate-thyroglobulin conjugate was generously provided by Dr. James Buckner (USDA-ARS Biosciences Research Laboratory, Fargo, ND). The antiserum had a 4-times higher affinity for ecdysone than it did for 20-hydroxyecdysone (13). Results are expressed as picograms ecdysone equivalents per milliliter of culture medium.

Chitin detection. Cell aggregates from the BRL-AG-2 line and pieces of adult cuticle including the underlying epidermal cell layer were fixed in Dulbecco's phosphate buffered saline (DPBS) containing 0.5% glutaraldehyde overnight at 4° C (there were no post-fixation osmium treatments). Samples were rinsed in DPBS, dehydrated in a graded ethanol series, and embedded in Lowicryl HM20 either at −20° C followed by ultraviolet light polymerization in a nitrogen atmosphere according to the manufacturer's instructions (Chemishe Werke Lawi GnbH, Postfach 1660, D-8264 Waldraiburg, Germany) or at 4° C according to (1). Thin sections (80 to 90 nm) were cut using a diamond knife and mounted on bare 200- and 300-mesh nickel grids.

For chitin detection, thin sections were stained either with biotinylated wheat germ agglutinin (WGA) followed by streptavidin-gold (20 nm diameter) (26) or with 15 nm colloidal gold (Ted Pella, Redding, CA) conjugated to chitinase (7) (from *Streptomyces griseus*, Sigma C1525). Controls involved the inclusion of 0.2 *M N*-acetylglucosamine (with the WGA-biotin) or 4 mg/ml *N,N,N*-triacetyl-chitotriose (with the chitinase-gold incubations) (3); as well as staining sections with gold conjugated goat anti-mouse IgG antibody alone (Ted Pella). In addition, some sections were pretreated with 1 mg/ml of nonconjugated chitinase for 4 h at 28° C before staining with the gold conjugated chitinase reagent.

Confluent cultures of BRL-AG-2, BRL-AG-3C, and the cockroach line UM-BGE-4 (2 wk old) were also labeled for 3 days with 0.8 μCi/ml of [acetyl-1-^{14}C] *N*-acetyl-D-glucosamine (ICN, Irvine, CA). The quantity of labeled sugar available was limited, so only one 25-cm^2 flask of each culture was labeled. The cells were harvested, pelleted, and the incorporation of labeled sugar into KOH (1.5 *M*, 100° C for 2 h) resistant material was determined (27). The results were converted to counts per minute (cpm) per milligram of protein.

Results

Establishment of continuous cell lines. We succeeded in establishing five continuous cell lines from embryonic tissue of the cotton boll weevil. These lines have now been subcultured in excess of 60 times. Subcultures of all five lines were routinely established on a weekly basis using a ratio of cell suspension to fresh medium of 1:3 to 1:4. Population doubling times for lines BRL-AG-1, BRL-AG-3A, BRL-AG-3C, and BRL-AG-4 were approximately 20 to 22 h. Lines BRL-AG-1 and BRL-AG-4 were elongate and fibroblastoid in appearance and grew as a single attached cells (Fig. 1 *A,E*). Line BRL-AG-4 had a pronounced tendency to form local, multilayered piles of cells. Line BRL-AG-3A was a mixture of both fibroblastoid and epithelioid type cells (Fig. 1 *C*), whereas line BRL-AG-3C grew primarily as single attached epithelioid cells which were often large and highly flattened (Fig. 1 *D*). Line BRL-AG-2 grew as a mixture of single attached cells and loosely attached or free-floating cell aggregates (Fig. 1 *B*) which made accurate cell counts impossible. Initially these cell aggregates tended to form large hollow vesicles (Fig. 1 *F*), but with continued passage the aggregates became smaller and more compact. All five lines tested negative for mycoplasma contamination when examined by UV microscopy after Hoechst 33258 staining. Samples of these lines from early passages (11 to 16) were frozen in 10% DMSO in Excell 401 and stored at −135° C.

Chromosome number. At least 50 chromosome spreads were counted for each cell line (Passage 26 to 30) and the results are displayed in Fig. 2. The normal haploid chromosome number in the insect is 21 (41). In all the boll weevil lines, except BRL-AG-4, the chromosome counts clustered around either the haploid or diploid number with some examples of polyploidy. Line BRL-AG-4 exhibited a modal aneuploid chromosome number of 36.

Isozyme patterns. Six different isozyme systems were used to certify the identity of the established cell lines. The isozyme patterns

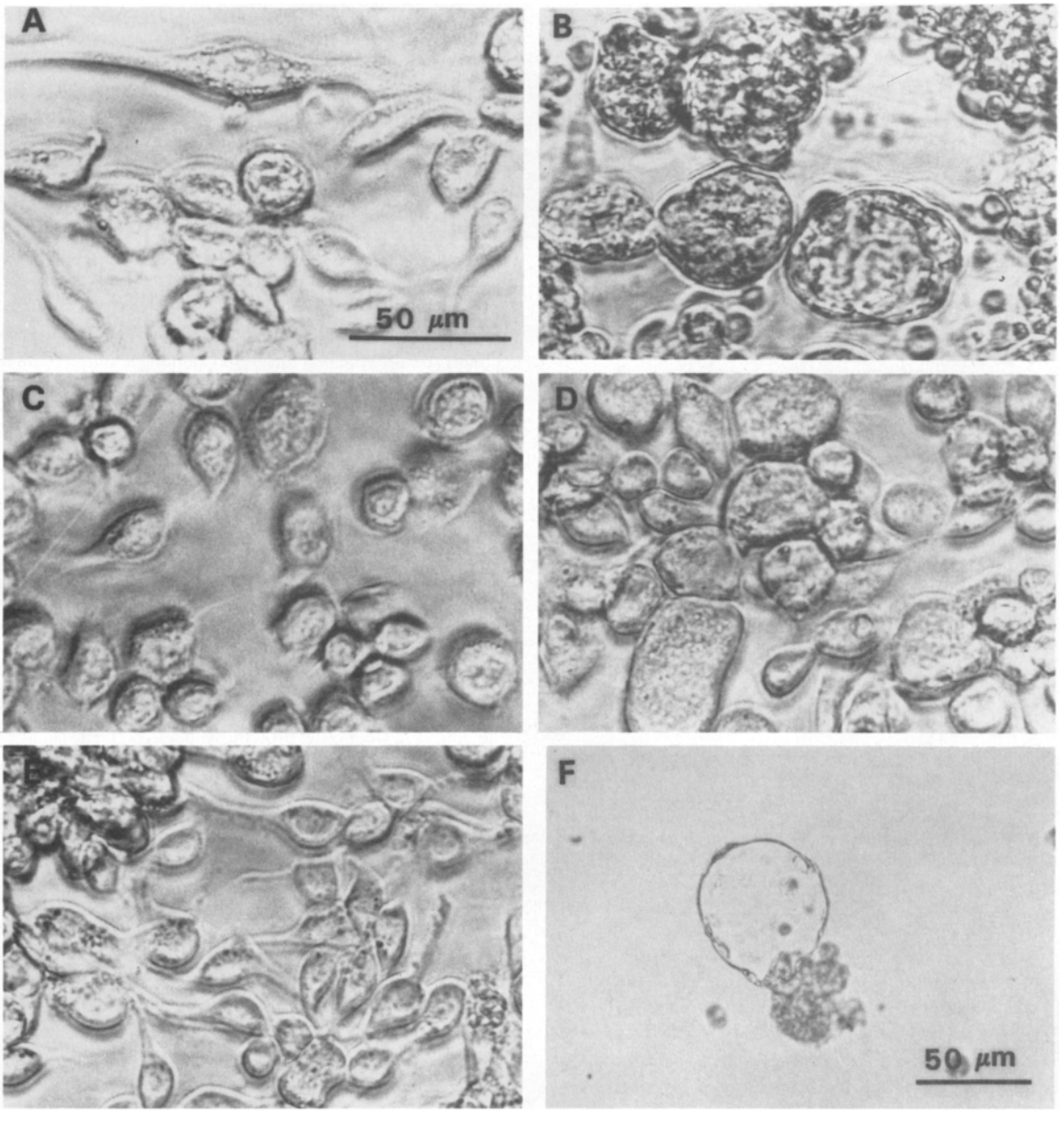

FIG. 1. Light photomicrographs of established cell lines from embryos of the cotton boll weevil. *A*, BRL-AG-1; *B*, BRL-AG-2 cell aggregates, late passage; *C*, BRL-AG-3A; *D*, BRL-AG-3C; *E*, BRL-AG-4; *F*, BRL-AG-2 hollow vesicle formed from cell aggregate, early passage. Magnification on *A–E* is the same as indicated by bar in *A*. *Bar* = 50 μm.

of the boll weevil cell lines were compared to that of boll weevil egg extract and lysates from two cockroach cell lines, UMBGE-2 and UMBGE-4 (24), which were being maintained in the laboratory during this time. All of the isozyme systems, except for Got, showed that the boll weevil cells were distinct from the cockroach cell patterns and similar to the weevil egg patterns (Fig. 3). In addition, the Pgm and Got isozyme systems exhibited differences between some of the individual boll weevil cell lines (Fig. 3 *E,F*).

Antibacterial products. Table 1 illustrates that when lines BRL-AG-1, BRL-AG-3A, and BRL-AG-3C (all at Passage 47 to 48) were exposed to an acetone extracted *E. coli* powder they produced detectable levels of an antibacterial factor(s) which was not present in the Excell 400 medium. All four of the attached cell lines had previously been observed to contain populations of cells capable of phagocytosing fluorescent labeled latex beads of either 0.2 or 1.0 μm diameter (data not shown). The antibacterial factor(s) released by the cells was found to be stable to freezing and to heat treatment (100° C for 5 min), but was inactivated by incubation with 1 mg/ml trypsin (37° C for 2 h; Sigma). All of the antibacterial activity was apparently due to a compound(s) of molecular weight greater than 10 000 (determined by retention in a Centricon-10 tube, Amicon, Beverly, MA).

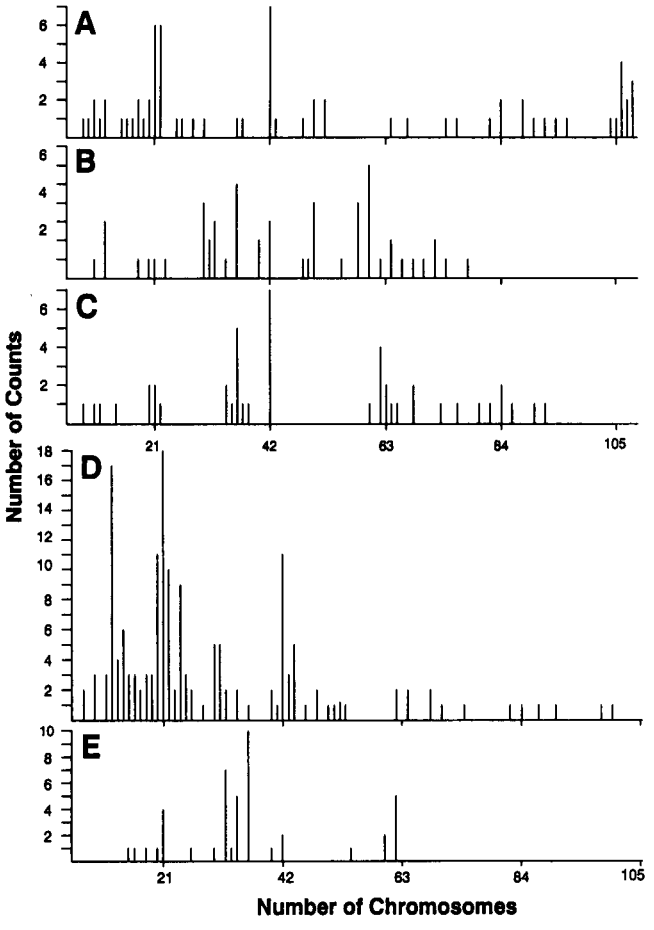

FIG. 2. Histograms of the chromosome counts made on Giemsa-stained chromosome spreads of the cotton boll weevil cell lines. *A*, BRL-AG-1; *B*, BRL-AG-2; *C*, BRL-AG-3A; *D*, BRL-AG-3C; *E*, BRL-AG-4.

Ecdysteroid production. The highest amount (131.8 pg/ml) of ecdysteroid RIA activity was found in the media from the BRL-AG-2 cell line (Table 2). The other lines released only 2 to 7 pg/ml of ecdysteroid into the medium. Fractionation of the media from BRL-AG-2 cells by HPLC followed by RIA showed that most of the activity co-migrated with ecdysone (Fig. 4 *A*, *B*). After correcting for background RIA activity, 48.5 pg/ml of ecdysone was found and it accounted for 70.3% of the total activity among the fractions. The remaining activity was associated with 20-hydroxyecdysone (4.8 pg/ml for 7.0%), makisterone A (3.0 pg/ml for 4.3%), and polar materials (4.7 pg/ml for 6.8%). We recovered 69% of the total ecdysteroid (RIA activity) that was applied to the HPLC column.

Chitin detection. Examination of thin sections of line BRL-AG-2 showed the presence of several morphologically distinct forms of extracellular material (ECM). One form of ECM was similar in appearance (electron density and textural structure) to typical basement membrane. This form showed no binding of the *N*-acetylglucosamine specific WGA lectin. The other forms of ECM are of a more fibrous and reticulated nature and vary in the degree of condensation from region to region and from cell aggregate to aggregate. This extracellular material did not form distinctly layered structures like those sometimes seen in the cockroach UM-BGE-4 line (34,50). Of this general category of ECM, the less condensed or looser accumulations showed the most frequent binding of the WGA stain (Fig. 5 *A*). The binding of the WGA varied in its occurrence and intensity among the many cell aggregates examined. The amount of this ECM and its intensity of staining with WGA was noticeably increased if the cell aggregates were first treated with two pulses of 20-hydroxyecdysone (0.8 μg/ml for 24 h, then 8 μg/ml for 48 h). To determine whether this WGA-positive material was chitin, a chitinase-gold conjugate was prepared and tested on sections of adult weevil cuticle (Fig. 5 *B*). The staining was specific for cuticle in the tissue sections and was almost totally inhibited by pretreatment of the sections with nonconjugated chitinase (Fig. 5 *B inset*) and was partially inhibited by the incorporation of *N*-acetyl chitotriose into the chitinase-gold staining solution (data not shown). When the BRL-AG-2 cells were examined, the WGA-positive type of ECM was also stained by the chitinase-gold (Fig. 5 *C*). Another form of material that demonstrated affinity for the WGA and chitinase-gold probes was observed in cytoplasmic inclusion bodies (Fig. 5 *D*). This material appeared variously as electron dense fibers, particles, and clumps within a clear, electron transparent vacuolar structure. The staining of this material was also highly variable from cell to cell.

It was determined by metabolic labeling that line BRL-AG-3C incorporated very little ^{14}C-labeled *N*-acetyl-D-glucosamine into alkaline-resistant material (499 cpm/mg protein), whereas both the cockroach line UM-BGE-4 and the boll weevil aggregate line BRL-AG-2 incorporated substantial amounts of radiolabel (3968 cpm/mg and 2421 cpm/mg protein, respectively). Resistance to alkaline treatment is a presumptive though not conclusive test for chitin. The UM-BGE-4 line was used as a positive control as it has been shown to produce chitin (33,34).

DISCUSSION

We were fortunate in that the pH and osmotic concentration of boll weevil hemolymph was found to be close to that of the commercially available Excell 401 medium so that no adjustments to the medium were needed. This relatively inexpensive, serum-free, low-protein culture medium gave excellent results in our attempts to initiate embryonic cell lines with a relatively rapid outgrowth (in 3 to 5 wk) of cells in the primary cultures. The four boll weevil cell lines which grew as single attached cells were fairly similar in morphology and growth characteristics with other insect cell lines. These lines all contained aneuploid and polyploid populations by Passage 30. Aneuploidy and polyploidy are common among Lepidopteran cell lines (32), although the two lines from the southern corn rootworm (Coleoptera) were reported to be 65% diploid (31). Barcenas et al. (2) observed polyploidy during early passage of their two boll weevil cell lines. Lines BRL-AG-1, BRL-AG-3A, BRL-AG-3C, and BRL-AG-4 have been successfully cloned (4, 4, 14, and 5 clones for each parent line, respectively) using cell dilution combined with gamma irradiated homologous feeder cells, although these cloned lines have not yet been characterized.

Line BRL-AG-2 contained cell aggregates/vesicles, a feature reported in at least three other insect lines, the German cockroach UM-BGE-4 line (an embryonic line which produces chitin) (24), the *Chironomus* midge line (embryonic, produces chitin) (52), and the cabbage looper IAL-TND-1 line (from imaginal discs) (29,30). Ini-

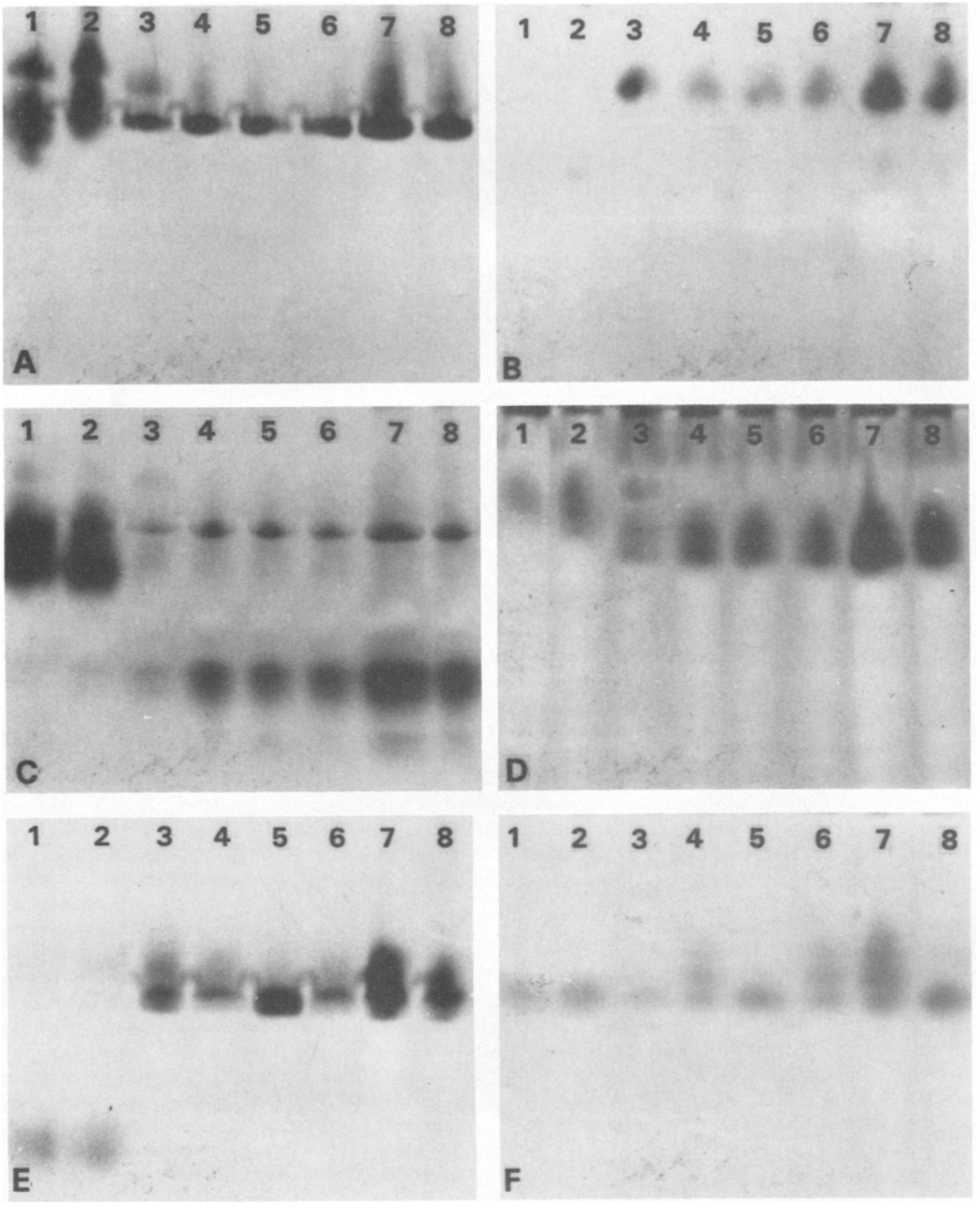

FIG. 3. Isozyme patterns of the cotton boll weevil cell lines and two German cockroach cell lines. The enzyme systems tested for were: *A*, G6pdh; *B*, Idh; *C*, Hk; *D*, Mdh; *E*, Pgm; *F*, Got. The cell lines tested were: *1*, UMN-BGE-2 (cockroach); *2*, UMN-BGE-4 (cockroach); *3*, boll weevil eggs; *4*, BRL-AG-2; *5*, BRL-AG-3A; *6*, BRL-AG-4; *7*, BRL-AG-1; *8*, BRL-AG-3C.

tially, BRL-AG-2 produced hollow multicellular vesicles which were very similar to those reported from the UM-BGE-4 German cockroach line. With continued passage however, line BRL-AG-2 produced, almost exclusively, compact aggregates of cells similar to those reported in the IAL-TND-1 line. As the three previously described aggregate or vesicle forming insect cell lines have been reported to synthesize low levels of ecdysteroids (28,35,46,49), we were interested to determine whether any of the boll weevil lines,

TABLE 1

RELEASE OF ANTIBACTERIAL ACTIVITY FROM BOLL WEEVIL CELL LINES FOLLOWING EXPOSURE TO DEAD *E. COLI*

Sample	Antibacterial Activity[a]
Excell 400 medium	−
BRL-AG-1[b]	
control	−
induced	+
BRL-AG-2	
control	−
induced	−
BRL-AG-3A	
control	−
induced	+
BRL-AG-3C	
control	−
induced	+
BRL-AG-4	
control	−
induced	−

[a] Based on the presence of a clear zone around sample wells in agar plates with *E. coli* as the test organism.

[b] Medium collected from confluent cultures either with or without a 48-h exposure to acetone extracted *E. coli*.

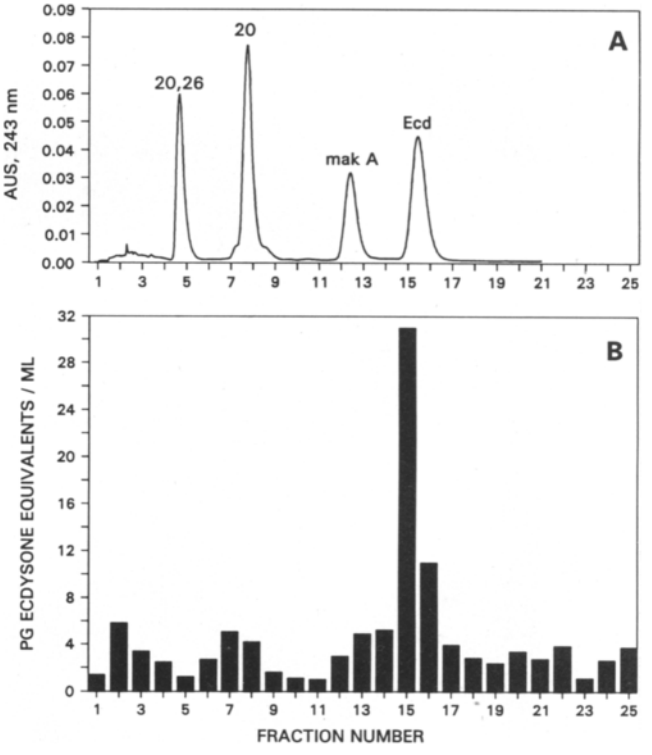

FIG. 4. HPLC separation of the ecdysteroids extracted from the BRL-AG-2 cell line medium by 60% methanol. *A*, ecdysteroid standards separated by HPLC- 20,26 = 20,26-hydroxyecdysone; 20 = 20-hydroxyecdysone; *mak A* = makisterone A; *ecd* = ecdysone. *B*, HPLC separation of culture medium extract followed by RIA of the individual fractions.

especially BRL-AG-2, produced ecdysteroids. RIA assays of spent culture medium indicated that BRL-AG-2 did release detectable levels of ecdysteroids, in the form of ecdysone and lower quantities of 20-hydroxyecdysone. These molting hormones were present at levels below the physiologic range (10 ng/ml to 2 μg/ml) generally reported for insect hemolymph (16,44). Both the UM-BGE-4 cockroach line and the cell line from *Chironomus* were reported to produce ecdysone which was released into the culture medium (46,49). The IAL-TND-1 cell line was found to produce 20-hydroxyecdysone, though only a small portion was released into the culture medium (28).

The cockroach vesicle-forming cell line has also been reported to produce the polysaccharide chitin (33), a major component of insect cuticle. Production of chitin by UM-BGE-4 was increased by exposure of the cells to physiologic levels of the molting hormone, 20-hydroxyecdysone (34,50). Electron micrographs of BRL-AG-2 cell aggregates exhibited small amounts of associated extracellular material which was WGA positive and which seemed to increase both in quantity and WGA staining after 20-hydroxyecdysone treatment. Electron micrographs of gold-conjugated chitinase stained sections suggested that chitin is present in the ECM and certain cytoplasmic inclusion bodies. The incorporation of radiolabeled *N*-acetyl-D-glucosamine into alkaline-resistant material supports the contention that line BRL-AG-2 produces chitin, although more rigorous testing (27) is needed for confirmation.

TABLE 2

TOTAL ECDYSTEROIDS RELEASED INTO THE CULTURE MEDIUM BY THE COTTON BOLL WEEVIL CELL LINES

Cell Line	Ecdysone Equivalents,[a] pg/ml
BRL-AG-1	6.7 ± 4.5
BRL-AG-2	131.8 ± 45.6
BRL-AG-3A	2.0 ± 0.5
BRL-AG-3C	5.4 ± 1.6
BRL-AG-4	4.0 ± 2.3

[a] The means ± standard deviation were based on four replicate samples obtained from separate cell line passages. The means were corrected for background by subtracting the value for RIA positive material found in Excell 401 medium.

It would be interesting to know whether any of the aggregate or vesicle-forming insect cell lines that can produce chitin are also capable of producing any of the exoskeletal cuticle proteins which are believed to be responsible for the specialized nature of different insect cuticles (43,51). It has been reported that 20-hydroxyecdysone treatment does induce the IAL-TND-1 line to produce new proteins of undetermined function (29). We have observed that treatment of line BRL-AG-2 with 20-hydroxyecdysone induced the synthesis of several proteins which are currently being tested with anti-cuticle protein antibodies (48). A cell culture system capable of producing cuticle proteins might be useful for screening potential insect control compounds for their ability to interfere with normal cuticle formation.

Antibacterial hemolymph proteins induced by microorganisms or their components have been reported in a variety of insects, includ-

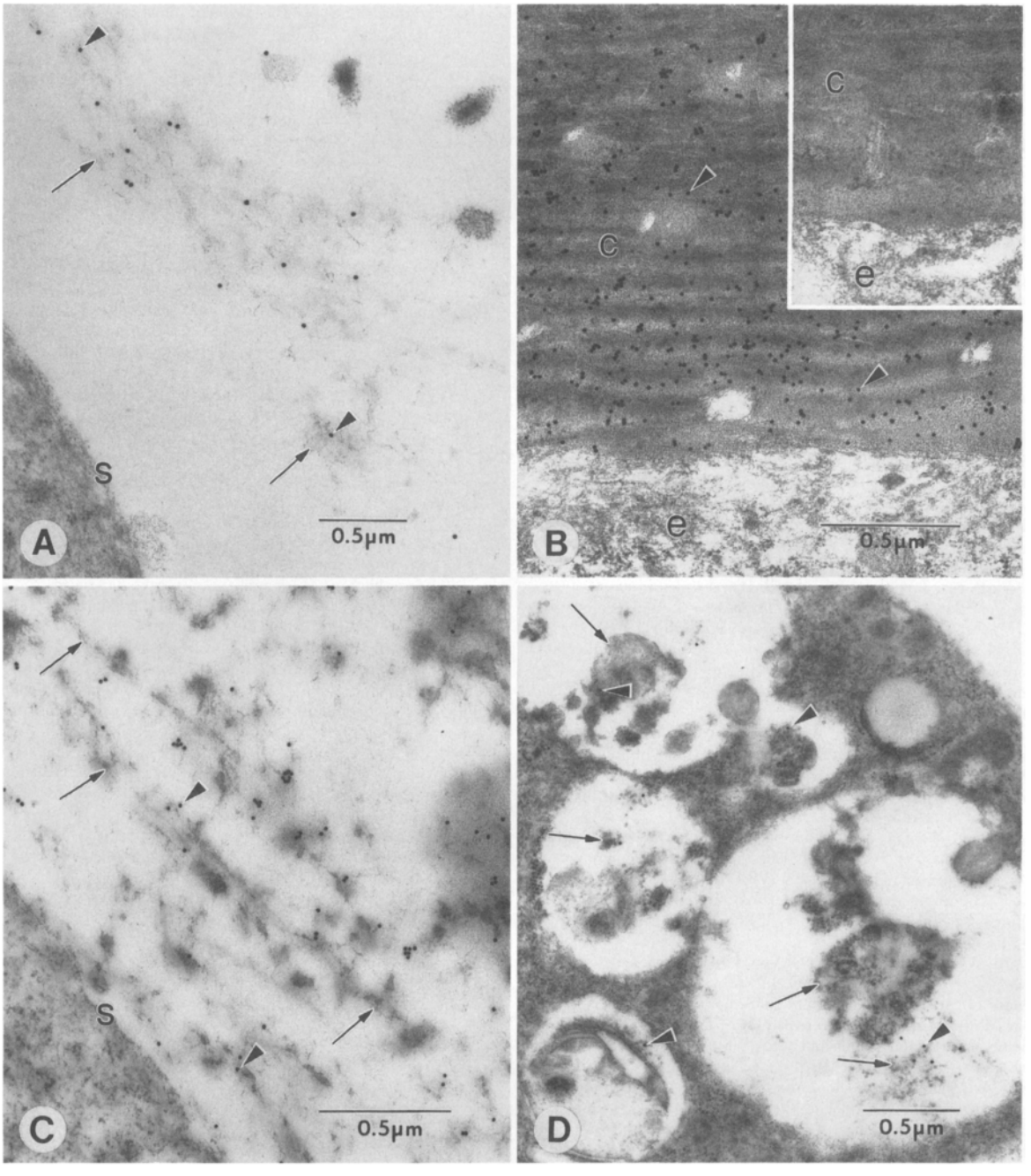

FIG. 5. Transmission electron micrographs of adult boll weevil cuticle and line BRL-AG-2 cell aggregates. *A*, extracellular material (*arrows*) near the cell surface (s) of 20-hydroxyecdysone treated BRL-AG-2 aggregate stained with WGA-biotin and streptavidin-gold (*arrowheads*; 20 nm diameter particle size); *B*, developing adult weevil cuticle (*c*) and associated epidermal cells (*e*) stained with chitinase-gold (*arrowheads*; 15 nm diameter particle size). *Inset* (same magnification as *B*) shows that the cuticle affinity for chitinase-gold is destroyed by pretreatment with chitinase; *C*, BRL-AG-2 cells treated with 20-hydroxyecdysone showing extracellular material (*arrows*) stained with chitinase-gold (*arrowheads*); *D*, inclusions within the cytoplasm of a 20-hydroxyecdysone treated BRL-AG-2 cell exhibiting heterogeneous accumulations (*arrows*) which often demonstrated an affinity for the chitinase-gold label (*arrowheads*).

ing the darkling beetle *Eleodes* (5,45). In the case of the flesh fly *Sarcophaga peregrina*, three antibacterial proteins have also been isolated from an established cell line (36). Hemolymph proteins from small insects, such as the cotton boll weevil, are difficult to study due to the small volumes of hemolymph fluid in these insects. We were curious about the potential of the boll weevil cell lines for the production of antibacterial proteins, which could then be purified for further study. The antibacterial factor(s) observed in me-

dium from three of the boll weevil cultures after induction by dead bacteria were probably protein in nature with a molecular weight greater than 10 000. These characteristics suggest that the protein(s) may be of the attacin type (5,21,42), although more work would be needed to confirm this structural relationship. It has been suggested (40) that the active portions of these insect proteins might be useful as antibacterial factors or antitumor agents if inserted into transformed organisms.

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