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Direct Mitogenic Effects of Insulin, Epidermal Growth Factor, Glucocorticoid, Cholera Toxin, Unknown Pituitary Factors and Possibly Prolactin, but not Androgen, on Normal Rat Prostate Epithelial Cells in Serum-free, Primary Cell Culture[1]

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ABSTRACT

Selective nutritive conditions were used to isolate normal epithelial cells from fibroblasts in primary cell cultures prepared from adult rat prostate. The pure population of normal epithelial cells proliferated at an exponential rate on a simple polystyrene substratum with doubling times of 35 to 50 hr for 10 to 12 days in the absence of high epithelial cell density, other cell types, or added extracellular matrix elements. Optimization of the nutritive environment allowed direct analysis of the hormone:growth factor requirements for sustained proliferation of the isolated epithelial cells in serum-free medium. An *in situ* videometric method was used to assay the effect of over 30 known hormones and growth factors on proliferation of the prostate epithelial cell population. The results revealed direct mitogenic effects of insulin, epidermal growth factor, glucocorticoid, cholera toxin, one or more unidentified factors from bovine pituitary, and possibly prolactin. No direct mitogenic effect of androgen on isolated prostate epithelial cells could be demonstrated. Radioimmunoassay of androgen in the primary cultures showed that endogenous androgen was about 34 pM on Day 1 of culture and thus probably too low to mask a response to exogenous androgen. Deletion of any single active growth factor did not reveal an androgen response. The results demonstrate a multihormonal control of normal prostate epithelial cell maintenance and proliferation without the direct participation of androgen.

INTRODUCTION

Normal prostate epithelial cell maintenance, proliferation, and function are strictly pituitary dependent (18, 23, 58). It is well established that the primary role of pituitary is indirect and mediated by androgen that is produced by the testes. Hypophysectomy or orchiectomy caused a rapid decline in prostate functions, followed by a loss of cellularity and involution of the gland. Gonadotropin or androgen injections caused epithelial cell growth and restoration of the functional activities of the prostate. A supportive role of pituitary factors other than gonadotropin in androgen-dependent prostate cell growth was suggested by experiments that indicated that prostatic atrophy is greater following hypophysectomy than atrophy following orchiectomy in the dog (23) and that the prostatic response to androgen was less in magnitude in hypophysectomized rats than in rats that had undergone orchiectomy alone (18, 60). Prolactin has been the top candidate for this role (2, 9, 15, 52), although earlier studies also suggested a role of growth hormone and ACTH[3] in prostate cell growth (24, 34, 58). Much recent progress has been made on the role of androgen in expression of specific gene products that are characteristic of differentiated, secretory cells of the prostate (21, 41, 42, 44). The profile of high-affinity androgen and prolactin receptors and androgen and prolactin metabolism in normal differentiated prostate tissue (1, 26, 59, 61) constitutes strong, circumstantial evidence that androgen and prolactin may act directly on prostate epithelial cells to maintain expression of specific prostate gene products. The role of androgen, prolactin, and other hormones in basic maintenance and proliferation of prostate epithelial cells is much less conclusive. Changes in hormone receptor levels and hormone receptor metabolism during atrophy after orchiectomy and during regeneration after androgen treatments are difficult to interpret, because the gland is a mixture of epithelial cells at various stages of regression, proliferation, and differentiation after these treatments (32). Androgens prolong maintenance and elicit epithelial cell hyperplasia in isolated prostate tissue in organ culture (4, 28), but mitogenic activity measured by an increase in cell number is questionable. Hoisaeter *et al.* (22) have cast doubt on whether the effects of androgen in organ culture occur by currently established mechanisms (59) of androgen:receptor interactions, *e.g.*, binding to a cytoplasmic receptor and subsequent migration into the nucleus. The evidence for a direct mitogenic effect of androgen on isolated prostate epithelial cells is also weak. The number of reports that have failed to demonstrate a mitogenic effect of androgen on isolated prostate cells (6, 8, 10, 27, 31, 33, 48–50) outnumbers the positive reports (11, 46, 55). Experimental systems to test the role of androgen on proliferation of isolated prostate epithelial cells to date are less than definitive on several grounds: (*a*) although close to the animal chronologically, primary cell cultures derived from prostate tissue are usually contaminated with nonepithelial cell types (10, 36); (*b*) although relatively pure, empirically derived prostate epithelial cell lines are of unknown origin, chronologically removed from normal prostate tissue, and sometimes karotypically abnormal (27, 31); and (*c*) both primary and cell line cultures usually require the presence of serum which can mask and modify the proliferative response of epithelial cells to androgen, other hormones, and environmental factors (20). Recently, we reported the isolation by selective nutritive conditions of prolif-

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[3] The abbreviations used are: ACTH, adrenocorticotropic hormone; EGF, epidermal growth factor; WAJC, nutrient medium developed at W. Alton Jones Cell Science Center; MCDB, nutrient medium developed at the Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO; NIAMDD, National Institute of Arthritis, Metabolism, and Digestive Diseases.

erating adult rat ventral prostate epithelial cells in serum-containing primary culture (35). Here, we report conditions that support the rapid and sustained proliferation of isolated normal prostate epithelial cells directly from the gland in the absence of serum. The proliferative response of the cell population to androgen, hormones, and other nonnutritive hormone-like factors was examined in serum-free medium. The results revealed direct mitogenic effects of insulin, EGF, glucocorticoid, cholera toxin, prolactin, and unknown pituitary-derived factor(s) on prostate epithelial cell proliferation. No positive effect of androgen could be demonstrated.

MATERIALS AND METHODS

Materials. Numbers in parentheses following reagents are catalogue numbers. Sprague-Dawley rats were obtained from Taconic Farms (Germantown, NY). Inbred Lewis rats were from the Trudeau Institute (Saranac Lake, NY). Collagenase (type II) was from Worthington Biochemicals (Freehold, NJ). The sources for individual components in culture media are listed in Table 1. Nutrient Media F-12K (78-0531), 199 (400-1200), F-12, (430-1700), Eagle's minimal essential medium (410-1500), and Dulbecco's modified Eagle's medium (430-1600) were obtained from Grand Island Biological Co. (Grand Island, NY). Horse serum was from Flow Laboratories (McLean, VA). Nylon screen was obtained from Tetko, Inc. (Elmsford, NY). The following were obtained from Sigma Chemical Co. (St. Louis, MO): α-tocopherol (T 3251); α-tocopherol acetate (T 3376); bovine insulin (I 5500); cholera toxin (C 3012); cholesterol (CH-K); dexamethasone (D 1756); dihydrotestosterone (A 8380); epinephrine (E 4250); ethanolamine (A 5629); fatty acid-free bovine serum albumin (A 0281); histamine (H 7250); oxytocin (O 3251); ovine prolactin (L 4876); phosphoethanolamine (P 0503); progesterone (P 0130); prostaglandin $F_{2\alpha}$ (P 5890); prostaglandin E_1 (P 5515); prostaglandin E_2 (P 5640); retinoic acid (R 2625); serotonin (H 5755); soybean phosphatidylcholine (P 5638); spingomyelin (S 7004); testosterone (T 1500); thyrotropic hormone (TS-10); triiodothyronine (T 2752); tyramine (T 2879); and vasopressin (V 2875). The following were from Collaborative Research (Waltham, MA): EGF (40001); fibroblast growth factor (40002); multiplication-stimulating activity (40004); thrombin (40007); and transferrin (40204). The following were from Calbiochem (La Jolla, CA): follicle-stimulating hormone (34411); luteinizing hormone (43855); somatotropic hormone (56741); and thyrotropin-releasing factor (609401). Luteinizing hormone-releasing hormone (337702) was from Beckman Instruments, Inc. (Palo Alto, CA). Rat prolactin (NIAMDD PRL-B-3) and sheep prolactin (NIAMDD O-PRL-15) were obtained from the NIAMDD hormone distribution program. [^{3}H]Dihydrotestosterone, [^{3}H]testosterone, and the kit (TRK 600) for immunoassay of both androgens were obtained from New England Nuclear (Boston, MA). Peroxidase-conjugated goat anti-rabbit IgG antiserum was from Cappel Laboratories (Cochranville, PA), and Hanker-Yates stain was from Polysciences, Inc. (Warrington, PA). Plasticware was from Lux Scientific Corp. (Newbury Park, CA) and Corning Glass Works (Corning, NY).

Dissociation of Prostate Tissue for Primary Culture. Sprague-Dawley and inbred Lewis rats were used as test animals. Ventral prostate lobes of 10- to 14-week-old rats were removed, freed of connective tissue, weighed, and minced into fragments of approximately 1.0 mm with scissors. On average, prostates weighed 0.4 to 0.6 g/animal. Tissue pieces were placed in 10-ml/g of wet tissue weight of a solution containing 675 units of collagenase/ml in Solution I [30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid hydroxide (pH 7.6):4.0 mM glucose:2.0 mM KCl:12 mM NaCl:1.0 mM KH_2PO_4:3.3 μM phenol red]. The tissue suspension was aspirated 3 times through a 14-gauge cannula and then incubated for 1 hr at 37° with constant shaking. After cooling to 4°, the suspension was aspirated 3 more times to dissociate the tissue. The cell suspension was then passed through a coarse screen (wire mesh, 1 mm) to remove debris and cell aggregates. The screen was washed with an equal volume of Solution I containing 5% horse serum (Solution II). Cells were collected by centrifugation at 100 × *g* for 5 min at 4°. After resuspension in 5.0 ml of Solution II/g of original tissue, the cell suspen-

Table 1
Composition of nutrient media

Component	Source	Concentration (M)[a] F-12K	WAJC 404
L-Alanine	S[b]	2.0E-4	1.0E-4
L-Arginine·HCl	S	2.0E-3	1.0E-3
L-Asparagine·H_2O	S	2.0E-4	1.0E-4
L-Aspartic acid	S	2.0E-4	3.0E-5
L-Cysteine-HCl·H_2O	S	4.0E-4	
L-Cystine	S		2.4E-4
L-Glutamine	S	2.0E-3	6.0E-3
L-Glutamic acid	S	2.0E-4	1.0E-4
L-Glycine	S	2.0E-4	1.0E-4
L-Histidine-HCl·H_2O	S	2.0E-4	8.0E-5
L-Isoleucine (allo-free)	S	6.0E-5	1.5E-5
L-Leucine	S	2.0E-4	5.0E-4
L-Lysine·HCl	S	4.0E-4	1.0E-4
L-Methionine	S	6.0E-5	3.0E-5
L-Phenylalanine	S	6.0E-5	3.0E-5
L-Proline	S	6.0E-4	3.0E-4
L-Serine	S	2.0E-4	6.0E-4
L-Threonine	S	2.0E-4	1.0E-4
L-Tryptophan	S	2.0E-4	1.5E-5
L-Tyrosine	S	6.0E-5	1.5E-5
L-Valine	S	2.0E-4	3.0E-4
D-Biotin	S	3.0E-7	6.0E-8
Folic acid	S	3.0E-6	1.8E-6
Lipoic acid	S	1.0E-6	1.0E-6
Niacinamide	S	3.0E-7	3.0E-7
Pantothenic acid (hemicalcium salt)	S	1.0E-6	1.0E-6
Pyridoxine·HCl	S	3.0E-7	3.0E-7
Riboflavin	S	1.0E-7	1.0E-7
Thiamine·HCl	S	1.0E-6	1.0E-6
Vitamin B_{12}	S	1.0E-6	3.0E-7
Adenine	S		1.8E-4
Hypoxanthine	S	3.0E-5	
Thymidine	S	3.0E-6	3.0E-6
Sodium acetate	A		3.7E-3
Choline·HCl	S	1.0E-4	1.0E-4
D-Glucose	B	7.0E-3	6.0E-3
i-Inositol	S	1.0E-4	1.0E-4
Putrescine·2HCl	S	2.0E-6	1.0E-6
Sodium pyruvate	S	2.0E-3	1.0E-4
$CaCl_2·4H_2O$	A	9.2E-4	1.3E-4
$MgCl_2·6H_2O$	J	5.2E-4	6.0E-4
$MgSO_4·7H_2O$	A	1.6E-3	
KCl	A	3.8E-3	1.5E-3
KH_2PO_4	B	4.3E-4	
$Na_2HPO_4·7H_2O$	A	8.1E-4	2.0E-3
NaCl	A	1.3E-1	1.3E-1
$CuSO_4·5H_2O$	J	1.0E-8	1.0E-9
$FeSO_4·7H_2O$	J	3.0E-6	1.5E-6
$MnSO_4·5H_2O$	J		1.0E-9
$(NH_4)_6Mo_7O_{24}·4H_2O$	J		1.0E-9
$NiCl_2·6H_2O$	J		5.0E-10
H_2SeO_3	J		3.0E-8
$Na_2SiO_3·9H_2O$	F		5.0E-7
$SnCl_2·2H_2O$	J		5.0E-10
NH_4VO_3	J		5.0E-9
$ZnSO_4·7H_2O$	J	5.0E-7	5.0E-7
$NaHCO_3$	J	3.0E-2	1.4E-2
Carbon dioxide		5%	5%
4-(2-Hydroxyethyl)-1-piperazineethane sulfonic acid (free acid)	R		2.8E-2
Phenol red	S	8.3E-6	3.3E-6
NaOH	A		1.0E-2
pH			7.65

[a] Concentrations are expressed in molar units (M). For example, "2.0E-4" means 2.0×10^{-4} M.

[b] S, Sigma Chemical Co., St. Louis, MO; A, Alpha Products, Thiokol Ventron Division, Danvers, MA (ultrapure grade); B, J. T. Baker Chemical Co., Phillipsburg, NJ (Ultrex grade); J, Johnson Matthey Chemicals, Ltd., London, England (obtained through Alpha Products); F, Fisher Scientific, Fair Lawn, NJ; R, Research Organics, Cleveland, OH.

sion was passed successively through nylon screen filters with mesh sizes of 253, 160, 100, and 41 μm, respectively, to obtain a suspension of predominately single cells. Screen filters were washed successively with an equal volume of Solution II. Cells were collected by centrifugation and resuspended in 10 ml of Solution I/g of original tissue. The cell suspension was then diluted with Solution I or other media compatible with the individual experiment. Cell suspensions (about 50 μl) containing the number of cells indicated in the text were inoculated into 35-mm plastic tissue culture Petri dishes containing 2 ml of the medium indicated in the text. Cultures were incubated in a humidified atmosphere of 5% carbon dioxide and 95% air at 37°. The culture medium was changed on Days 3, 5, 7, and 9 of culture unless otherwise indicated. Average cell yields were 5×10^7 cells/g, wet weight, of prostate tissue. Cell number in the primary suspension was estimated by Coulter count.

Preparation of Culture Media. Experimental nutrient media were prepared from controlled and dated stocks made from high-quality single chemicals as described previously (35, 38). Commercially obtained formulas were prepared from powder according to manufacturer's instructions. Nutrient medium formulas and sources for chemicals for Media F-12K and WAJC 404 are listed in Table 1. For discussion of nutrient media other than the WAJC series, see Ref. 20. Medium WAJC 401 was described previously (35) and contained (*a*) 240 μM cysteine instead of the 240 μM cystine; (*b*) 500 μM instead of the 100 μM pyruvate; and (*c*) 30 μM instead of the 130 μM $CaCl_2 \cdot 4H_2O$ in Medium WAJC 404 (Table 1). Single hormones and growth factors were prepared in 20, 500, or 1000× stock solutions described below and added to the nutrient media at concentrations indicated in the text. Extracts of bovine pituitary were prepared as described (35). Freeze-dried cholera toxin received from the supplier was dissolved in sterile water to yield a concentration of 10 μg protein/ml. The stock solution also contained 50 μM Tris HCl (pH 7.5), 2 mM NaCl, 30 μM NaN_3, and 10 μM disodium EDTA. Freeze-dried EGF received from the supplier was dissolved in sterile water at 10 μg protein/ml. Testosterone, dihydrotestosterone, and dexamethasone were dissolved at 1.0 mM in 70% ethanol. Insulin was dissolved at 2.5 mg/ml in 30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid hydroxide (pH 3.0) and 100 mM NaCl. Prolactin was prepared at 1 mg/ml in 100 mM $NaHCO_3$. Stock solutions of insulin, prolactin, and bovine pituitary extract were sterilized by membrane filtration (Gelman Acrodisc, 0.2 μm, No. 4192; Gelman Sciences, Ann Arbor, MI).

Immunochemical Staining for Keratin in Prostate Epithelial Cells. Human strateum corneum keratin was isolated, and antiserum was prepared in rabbits as described by Sun and Green (53). Primary cultures of prostate epithelial cells were prepared on glass coverslips and fixed in cold acetone as described previously (39). Fixed cells were stained for the presence of keratin by the double-antibody immunoperoxidase method (47).

Videometric Measurement of Cell Number. The number of cells attached to Petri dishes was estimated *in situ* by a videometric method (5, 57). Cells were fixed with a solution of methanol:acetic acid (3:1) and then stained with a solution of Harris hematoxylin followed by 1 mM lithium carbonate. Petri dishes containing fixed and stained cells were placed under the camera of an Artek Model 980 cell counter (Artek Systems Corp., Farmingdale, NY) equipped with an Artek Model 890 light box. Optimum settings for the cell counter were determined experimentally. Specific settings were: mode, "object area"; image, "positive"; and sensitivity, "auto." Aperture area was set in circular mode at 730 units on the Vernier scale. At this setting, the aperture field covered 729 sq mm of a Petri dish with a total surface area of 800 sq mm. The camera lens (standard 50 mm) was set at "f8" at a distance of 10.5 cm from the Petri dish. The light box was set on "bright field." Measurements are reported throughout the text in units of 10 × sq mm read directly from the instrument at the above settings.

Karyological Analysis. The number of chromosomes present in prostate epithelial cells was determined by the method described in Ref. 62.

Immunoassay of Endogenous Androgen. The presence of testosterone and dihydrotestosterone in the prostate cell inoculum and bovine pituitary extract was determined by competitive binding immuoassay with Amersham Kit TRK 600 according to the manufacturer's instructions.

RESULTS

Measurement of Cell Proliferation. Cell proliferation was measured in primary cell cultures by the *in situ* video method described in "Materials and Methods." Chart 1 shows the relationship of the measurement with cell number within the range of cell densities used in the study. Cell number was estimated separately by removing the cells with trypsin, followed by direct count on a Coulter Counter. Chart 2 shows the linear correlation of the videometric method to direct cell count and DNA synthetic activity over time in the primary cultures.

Optimization of Nutrient Requirements for Prostate Epithelial Cell Proliferation. Rat ventral prostate tissue was prepared for culture as described in "Materials and Methods." Epithelial cells were isolated in serum-containing primary cultures by the use of selective nutritive conditions (35). A specific set of nutrients (Medium WAJC 401) containing cholera toxin (130 pM), insulin (833 nM), EGF (1.6 nM), prolactin (43 nM), dexamethasone (1 μM), bovine pituitary extract (25 μg/ml) and 5% (v/v) horse serum supported the rapid proliferation of prostate epithelial cells without extensive overgrowth with fibroblasts. Dialysis of the horse serum resulted in a significant loss of multiplication-stimulating activity (Chart 3). A closer examination of the serum requirement indicated that a level of dialyzed horse serum equivalent in protein content to native serum was inhibitory to cell proliferation. A reduction in the concentration of dialyzed serum resulted in a significant increase in cell growth (Chart 3). Epithelial cell proliferation occurred at a detectable rate in the absence of serum (Chart 3). At this point, we performed tests by Ham and McKeehan (20) to determine whether individual nutrients in Medium WAJC 401 were deficient or in excess. A significant difference between Medium WAJC 402 and its precursors and most

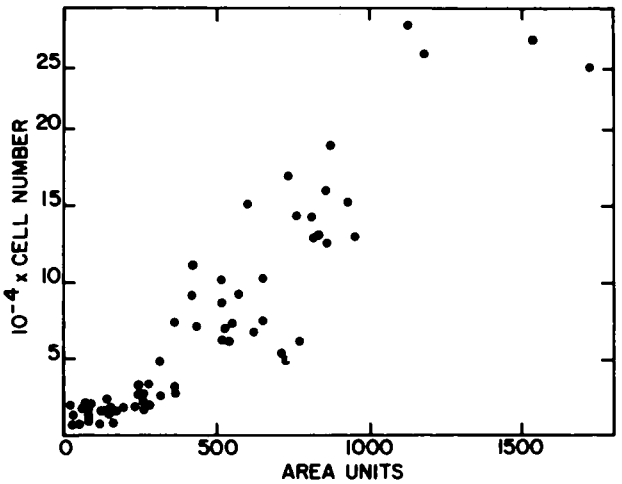


Chart 1. Relationship between area units and cell number. Cells from replicate 35-mm Petri dishes were counted directly and also analyzed by the videometric procedure described in "Materials and Methods." Data are pooled from multiple experiments and conditions from the study where both cell number and area units were measured. The indicated data are area units read directly from the cell counter and cell counts determined by Coulter Counter. An area reading of 500 units indicates that 50 sq mm of an aperture field of 729 sq mm were covered by stained cells. The total surface area of the 35-mm plastic Petri dish is about 800 sq mm. The data indicate that 500 area units are equivalent to about 70,000 cells estimated by direct count.

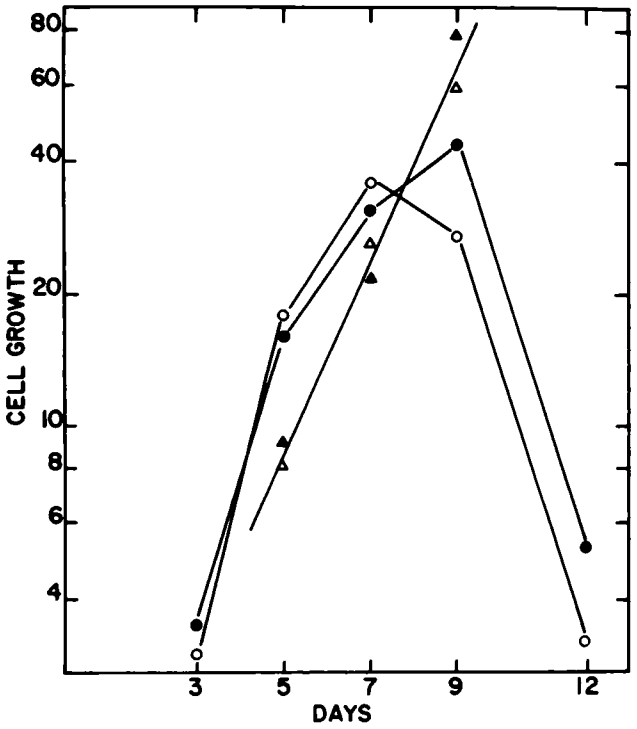


Chart 2. Time curve for prostate cell growth in primary culture measured in area units, direct cell count, and DNA synthesis. The data are from 2 separate experiments. In one experiment (▲, △), cell counts and area measurements were made on replicate 35-mm Petri dishes on the indicated day of primary culture. DNA synthesis and area measurements were made on replicate dishes in the second experiment (●, ○). DNA synthesis was estimated by incorporation of [^{3}H]thymidine (20 Ci/mmol, 1.0 μCi/ml) into acid-insoluble product for 3 hr. The cultures contained Medium WAJC 401, growth factors, and 5% horse serum and were inoculated with 200,000 cells. ●, △, 10^{-1} × area units; ▲, 10^{-3} × cell number; ○, 10^{-2} × cpm of [^{3}H]thymidine incorporation.

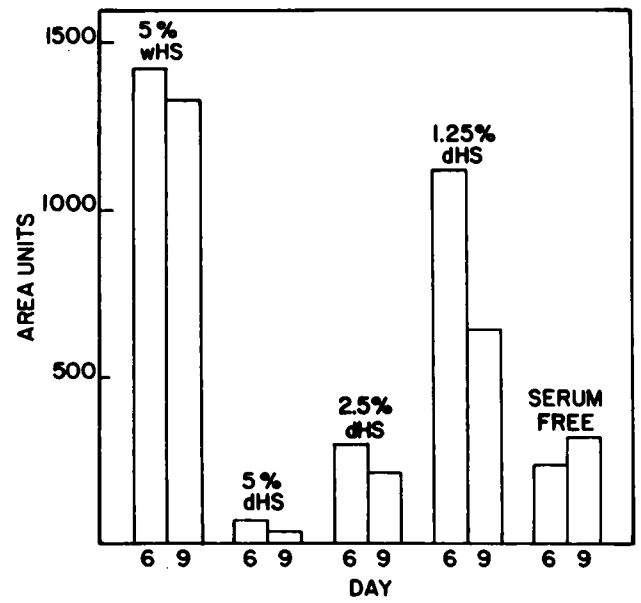


Chart 3. Effect of dialysis on growth activity of horse serum in Medium WAJC 401 plus growth factors. Horse serum was dialyzed against deionized water overnight, freeze dried, and reconstituted in Solution I to 50 mg, dry weight, per ml. The whole horse serum used in this experiment contained 70 mg protein/ml. Additions are expressed in volume percentage. Cell number was estimated in area units on the day and under the condition indicated. Cell inoculum was 400,000 cells/35-mm dish. *wHS*, whole horse serum; *dHS*, dialyzed horse serum.

other nutrient media is a low calcium concentration which contributes to its suppression of fibroblast proliferation (43, 57). Therefore, we first examined the calcium requirement for cell proliferation at low concentrations of dialyzed horse serum protein and in serum-free Medium WAJC 401 (Chart 4). The results indicated that the level of calcium in Medium WAJC 401 (30 μM) was deficient. Prostate cell proliferation increased with increasing calcium and reached a plateau at about 130 μM. Further increases in calcium up to 1.0 mM had a slight inhibitory effect on cell proliferation. Increased levels of calcium in the medium had neither massive negative effects on cell proliferation nor increased expression of specific epithelial cell products as has been reported for keratinocytes and bronchial epithelial cells (30, 43, 57). The calcium concentration in Medium WAJC 401 was adjusted to 130 μM, and the new medium was designated Medium WAJC 402.

The proliferative response of epithelial cells to other nutrients in serum-free primary culture was then examined. Chart 5 shows that the cysteine level in Medium WAJC 402 (240 μM) was too high. The rate of cell proliferation was improved by reduction of

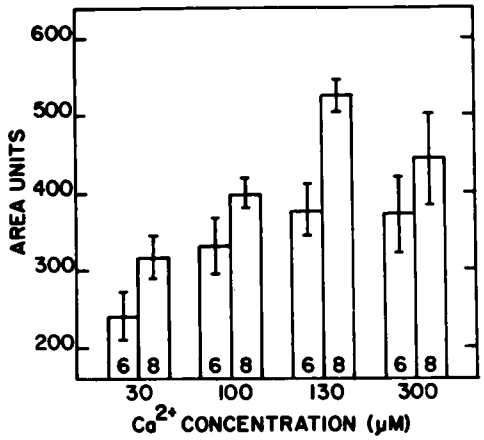


Chart 4. Effect of Ca^{2+} concentration on prostate cell proliferation in serum-free Medium WAJC 401 and growth factors. The indicated amount of $CaCl_2 \cdot 4H_2O$ was added to $CaCl_2 \cdot 4H_2O$-deficient WAJC 401 plus growth factors. Area measurements were made on Days 6 and 8 of primary culture as indicated. *Columns*, mean of triplicate dishes; *bars*, S.D. Inoculum was 400,000 cells.

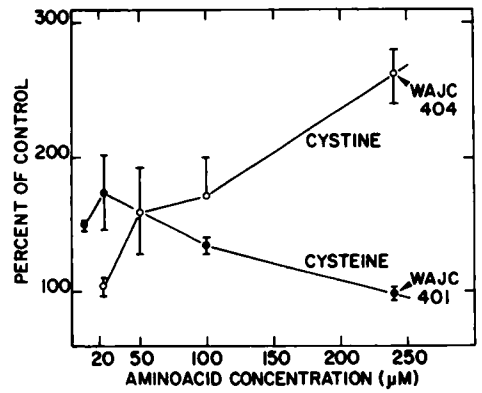


Chart 5. Effect of cysteine and cystine on prostate cell proliferation. The indicated amounts of cysteine or cystine were added to cysteine-deficient Medium WAJC 401 containing 130 μM $CaCl_2 \cdot 4H_2O$ and growth factors. Area units were measured on Day 8 of culture. The data are expressed as percentage of the area units at 240 μM cysteine, the level in Medium WAJC 401. Inoculum was 400,000 cells. The level of amino acid in Medium WAJC 401 and Medium WAJC 404 is indicated. *Points*, mean of triplicate Petri dishes; *bars*, S.D.

the cysteine level to 50 μM or less. Additional experiments indicated that the requirement for cysteine carbons may be masked by the reduced form in which they are introduced to the medium (Chart 5). Epithelial cell proliferation was further improved by the replacement of cysteine with 240 μM cystine (Chart 5). A dose reponse to sodium pyruvate indicated that the level of pyruvate was also too high in Medium WAJC 402 (results not shown). The sodium pyruvate concentration was reduced from 500 to 100 μM in subsequent test media. Amino acids other than cysteine (or cystine), glutamine, alanine, aspartate, glutamate, and glycine were examined as a group at levels 0.1, 0.5, 1, 5, and 10 times the normal concentration in Medium WAJC 402 (results not shown). The proliferative response to alanine, asparate, glutamate, and glycine as a group was similarly examined. The proliferative response to vitamins biotin, folic acid, lipoic acid, niacinamide, pantothenic acid, pyridoxine, thiamine, and vitamin B_{12} was examined also as a group. The response to glutamine, pyruvate, adenine, inositol, lipoate, thymidine, putrescine, magnesium ions, and riboflavin was examined individually (results not shown). The results indicated that the levels of these nutrients in Medium WAJC 402 were optimum for proliferation of prostate cells in serum-free primary culture. From the above comprehensive results, an optimized nutrient medium was formulated and designated as Medium WAJC 404 (Table 1). Medium WAJC 404 was used subsequently to study the proliferative response of prostate epithelial cells to hormones and other nonnutritive factors under serum-free conditions.

Proliferation of Prostate Epithelial Cells in Serum-free Primary Culture. Serum-free Medium WAJC 404 and cholera toxin, insulin, EGF, dexamethasone, prolactin, and pituitary factors (hereafter referred to as "growth factors") specifically supported pure primary cultures of epithelial cells (Figs. 1 to 3). The purity of primary cultures was estimated by colony morphology after fixation and staining (Fig. 1), cell morphology under the light microscope (Figs. 2 and 3), and immunochemical staining for the presence of keratin (Fig. 3). The presence of epidermal keratin is a specific marker for cells of epithelial origin from various tissues (16, 54). Addition of serum promoted the appearance of larger, but fewer epithelial cell colonies (Fig. 1*d*) concurrent with an increase in fibroblasts (Fig. 2). The ability to support epithelial cell proliferation in the absence of both fibroblasts and serum was specific to the nutrient medium (Fig. 1). Other sets of nutrients (*e.g.*, Media 199, F-12, Eagle's, MCDB 104, and MCDB 202; see Ref. 20 for formulas) yielded results similar to those of Medium F-12K. However, the growth factors were essential for support of epithelial cells independent of the composition of the nutrient medium and in addition to the presence of fibroblasts and serum (Fig. 1). Although Medium F-12K containing serum and growth factors supported a mixture of epithelial cells and fibroblasts (Fig. 1*e*), omission of the growth factors yielded pure cultures of fibroblasts (Fig. 1*f*).

Total cell proliferation occurred in serum-free Medium WAJC 404 containing growth factors at rates nearly equal to the rates in serum-containing medium (Chart 6). Addition of horse serum increased the total cell number in the primary cultures to a variable extent (Chart 6; Table 2). Since the increase in cell number due to serum was accompanied by an increase in fibroblasts in the cultures (Figs. 1 and 2), we concluded that serum-free Medium WAJC 404 and the growth factors supported proliferation of the epithelial cell population at rates equal to rates

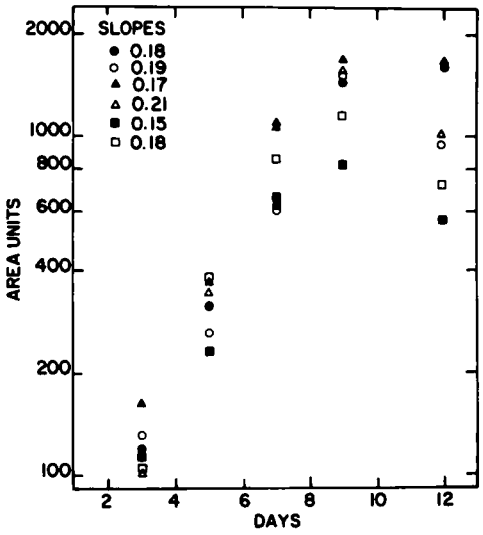


Chart 6. Time course of prostate cell proliferation in primary culture. The cell inoculum was 400,000 cells into Medium WAJC 404 plus growth factors and the amount of horse serum indicated below. Area measurements of cell growth were made on the day indicated. Data are the mean of triplicate dishes. The indicated slopes were determined by linear least-squares analysis of each set of data. ●, 5% whole horse serum; ○, 5% dialyzed horse serum; ▲, 1.25% whole horse serum; △, 1.25% dialyzed horse serum; ■, no serum; □, no serum with medium change daily. Whole horse serum contained about 70 mg protein/ml, and dialyzed serum contained 50 mg nondialyzable solids (dry weight)/ml.

Table 2

Effect of inoculation density on prostate epithelial cell proliferation

Cell population-doubling rate at each condition was calculated between Days 5 and 9 of culture from the slope of cell number in area units *versus* time.

Condition	Initial cell density (10^{-4} cells/sq cm/ml)	Doubling time (hr)
Serum free	1.25	35
	2.5	59
	5.0	53
Dialyzed horse serum (100 μg/ml)	1.25	27
	2.5	40
	5.0	39

in the presence of serum. On Day 6 of culture, essentially 100% of the epithelial cells incorporated [^{3}H]thymidine into nuclear DNA during a 24-hr exposure to the isotope (results not shown). The rate of epithelial cell proliferation in serum-free culture decreased with increasing initial cell density under standard conditions which involved a medium change every other day after Day 3 (Table 2). Daily changes of culture medium after Day 3 enhanced the growth rate by about 20% at an initial cell density of 1.3×10^4 cells/sq cm/ml (Chart 6). On Day 6, 60% of the cells exhibited the normal diploid rat karyotype of 42 chromosomes (Chart 7). During Days 9 to 12, the population growth rate sharply declined, and extensive cell death was apparent (Chart 6). The decline was independent of cell density and medium changes.

Hormone Requirements for Maintenance and Proliferation of Prostate Epithelial Cells. In the absence of serum, prostate epithelial cell proliferation was markedly stimulated by an extract of bovine pituitary (Fig. 1, *a* and *c*; Chart 8). The dose response exhibited an optimum at about 25 μg protein/ml. Crude horse serum protein did not modify the dose response to pituitary extract, although serum factors had a stimulatory effect on total

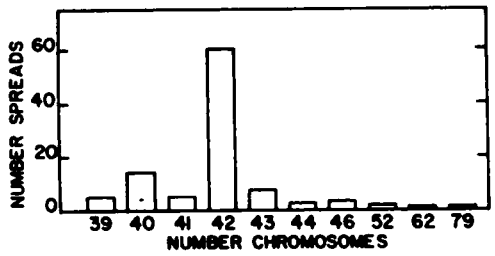
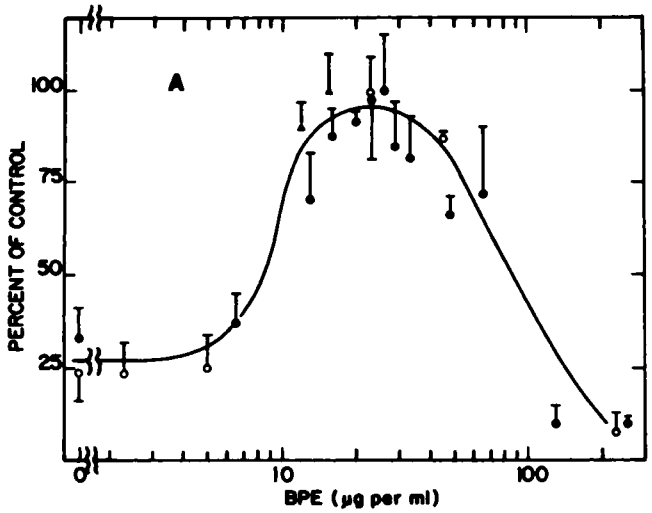


Chart 7. Karyotype of prostate epithelial cells in primary culture. Dissociated cell suspensions were prepared from ventral prostate for primary culture and inoculated into T-25 plastic culture flasks containing 5.0 ml of Medium 404 plus growth factors. Karyotypes were performed on cells in serum-containing and serum-free medium on Day 7 (45). The number of chromosomes from 100 metaphase cells ("spreads") was determined manually. The data indicated are from serum-containing cultures. Serum-free cultures yielded similar results.

cell number in the presence of pituitary factors (Chart 8*B*). A rapid rate of epithelial cell proliferation was dependent on cholera toxin at pM levels (Chart 9) and on insulin in the nM to μM range (Chart 10). Dexamethasone or hydrocortisone at nM levels yielded a 2- to 3-fold stimulation of cell number (Chart 11), while EGF at pM to nM levels stimulated the proliferation rate about 3-fold (Chart 12). The effect of prolactin was variable from experiment to experiment. Chart 13 shows a representative experiment. The following hormones and growth factors neither had effect on the rate of epithelial cell proliferation when added to medium containing the defined growth factors nor replaced or spared the requirement for the pituitary extract: epinephrine; ethanolamine; fatty acid-free bovine serum albumin; fibroblast growth factor; follicle-stimulating hormone; histamine; luteinizing hormone; luteinizing hormone-releasing hormone; multiplication-



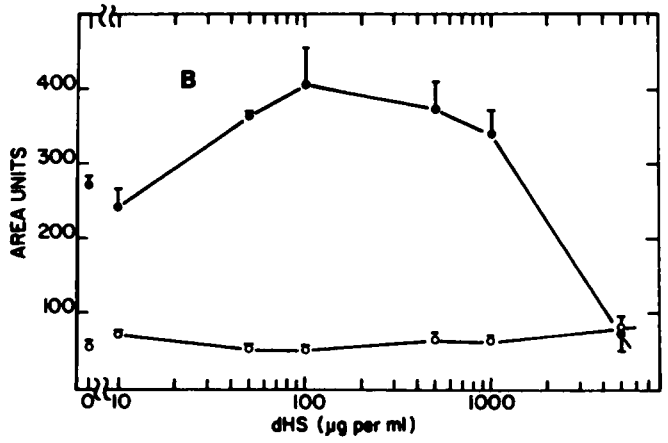


Chart 8. Effect of bovine pituitary extract on prostate cell proliferation. In *A*, the indicated amounts of bovine pituitary extract (*BPE*) were added to Medium WAJC 404 containing all growth factors except the pituitary extract. Area units were measured on Day 9 of culture and expressed as percentage of area units at 25 μg pituitary protein/ml. Different symbols indicate different experiments. *Points*, mean of triplicates; *bars*, S.D. *B*, effect of horse serum protein on requirement for bovine pituitary extract. The indicated amount of dialyzed horse serum protein (*dHS*) was added to Medium WAJC 404 containing all growth factors (●) or all growth factors except the pituitary extract (○). Area measurements were made on Day 7 of culture. *Points*, mean of triplicates; *bars*, S.D. Cell inoculum in both *A* and *B* was 200,000 cells.

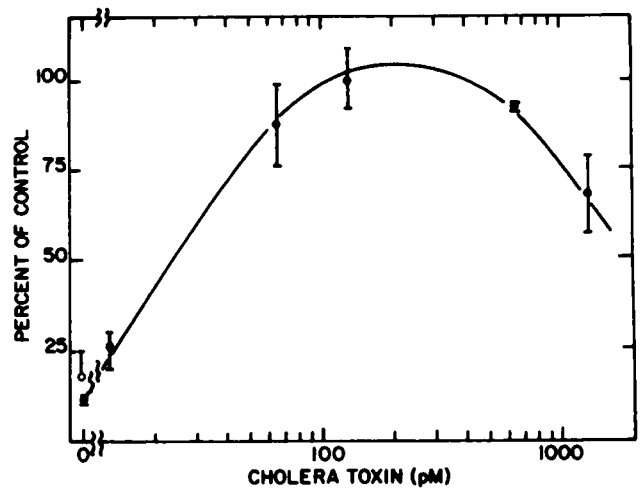


Chart 9. Effect of cholera toxin on prostate epithelial cell proliferation. The indicated amounts of cholera toxin were added to Medium WAJC 404 plus growth factors minus cholera toxin. Cells were fixed on Day 9, and area units were determined. Inoculum was 200,000 cells. *Points*, mean of triplicates and expressed as percentage of area units at 130 pM cholera toxin; *bars*, S.D.

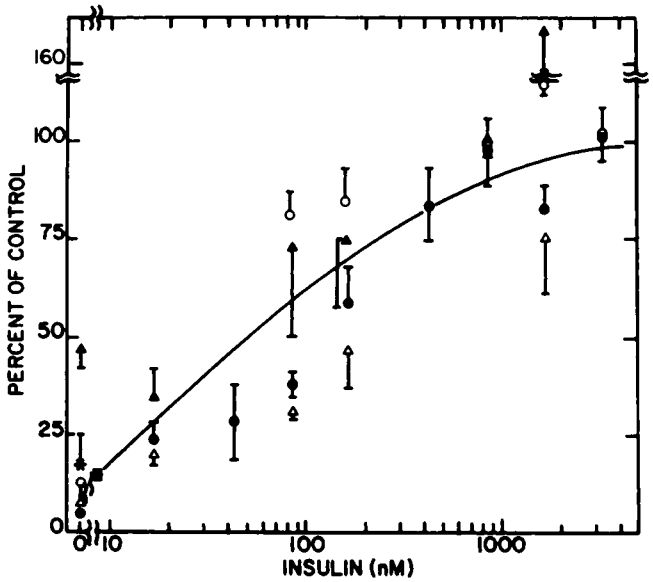


Chart 10. Dose response of prostate epithelial cell proliferation to insulin. Conditions were as described in Chart 9, except that insulin was omitted rather than cholera toxin. Different symbols indicate different experiments. Control was area units at 833 nM insulin.

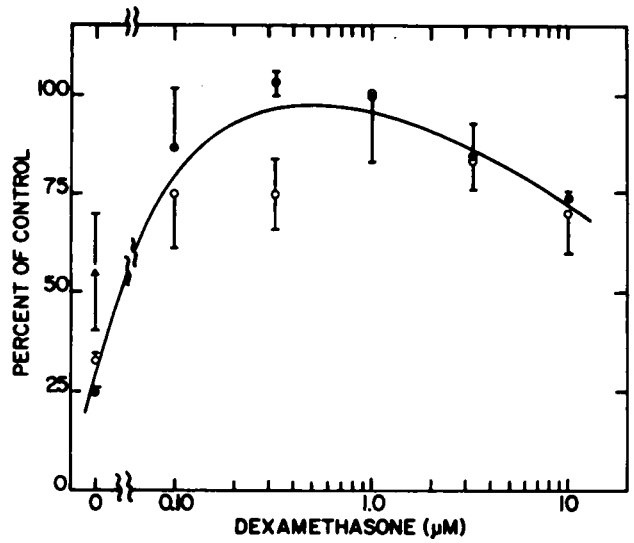


Chart 11. Effect of dexamethasone on prostate epithelial cell proliferation. Conditions were as in Chart 9, except that dexamethasone was omitted rather than cholera toxin. Control was area units at 1 μM dexamethasone.

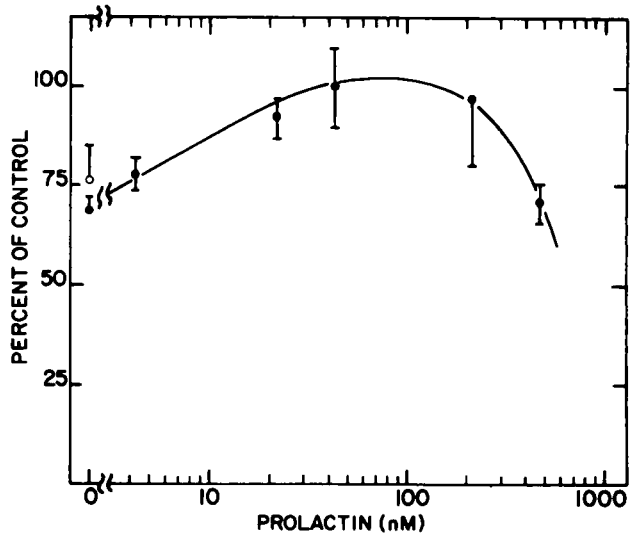


Chart 13. Effect of prolactin on prostate epithelial cell proliferation. Conditions were as in Chart 9, except prolactin was omitted instead of cholera toxin. Control was area units at 43 nM prolactin.

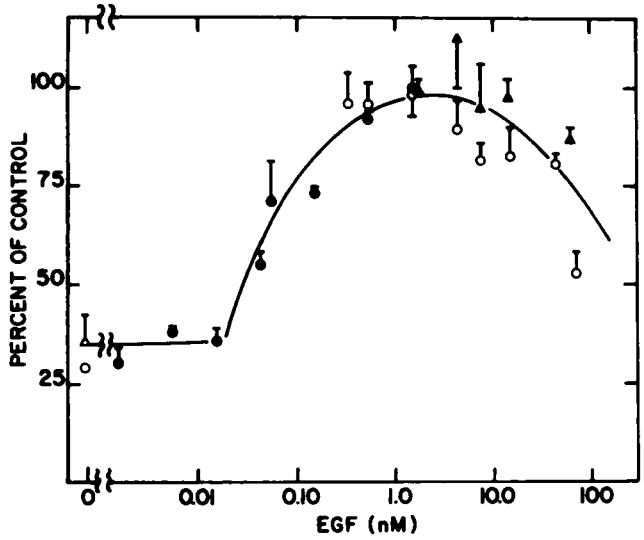


Chart 12. Effect of EGF on prostate epithelial cell proliferation. Conditions were as in Chart 9, except that EGF was omitted instead of cholera toxin. Control was area units at 1 nM EGF.

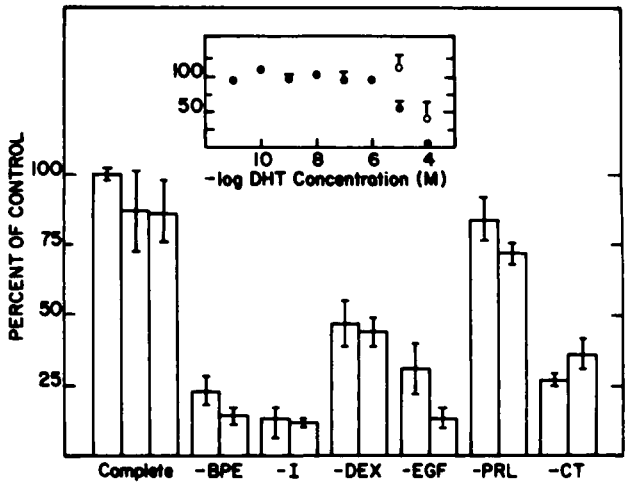


Chart 14. Effect of dihydrotestosterone (*DHT*) on prostate epithelial cell proliferation. Dihydrotestosterone was added at a concentration of 1 nM and 1 μM to Medium WAJC 404, bovine pituitary extract (*BPE*), insulin (*I*), dexamethasone (*DEX*), EGF, prolactin (*PRL*), and cholera toxin (*CT*). The single factor indicated was omitted. The *left column* of each pair represents cultures containing 1 nM, and the *right column* represents those containing 1 μM dihydrotestosterone. Concentrations of 10 nM and 10 μM dihydrotestosterone were also examined with no positive effect, but data are not shown. Area units were determined on Day 9 of culture. Control (100%) was the complete medium with no dihydrotestosterone added (*leftmost column*). Data are expressed as percentage of control. *Columns*, mean of triplicate dishes; *bars*, S.D. Inoculum was 400,000 cells. In the *inset*, dihydrotestosterone was added at the indicated concentration to Medium WAJC 404 plus growth factors. Area units were determined on Day 9 of culture. The *ordinate* is expressed as percentage of area units in the absence of added dihydrotestosterone. O, effect of the amount of ethanol introduced with the dihydrotestosterone (*DHT*).

stimulating activity; oxytocin; phosphoethanolamine; platelet-derived growth factor; progesterone; prostaglandins $F_{2\alpha}$, E_1, or E_2; retinoic acid; serotonin; somatotropic hormone; thrombin; thyrotropic hormone; thyrotropin-releasing factor; thyroxine; transferrin; tyramine; and vasopressin. Soybean phosphatidylcholine liposomes prepared with cholesterol and α-tocopherol (57) also had no stimulatory effect in the presence or absence of pituitary factors.

Effect of Androgen on Cell Proliferation. Up to 1 μM dihydrotestosterone (or testosterone) had no effect on prostate epithelial cell proliferation in serum-free medium (Chart 14). Concentrations of androgen above 1 μM were inhibitory. To test whether any one of the hormones and growth factors already present masked or substituted for an androgen requirement, each growth factor was deleted in the presence of androgen (Chart 14). Androgen did not replace the stimulatory effect of pituitary factors or any other single mitogenic hormone or factor presently in the primary culture system. Thus, either the epithelial cells were unresponsive to androgen, or endogenous androgen was present at sufficient concentration to mask the effect of exogenous androgen.

Measurement of Endogenous Androgen in Prostate Cells and Pituitary Protein. The cell inoculum and crude pituitary protein are the remaining sources of endogenous androgen in

Table 3

Measurement of testosterone-dihydrotestosterone in primary prostate cell suspensions and bovine pituitary extract

Experiment	Testosterone:dihydrotestosterone pg/10^6 cells	Testosterone:dihydrotestosterone pg/mg protein	Concentration in primary culture (pM)[a]
Primary cell suspension			
1	107 ± 29[b] (3)[c]		37
2	116 ± 44 (6)		40
3	56 ± 10 (6)		19
Mean	93 ± 32		32 ± 11
Bovine pituitary extract			
1		16.4 (1)	1.3
2		21.8 ± 4.7 (3)	1.7
Mean		19.1 ± 3.8	1.5 ± 0.3

[a] Concentration contributed by the cell inoculum and by the pituitary extract is based on 200,000 cells/dish and 23 μg protein/ml, respectively.
[b] Mean ± S.E. of the number of determinations.
[c] Numbers in parentheses, number of determinations.

Table 4

Effect of charcoal extraction on growth activity of bovine pituitary extract

Cell proliferation in the absence and presence of the indicated extract was measured in primary culture by videometry as described in "Materials and Methods." Pituitary extract was treated with dextran-coated charcoal where indicated. Standard [^{3}H]dihydrotestosterone (170 pmol) was also added to native extract, and the extract was then treated with charcoal.

Addition	Area units (Day 7)	[^{3}H]Dihydrotestosterone (cpm/ml)
No pituitary extract	77 ± 22[a]	
Native pituitary extract	383 ± 40	53,000
Charcoal-treated extract	598 ± 40	2,430

[a] Mean ± S.D. of triplicates.

the primary cultures. Therefore, androgen was measured in the initial prostate cell suspension used to establish primary cultures and in the pituitary extract by competitive-binding radioimmunoassay (Table 3). The dissociated prostate cells that were inoculated into primary culture contained about 93 pg androgen/10^6 cells, and the pituitary extract contained 19 pg/mg protein. From these results, it can be estimated that 2.0×10^5 dissociated prostate cells and 23 μg pituitary protein/ml contribute not more than 34 pM androgen to the primary cultures on Day 1 of culture. Extraction of pituitary extract with dextran-coated charcoal, which removed 95% of a [^{3}H]dihydrotestosterone standard, actually enhanced the growth-stimulatory activity of the extract by 56% (Table 4). This is the opposite result to that expected if endogenous androgen or other steroid were masking the requirement for androgen for epithelial cell proliferation.

DISCUSSION

Direct Hormonal Control of Prostate Epithelial Cell Proliferation. Hypophysectomy and orchiectomy followed by gonadotropin or androgen treatment clearly indicate that prostate epithelial cell proliferation is testes or androgen dependent (18, 23, 58, 60). Growth hormone, prolactin, and ACTH have been suggested as additional pituitary factors involved in prostate cell proliferation (24, 34, 58), although others report no effect of the hormones or adrenalectomy on prostate wet weight or expression of specific prostate functions (9, 18, 45, 58). Conventional organ ablation followed by hormone therapy in animals cannot distinguish whether hormones act directly on prostate epithelial cells or their action is mediated by other factors that act directly on the prostate cells. Recent advances in biochemical characterization of the complex hormonal and nutritional role played by crude serum in cell cultures (3, 20) now allow the maintenance of different cell types *in vitro* away from the uncontrolled influences of other cells and the circulation in whole animals. Therefore, the nutritional and hormonal elements that act directly on prostate epithelial cells can be identified, and their mechanism of action can be studied under defined and controlled conditions. In the present study, we used a novel videometric method to rapidly and accurately estimate cell number from replicate cell cultures. The method does not require harvesting and destruction of the cells or execution of other manipulations beyond fixation and staining after the incubation was terminated. The method had the following advantages for the objective of the present study. (*a*) It was practical for measurement of cell numbers from large numbers of replicate assays. The method can be easily automated and interfaced with a computer. (*b*) The method was nondestructive and allowed subsequent examination of the assay results. (*c*) The method was more accurate than harvesting cells and counting by Coulter Counter or hemocytometer. Variation in yields during the cell collection procedure or heterogeneity in the cell suspension due to cell aggregation or clumping did not affect the results. (*d*) The method allowed determination of cell numbers that were too low to be accurately counted by other methods. Direct count after harvesting becomes unreliable at about 10,000 cells/dish (Chart 1). The measurement of cell proliferation at low cell density allowed the maintenance of near steady-state conditions among assays without continuous changes of culture medium. It also allowed measurement of multiple rounds of cell division in single test cultures on a relatively small surface area and within a small volume of culture medium.

We then used selective nutritive conditions to isolate normal proliferating prostate epithelial cells from prostate fibroblasts (35). Further quantitative optimization of nutrients in the presence of a specific set of hormones and growth factors resulted in the elimination of the requirement for crude serum, the major source of endogenous nutrients and hormones in the *in vitro* system. For a period of 9 to 12 days after removal of the prostate gland from the animal, a normal, pure population of prostate epithelial cells expanded exponentially at doubling times of 35 to 50 hr on a simple negatively charged polystyrene substratum. Proliferation proceeded independently of serum, preexisting extracellular matrix, and other cell types. In addition, the proliferation rate occurred at relatively low cell densities and was relatively independent of frequent changes of the culture medium. Under the assay conditions, the area covered by the cell monolayer rarely exceeded 15% of the available substratum. Thus, endogenous, cell-derived factors were unlikely contributors to proliferation rate of the cell population. If it is assumed that the observed population growth rates represented a uniform rate of cell division of progeny from single cells in the initial cell suspension prepared from the gland, then it can be estimated that about 4% of the prostate cells gave rise to the proliferative cell population. Immunochemical analysis revealed that essentially 100% of the population exhibited keratin antigen, a specific property of epithelial cells. Cells containing prostatic binding protein, a specific androgen-dependent marker of differentiated secretory cell function (36, 39), were rare and only detectable in very early stages of the primary culture. Currenty, we cannot distinguish whether the proliferating cell population arises from undifferentiated basal

("reserve") or dedifferentiated secretory epithelial cells in the prostate gland. Simultaneous immunochemical staining for prostatic binding protein and autoradiography of [^{3}H]thymidine incorporation revealed that some differentiated secretory cells can undergo DNA synthesis in primary culture. The fate of former secretory cells could not be determined beyond the initial round of DNA synthesis to determine whether they gave rise to daughter cells and further progeny.

Analysis of the growth-stimulatory effect of over 30 nonnutritive hormones and growth factors on prostate epithelial cells in primary culture showed that insulin, EGF, glucocorticoid, cholera toxin, prolactin, and one or more unidentified, nondialyzable factors from bovine pituitary were direct mitogens for the isolated prostate epithelial cells.

Insulin, Glucocorticoid, and EGF. Like several cultured mesenchymal and epithelial cell types, prostate epithelial cell proliferation required near μM amounts of insulin, nM amounts of glucocorticoid, and pM to nM amounts of EGF (3). Similar results have been reported for explants of prostate acini (8) and prostate cell lines (27, 31). The requirement for insulin at near μM concentrations in most cell types is thought to reflect the low affinity interaction of the insulin molecule with high affinity receptors for insulin-like growth factors (somatomedins) separate from or current with interactions of insulin with its own receptor (25). Somatomedins mediate many of the anabolic effects (including cell proliferation) of growth hormone *in vivo* (for review, see Ref. 12). We have confirmed separately that purified multiplication-stimulating activity, a somatomedin from cultured rat liver cells, can spare, but does not replace, the insulin requirement reported here for prostate epithelial cells (results not shown). The insulin or somatomedin requirement for prostate epithelial cells *in vitro* may explain reports of an enhancing effect of growth hormone on prostate cell proliferation *in vivo* (24, 34).

The mitogenic activity of glucocorticoids on isolated prostate epithelial cells is consistent with an enhancement of ACTH via the adrenals on prostate after androgen treatment of hypophysectomized, orchiectomized animals (58). In several cell types that require the insulin:glucocorticoid:EGF triad *in vitro*, glucocorticoid regulates the EGF receptor concentration and, in fibroblasts, appears to promote release of autocrine growth factors into the medium (for review, see Ref. 13). EGF is concentrated in the male submaxillary gland in mice and the prostate in guinea pigs (for review, see Ref. 7). Although submaxillary levels of EGF are androgen dependent, the plasma level of normal males, orchiectomized males, and normal females is equal. Thus, the physiological role of EGF in control of proliferation of its many target cell types, which appears to include prostate epithelial cells, is obscure.

Prolactin. Our results are suggestive that prolactin is directly mitogenic for isolated prostate epithelial cells. Effects varied from a near-absolute requirement to the small stimulation shown in Chart 13. Commercial ovine and rat prolactin from the NIH hormone distribution program gave similar results. Thus, evidence that prolactin is a direct mitogen for prostate epithelial cells is tentative. The variable prolactin response may be due in part to the necessary presence of crude pituitary extract which is an uncontrolled source of endogenous prolactin which may mask a complete response to external purified prolactin. Purification and replacement of the unknown pituitary-derived growth factor(s) with purified factors will resolve this issue.

Cholera Toxin. Similar to skin (19) and mammary epithelial cells (56), cholera toxin is a potent mitogen for isolated prostate epithelial cells. Cholera toxin exerts its effects in most cells by catalyzing ADP ribosylation of the GTP-binding regulatory component of the adenylate cyclase [ATP pyrophosphate lyase (cyclizing); EC 4.6.1.1.] which causes accumulation of intracellular cyclic AMP (for review, see Ref. 40). Therefore, our results suggest a positive influence of elevated cyclic AMP on prostate epithelial cell proliferation. Since both structural and functional parallels are existent between cholera toxin and several hormones (14), the mitogenic effect of cholera toxin may be mimicking the requirement for more physiological but undiscovered prostate mitogens.

Pituitary Factors. Pituitary extract is the remaining undefined source of mitogenic factors that act directly on normal prostate epithelial cells in primary culture. Attempts to replace or spare the requirement for the pituitary factor(s) with characterized pituitary hormones were unsuccessful. In addition, over 30 other potential growth factors did not modify the requirement for pituitary factor(s). Although we cannot conclusively eliminate the tested factors as mitogens for prostate epithelial cells, the results suggest that none of the defined factors or agents with similar function is the single most active agent in the pituitary extract. Since crude pituitary extract is maximally active at about 20 μg protein/ml and since high levels of serum protein cannot replace or spare the requirement for pituitary extract (Chart 8), the principle mitogenic activity in pituitary extract is unlikely due entirely to blood-derived factors in the pituitary tissue. Our results suggest a direct role of one or more uncharacterized mitogens for prostate epithelial cells that are concentrated in pituitary.

Androgen Not Directly Mitogenic for Isolated Prostate Epithelial Cells. Although prostate epithelial cell proliferation *in vivo* is clearly dependent on androgen via the pituitary-testes axis, we failed to demonstrate a direct effect of androgen on proliferation of isolated epithelial cells in serum-free primary culture. Direct analysis revealed that, barring extremely localized endogenous androgen concentrations and androgen stability, androgen was too low in the primary cultures to have an impact on the observed rates of cell proliferation. The results indicated an endogenous level of 34 pM on Day 1 of culture that was primarily contributed by the cell inoculum. If the endogenous androgen on Day 1 were 100% sequestered in the actively proliferating cells, it would be diluted continuously by nearly 70-fold by Day 9 of culture, given a population growth rate of 0.8 cell generation/day (doubling time, 1.3/day; Table 2). During this period, the cell population showed no decline in proliferation rate (Chart 6). Extensive medium changes caused a small stimulation of population growth rate, an opposite effect to that expected if build-up of cell-produced androgen or other factors in the medium were critical to cell proliferation. The inactivity of androgen upon deletion of any other single defined growth factor or pituitary extract in the primary cultures also argued against the possibility that the other hormones masked or substituted for a requirement for androgen.

In summary, the results demonstrate a multihormonal control of prostate epithelial cell maintenance and proliferation without direct participation of androgen. Responsiveness of isolated cells *in vitro* to specific hormones and growth factors is dependent on the nutritive environment (20, 37). Responsiveness to hormones is also dependent on the composition and spatial configuration

of nondiffusible elements of the cellular environment (*e.g.*, the matrix, substratum, and other cells) (17). Therefore, the results do not rule out a mitogenic role of androgen directly on prostate epithelial cells under alternate conditions *in vitro* or *in vivo*. However, the present results support the possibility that the mitogenic activity of androgen *in vivo* may be indirect and mediated by one or more factors with similar structure or function to insulin, glucocorticoid, EGF, cholera toxin, prolactin, and yet unidentified, nondialyzable factor(s) that are concentrated in the pituitary. A similar indirect mechanism has been proposed for the role of estrogen in proliferation of estrogen-dependent tissue (29, 51).

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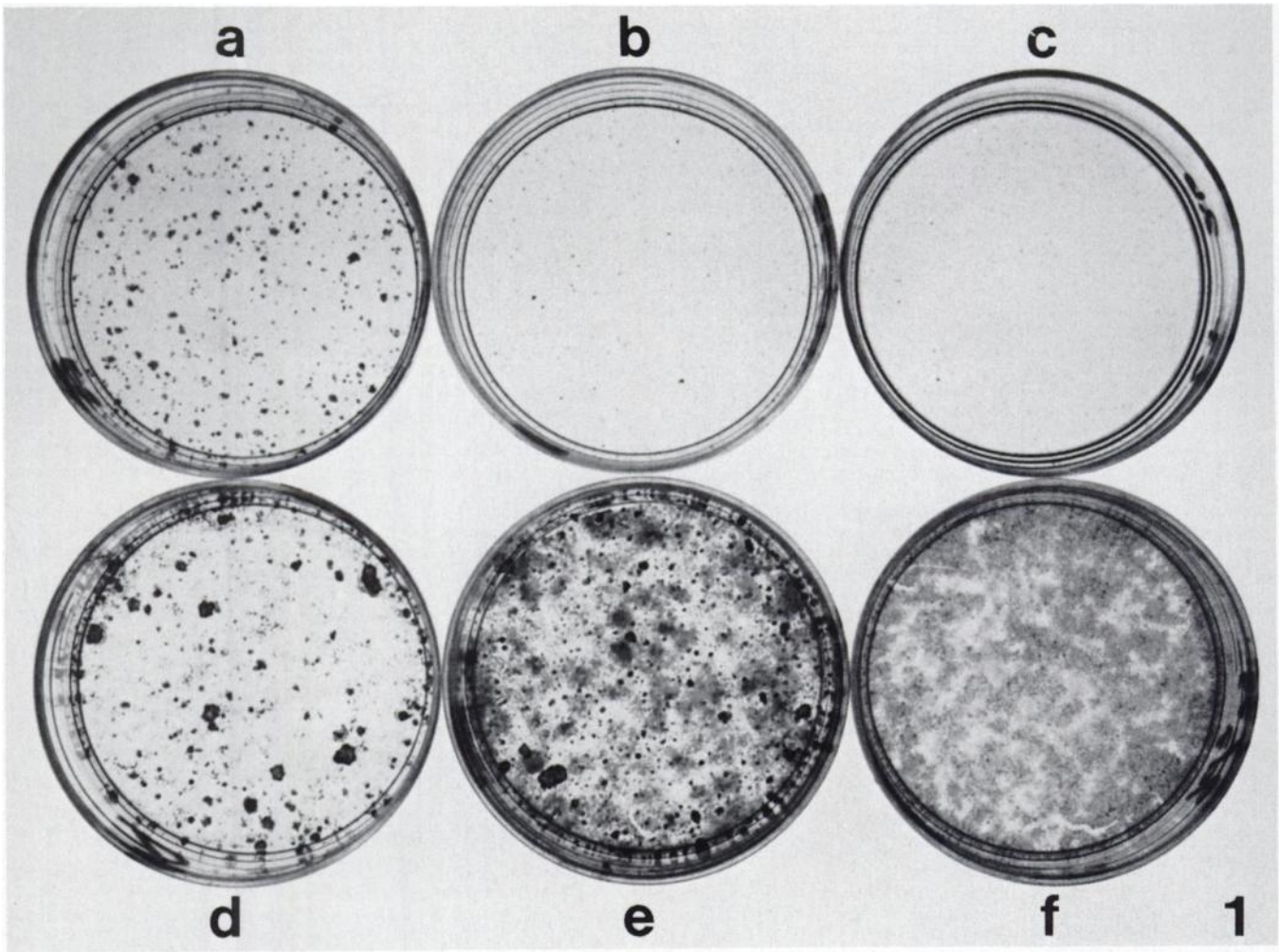

Fig. 1. Normal rat prostate cells in primary culture. Rat ventral prostate cells were prepared for primary culture as described in "Materials and Methods." Cells (200,000) were inoculated into 35-mm plastic Petri dishes containing the indicated medium. Native horse serum was present at 5% (v/v) where indicated. After 7 days of incubations, cells were fixed and stained ("Materials and Methods"). Colonies of epithelial cells appear as densely packed areas relative to the diffuse areas of fibroblasts. *Dish a* is a pure culture of epithelial cells, and *Dish f* is a pure culture of fibroblasts. *Dish a* contained 572 area units of stained cells of a total culture surface area of 7291 units that was scanned by the cell counter aperture. *a*, Medium WAJC 404 plus growth factors; *b*, Medium F-12K plus growth factors; *c*, Medium WAJC 404 plus all growth factors except bovine pituitary extract; *d*, Medium 404 plus growth factors plus horse serum; *e*, Medium F-12K plus growth factors plus horse serum; *f*, Medium F-12K plus horse serum.

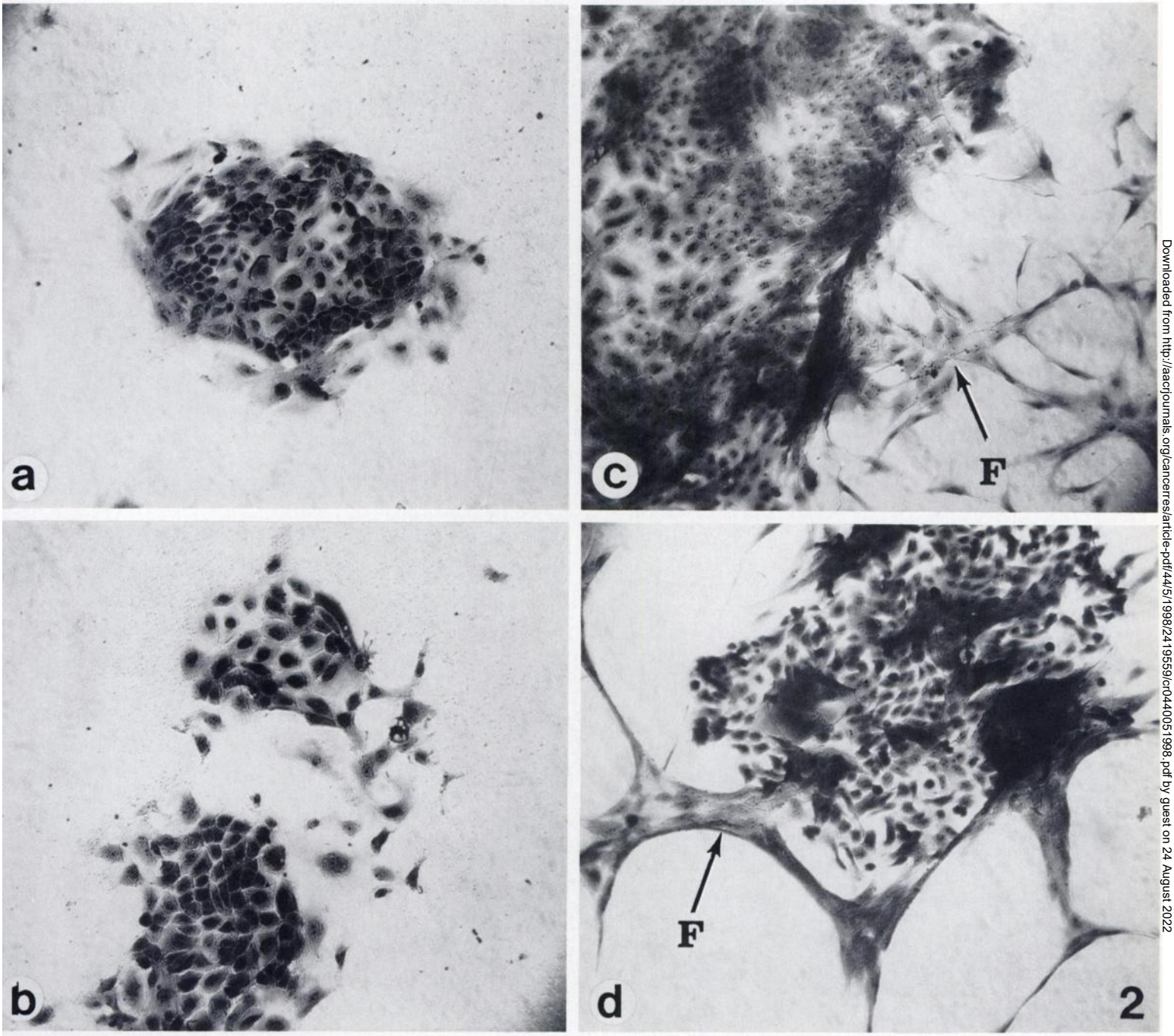

Fig. 2. Rat prostate cells in serum-free and serum-containing primary culture. *Fields a* and *b* are from *Dish a* and *Fields c* and *d* are from *Dish d* of Fig. 1. Elongated fibroblasts (*F*) can be easily distinguished from the tightly clustered epithelial cells. × 100.

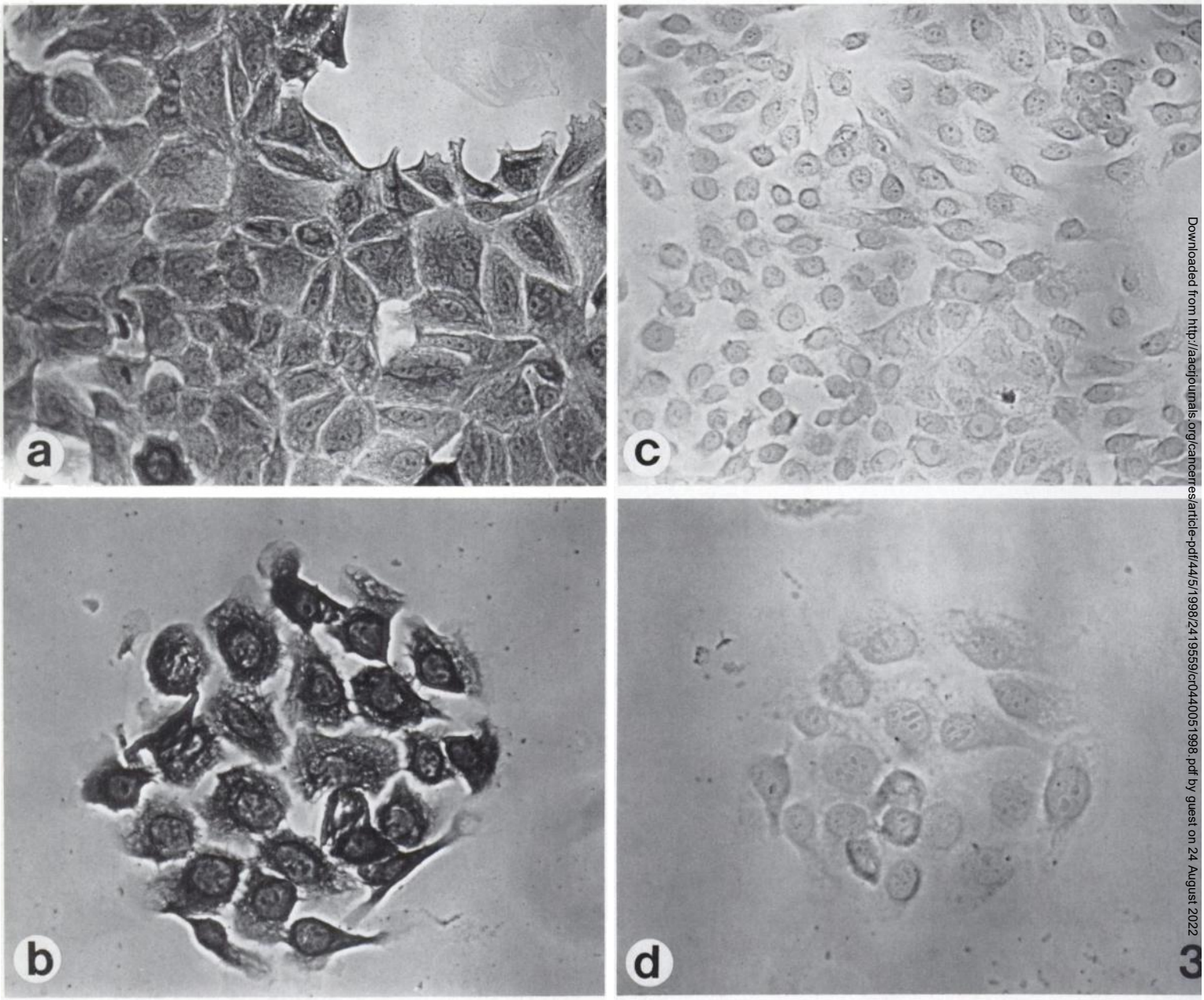

Fig. 3. Immunochemical staining of keratin associated with prostate cells in primary culture. Prostate cells were stained on Day 6 of primary culture with rabbit antiserum against human keratin ("Materials and Methods"). Both large (*a*) and small (*b*) colonies of epithelial cells stain intensely, relative to cells treated with normal rabbit serum (*c* and *d*). *a* and *c*, × 248; *b* and *d*, × 387.