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Promotion of Growth and Differentiation of Rat Ductular Oval Cells in Primary Culture[1]

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ABSTRACT

Oval cells emerging in rat liver at the early period of 3-methyl-4-dimethylaminoazobenzene treatment constitute a mixed epithelial cell compartment with respect to α-fetoprotein (AFP) and cytokeratin differential expression, and include a subpopulation which exhibits a phenotype intermediate between ductular cells and hepatocytes (Germain *et al.*, Cancer Res., *45:* 673–681, 1985). In the present study we have examined the developmental potential of ductular oval cells in primary culture and after *in vivo* transfer. The use of monoclonal and polyclonal antibodies directed against cytokeratins of M_r 39,000 (CK39), 52,000 (CK52), and 55,000 (CK55) and vimentin, and also monoclonal antibodies against exposed surface components of oval cells (BDS_7) and normal hepatocytes (HES_6) allowed us to establish the ductular phenotype of the oval cells. A highly enriched preparation of oval cells was obtained by perfusion/digestion of the liver with collagenase, treatment of the cell suspension with trypsin and DNase, selective removal of hepatocytes by panning using the anti-HES_6 antibody, and cell separation by isopyknic centrifugation in a Percoll gradient. The procedure yielded about 8 × 10^7 cells, of which 95% expressed CK39, CK52, and BDS_7, 84% γ-glutamyl transpeptidase, and 5% albumin and AFP. The primary response of cultured oval cells to various combinations of growth and differentiation promoting factors was evaluated with respect to their capacity to initiate DNA synthesis as measured by [^{3}H]thymidine labeling from day 1 to 3, and/or to produce albumin and AFP and express tyrosine aminotransferase. Culture in the presence of either serum or clot blood extract resulted in a low proliferative activity with less than 5% of the nuclei being labeled. Over a 5-day period, fusion of a large portion of the oval cells led to multinucleated cells. When the cells were cultured in the presence of an elaborate combination of supplements [minimum essential medium containing 1 mM pyruvate, 0.2 mM aspartate, 0.2 mM serine, 1 mM tyrosine, 1 mM proline, 1 mM phenylalanine and supplemented with 20% clot blood extract, 10 ng/ml oxidized bile acids, 17 μM bilirubin, 10 ng/ml cholera toxin, 1 μM dexamethasone, 2.5 μg/ml insulin, 50 mM β-mercaptoethanol, and 5 μg/ml transferrin (medium MX)], the labeling index increased to around 30% and the level of cell fusion greatly decreased. The addition of dimethyl sulfoxide further enhanced the initiation of DNA synthesis, while sodium butyrate acted as an inhibitor. Cell morphology varied depending on the combinations of factors used. Most of the oval cells still expressed the three cytokeratins, BDS_7, γ-glutamyl transpeptidase, and some vimentin after 5 days in culture. Culture in medium MX in the absence of sodium butyrate resulted in the emergence of a small subpopulation of BDS_7 negative/γ-glutamyl transpeptidase positive fusiform cells, which by day 5 constituted a distinct epithelial cell population that expressed CK39 and vimentin and which could be subcultured. The addition of dimethyl sulfoxide or sodium butyrate at day 1 in the presence of dexamethasone differentially modified the phenotype of the ductular oval cells cultured in growth factor-supplemented medium. Of major interest was the finding that the presence of sodium butyrate and dexamethasone led to a massive production of albumin over a 3-day period and a progressive induction of tyrosine aminotransferase activity. Fourteen days after the injection of freshly isolated oval cells into the fat pads of syngeneic rats, the cells were arranged as clusters similar to bile ductular structures. These findings indicate that oval cells isolated from 3-methyl-4-dimethylaminoazobenzene-treated rat liver which exhibit the phenotypic characteristics of bile ductular epithelial cells after *in vivo* transfer, produce albumin, cease AFP production, and express tyrosine aminotransferase activity in primary mass culture, properties of facultative hepatocytic precursors.

INTRODUCTION

The development of hepatocellular carcinomas in rats is a progressive process characterized by the successive appearance of distinct populations of epithelial cells which exhibit altered phenotypes (1–5). Among the first cellular changes induced by most chemical carcinogens is the emergence of a cell population morphologically defined as oval cells (1). From *in situ* observations that oval cells appear initially in the portal areas of the liver, form elaborate duct-like structures, and express bile ductular markers, it is generally believed that this nonparenchymal epithelial cell population derives from cells of the terminal biliary ductules (6–8). However, several analyses performed on rat liver tissue slices and/or enriched oval cell preparations using GGT[4], AFP, albumin, cytokeratins, and also various sets of monoclonal antibodies against undefined cellular constituents have suggested that oval cells constitute a heterogeneous compartment containing subpopulations of epithelial cells of distinct phenotype with different developmental potential (4, 9–14). In the azo-dye rat model, the demonstration of cells phenotypically intermediate between oval cells and hepatocytes has led to the hypothesis that oval cells may rapidly give rise to hepatocytes (7, 15, 16). Similarly, several *in vitro* studies have indicated that it is possible to establish phenotypically distinct nonparenchymal epithelial cell lines from normal and CDE-fed rat liver which variably express few hepatocytic traits (17–19). Although such cell lines are apparently clonogenic, our recent cell typing analysis has indicated that, while some lines exhibit a bile ductular phenotype, others might derive from the Glisson capsula (20). In the light of these proposals, it becomes of particular interest to more precisely assess the phenotype variability of oval cells *in situ* and to obtain them as purified preparations in order to examine their developmental potential in primary culture and after *in vivo* transfer.

In the present study, this question has been addressed using the 3′-Me-DAB treated rat liver model and a three-step experimental approach: (*a*) in addition to metabolic enzymes and plasma proteins as liver cell type specific markers (21), we used polypeptides of the intermediate filaments (22–24) and compo-

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[4] The abbreviations used are: GGT, γ-glutamyltranspeptidase; AFP, α-fetoprotein; AP, alkaline phosphatase; BDS_7, bile duct surface component; CDE diet, choline deficient/DL-ethionine-supplemented diet; CK39, CK49, CK52, CK55, cytokeratins of M_r 39,000, 49,000, 52,000, and 55,000, respectively; CLEX, clot blood extract; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HES_6, hepatocyte surface component; MEM, minimum essential medium; medium MX, MEM containing 1 mM pyruvate-0.2 mM aspartate-0.2 mM serine-1 mM tyrosine-1 mM proline-1 mM phenylalanine, and supplemented with 20% CLEX, 10 ng/ml oxidized bile acids, 17 μM bilirubin, 10 ng/ml cholera toxin, 1 μM dexamethasone, 2.5 μg/ml insulin, 50 mM β-mercaptoethanol, and 5 μg/ml transferrin; medium MS, medium MX without cholera toxin, biliary acid, and bilirubin; 3′-Me-DAB, 3-methyl-4-dimethylaminoazobenzene; PO, peroxidase; TAT, tyrosine aminotransferase, Ig, immunoglobulin; dThd, thymidine.

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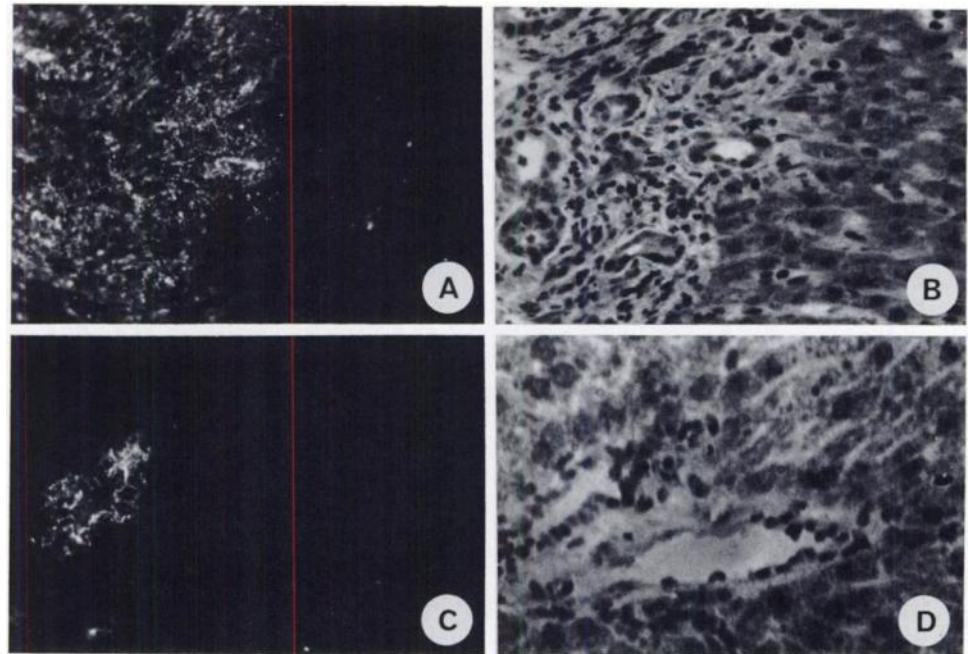

Fig. 1. Immunofluorescence microscopy of frozen sections of 3'-Me-DAB-treated (*A*) and normal (*C*) rat livers using anti-BDS$_7$ antibody. Hematoxylin and eosin stainings of adjacent tissue sections are shown in *B* (treated) and *D* (control). × 250.

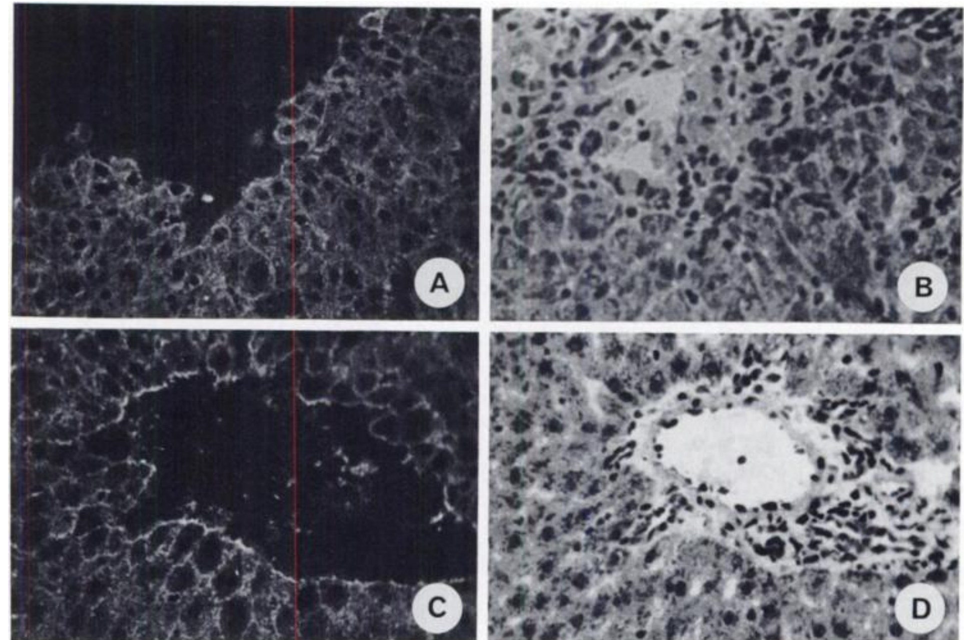

Fig. 2. Immunofluorescence microscopy of frozen sections of 3'-Me-DAB-treated (*A*) and normal (*C*) rat livers using anti-HES$_6$ antibody. Hematoxylin and eosin stainings of adjacent tissue sections are shown in *B* (treated) and *D* (control) × 250.

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nents expressed on the cell surface (25, 26) as complementary and highly reliable cell lineage-dependent markers of tissue differentiation to obtain a more precise definition of ductular *versus* hepatocytic cell phenotypes. The cell typing assay relied on the use of monoclonal and polyvalent antibodies recently produced in our laboratory against cytokeratins and vimentin and monoclonal antibodies against exposed surface components of oval cells and hepatocytes (20); (*b*) we developed a dispersion and purification procedure for oval cells which incorporates panning with a monoclonal antibody and isopyknic centrifugation in a Percoll gradient to remove hepatocytes and mesenchymal cells; and (*c*) we examined the growth and differentiation potential of highly enriched ductular oval cells in primary culture following the addition of various combinations of growth and differentiation promoting factors. We also examined the developmental potential of ductular oval cells *in vivo* after transfer into the fat pads of syngeneic normal rats. We find that highly enriched preparations of 3'-Me-DAB-derived oval cells exhibit phenotypic features of a relatively homogeneous nonparenchymal epithelial cell population and form bile ductular structures after *in vivo* transfer. In response to particular medium supplements, these enriched oval cells can be induced to initiate DNA synthesis in primary culture, produce albumin, cease to produce AFP, and express TAT activity in the absence of cell growth activation.

MATERIALS AND METHODS

Materials

Bilirubin, bile acids (oxidized), cholera toxin, palmitic acid, steraric acid, DMSO, and sodium butyrate were purchased from Sigma Chemical Co., St. Louis, MO. Percoll and Sephacryl S-300 were obtained from Pharmacia Fine Chemicals, Tissue tek O.C.T. compound and Lab-tek 100- × 25-mm Petri dishes were bought from Canlab, Ste-Foy, Québec, Canada. Collagenease and DNase I were obtained from Boehringer

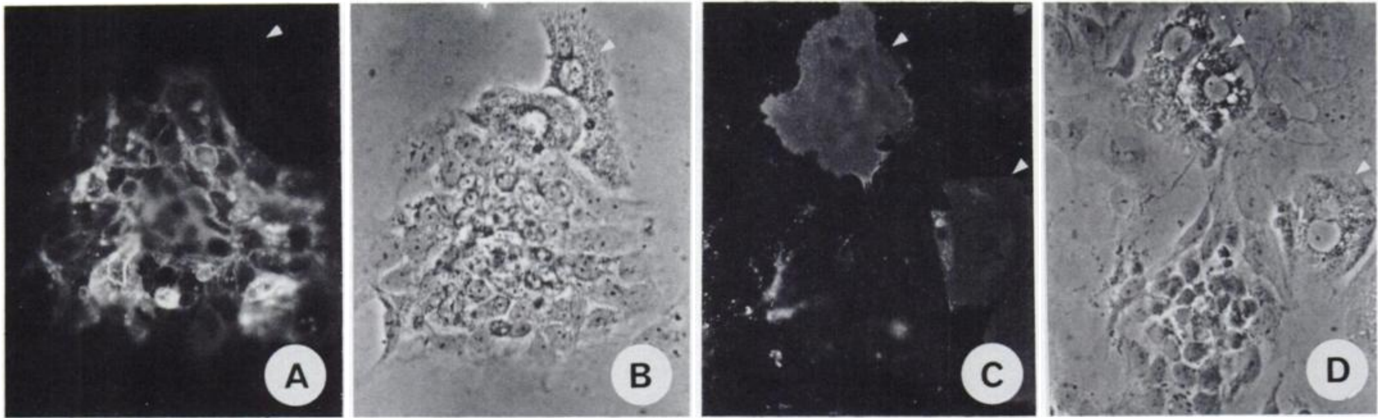

Fig. 3. Immunofluorescence microscopy of 24-h cultured partially purified oval cells using anti-BDS_7 (*A*) and anti-HES_6 (*C*) antibodies. *B* and *D* are phase contrast micrographs of *A* and *C*, respectively. Note that anti-BDS_7 antibody stains only bile ductular cells while anti-HES_6 antibody stains only hepatocytes. Hepatocytes are denoted by *arrowheads*. × 400.

Table 1 *Histochemical and immunohistochemical characteristics of oval cell-enriched populations*

Cells isolated from the liver of 3'-Me-DAB-treated adult rats were fractionated by differential centrifugation at 25 × *g* for 5 min at 4°C to obtain a mixed cell type supernatant fraction containing highly dispersed oval cells and a pellet containing mainly oval cell aggregates and hepatocytes. Oval cells from the supernatant were purified by a selective attachment of the sinusoidal cells onto tissue culture dishes, and hepatocytes were removed by panning using the anti-HES_6 and isopyknic centrifugation in a Percoll gradient. Oval cells from the pellet were purified by treatment of the cells in suspension with trypsin and DNase I, cell panning, and isopyknic centrifugation. Cells from each final fraction were seeded on slides using a cytocentrifuge and assayed histochemically for GGT, AP, and PO, and immunocytologically for HES_6 and BDS_7.

	% positive cells														
Cell fraction	GGT			ALKP			PO			HES_6			BDS_7		
Supernatant	88[a]	73	82	97	—[b]	83	0	4	6	0	0	3	99	88	79
Pellet	85	81	95	94	—	95	0	2	1	0.5	0.5	1	99	89	92

[a] Each vertical column represents a separate experiment; 500 cells were counted on each slide.
[b] Not determined.

Mannehim, Montreal, Québec, Canada. Hoechst was purchased from American Hoechst Corporation, NJ. Minimum essential medium was obtained from Flow Laboratories, Inc., Mississauga, Ontario, Canada. CLEX was obtained from Dextran Products, Ltd., Scarborough, Ontario, Canada. β-Mercaptoethanol was purchased from Bio-Rad, Mississauga, Ontario, Canada. Transferrin was obtained from Collaborative Research, In., Lexington, MA. Fluorescein isothiocyanate-conjugated goat anti-rabbit, rabbit anti-mouse Ig or IgM antibodies were purchased from Rego Industries, Québec, Canada. Fluorescein isothiocyanate-conjugated goat anti-guinea pig was obtained from Kirkegaard and Perry Laboratories, Gaithersburg, MD. Rhodamine-conjugated goat anti-rabbit and rabbit anti-mouse antibodies were a gift from Dr. Jean-Paul Valet, CHUL, Ste-Foy, Québec, Canada.

Animals

Young adult male Fischer 344 rats, 100–150 g (Charles River Breeding Laboratories, Inc., Wilmington, MA) were fed a solid diet containing 0.06% of 3'-Meb-DAB *ad libitum* until killing at 4 weeks (10).

Production of Monoclonal Antibodies to Hepatocyte and Oval Cell Exposed Surface Components

Hepatocytes (anti-HES_6). BALB/c mice were immunized by one i.p. injection in complete Freund's adjuvant of 10^7 hepatocytes isolated from the liver of normal adult Fischer 344 rats by our modifications (27, 28) of the two-step collagenase perfusion method (29). After 3, 6, and 9 weeks, 10^6 hepatocytes in saline were injected i.v. Hybridomas were generated as previously described (20).

Oval Cells (anti-BDS_7). Mice were immunized using an enriched preparation of oval cells obtained by liver perfusion and digestion (see below) of livers from 3'-Me-DAB-treated rats. The cells were first incubated for 30 min at 23°C with mouse polyclonal anti-rat hepatocyte antibody (20). Both anti-BDS_7 and anti-HES_6 antibodies were IgM.

Antibodies to Cytokeratins and Vimentin

The production of monoclonal anti-CK55 and polyclonal anti-CK52 and vimentin antibodies has been described in detail (10, 20). The anti-CK39 was generously provided by Dr. W. W. Schmidt, Nashville, TN (30, 31).

Panning Assay

The IgM fraction of anti-HES_6 ascitic fluid was extracted by precipitation with 50% ammonium sulfate. The precipitate was resuspended in 150 mM NaCl containing 10 mM sodium phosphate (pH 7.0) and dialyzed overnight against the same buffer. The sample was separated by filtration on a 2.5- × 90-cm Sephacryl S-300 superfine column equilibrated with the dialysis buffer. IgM eluted as a single peak in the void volume. The preparation of antibody-coated dishes was based on the procedure of Tsoi *et al.* (32).

Tissue Section Preparation

The analysis was done according to procedures previously described in detail (10, 20).

Liver Cell Dispersion

Liver cells were isolated from rats maintained for 4 weeks on a 3'-Me-DAB diet by the two-step collagenase perfusion method (10, 20) except that recirculation with collagenase was for 15 min. After perfusion, the liver was minced in 30 ml of the collagenase solution and incubated for 10 min at 37°C in a shaking water bath. The tissue digest was then subjected to gentle titration (8–10 times) with a 10-ml pipet and filtered through a Nitex screen with pore diameter of 100 μm. Tissue fragments retained on the filter were incubated for 10 more min in 10 ml of the enzyme solution and filtered. Both filtered cell suspensions were combined and diluted to a final volume of 350 ml in cold HEPES buffer.

Oval Cell Purification

The cell suspension was centrifuged at 25 × *g* for 5 min at 4°C yielding a cell pellet containing parenchymal cells and aggregated ductular/oval cells. The supernatant included sinusoidal cells and nonaggregated oval cells. Two different methods were used to purify oval cells present in the supernatant and the pellet.

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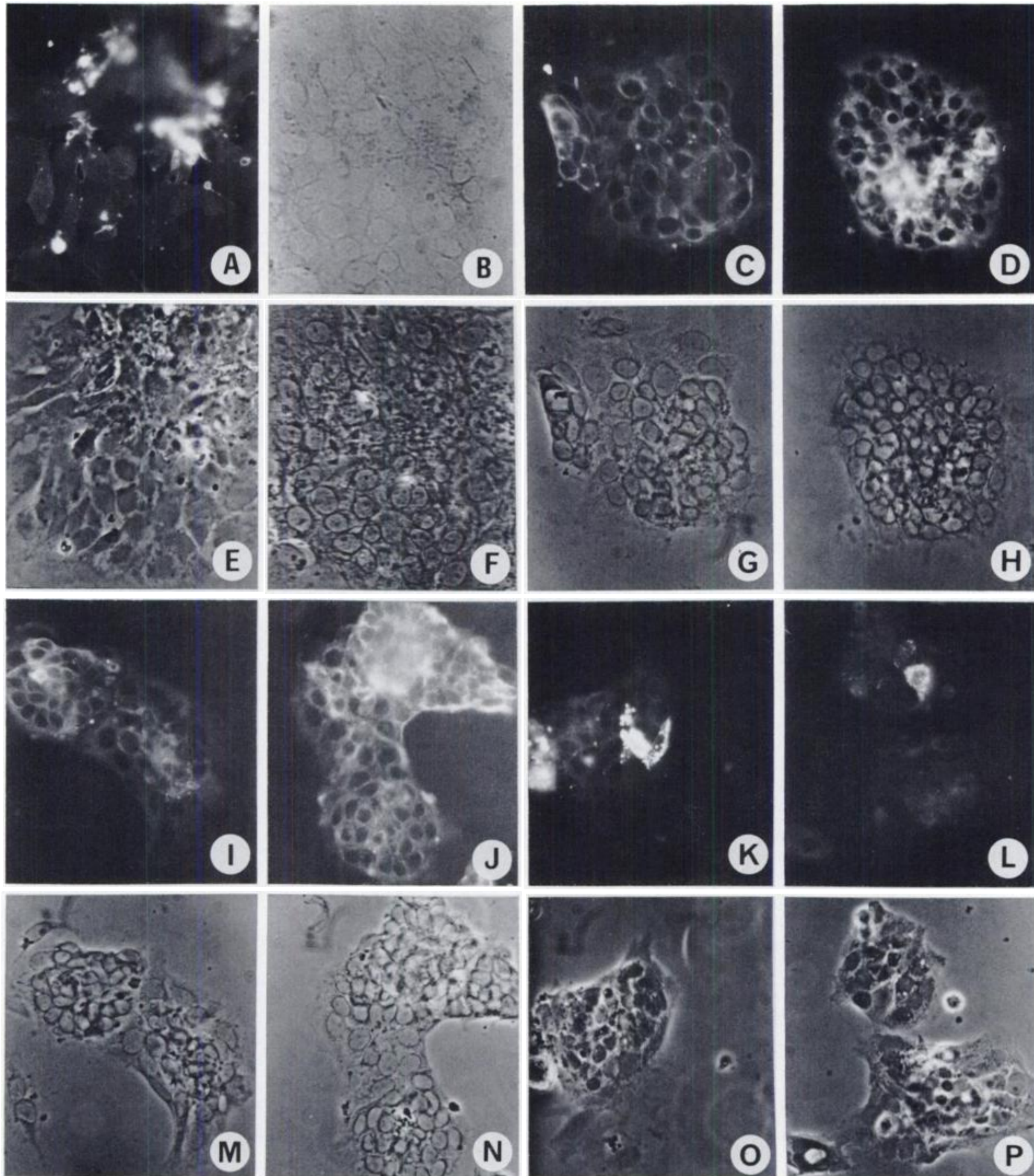

Fig. 4. Immunofluorescence microscopy of 24-h cultured purified oval cells using anti-BDS₇ (*A*), anti-CK55 (*C*), anti-CK52 (*D*), anti-CK39 (I), antivimentin (*J*), anti-AFP (*K*), and antialbumin (*L*) *antibodies*. *B* is GGT staining. E–H and M–P are phase contrast micrographs of A–D and I–L, respectively. Note that the cells were fixed either in paraformaldehyde (*A, E, K, L, O, P*) or acetone (*B–D, F–J, M, N*). × 400.

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Supernatant

The supernatant was centrifuged at 200 × *g* for 10 min and 10-ml portions of the unsedimented cells were placed in 100-mm tissue culture Petri dishes to remove the adherent small cells (Kupffer and endothelial cells). After 15 min at room temperature the dishes were rinsed five times with 5 ml of HEPES buffer. The recovered cells were centrifuged at 200 × *g* for 10 min and suspended in MEM-HEPES. For panning, the suspension was layered onto the anti-HES antibody-coated Petri dishes. The supernatant containing unattached cells was gently poured off, and the surface was washed five times with HEPES buffer. The cells in the washes were pooled with the supernatant, centrifuged at 200 × *g* for 10 min, resuspended in MEM-HEPES, and layered onto a discontinuous gradient of 50, 70, and 90% isoosmotic Percoll (12) in MEM-HEPES. The cell band enriched in oval cells was collected and washed twice in MEM-HEPES.

Pellet

The cell pellet was washed in HEPES buffer and resuspended in HEPES buffer containing 0.05% collagenase-0.02% EDTA-0.25% trypsin. After a 25-min incubation at 37°C in a shaking water bath, an equal volume of MEM-HEPES containing 10% fetal calf serum was added and the cell suspensions centrifuged at 200 × *g* for 10 min. The cell pellets were then resuspended in 10 ml of MEM-HEPES containing 25 μg/ml DNase I. After a 10-min incubation at room temperature, the suspension was diluted to 50 ml with MEM-HEPES and centrifuged at 200 × *g* for 10 min. The pellet was suspended in MEM-HEPES and 10 ml of the cell

Table 2 *Effect of medium supplements on DNA synthesis in cultured oval cells*

Highly enriched oval cells from the pellet fraction (see Table 1) were seeded on 35-mm tissue culture dishes coated with fibronectin (26), in modified MEM supplemented with CLEX alone, or with mixtures of growth-promoting factors (see Footnote 4 for detailed compositions). The percentage of labeled nuclei was measured by [^{3}H]dThd incorporation between 24 and 72 h of culture. The extent of cell fusion was monitored by the distinct arrangements of nuclei and CK55 filaments.

Supplements	% labeled nuclei	Induced cell fusion[a]
CLEX	6	+
Medium MX	30	
Medium MX + DMSO	41	
Medium MS + DMSO	16	
Medium MS + DMSO + EGF	50	
Medium MX + SB[b]	2	−
Medium MS + SB	2	−
Medium MS + SB + EGF	4	−

[a] Dependent on cell density.
[b] SB, 3.75 mM sodium butyrate.

suspension containing 1 × 10^6 hepatocytes were added to each anti-HES$_6$ antibody-coated dish. The dish was incubated for 30 min at room temperature, swirled gently to resuspend the cells, and incubated for an additional 30 min. At the end of the incubation, the nonadherent cells were removed as described above. Cell diameters were measured with an eyepiece micrometer.

Histochemical Assays

Slides were prepared with cells obtained at each purification step with a cytocentrifuge and stored at −20°C. The cells were fixed in cold acetone and tested for GGT (10, 33) AP (34), and PO (35). In each preparation the percentage of positive cells was determined by examination of 500 cells.

Indirect Immunofluorescence

Single-Antibody Reaction. Analysis by indirect immunofluorescence staining of cytokeratins, vimentin, BDS$_7$, HES$_6$, and AFP in cells in culture or tissue sections was performed as described before (10, 20). In the case of albumin, cells were fixed in 4% paraformaldehyde as described (36) except that glutaraldehyde was omitted. Cell nuclei were also labeled with Hoechst reagent 33258 (10, 37) following the immunofluorescence staining.

Double-Antibody Reaction. The single-antibody reaction involving the use of fluorescein-tagged anti-Ig was repeated, this time with the rhodamine-labeled anti-Ig (10). Slides were examined with appropriate light filters. Control experiments revealed no cross-reaction between antibodies.

Oval Cell Culture

In most experiments cells were seeded on 35-mm tissue culture dishes or 24-well culture plates containing glass coverslips precoated with fibronectin (27, 28), MEM supplemented with 1 mM pyruvate-0.2 mM aspartate-0.2 mM serine-1 mM tyrosine-1 mM proline-1 mM phenylalanine (38), and various combinations of growth and differentiation-promoting factors. In most experiments, the culture medium contained CLEX (39), which supports the growth of T51B cells, a propagable hepatic epithelial cell line expressing cytokeratins and BDS$_7$ markers of bile ductular cells (20). Insulin, EGF, dexamethasone, and DMSO are modulators of cultured differentiating rat hepatocytes (40, 41). β-Mercaptoethanol and cholera toxin are known modulators of cell growth and differentiation (42). Transferrin is usually required for cell growth under serum-free conditions (43). Sodium butyrate is a strong differentiation-promoting factor (44). Some propagable hepatic epithelial cells can grow in serum-free medium containing fatty acids (45). Bile ductular epithelial cells are exposed to biliary acids and bilirubin *in vivo.* In a few experiments, cells were seeded in serum containing medium.

DNA Synthesis

In most experiments, cells were pulse labeled for 48 h with [^{3}H]dThd (1 μCi/ml) 1 day postseeding. On day 3 of culture, cells were fixed with 100% cold acetone at −20°C for 10 min and processed for immunofluorescence staining and autoradiography as described previously (46) but without drying. In a few experiments, cells were pulse labeled for 24 h at various times postseeding.

Albumin and AFP Production

Medium from oval cells cultured in the presence of various hormonal combinations was changed daily and kept for radioimmunoassay of albumin and AFP. Pure rat albumin and AFP were radiolabeled with ^{125}I and supernatant AFP and albumin were measured by double antibody assay (47).

Tyrosine Aminotransferase Activity

Oval cells cultured in defined medium were assayed for TAT activity as described before (46, 48).

Cell Transfer *in Vivo*

Oval cells were injected into the interscapular and axillary fat pads of 100- to 125-g Fischer 344 rats according to described procedures (49); 1 × 10^6 cells were injected in MEM-HEPES supplemented with brain homogenate (49). Fourteen days after cell transfer, the animals were sacrificed and transplant sites were removed and cut into small pieces.

Electron Microscopic Examination

Tissue pieces were fixed in 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer for 2–24 h, postfixed in 1% osmium tetroxide, dehydrated in alcohol, and embedded in Epon. Thin sections were cut and

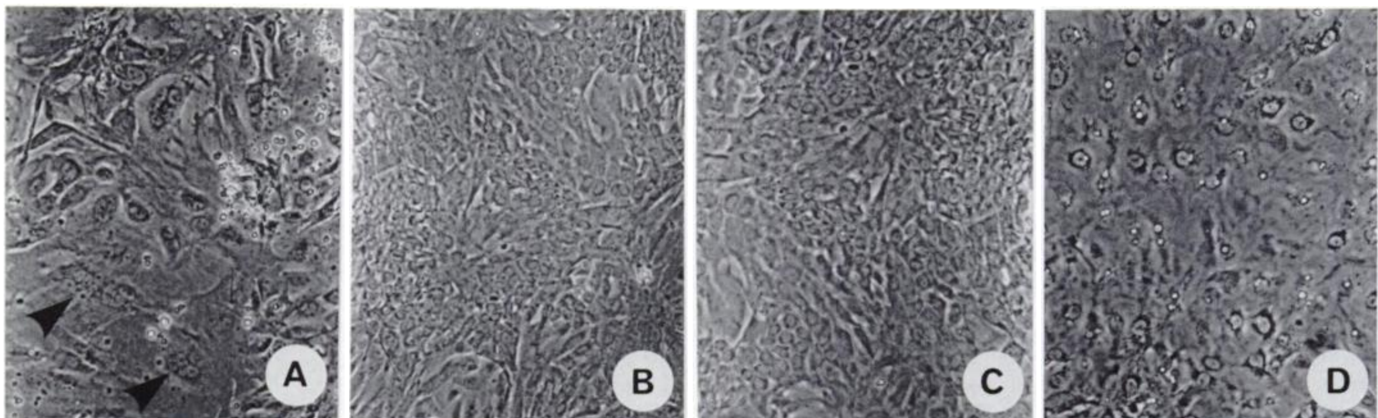

Fig. 5. Phase contrast micrographs of oval cells cultured for 5 days under various conditions. *A*, modified MEM (see "Materials and Methods") containing 20% CLEX, fatty acids, insulin, dexamethasone, and β-mercaptoethanol; *B*, medium MX; *C*, medium MK supplemented with DMSO; *D*, medium MX supplemented with sodium butyrate. × 100. *Arrowheads*, multinucleated cells.

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Table 3 *Kinetics of initiation of DNA synthesis in oval cells in primary culture*

Oval cells were seeded onto fibronectin-coated plastic dishes, cultured for 5 days in medium MX (see Table 2), and pulse (24 h) labeled daily with [^{3}H]dThd. While at least 95% of the cells were BDS_7^+ and largely GGT^+ at time of seeding (Table 1), a small subpopulation of BDS_7^-/GGT^+ cells emerged at day 1 and comprised about 15% of the population at day 5. These cells were identified by double labeling with [^{3}H]dThd and anti-BDS_7 antibody (Fig. 6).

Time in culture (days)	% labeled nuclei	
	BDS_7^+/GGT^+	BDS_7^-/GGT^+
1	5	8
2	16	56
3	16	83
4	9	92
5	7	70

stained with uranyl acetate and lead citrate and examined in a Zeiss E. M. 9-A electron microscope.

RESULTS

Reactivity of Antibodies to Cytokeratins, Vimentin, and Surface-exposed Components. We previously reported that antibodies against CK52, CK55, vimentin, AFP, and albumin can resolve three subpopulations of oval cells in 3′-Me-DAB-treated rat liver (20) and that the same antibodies can readily be used in combination with anti-BDS_7, and anti-HES_6 antibodies to define the cell of origin of normal rat liver epithelial cells in primary and long-term established cultures (20). We further examined the specificity of the anti-BDS_7 and anti-HES_6 antibodies. Anti-BDS_7 reacted with ductular oval cells in rat livers treated with 3′-Me-DAB for 4 weeks and bile ductular cells in normal rat livers with no reaction with any other cell types, including hepatocytes (Fig. 1). In contrast, anti-HES_6 reacted specifically with hepatocytes in 3′-Me-DAB-treated and normal rat livers (Fig. 2). The specificity of the antibodies was confirmed on partially purified preparations of oval cells from 3′-Me-DAB rat livers which were maintained for 1 day in primary culture (Fig. 3). As expected, oval cells were stained only by anti-BDS_7, while anti-HES_6 antibody reacted only with hepatocytes. The antibodies decorated both non- and formaldehyde-fixed cultured cells (20), demonstrating that they recognize exposed cell surface determinants.

Oval Cell Isolation and Purification. During the purification procedure, oval cells were identified by three criteria (7, 12, 14): (*a*) a cell diameter smaller than 15 μm; (*b*) the presence of GGT activity; and (*c*) the absence of PO activity. Oval cells dispersed from 3′-Me-DAB treated rat livers tend to form large aggregates. The first centrifugation step yielded around 3.5×10^8 nonparenchymal cells in the supernatant (13% GGT^+) and 5×10^8 in the pellet (41% GGT^+). Hepatocytes, which were mostly GGT^- and all expressed HES_6, constituted 9% of the supernatant and 23% of the pellet.

The supernatant also contained a high percentage of Kupffer and endothelial cells, but most of these mesenchymal cells rapidly attached to untreated plastic dishes. Hepatocytes contaminating the nonattached cell suspension were then eliminated by panning with anti-HES_6 antibody. At this point, the supernatant was enriched in oval cells as shown by the increase in GGT^+ cells (41%) but was still contaminated with some Kupffer and endothelial cells and nonviable hepatocytes. After isopyknic centrifugation on a Percoll gradient, there was less than 1% hepatocytes (HES_6^+) and less than 6% Kupffer (PO^+) cells. On the average, 90% of the cells were positive for BDS_7 and AP (Table 1). The viability was 85–90%.

The crude cell pellet consisted of large aggregates of oval cells, single hepatocytes, and endothelial and Kupffer cells. The percentage of GGT^+ cells was much higher in this fraction than in the supernatant. Trypsin digestion of the pellet reduced the number of hepatocytes by at least 50%. The antibody panning step worked well when the number of hepatocytes was less than 10^6 and the total number of cells was 10^7. The oval cell-enriched preparation obtained after panning still contained nonviable hepatocytes and some sinusoidal cells, which could be removed by isopyknic centrifugation. The yield of oval cells was 8.2×10^7/liver (84% GGT^+), with less than 2% Kupffer cells and less than 1% hepatocytes (Table 1). Around 95% of the cells were BDS_7^+ and AP^+ (Table 1). More than 85% of the cells excluded trypan blue. The rest of the experiments were performed on these highly enriched oval cell preparations.

Phenotypic Properties of Purified Oval Cells after 24 h of Culture. Oval cells attached as colonies and after 24 h of culture exhibited a typical epithelial cell morphology with a compact cytoplasm (Fig. 4). In fact, immunocytological analyses at various cell-seeding densities revealed that most of the cells contained the three cytokeratins, CK55, CK52, and CK39 and vimentin while few cells still contained albumin and AFP (Fig. 4). Double immunofluorescence staining showed that the same cells produced albumin and AFP and that these cells expressed BDS_7. At least 95% of the cells were BDS_7^+/GGT^+ cells.

Growth Promotion and Associated Phenotypic Modifications of Oval Cells in Primary Culture. The growth potential of oval cells was evaluated by measuring the percentage of [^{3}H]dThd-labeled nuclei over a 5-day culture period. In the presence of serum or CLEX alone, oval cells exhibited a low proliferative activity, with 6% of nuclei labeled following a 2-day [^{3}H]dThd incorporation (Table 2). A large proportion of the oval cells rapidly fused so that by day 5 they formed multinucleated cells (Fig. 5*A*). The extent of fusion was more pronounced at low cell-seeding density, as assessed by the alterations in the arrangements of nuclei and the staining patterns of CK55 filaments.

Oval cells attached rapidly and massively to fibronectin-pre-

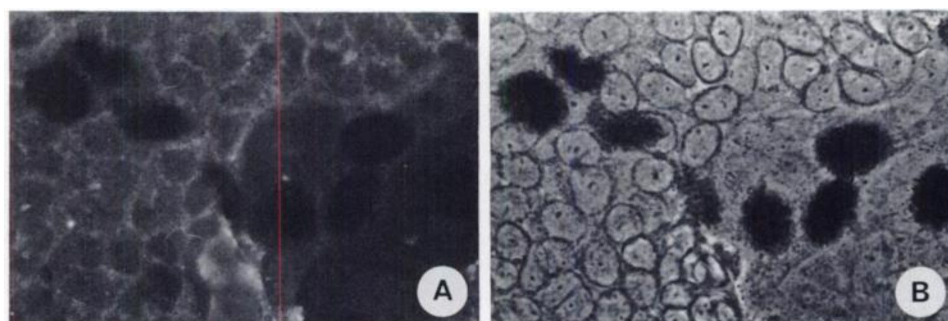

Fig. 6. Immunofluorescence staining using anti-BDS_7 antibody (*A*) coupled with autoradiography of 3-day cultured oval cells. Cells were cultured in modified MEM (see "Materials and Methods") containing 20% CLEX and labeled with [^{3}H]dThd for 48 h. Note that cells were fixed in acetone.

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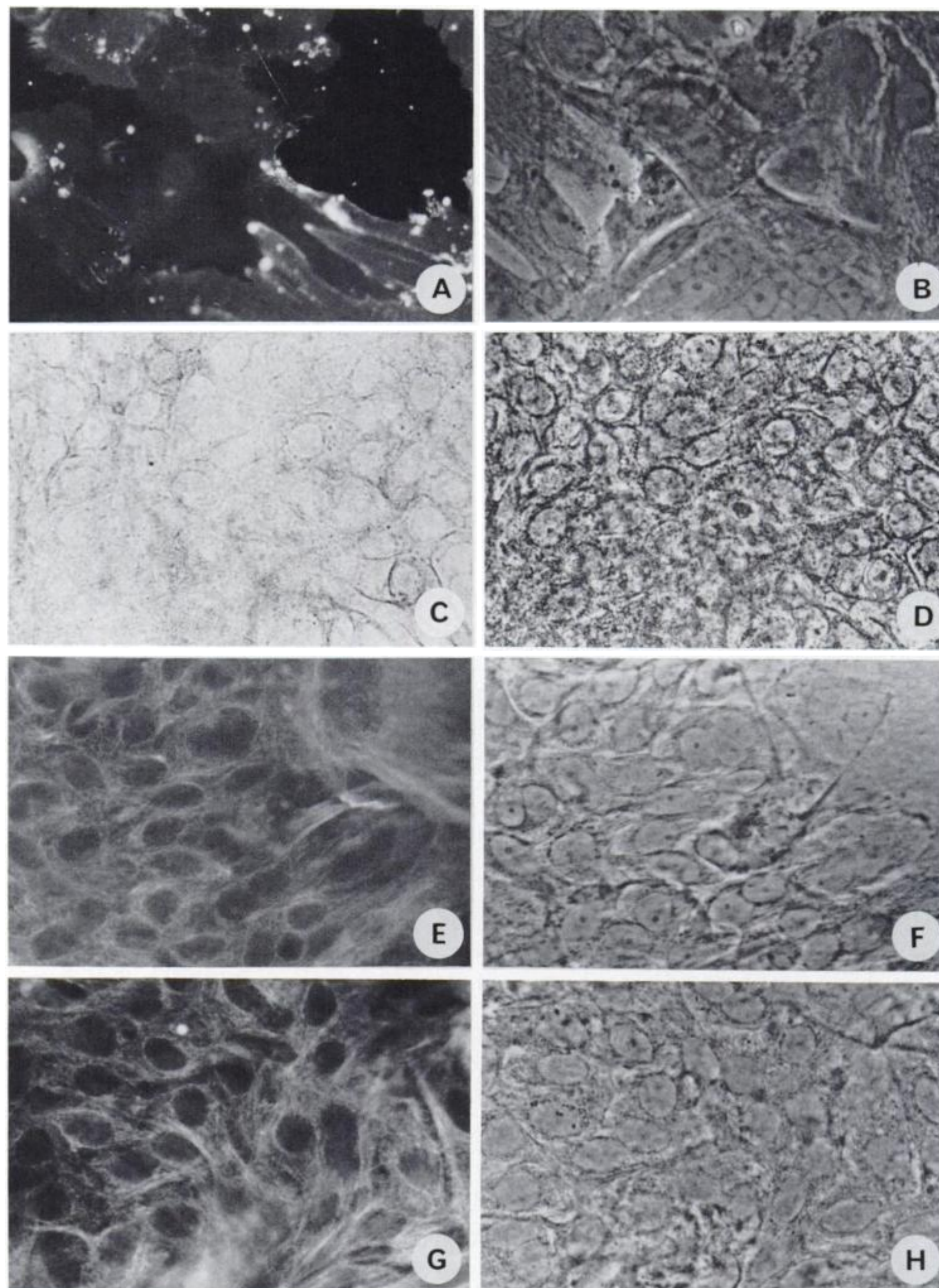

Fig. 7. Immunofluorescence microscopy of oval cells cultured 5 days in medium MX using anti-BDS_7 (*A*), anti-CK55 (*E*), and anti-CK52 (*G*) antibodies. *C* is GGT staining. *D*, *D*, *F*, and *H* are phase contrast micrographs of *A*, *C*, *E*, and *G*, respectively. Note that the cultured oval cells were fixed in either paraformaldehyde (*A*–*D*) or acetone (*E*–*H*). × 400.

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coated plastic dishes. This allowed an evaluation of their growth response in serum-free medium supplemented with various combinations of growth- and differentiation-promoting factors. The addition at day 0 of medium MX increased the labeling index to 30% (Table 2). Daily pulse labeling over the 5-day culture period revealed that DNA synthesis was initiated around day 2 (Table 3). The addition of DMSO to medium MX further increased the labeling index to about 40%, whereas sodium butyrate led to an inhibition of DNA synthesis (Table 2). The use of medium MS in the presence or absence of DMSO resulted in a reduction of the labeling index to 16% (Table 2). Still, the addition of EGF to medium MS in the presence of DMSO yielded a labeling index of 50%. Sodium butyrate again abolished essentially all growth activity.

Different medium supplements caused major variations in oval cell morphology (Fig. 5, *B*–*D*). For example, in the presence of sodium butyrate the oval cells rapidly spread so that at days 4–5 the monolayer appeared confluent in spite of the fact that cell growth was inhibited (Fig. 5*D*). In the presence of medium MX alone, a small (1%) subpopulation of BDS_7^-/GGT^+ cells emerged at day 1 and expanded rapidly after day 2 in culture so that by day 5 they represented about 15% of the oval cell population. Double labeling with [^{3}H]dThd and anti-BDS_7 antibody (Fig. 6) revealed a labeling index of 70% for these BDS_7^-/GGT^+ cells at day 5 (Table 3). In the presence of DMSO the expansion of this small subpopulation was delayed by 1–2 days. Although these BDS_7^-/GGT^+ cells had a fusiform shape they expressed CK39 and vimentin. They did not produce albumin or AFP. These cells could be subcultured and still expressed GGT at the second passage. Very few GGT^- cells expressing only vimentin were observed throughout the 5-day period indicating that the number of mesenchymal cells remained extremely low in these primary cultures.

The highly predominant and typical ductular oval cells that constituted the mass primary culture at day 1 after seeding in the presence of medium MX (Fig. 4) still expressed cytokeratins,

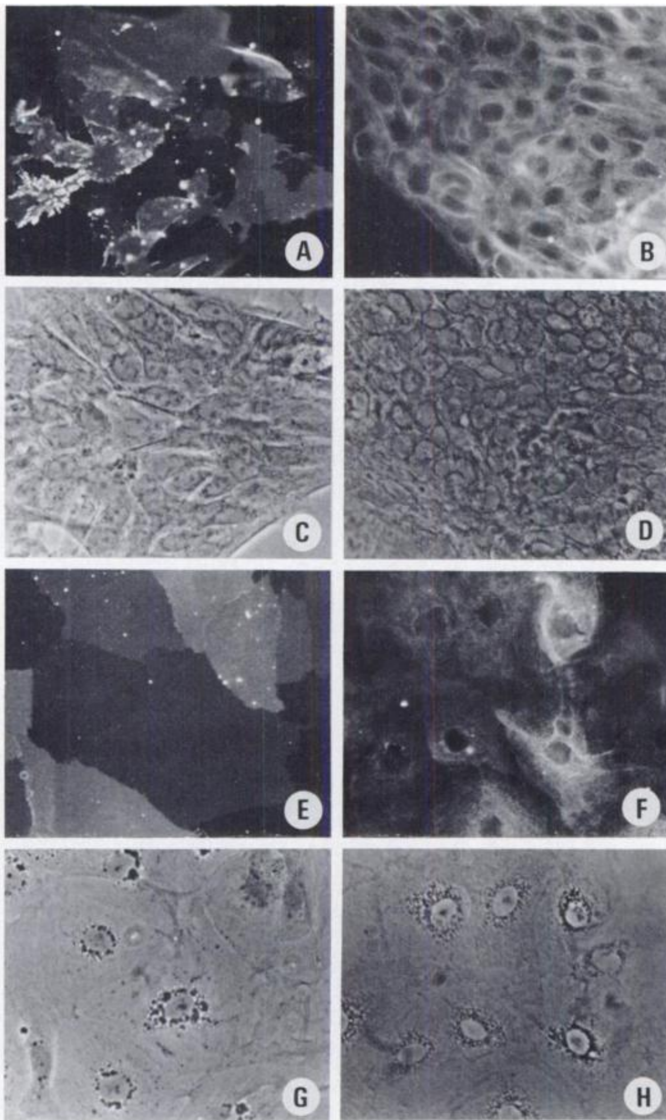

Fig. 8. Immunofluorescence microscopy of oval cells cultured 5 days in medium MX supplemented with DMSO (*A–D*) or sodium butyrate (*E–H*) using anti-BDS_7 (*A* and *C*) and anti-CK52 (*B* and *F*) antibodies. *C, D, G,* and *H* are phase contrast micrographs of *A, B, E,* and *F,* respectively. Note that cells were fixed in either paraformaldehyde (*A, C, E, G*) or acetone (*B, D, F, H*). × 400.

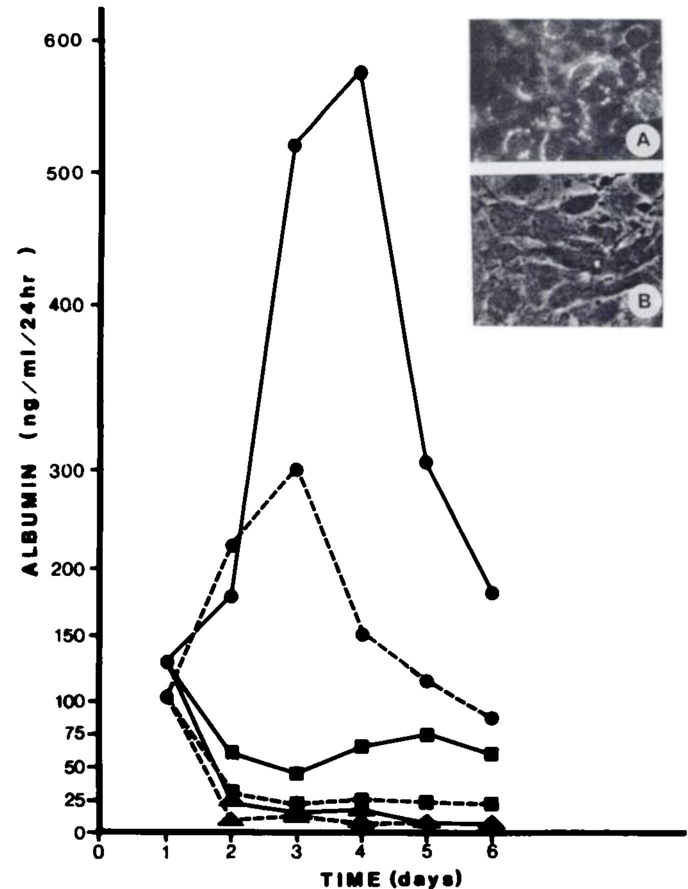

Fig. 9. Albumin production by oval cells cultured in different media: medium MX without dexamethasone (▲- - - ▲); medium MX (▲ —— ▲), medium MX with DMSO without dexamethasone (■- - - ■), medium MX with dexamethasone and DMSO (■ —— ■); medium MX with sodium butyrate without dexamethasone (● - - - ●); medium MX containing dexamethasone and sodium butyrate (● —— ●). *Insets*: immunofluorescence microscopy using anti-rat albumin monoclonal antibody (*A*) of oval cells after 3 days in primary culture in presence of medium MX supplemented with sodium butyrate; *B*, phase contrast micrograph of *A*. × 400.

GGT and BDS_7 at day 5 (Fig. 7). In the medium MX supplemented with DMSO or sodium butyrate, the oval cells expressed variable levels of cytokeratins and BDS_7 (Fig. 8).

Differentiation Promotion of Oval Cells in Primary Culture. The potential transition of oval cells into hepatocytic cells was assessed by first measuring albumin and AFP production in the culture medium. In presence or absence of DMSO supplemented to medium MX, albumin production remained low over a 6-day culture period (Fig. 9). In contrast, upon the addition of sodium butyrate without dexamethasone, the oval cells initiated a massive production of albumin which reached a maximum at day 3 (Fig. 9). The combined addition of sodium butyrate with dexamethasone further increased the albumin production (Fig. 9). The AFP production decreased to an undetectable level at day 2. Indirect immunofluorescence analysis showed that the variation in the percentage of albumin-producing cells followed the secretion curve (Fig. 9, *inset*), so that by day 3 up to 40% of the oval cells were positive and 8% by day 6 of culture.

To further document the promoting effect of sodium butyrate on the expression of hepatocytic functions, TAT induction was measured at various times postseeding in medium MX. Sodium butyrate added to dexamethasone progressively induced TAT activity to about 4 milliunits/mg protein at day 6 of culture.

***In Vivo* Transfer of Oval Cells.** Fourteen days after injection into the fat pads of syngeneic rats (Fig. 10), oval cells were mainly arranged as clusters that resemble the bile ductular structures of normal rat liver. Electron micrographs showed microvilli in the lumen of the ductular structure (Fig. 10*A*). The cells exhibited the typical ultrastructural features of bile ductular cells (few mitochondria, dense intermediate filaments, desmosomes) (Fig. 10, *A* and *B*). They stained with anti-CK55, anti-CK52, and anti-BDS_7 antibodies and thus had a bile ductular cell phenotype (Fig. 10, *C–F*).

DISCUSSION

The present results demonstrate that 3′-Me-DAB rat liver-derived oval cells exhibit a phenotype characteristic of bile ductular cells, based on the differential expression of GGT, cytokeratins, and surface-exposed components. We also show that a highly enriched preparation of oval cells can be obtained from the liver by cell panning and isopyknic centrifugation and that ductular cells in primary culture in the presence of growth- and differentiation-promoting factors can express phenotypic features of facultative hepatocytic precursors.

The reference marker for bile ductular cells in normal rat liver

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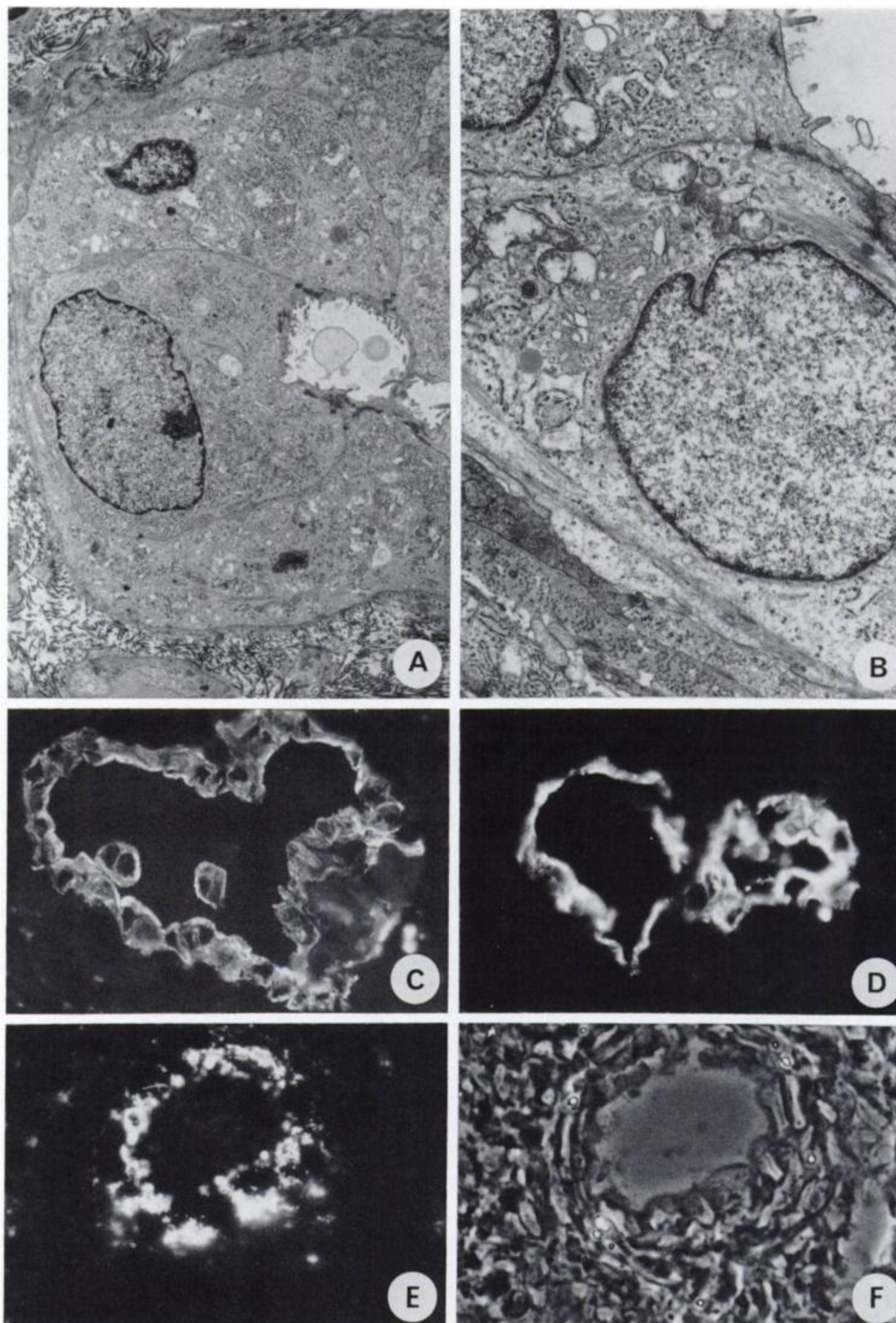

Fig. 10. Electron (*A* and *B*) and immunofluorescence (*C*, *D*, *E*) microscopy of transplant sites 14 days after oval cell injection into fat pads. Oval cells form typical bile ductular structures with microvilli in the lumen (*A*, × 900) and exhibit ultrastructural characteristics of ductular cells (*B*, × 4000). Immunofluorescence microscopy of frozen sections of transplantation sites using anti-CK55 (*C*), anti-CK52 (*D*), and anti-BDS$_7$ (*E*) antibodies. *F* is a phase contrast micrograph of *E*. × 400.

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is GGT (50–52), a surface exposed glycoprotein (53). However, it is well established that various hormonal and chemical treatments can induce GGT activity in hepatocytes (1, 3). In line with the immunological approach reported by others (13, 54), we have generated monoclonal antibodies of high specificity for distinct surface-exposed components of oval cells derived from livers of 3′-Me-DAB-treated rats and of hepatocytes of normal rats. Such high specificity can be explained by the fact that the antibodies are directed against surface components which are expressed as cell lineage-dependent markers (25, 26, 55). In this respect, cytokeratins can also be used as cell-lineage markers (22, 23). Previous data from our (10, 20) and other (31) laboratories have shown that normal and oval ductular cells express CK39 and CK52, whereas CK55 is present in both ductular cells and hepatocytes. Therefore, the combined use of antibodies against surface-expressed components, cytokeratins, and metabolic markers provides a good tool to examine the epithelial cell lineage relationship in normal and preneoplastic rat livers.

Cell-separation procedures recently developed to select oval cells from cell preparations of preneoplastic rat liver have relied on differences in cell density, cell surface charge, or cell size (10, 12, 50, 56, 57). In the CDE model system, oval cells can be dispersed as single cells, and since they are smaller than hepatocytes, the two cell populations can be readily separated by centrifugal elutriation (56). However, 3′-Me-DAB rat liver-derived oval cells are mostly dispersed as aggregates and sediment with hepatocytes (10). The present procedure has therefore been designed to first lyse a large proportion of the freshly dispersed

hepatocytes with trypsin and then remove the remaining viable hepatocytes by panning. At this point the anti-BDS_7 antibody could have been used to select oval cells from the supernatant. In practice, however, this step was replaced by a single isopyknic centrifugation in a Percoll gradient, which removed essentially all the remaining nonoval cells. Moreover, unlike Pronase, trypsin allows the rapid and massive attachment of oval cells to culture substratum.

The phenotype of oval cells purified from the liver of CDE-fed rats apparently differs from those of rats fed 3′-Me-DAB. Oval cells purified from CDE-rat liver by centrifugal elutriation after dispersion with Pronase have a median diameter of 13.1 μm (56) and are 93% GGT^+, 61% $albumin^+$, and 29% AFP^+ (56). These are phenotypic features of transitional cells. In comparison, oval cells obtained from livers of rats fed 3′-Me-DAB with the cell purification strategy described here have a diameter of 9 μm, with only 5% AFP^+ and $albumin^+$. Based on the BDS_7 and GGT data, most of these cells constituted a typical population of bile ductular epithelial cells. It would therefore be of particular interest to type the CDE-rat liver derived AFP^+/albumin positive cells on the basis of the differential expression of cytokeratins and surface-exposed components.

To our knowledge, this is the first *in vitro* demonstration of a massive induction of hepatocytic traits in ductular cells. Among the three differentiating agents tested, sodium butyrate was the most potent, and the induced effect seems biologically significant. In fact, if one takes into account the difference in cell size and percentage of albumin-containing cells, the peak value of albumin production for cultured oval cells is at least 10% of that of cultured differentiating rat hepatocytes (40). Similarly, the specific activity of the induced TAT in oval cells at day 5 of culture reaches the basal level of cultured hepatocytes (40). Although the mechanism of the butyrate action on gene regulation is not clearly established, previous studies of several cell types have indicated that it acts specifically in that it induces or suppresses the expression of only certain genes (see Ref. 44). Our data with sodium butyrate supports this view and further indicate that the selective induction of albumin production and TAT activity is part of a general "program" of hepatocyte differentiation, which also includes the loss of AFP production and the inhibition of DNA synthesis (40).

The 3′-Me-DAB rat liver-derived BDS_7^+/GGT^+ oval cells forming the mass monolayer culture have a limited growth capacity (Table 3), but it is worth noting that the few BDS_7^-/GGT^+ cells which emerge in the absence of sodium butyrate can be subcultured. Further attempts to establish cell lines have indicated that at least another phenotypically distinct epithelial cell type is present as a very minor subpopulation in the original oval cell preparation.[5] Considering the known heterogeneity of the oval cell compartment (10–14, 17, 58), such cell lines may arise from distinct oval subpopulations which have different developmental potentials. The emergence of oval cell lines can be compared to the outgrowth of "propagable hepatic epithelial cells" derived from monolayer cultures of normal rat hepatocytes (17, 58, 59–66), which arise as clonogenic colonies, variably express few hepatocytic functions, and are thought to originate from terminal bile ductules (59). We have recently provided evidence that on the basis of GGT, cytokeratin, and surface-component selective expression, normal propagable hepatic epithelial cells of distinct morphology belong to different cell lineages; *i.e.*, some of them are of bile ductular origin, while others might originate from the Glisson capsula (20). Further analysis using this cell typing approach should establish whether propagable hepatic epithelial cell lines and oval cell-derived lines share a common cell of origin.

The present findings on the induction of typical hepatocytic traits in oval cell primary culture support the hypothesis that cells from "terminal biliary ductules constitute facultative hepatocytic stem cells" (59). Such a proposal is certainly quite attractive, but in order to be fully established as a putative differentiation pathway of hepatocytes, one must be able to show that the same cellular transition can take place at any period of liver development, particularly in the embryo at the early time of hepatic tissue formation. Experiments are in progress in our laboratory to assess this possibility by performing sequential *in situ* and *in vitro* analyses of the differential expression of albumin, AFP, cytokeratins, and surface-exposed components during the days 11–16 fetal period, *i.e.*, at the time of bile ductular structure formation.

The strategy developed here constitutes an attractive approach to define the actual role of ductular oval cells and hepatocytic cells in the process of chemical hepatocarcinogenesis. Considering the wealth of *in situ* data obtained on the sequential cellular events associated with the emergence of tumors (1–9, 15, 16, 67–69), it becomes possible to address direct questions about initiation *versus* promotion by using *in vivo*/*in vitro* model systems.

[5] N. Marceau and M. Noël, unpublished observations.

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