Reorganization of Porcine Thyroid Cells into Functional Follicles in a Chemically Defined, Serum- and Thyrotropin-free Medium

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ABSTRACT In the serum-free, chemically defined medium NCTC 109, freshly isolated porcine thyroid cells aggregate and form functional follicles in culture even in the absence of thyrotropin. The follicular pattern observed under light and electron microscopy express the main morphological characteristics of in vivo thyroid cells. Follicles are large, replete with dense colloid, and the apical pole of cells is characterized by well-developed microvilli and the presence of aminopeptidase N. The index of iodide transport activity (^{125}I-C/M ratio) decreases vs. days of culture to a resting value of about 1 or 2 at day 2. Addition of thyrotropin (200 μU/ml final concentration) at day 4 is followed by a 10-fold increase in iodide transport activity within 24 h and a 40-fold increase 4 d later. Incorporation and organification of iodide are dose dependent between 0 and 250 μU/ml thyrotropin; highest concentrations (4,000–16,000 μU/ml) are significantly inhibitory. In the absence of thyrotropin each cell synthesizes 8.2 pg thyroglobulin/d. Acute stimulation by thyrotropin at day 4 resulted in a slight decrease in the quantity of thyroglobulin present in the cell layer but in an increase in the total amount of thyroglobulin recovered in both cells and medium, reaching 34.3 pg/cell/d. The protein exported into the medium is thyroglobulin, as shown by SDS PAGE and immunological properties. Here we demonstrate that porcine thyroid cells can be maintained in culture as resting, highly differentiated, follicular-associated cells, sensitive to acute stimulation by thyrotropin.

A model system of thyroid cells in culture able to reproduce the main characteristics of the thyroid gland should resemble in vivo follicles with respect to expression of morphological differentiation of follicle-associated cells (26), expression of the specific thyroid metabolism leading to synthesis of thyroglobulin (Tgb) and thyroid hormones (43), and simulation of the events following the interaction between thyroid cells and their physiological stimulator, thyrotropin (TSH) (9).

On the basis of light microscope examination, the reorganization of isolated TSH-stimulated cells into follicle-like structures has been reported in primary cultures of thyroid tissue from sheep (36), lamb (34), hog (13, 14, 32), steer (59), dog (65), and man (8) as well as in subcultures of thyroid cells of hog (21), rat (2), and man (8). The polarized character of these epithelial cells and the nature of the three-dimensional follicular pattern were confirmed in certain cases by electron microscopy (8, 15, 32).

The metabolic properties of thyroid cells in vitro have been studied in a general fashion by the same authors. In primary culture, a rapid decrease in thyroxine (T_4) production (29), Tgb synthesis (32, 52), and iodide binding (37) are observed. The loss of these activities was retarded but not prevented by exposure of cells to TSH (37). However, in the presence of TSH, porcine thyroid cells actively trap iodide (17), synthesize Tgb (13, 32, 41), and produce T_4 (41) after several days in culture. (For a review, see Lissitzky et al. [42]).

Some regulatory events relating to TSH action have also been described in support-anchored thyroid cell culture systems and in isolated follicles in suspension. Acute effects observed in response to TSH include (*a*) formation of pseudopods (48, 67); (*b*) apical migration of lysosomes (48); (*c*) depletion of luminal colloid (48); (*d*) changes in cell morphology from epithelial to stellate-shaped (55); (*e*) increase in iodide efflux (17); and (*f*) increase in Tgb mRNA levels (7). Chronic effects

THE JOURNAL OF CELL BIOLOGY · VOLUME 93 MAY 1982 479-488

of TSH have also been reported including (*a*) desensitization to high doses of the hormone (53) and (*b*) regulatory effects of iodide on iodide trapping (18).

However, despite the above studies, the importance of TSH on the in vitro reorganization of thyroid cells into follicles remains unclear. In hypophysectomized chick (25, 66) and decapitated rabbit embryos (33), the thyroid gland develops normally in the absence of TSH. It thus appears that, in vivo, the morphogenesis of functional thyroid follicles is not dependent upon thyrotropin stimulation. Several in vitro studies have also indicated that thyroid follicles could be formed from isolated thyroid cells cultured without TSH (5, 44) in the presence of gelatin (45, 60), after the interaction of epithelial and mesenchymal cells (30), or using clusters of rat thyroid cells in suspension (48).

A second point which remains to be clarified is the influence of TSH on Tgb synthesis. In relation to previous studies in vivo or using tissue slices (for review, see Dumont [9]), comparable increases in Tgb production in response to TSH have not been obtained in cell culture (41).

A third point relating to follicle formation in vitro is the influence of serum which has been used as a medium supplement in all the cell culture systems mentioned herein, with the exception of three recent studies (8, 21, 48). The general interest in using serum-free media has been recently emphasized (4, 12) and preliminary studies indicated the long-term preservation of thyroid cell differentiation in serum-free media (20) or in low serum media (1).

Here we describe a new thyroid cell culture system. Porcine thyroid cells isolated by trypsin were shown to aggregate and reorganize into follicles in a serum- and TSH-free medium. The medium was entirely synthetic and no limiting factor was apparent before day 10 in culture. Under these conditions, the cells express the main morphological and metabolic characteristics of fully differentiated thyroid cells in vivo, and they are sensitive to acute stimulation by TSH which quadruplicates Tgb production and increases iodide transport 40 times.

MATERIALS AND METHODS

Materials

Soybean trypsin inhibitor and poly-L-lysine (Type I-B) were products of Sigma Chemical Co. (St. Louis, MO). Trypsin (1:250) was supplied by Gibco Bio-Cult Ltd. (Scotland). Porcine TSH (Endo, 0.5 U/mg) was obtained from Organon (St. Denis, France).

Falcon plastic tissue culture-ware was used throughout, with the exception of 16 mm-microwell clusters which were a product of Costar (Costar, Data Packaging, Cambridge, MA). Culture surfaces were coated with poly-L-lysine according to McKeehan and Ham (35) and stored dry. Eagle's medium with Earle's salts and nonessential amino acids (10) and NCTC 109 medium (12) were supplied by Eurobio (Paris) and Biopro (Lille, France). Pure porcine Tgb was a generous gift of Dr. C. Marriq (U 38 de l'Institut National de Santé et de la Recherche Medicale).

Isolation and Culture of Cells

Cells were isolated from adult porcine thyroid glands according to Tong et al. (62). We currently use a discontinuous method (14, 17). Cells were seeded at a concentration of $5 \times 10^5/cm^2$ of poly-L-lysine-treated culture surface and incubated at 37°C under a humidified atmosphere of 95% air–5% CO_2. Soybean trypsin inhibitor was added to Eagle's or NCTC 109 medium at a final concentration of 0.25 mg/ml only during the first day of culture. 1% Gibco antibiotic-antimycotic solution was used throughout. Media were changed at day one.

A single dose of TSH was added to cell culture media at day 4 to yield a concentration of 200 μU/ml. Where appropriate, variable doses of TSH ranging from 0 to 16 mU/ml were added at day 4.

Morphological Controls

Each day, cell cultures were observed under light microscopy. Cells were photographed under phase-contrast or fluorescence or Nomarski optics. For electron microscopy, cells were collected by centrifugation after scraping with a rubber policeman and then fixed and processed as previously described (15).

Aminopeptidase N Localization

Cells cultured on poly-L-lysine-treated glass cover slip supports were fixed in formaldehyde 4% in PBS, treated by acetone for membrane permeabilization, and labeled by indirect fluorescence technique using antibodies directed specifically against aminopeptidase N. All procedures were performed according to Feracci et al. (22, 23).

Cell-layer Treatment

Where appropriate, cell layers fixed on the plastic flasks and decanted media were stored at −20°C. Cells were then thawed, collected with a rubber scraper in distilled water, transferred into lyophilization tubes, and freeze-dried. The dessicated cell layer content was homogenized in 1 ml of distilled water, and aliquots were used for Tgb and DNA content determination. DNA was estimated according to McIntyre and Sproull (47).

Gel Electrophoresis

PAGE were performed in 0.1% SDS on 5% (wt/vol) acrylamide slab separating gel (80 × 100 × 2.5 mm) according to Ferro-Luzzi Ames (24) using 3.75% (wt/vol) stacking gel and the Tris-glycine buffer system of Laemmli (38). Denaturation of samples, protein migration, fixation, and staining with Coomassie Blue were performed according to Barber and Crumpton (3). Destaining was performed in methanol-acetic acid-water (7:7:86, by vol) for 48 h. Samples of cell culture media (25 μl) were run in parallel with pure 19 S thyroglobulin (M_r of the 12 S subunit, 330,000).

Active Transport of Iodide

Two methods were used: (*a*) First method: ^{125}I-C/M (cell to medium) ratios were determined according to Rodesch and Dumont (56) with slight modifications (17). All incubations were performed in NCTC 109 medium with 1 mM $Na^{127}I$, in the presence of 2 mM methylmercaptoimidazole, an inhibitor of iodide organification. (*b*) Second method: the uptake of iodide by thyroid cells *in situ* was examined using the technique described by Planells et al. (51), with minor modifications (8). Iodide efflux was measured according to Fayet and Hovsépian (17).

Radioimmunoassay (RIA) of Tgb

Tgb was measured by a solid-phase RIA using rabbit antiserum which had been adsorbed passively onto polystyrene tubes (11). The tubes were coated with 0.5 ml of diluted (1:100,000) porcine Tgb antiserum in a coating buffer ($NaHCO_3$:35 mM; Na_2CO_3:15 mM; NaN_3:3 mM; pH 9.6). The tubes were left overnight at room temperature, rinsed once with Dulbecco phosphate-buffered saline (PBS) plus 0.5% (wt/vol) bovine serum albumin (BSA), and three times with PBS. Tgb labeling with ^{125}I was performed according to Thorell and Johansson (61) using lactoperoxidase and H_2O_2 as the oxidizing agent. The labeled protein was separated from iodide by Sephadex G-25 gel filtration. The label, standards or unknowns were added to a final volume of 0.5 ml in PBS/BSA. The tubes were left for 24 h at room temperature. PBS/BSA was added to fill tubes which were then decanted, rinsed three times with PBS, and counted.

RESULTS

Reorganization of Porcine Thyroid Cells into Follicles in the Absence of TSH

The majority of population of trypsin-dissociated porcine thyroid cells was composed of single cells or coupled cells with sparse aggregates of less than ten cells. No intact follicles were observed (Fig. 1*A*). As previously demonstrated by microcinematography, some macrophages and fibroblasts were present but these cells were scarce and their number absolutely negligible as compared to epithelial thyroid cells (19). When the freshly isolated porcine thyroid cells were cultured in NCTC 109 medium, the cells plated onto poly-L-lysine-coated surfaces aggregated within 12–24 h (Fig. 1*B*) and reorganized into follicles from day 2 to day 13. Under the light microscope, at day 4 most cells were formed into follicles (Fig. 1*C*). The cell

layer was composed of plastic-anchored structures that are a single follicle thick.

When the freshly isolated porcine thyroid cells were cultured in Eagle's medium onto poly-L-lysine-coated culture-ware surfaces, the cells developed as a monolayer (Fig. 1 *D*) following an initial period of cellular aggregation of ~12–24 h similar to the aggregation period described in NCTC 109 medium.

The inclusion of TSH (200 μU/ml) in culture media from the onset of culturing resulted in a similar follicular pattern both with NCTC 109 and with Eagle's medium (Fig. 1 *E*).

Electron microscopy examination of cultures in NCTC 109 medium confirmed the presence of large follicles filled with electron-dense material. Well-developed microvilli were present along the apical membrane of the follicle-forming cells, indicating that the cellular polarity characteristics of intact thyroid tissue had been reestablished (Fig. 1 *F*). Aminopeptidase N, a marker of the apical pole of thyroid cells in vivo and in vitro (22, 23), was localized at day 4 mainly in the plasma membrane area directed towards the follicular lumen, indicating a high degree of morphological (Fig. 1 *G*) and molecular polarization (Fig. 1 *H*).

Effect of TSH on the Active Transport and Organification of Iodide

With control cultures (without TSH) in NCTC 109 medium, iodide transport activity (^{125}I-C/M ratio) decreased with time, reaching a resting value of between 1 and 2 after 3 days (see Figs. 2 and 3). Chronic stimulation of cells by inclusion of TSH in the medium from the onset of culture (Fig. 2) resulted, at all time points examined, in ^{125}I-C/M ratios higher than in controls. A systematic decline in iodide transport activity up to the second day in culture was observed, followed by an increase in the following days. In addition, when 4-d-old control cultures in NCTC 109 medium were exposed to TSH (200 μU/ml), a 10-fold increase in the ^{125}I-C/M ratio was observed after 24 h and a 40-fold increase 4 days later (Fig. 3).

The TSH-responsiveness of follicle-associated cells in NCTC 109 medium was further examined in terms of the dose-dependent stimulation of iodide uptake and organification. Fig. 4 shows that when 4-d-old cultures were exposed to various doses of TSH for 4 d and then labeled with ^{125}I iodide for a 6-h period, a dose-dependent increase in iodide uptake and organification was observed between 0 and 500 μU TSH/ml. A maximal increase in iodide trapping 11 times that of control values was obtained in response to 250–1,000 μU TSH/ml, and 50% of maximal stimulation was obtained with ~50 μU/ml. Concentrations of TSH >1,000 μU/ml produced a significant decrease in both iodide uptake and organification when compared to maximal levels. This inhibitory effect of high doses of TSH might reflect a desensitization of the cells to high doses of the hormone as recently described (53).

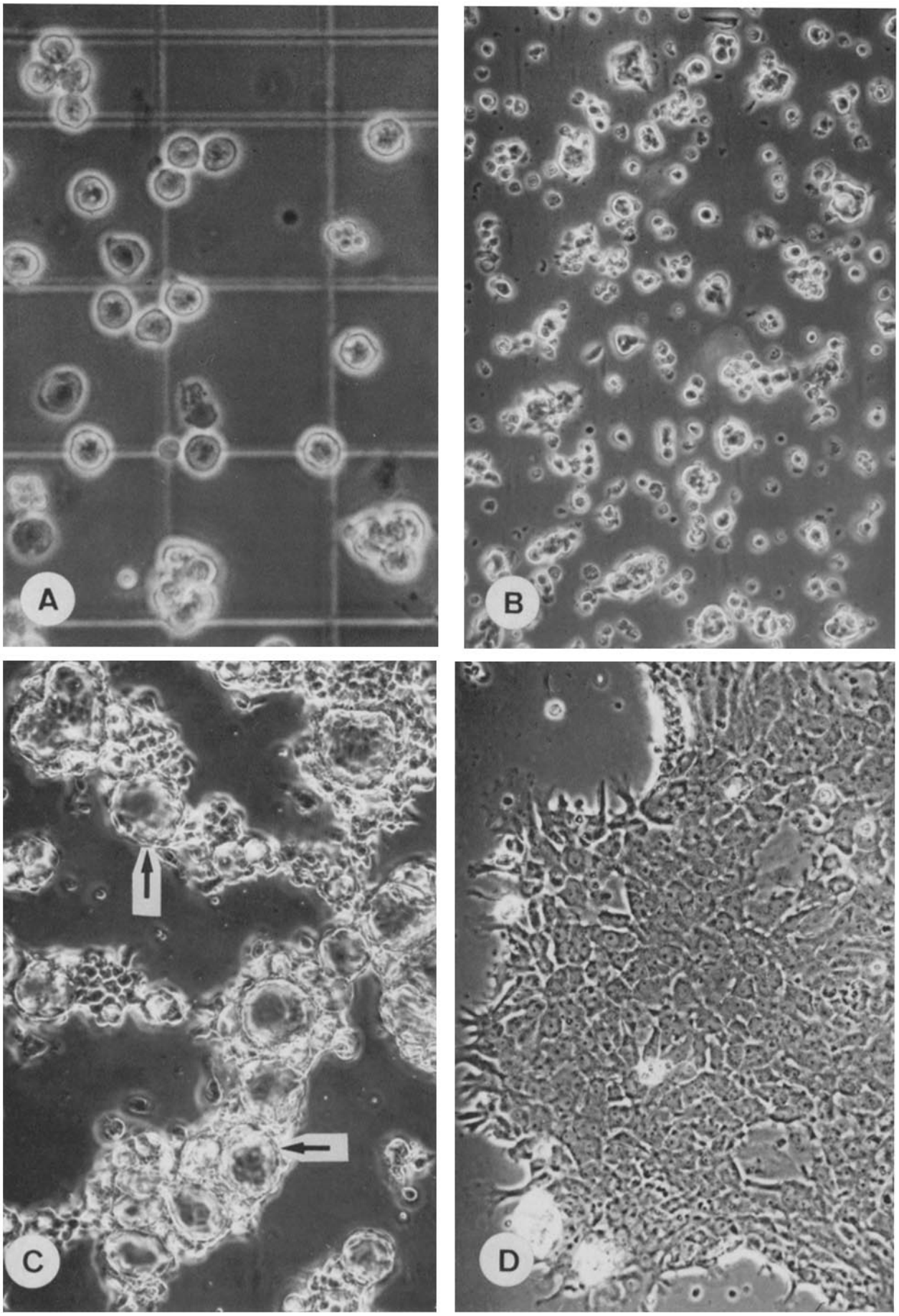
The above experiments demonstrate that the present culture system of thyroid cells reorganized into follicles in the absence of TSH mimics the thyroid gland in vivo in terms of the stimulatory effects of TSH on iodide trapping. It has long been known, however, that in vivo TSH has a biphasic action on iodide trapping by thyroid tissue (28). Before its stimulatory effect, TSH produces a transient decrease in the tissue-to-serum iodide concentration ratio. This effect occurs via an increase in the passive efflux of iodide from the cells. Fig. 5 illustrates that this phenomenon can also be observed with the present in vitro system when the acute effect of TSH on iodide uptake is examined. In this experiment, follicle-associated cells in culture were pretreated at day 3 with 50 μU TSH/ml in order to stimulate iodide transport activity and 2 mM methimazole (an inhibitor of iodide organification). After 24 h, the kinetics of iodide uptake was analysed over a 3-h period in the presence and absence of 1 mM perchlorate (a competitive inhibitor of iodide transport). In the absence of perchlorate, ^{125}I uptake reached equilibrium after ~2 h. As should be expected from the physiology of normal thyroid cells in vivo, addition of a saturating dose of TSH (16 mU/ml) at this equilibrium position resulted in a 50% efflux of trapped iodide within 30 min (Fig. 5).

Basal and TSH-stimulated Synthesis of Tgb

The levels of Tgb in culture media (Fig. 6 *a*) and in cell layers (Fig. 6 *b*) were estimated by RIA at various times between the onset of culture and day 13. The total amount of Tgb recovered is represented in Fig. 6 *c*, and all results are expressed in μg Tgb/μg DNA in the cell layer.

Fig. 6 *a* shows that cultures of porcine thyroid cells in NCTC 109 medium released large quantities of Tgb into the culture medium versus days in culture. Addition of TSH (200 μU/ml)

FIGURE 1 (*A*) Light microscope examination of freshly isolated thyroid cells obtained by trypsin from porcine thyroid tissue. Using a Thoma haemocytometer, controls of the cell suspension show isolated cells, coupled cells, and few aggregates of less than ten cells. × 1,200. (*B*) Aggregates of thyroid cells after one day of culture. Freshly isolated porcine thyroid cells were plated onto poly-L-lysine-coated culture plastic flasks in NCTC 109 medium plus 0.25 mg/ml soybean trypsin inhibitor and photographed after 12 h of culture. × 175. (*C*) Follicle formation from isolated thyroid cells in TSH-free synthetic medium (day 4 of culture). Cells from the same batch were plated onto poly-L-lysine-coated culture plastic flasks in NCTC 109 medium plus 0.25 mg/ml soybean trypsin inhibitor. At day 1, the medium was replaced with fresh NCTC 109 medium. Cells were organized into follicles from day 2 to day 13 of culture. Arrows indicate follicles. × 175. (*D*) Freshly isolated thyroid cells were plated onto poly-L-lysine-coated culture plastic flasks in Eagle's medium plus 0.25 mg/ml soybean trypsin inhibitor. At day 1, the medium was replaced with fresh Eagle's medium. At day 4, the cells formed a monolayer. × 175. (*E*) Same conditions as in C, but 200 μU/ml thyrotropin were added at the onset of culture. At day 4, follicles (arrows) resemble those obtained in the absence of thyrotropin. × 175. (*F*) Same conditions as in *C*. At day 4, cells were detached with a rubber scraper, centrifuged, fixed, and processed for electron microscopy. Cells are organized into follicles. Apical membranes are characterized by well-developed microvilli (*M*) directed towards the colloid-filled luminal space (*L*). × 3,000. (*G*) Freshly isolated thyroid cells were plated onto poly-L-lysine-coated glass surfaces in NCTC 109 synthetic medium plus 0.25 mg/ml soybean trypsin inhibitor. At day 1, the medium was replaced with fresh NCTC 109 medium. 5-d-old follicles observed under Nomarski optics. × 530. (*H*) Same culture and same field as in *G*. At day 5, cells were fixed in formaldehyde, permeabilized by acetonetreatment, and stained by an immunofluorescent sandwich technique using rabbit anti-aminopeptidase N IgG and fluorescein-conjugated goat anti rabbit IgG. Fluorescence linked to aminopeptidase N was mainly localized in the apical region of cell-forming-follicles (horizontal arrows). In some scarce cells, fluorescence was directed towards the medium (vertical arrows).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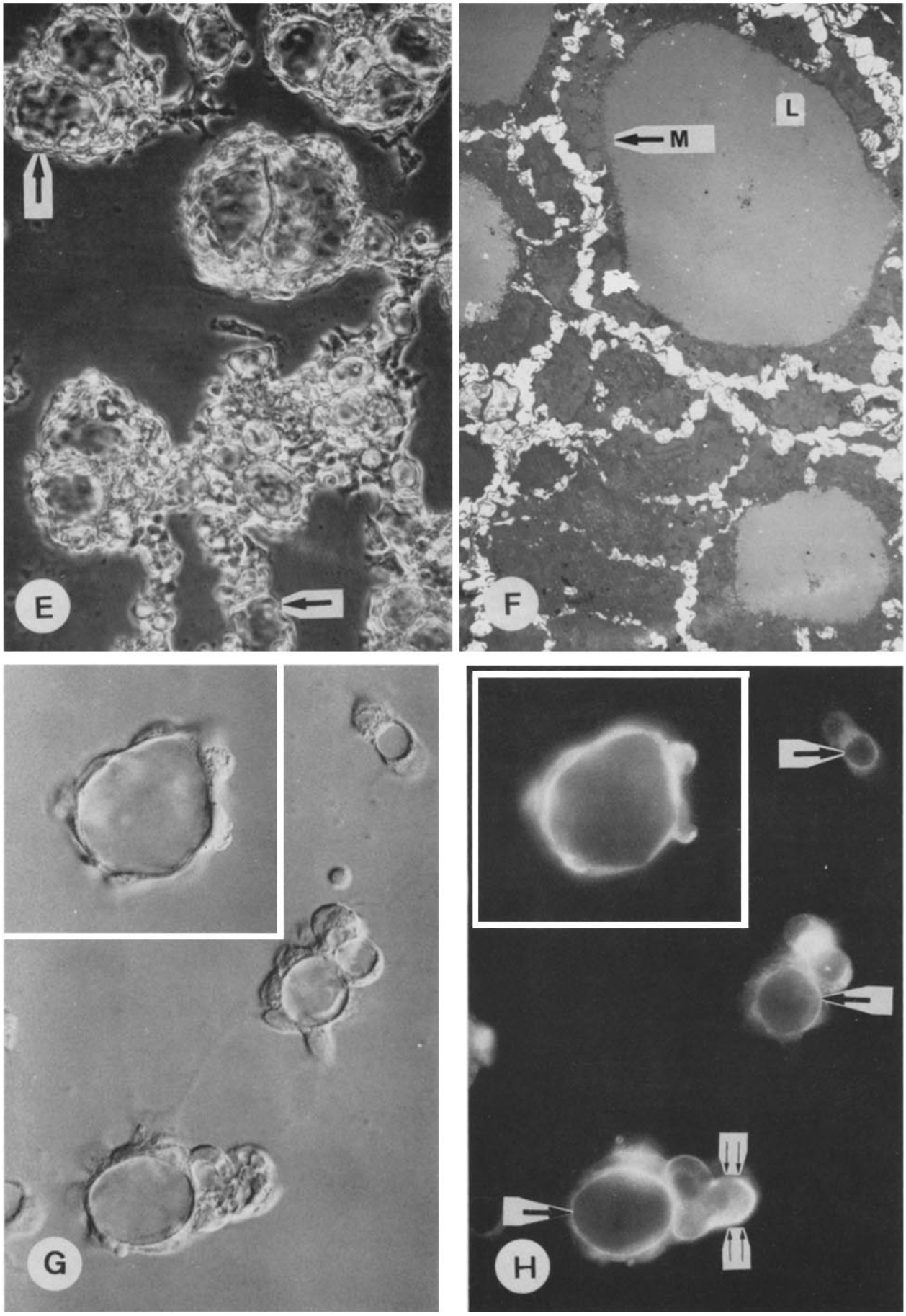
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at day 4 resulted in significant increases in the level of Tgb in the medium at days 6, 8, and 10, but not at day 13 where limiting factors in the medium may be interfering.

Fig. 6 *b* indicates that large amounts of Tgb were also present in the follicles of the cell layer portion, between days 2 and 13. In this case, addition of TSH (200 μU/ml) at day 4 of culture resulted in a small decrease in the level of Tgb present in the cell layer at day 6 and day 13. However, at days 8 and 10, this change was not significant.

Fig. 6 *c* represents the total production of Tgb, i.e., the sum of Tgb in the medium and in the cell layer. Assuming that 1 μg of DNA represents 62,500 cells (41), the rate of Tgb production may be calculated for control and TSH-stimulated cultures over the relatively linear period between day 4 and day 10. In control cultures, each cell produced ~8.2 pg of Tgb/d. After addition of TSH (200 μU/ml) at day 4, the amount of Tgb recovered was increased by a factor of 4.2, each TSH-stimulated cell producing 34.3 pg of Tgb/d.

These results demonstrate that in the present in vitro system, the production of Tgb was increased in response to TSH stimulation.

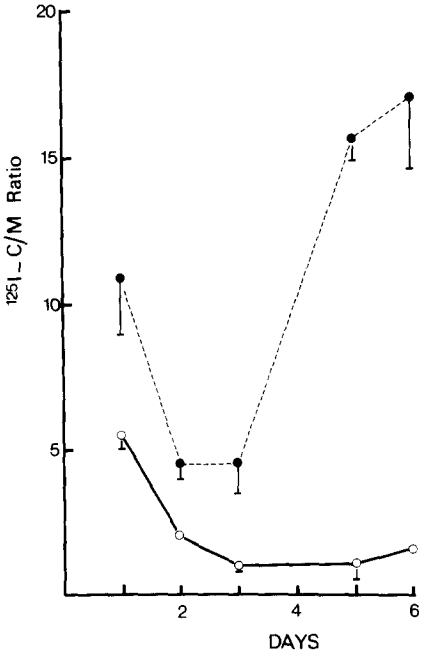

FIGURE 2 Effect of chronic TSH stimulation on iodide transport activity in primary porcine thyroid cell cultures. Iodide transport activity expressed as ^{125}I-C/M ratio was measured vs. days of culture. Cultures were performed either in NCTC 109 medium without TSH (solid line) or under chronic stimulation by 200 μU/ml TSH (dotted line). Each value is the mean of duplicate cultures.

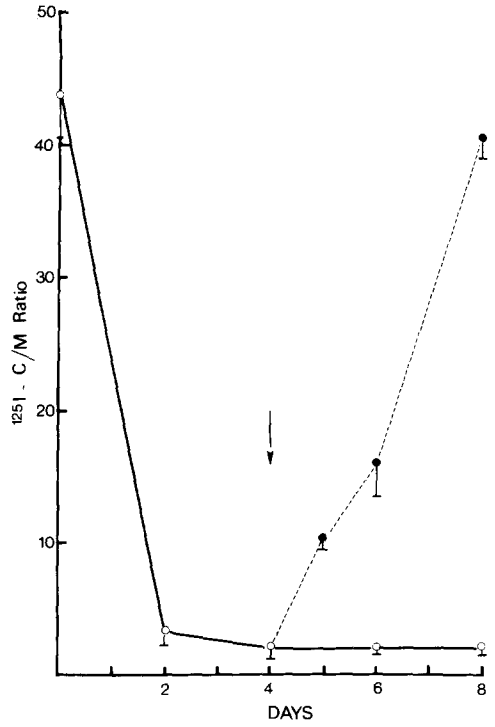

FIGURE 3 Effect of acute TSH stimulation on iodide transport activity. ^{125}I-C/M ratios were measured versus days of culture in NCTC 109 medium in the absence of TSH (solid line) or when an acute stimulation with 200 μU/ml TSH was performed at day 4 (dotted line). Each value is the mean of duplicate cultures.

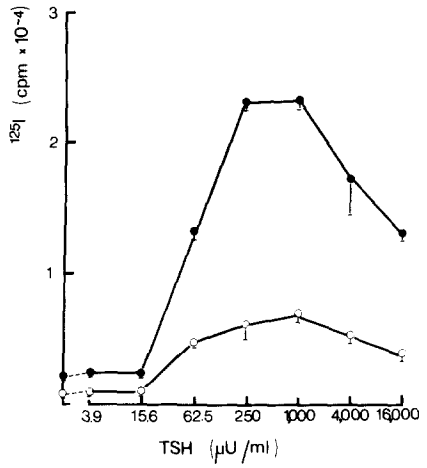

FIGURE 4 Modulation of iodide uptake and organification vs. doses of TSH acutely added to cell medium at day 4. In NCTC medium, at day 8 of culture, the ability of cells to take up and organify iodide was examined versus variable doses of TSH added at day 4. Total uptake (●), incorporation into trichloroacetic acid-insoluble material (○) over a 6-h period. Values are the means of independent cultures in triplicate. Results expressed as mean ± SD.

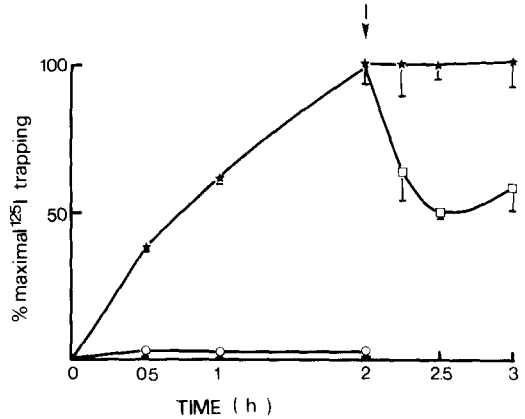

FIGURE 5 Efflux of iodide in TSH pretreated cultures. Follicle-associated cultures maintained in NCTC 109 medium were pretreated at day 3 with 50 μU/ml TSH and 2 mM Methimazole (MMI). After 24 h, the kinetics of iodide uptake by cell suspensions were analyzed over a 3-h period in the presence (○) and absence (★) of 1 mM perchlorate. At equilibrium, obtained near the second h, addition of 16 mU TSH/ml resulted in a fast release of iodide (□): 50% efflux within 30 min. Values are means ± SD of assays in triplicate.

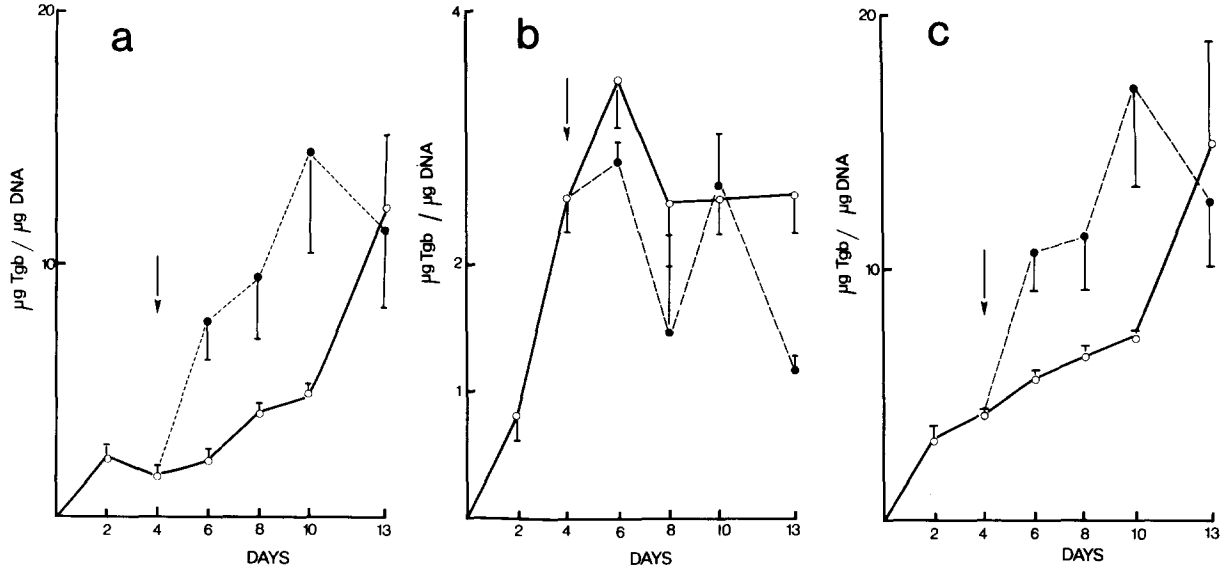

FIGURE 6 Basal and TSH-stimulated synthesis of Tgb. The levels of thyroglobulin (*Tgb*) were estimated by RIA between the onset of culture and day 13, in culture media (*a*) and in cell layers (*b*). The total amount of Tgb is represented in *c*. All results are expressed in μg Tgb/μg DNA in the cell layer. (*a*) Tgb is released into the culture media vs. days of culture (solid line). Addition of 200 μU/ml TSH at day 4 (arrow) resulted in an increase in concentration of Tgb in the medium (dotted line). (*b*) As expected from electron microscopy (Fig. 1 *F*), Tgb was also present in the follicles of the cell layer between day 2 and day 13 (solid line). Addition of 200 μU/ml TSH at day 4 of culture (arrow) resulted in a small decrease in the level of Tgb present in the cell layer (dotted line). (*c*) The total production of Tgb from the onset of culture to day 13 (solid line) was greatly influenced by the acute addition at day 4 of 200 μU/ml TSH (dotted line). Basal production of Tgb was 8.2 pg/cell/d and 34.3 pg/cell/d after TSH stimulation.

To confirm the presence in culture media of the large amount (500 μg/ml at day 6) of Tgb which has been measured by RIA, samples of culture media were subjected directly to SDS PAGE. As shown in Fig. 7, analysis of culture media yielded an electrophoretic pattern containing one major protein band which migrated in the same position as the porcine Tgb 12 S subunit that derived from the dissociation in SDS of the high molecular weight Tgb 19 S dimer. In control experiments, incubation of cells with radioactive [^{125}I]iodide resulted in the biosynthesis of a labeled protein showing an exclusion pattern in Sephadex G 200 gel filtration similar to that of pure porcine 19 S Tgb (not shown).

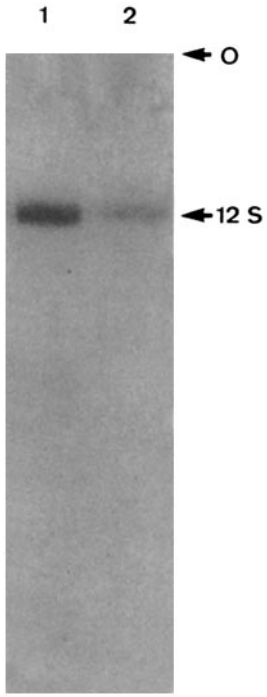

FIGURE 7 SDS PAGE of serum-free cell culture media. Lane *1*: NCTC 109 medium from a 10-day-old culture (50 μl). Lane *2*: Purified porcine thyroglobulin (8 μg Tgb in 50 μl). Arrow indicates the position of the 12 S subunit (M_r 330,000) of Tgb. *O*, origin of migration.

DISCUSSION

In previous studies performed in primary culture with surface-adhering thyroid cells derived from adult animals, TSH was found to be a constituent of culture media necessary to obtain the reorganization of cells into follicles (13, 34, 36). All the culture systems employed used serum-supplemented media and, in general, the period of follicular organization was followed by the spreading of cells into a monolayer. The use of cell suspension cultures in 10% serum-supplemented media gave the same results (32, 45, 46), and TSH was also found necessary for the reorganization of cells into follicles. In the absence of TSH, cells formed in suspension dysfunctional pseudofollicles similar to spherical, closed monolayer structures in which the apical pole of cells was oriented towards the medium (32, 45, 46).

Recent reports have indicated that a reduction of the serum concentration in, or its elimination from, the culture media had a permissive effect on the formation and the maintenance of thyroid follicles in vitro. Ambesi-Impiombato et al. (1, 2) described the long-term preservation of differentiation characteristics in a rat thyroid cell line (FRT_L) which was maintained in hormone (including TSH)-supplemented medium containing only 0.5% serum. With this low concentration of serum (0.5%), Nitsch and Wollman (48) prepared TSH-sensitive rat thyroid follicles in suspension culture from clusters obtained by differential filtration through nylon meshes. Follicles formed within 1 d from resealing of partly disrupted follicles. Increasing the serum concentration to 5% or using a few unusual batches of serum resulted in inversion of follicles in 1 or 2 d (49). Recently, Fayet and Hovsépian (21) showed

that, under serum-free conditions, dysfunctional monolayer cultures of porcine thyroid cells could be converted into functional follicle-associated cells in the presence of TSH. This latter result was confirmed and extended by Dickson et al. (8) using human thyroid cells isolated from goitres of patients with Graves's disease. With these human cell cultures, the use of serum-free culture conditions was critical for TSH-induced follicle formation. These data suggested that the use of a decreasing serum concentration may result in the limitation of the cell-substratum interaction, thus allowing cellular aggregation which is a prerequisite to follicle formation.

However, the use of poly-L-lysine that increases cell-substratum adhesion appears contradictory to the limitation of cell-substratum interaction for instance in suspension cultures. In fact, as we demonstrate here, trypsin-isolated thyroid cells aggregate on such psiively charged surfaces and form follicles. Furthermore, no monolayers were formed from follicle-associated cells even at day 13, the last day so far tested. One explanation for the above discrepancy may be that in the poly-L-lysine increasing cell-substratum interaction, the delay in obtaining cell fixation is shortened to <1 h. Thus, the highest cell densities are rapidly obtained, allowing efficient cell-cell interactions. The importance of cellular concentration at the onset of culturing (i.e., the number of cells per unit surface) in obtaining follicle formation has been previously mentioned (13, 14). Consequently, in the absence of serum, cell-cell interaction is favored as compared to cell-substratum interaction.

As shown by the results obtained here with Eagle's medium, serum-free conditions associated with poly-L-lysine treatment were obviously not sufficient to induce follicle formation. The formation of follicles in the absence of TSH required the use of a more complex medium. Comparative studies performed with Eagle's medium, RPMI 1640, Ham's F12, medium 199, and NCTC 109 demonstrated that the reorganization of cells into follicles was obtained only in NCTC 109 and, to a lesser extent, in medium 199. Coon's modified F12 medium was used by others for the survival of rat thyroid cells in suspension (48), but NCTC 109 is an easily commercially available medium that has been also selected previously to obtain a better activity in culture of beating heart cells (16) and in obtaining the growth of porcine cells in monolayers (20). In comparison to Eagle's medium, NCTC 109 contains additional coenzymes (NAD, NADH, CoA, FAD), nucleotides (UTP, deoxyguanosine, thymidine, 5-methyl cytosine), and a wider spectrum of vitamins and amino acids. The dramatic effects of NCTC 109 on the reorganization of cells into follicles, in comparison to Eagle's medium, suggests that one or more of these additional components is of importance in the reorganization process. In this respect, a closer definition of the metabolic requirements for follicle formation might be derived from a systematic study of the effect of deleting individual or groups of components from the full NCTC formula, and careful comparison with medium 199 and Coon's modified F12 medium.

With regard to the influence of TSH on follicle formation, the in vivo development of embryonic thyroid tissue was shown to be TSH-independent (25, 33, 66). Under in vitro conditions, Hilfer (30) demonstrated that chick embryo thyroid cells in monolayer culture will reorganize into follicles in the absence of TSH when co-cultured with mesenchymal cells derived from thyroid capsule or heart tissue. The epithelial cells themselves, however, seem to be responsible for the pattern of lobulation within the developing gland (31). Indeed, using trypsin-isolated thyroid cells from adult rat glands, Mallette and Anthony (44) described the reformation of follicles in the absence of added TSH under culture conditions of high cell density and limited cell mobility. The transitory formation of small follicular structures has been observed even in the absence of TSH (5), but these structures were not stable and were lost as the cells spread to form a monolayer. A similar situation has been observed in cultures derived from collagenase-treated dog thyroid tissue (54). The collagenase treatment released intact follicles from the tissue but, when these structures were placed in culture using serum-supplemented medium, the cells rapidly spread to form a monolayer with the concomitant loss of follicular architecture.

In our experiments we used freshly isolated thyroid cells obtained by the trypsinization procedure. The isolated cells have lost their microvilli (5), and the apical membrane marker aminopeptidase N was randomly distributed all over the cell surface (22, 23), indicating a loss of morphological and molecular polarity. The morphogenesis of follicles (5) and the kinetic studies in aminopeptidase localization versus follicle-formation (S. Hovsépian et al., manuscript submitted for publication) indicated that the mechanism of polarization of thyroid cells does not concern preferentially the small clumps of cells observed after trypsinization (Fig. 1*A*) but large aggregates obtained during the first day of culture. The mechanism of follicular formation in the present system is certainly very different from the process of resealing of open follicles described by Nitsch and Wollman (48). Furthermore, in this latter system, the possibility of an action of a small number of endogenous TSH molecules bound to the cellular receptors cannot be ruled out, especially because cells were isolated by collagenase treatment. In this work, cells were isolated by trypsin that destroys thyrotropin receptors (42); consequently, this argument is less consistent when applied to the present data but cannot be really circumvented. An important argument, indicating that TSH in vitro does not direct follicle formation, was given using thyroid cells cultured in the presence of gelatin according to the technique of follicle formation in suspension described by Mauchamp and Fayet (45). Follicle reconstruction in this case was not accompanied by any change in cyclic AMP levels (60).

With respect to normal in vivo morphology and physiology, the primary culture of isolated porcine thyroid cells in the serum-free NCTC 109 medium appears to represent a new model of the intact thyroid gland. The system requires trypsin-isolated porcine thyroid cells that can be obtained in amounts (62) compatible with biochemical studies, a commercially available medium, and a poly-L-lysine-treated cell culture support easy to prepare. Furthermore, the cell attached-stationary-culture system is easier to manage than the cell-suspension technique, especially with regard to cell culture medium changes and microscopic examination with immunofluorescence techniques. Unlike the situation with follicle-associated cultures in serum- and TSH-containing medium (15), all follicles obtained in the present study contained electron-dense colloid material. While the cells involved in a follicular structure showed well-developed polarity with characteristic apical microvilli, no evidence was found for the presence of basement membrane.

In resting cultures, the ^{125}I-C/M ratio fell to a steady basal value close to the theoretical level of 1 which indicates the passive diffusion of iodide into cells. A similar effect is observed in vivo in the absence of circulating TSH which follows hypophysectomy or T_4 administration (27). TSH stimulation of the cultures was shown to result in an initial efflux of iodide

followed by a dose-dependent increase in iodide uptake and organification. This biphasic pattern is characteristic of the in vivo response of the intact thyroid gland to TSH administration (28).

In addition to its effect on iodide trapping, TSH was found to have a stimulating action on the Tgb production of thyroid cells in culture. Studies on isolated thyroid cells have shown that TSH enhances protein synthesis (63, 64) probably via increased RNA transcription (6, 58). However, the classical in vivo (50) and in vitro (39, 40) response to TSH on the increase in protein synthesis has not been reproduced with previous cultured thyroid cell systems (41). Differences in Tgb messenger RNA levels were obtained (7) in thyroid cells cultured with or without TSH, but the nonstimulated cells in these studies were not fully stimulable by TSH and were partially dedifferentiated. In this study, the production of Tgb was increased from 8.2 pg/cell/d in resting cultures to 34.3 pg/cell/d after TSH stimulation. Noniodinated and poorly iodinated Tgb are known to fully dissociate into 12 S subunits under a variety of conditions and especially in the presence of detergents (43). While it must be expected that the culture media contained trace amounts of iodide contaminating the salts used for medium preparation, it is probable that the Tgb produced by the cells represents material containing very low amounts of organically bound iodine, i.e., fully dissociable into 12 S subunits in SDS PAGE. Indeed, the protein material present in the culture media migrated in SDS PAGE in the same position as the authentic 12 S porcine Tgb subunit (Fig. 7). In addition, its immunoreactivity towards antibodies to porcine thyroglobulin and its behaviour after Sephadex G-200 gel filtration demonstrate that the material detected in the culture media is the noniodinated or very poorly iodinated 17–18 S dimer of Tgb. Since ~0.5 mg Tgb/ml of culture medium can be recovered over a 6-d period, the primary cultures described in this study represent a source of noniodinated Tgb yielding sufficient quantities to permit structural studies. Regarding the level of Tgb in the follicles of the cell layer, this was shown to decrease slightly in the 48-h period after TSH stimulation. This result may, however, reflect a phenomenon equivalent to the known in vivo effects of TSH on endocytosis of colloid and reduction in luminal colloid (57). Once within the phagolysosome system, Tgb is digested to release free thyroid hormones. Detailed electron microscopic studies and T_4 estimations are underway to determine whether endocytotic response to TSH occurs in this system.

As previously observed (41), most of the produced Tgb molecules were released into the cell culture medium. This situation is abnormal when compared with the extremely small amount of blood-circulating Tgb in the animal. One explanation is that Tgb detected in the media is secreted by some cells exposing an orientation of their apical pole towards the medium as suggested by Fig. 1 *H*, vertical arrows. But in this hypothesis the very small percentage of misoriented cells would not explain that 80–90% of the total production of Tgb is exported into the medium. A second interpretation is linked to the disorders in membrane antigens topography accompanying the restructuring of polarity of cells in culture. According to the recent kinetic studies of the localization of aminopeptidase in cells vs. days of culture (S. Hovsépian, H. Feracci, S. Maroux, and G. Fayet. 1982. *Cell Tissue Res.* In press), the polarization of cells in terms of microvillus formation occurs between days 1 and 4 although the total disappearance of aminopeptidase from the basolateral membranes (which is not complete at day 5) will be achieved only at day 7. However, this argumentation does not explain the abnormal exportation of Tgb which is still observed after day 7 of culture according to Fig. 6 *c*. Consequently, the molecular aspects of the secretion of Tgb in this system is still unexplained.

In conclusion, our results show that porcine thyroid cells can be maintained, in vitro, as resting, highly differentiated, follicle-associated cultures even in the absence of TSH. The cells are sensitive to TSH and show some acute and long-term responses characteristic of the intact thyroid gland in vivo. To correlate morphological and biochemical data on the in vitro effects of TSH on thyroid cells, intact follicular architecture is required in both control and hormone-stimulated cultures, as is the case with in vivo studies. The primary culture of porcine thyroid cells in serum-free NCTC 109 medium satisfies this requirement and the system thus brings the facility and potential of in vitro experimentation to the investigation of both the morphological and biochemical responses of thyroid cells to TSH stimulation.

The authors thank Drs. S. Maroux and H. Feracci for their help in immunofluorescence studies and Miss M. Viot for technical assistance in electron microscopy controls.

This work was supported by Institut National de la Santé et de la Recherche Medicale ATP 58.78.90 and CRL 79.5.205.4.

Received for publication 12 May 1981, and in revised form 11 January 1982.

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