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Transplantation of Monodispersed Rat Thyroid Cells: Hormonal Effects on Follicular Unit Development and Morphology (40730)

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Cell transplantation techniques have long been used in the study of tumor biology (1) and recently to evaluate the growth and development of organized tissues following the injection of monodispersed suspensions of normal cells (2–6). In order to extrapolate the relationships observed with such models to the events occurring in the organized tissue *in situ* it is necessary to characterize the morphological properties of the structures which develop following transplantation of monodispersed cells. This report describes the morphology of the follicular units during their development in recipient animals following the inoculation of monodispersed rat thyroid cells and the response of these structures to changes in the hormonal status of the recipients.

Materials and methods. Animals. Donors and recipients were 4- to 5-week-old male rats of the inbred Fischer strain. Some recipients were thyroidectomized under light ether anesthesia the day before cell injection and were maintained on drinking water supplemented with 1% $CaCl_2$ for 24 hr after surgery to reduce the incidence of tetany.

Thyroid cell preparation. Details of the enzymatic dispersion technique have been reported (2, 3). Briefly, thyroid glands were dissected free from the trachea, pooled, and minced in chilled Medium 199. The minces were then incubated for 2 hr in a collagenase solution at 37°C followed by 1.25% Pronase for 90 min at 4°C and then passed through a 53-μm cloth filter to remove cell aggregates. A small amount of DNase was added to reduce cell clumping. Appropriate dilutions of known numbers of cells were prepared in fresh Medium 199 with the use of a hemocytometer and high-power phase microscopy. Only nucleated cells with intact plasma membranes were counted as morphologically "viable."

Cell transplantation. Aliquots containing 100,000 monodispersed thyroid cells in 0.06 ml Medium 199 and brain homogenate (1:1) (5, 6) were injected into the cephalic portion of the left and right inguinal white fat pads of thyroidectomized or nonthyroidectomized recipients. Seven recipient animals were placed in each of four experimental groups: thyroidectomized recipients fed low iodine diet (TX-LID), nonthyroidectomized recipients fed low iodine diet (NTX-LID), thyroidectomized recipients fed normal diet (TX-ND), and nonthyroidectomized recipients fed normal diet (NTX-ND).

Autopsy. Animals from each group were killed 1, 2, 4, 5, 7, 14, and 28 days after cell transplantation. The transplantation sites were removed and prepared for light or electron microscopy.

Preparation for microscopy. Tissues fixed in Hollande–Bouin's solution for light microscopic studies were dehydrated in a graded ethanol series, cleared in xylene, and embedded in JB-4 glycol methacrylate. Tissues used for immunohistochemical studies were fixed in 4% neutral buffered formalin, dehydrated, embedded in paraffin, and sectioned at 6 μm. For ultrastructural examination, monodispersed rat thyroid cells were fixed in 2.5% glutaraldehyde in 0.1 *M* sodium cacodylate buffer (320 mOs*m*, pH 7.2) for 1 hr on ice after completion of the final step in the enzymatic dispersion technique. A 0.2-ml aliquot of the glutaraldehyde-cell suspension was rapidly mixed with 0.2 ml of 15% bovine serum albumin. After centrifuging at 800*g* in plastic microfuge tubes

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for 3 min, the pellet was partially dried in a 37°C oven. The pellet was then diced into small pieces and fixation was completed in the same fixative–buffer solution for 30 min at room temperature.

Fixation of transplant sites for electron microscopic examination was initiated *in situ* by flooding the exposed sites with 2.5% glutaraldehyde in 0.1 *M* sodium cacodylate buffer. The sites were removed, cut into small pieces, and further fixed in the same solution for 1 hr at room temperature. All tissue pieces were postfixed in 1% osmium tetroxide. The tissue pieces were embedded in Epon–Araldite. Thin sections were cut and stained with uranyl acetate and lead citrate and examined in a Philips 300 electron microscope.

Immunohistochemistry. The peroxidase antiperoxidase (PAP) bridge technique was utilized to demonstrate the presence of thyrocalcitonin (TCT) containing "C" cells in the graft sites. It has been reported that human and rat TCT are structurally similar (7) and can cross-react in immunocytochemical techniques (8). Commercially available rabbit anti-human TCT serum was therefore used in these studies. Briefly, several drops of rabbit anti-human TCT (RAHTCT), diluted 1:80 with Tris-buffered saline, were placed directly on the tissue sections which were then incubated overnight in a moist chamber at room temperature. The sections were then sequentially treated with goat anti-rabbit IgG Serum and a rabbit PAP complex for 30 min each. Following thorough washing, the sections were incubated with 0.05% 3,3'-diaminobenzidine HCl–0.01% hydrogen peroxide solution for 10 min. The sections were counterstained with weak hematoxylin, dehydrated, and mounted.

The specificity of the primary antiserum (RAHTCT) was evaluated by replacing the RAHTCT with nonimmune rabbit serum or with RAHTCT which had been previously absorbed with increasing amounts of purified synthetic human thyrocalcitonin (HTCT). Briefly, 200 μl of 1:40 RAHTCT was incubated with 200 μl of HTCT at 37°C for 1 hr followed by incubation for 24 hr at 4°C. This mixture was then centrifuged at 5000 rpm for 20 min and the supernatant used to replace the RAHTCT in the PAP staining reaction.

Sections of liver, skin, parathyroid, sublingual gland, and parotid gland served as negative controls. Normal rat thyroid served as the positive control.

Results. Ultrastructure of monodispersed cells. Little ultrastructural evidence of cellular damage was detected in the overwhelming majority of enzymatically dispersed thyroid cells. No intact follicular structures were observed, and almost all of the cells were found to be dissociated (Fig. 1). Occasional small aggregates of cells still joined together by intact junctional complexes were seen (Fig. 2). Microvillous projections were still evident along the apices of these associated cells. In contrast, the dissociated cells had spherical profiles

FIG. 1. Electron micrograph of monodispersed rat thyroid cells. Several cells contain colloid droplets (wide arrows). Two cells are still attached at the position of the apical junctional complex (narrow arrows). ×4800.

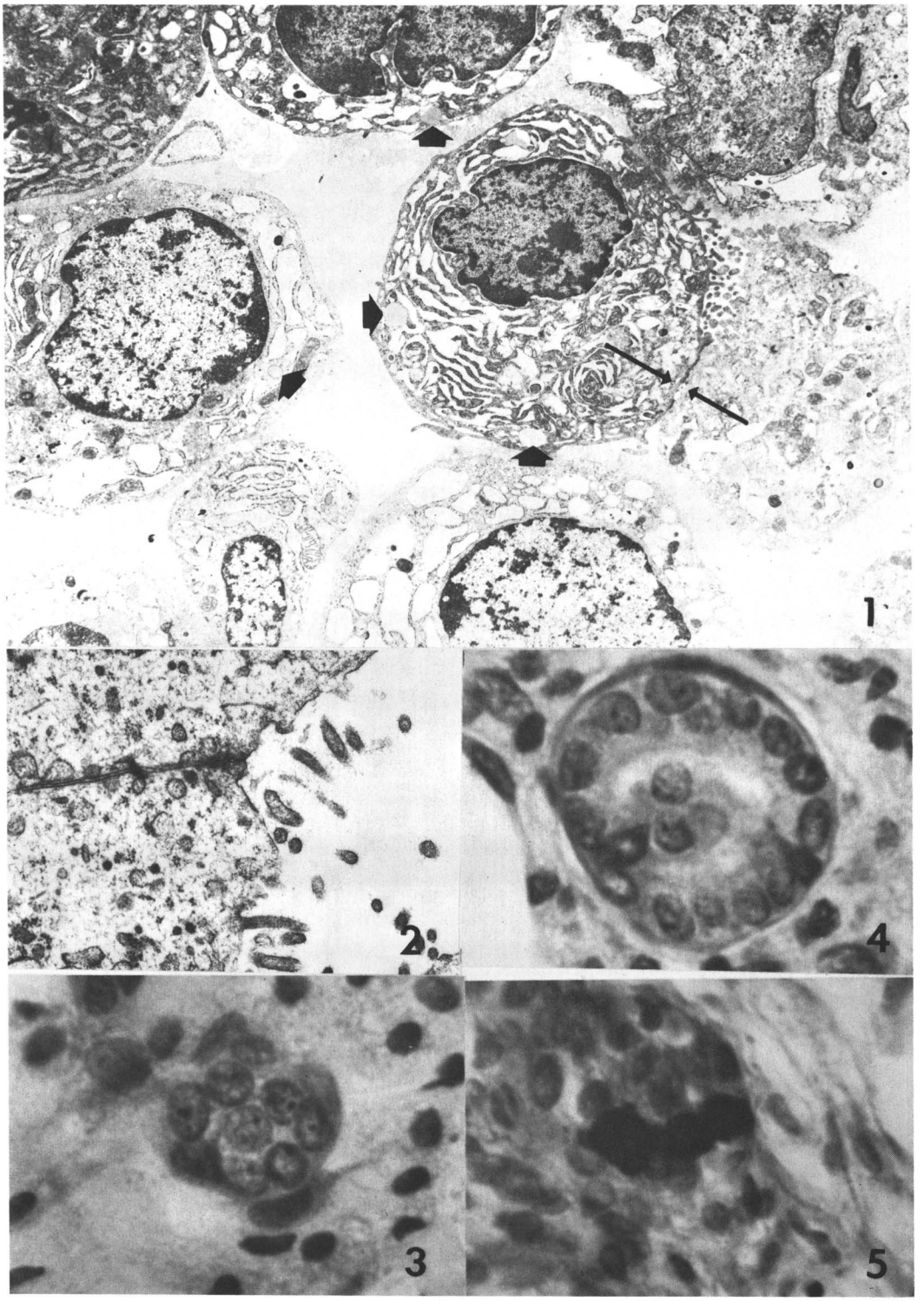
FIG. 2. Electron micrograph of junctional complex between two "associated" rat thyroid cells. Note the retention of microvilli. ×11,280.

FIG. 3. Solid, organized epithelial structure common in all graft sites at Days 4 and 5. These spheres are generally two or three cells in diameter. ×700, PAS + hematoxylin.

FIG. 4. "Follicular unit" with patent lumen 5 days after thyroid cell transplantation. The cell nuclei are basally located. ×750, PAS + hematoxylin.

FIG. 5. Immunoperoxidase positive C cells were present in graft sites 28 days after cell grafting. See text for details. ×760, weak hematoxylin counterstain.

FIGS. 6–9. Sections through graft sites from: TX-LID recipients, Fig. 6; NTX-LID recipients, Fig. 7; TX-ND recipients, Fig. 8; NTX-ND recipients, Fig. 9; 28 days after transplantation. Note PAS+ granulated cells in follicles from TX-ND recipients (arrows) Fig. 8. For comparisons of relative follicular diameters and epithelial cell heights all four figures were printed at the same magnification. ×340, PAS + hematoxylin.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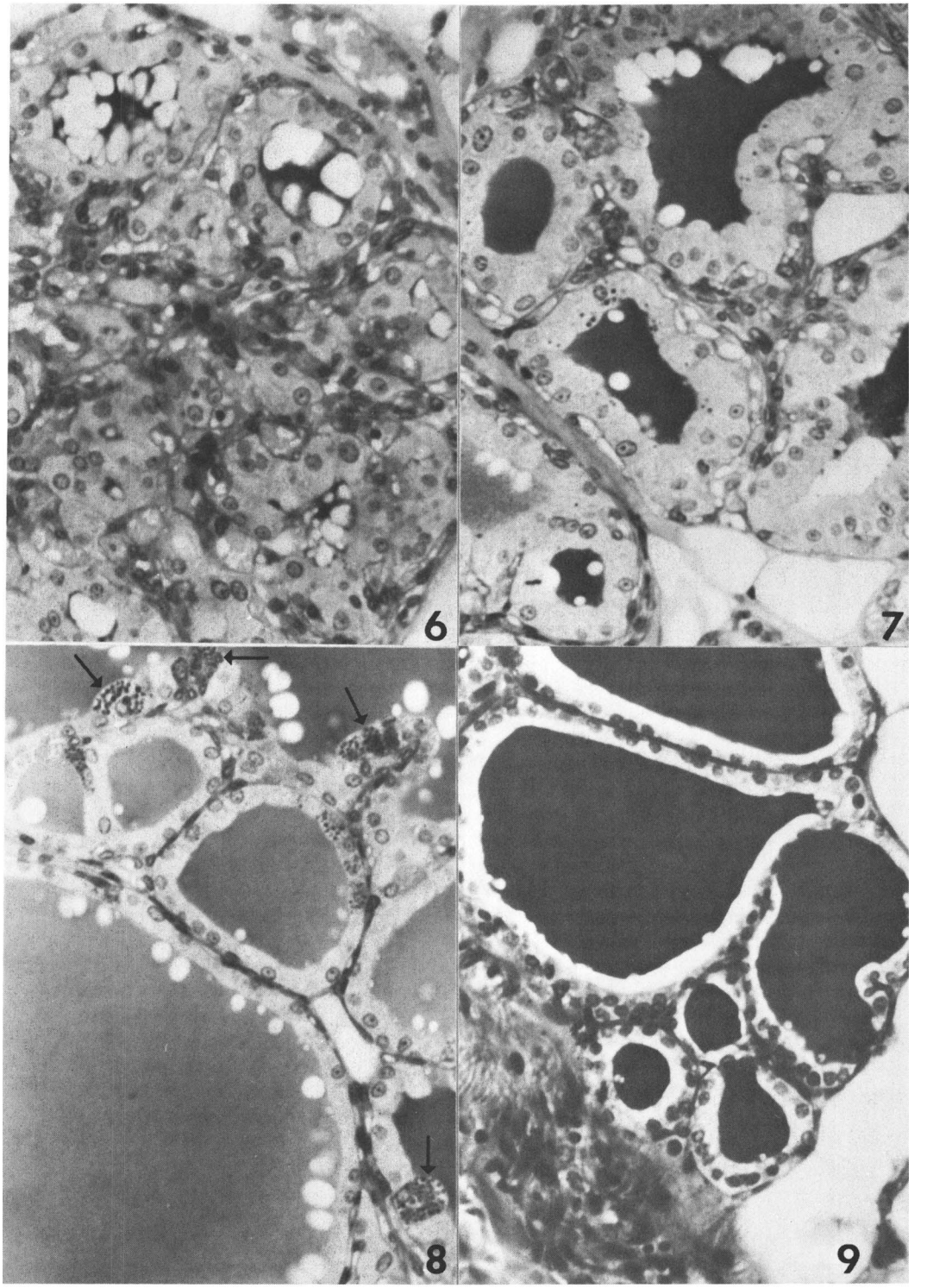
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free of microvilli. The mitochondria, Golgi apparatus, lysosomes, endoplasmic reticulum, and nuclei all appeared normal.

Transplant site morphology. As revealed by light microscopy, the chronological sequence of follicular development from Days 1 to 7 was identical for all four experimental recipient conditions. One and two days after grafting an intense acute inflammatory reaction, characterized by massive infiltration by polymorphonuclear leukocytes (PMNs), developed at the injection sites. The surrounding tissue was hemorrhagic and edematous. No organized epithelial structures were observed.

By Day 4 the acute inflammatory reaction was greatly reduced, but occasional PMNs remained. Solid spheres of organized epithelial cells, two to three cells in diameter, could be identified for the first time. The large, pale nuclei of the cells in these spheres often contained one or two prominent nucleoli.

By Day 5, these organized epithelial structures were plentiful in all transplant sites (Fig. 3). In addition, spherical structures consisting of a single layer of cuboidal epithelial cells surrounding an empty, central lumen (Fig. 4) were scattered throughout many of the transplant sites. The large, oval nuclei of the follicular cells were basally located in close apposition to the basement membrane. No periodic acid–Schiff positive (PAS+) colloid material was detectable at this early stage of follicular development.

On the seventh day following injection of monodispersed thyroid cells, morphologically normal follicular structures were present in all the sites examined. These follicular units were much more heterogeneous with respect to size and shape than those observed at Day 5. The cells of the epithelial lining were generally low columnar with a small nuclear to cytoplasmic ratio. The nuclei were still adlumenally positioned. Mitotic figures were plentiful. A fine network of capillaries developed in close association with each of the differentiating follicular units. PAS+ colloid-like material was present along the apical border of the epithelial cells of follicular units growing in TX-ND recipients. However, no PAS+ material was observed in follicular units in hosts of the other treatment groups at this time.

Morphological variation was more pronounced by Day 14. All follicular units still consisted of a single epithelial lining around a central lumen which contained PAS+ colloid material. In general, the epithelial cells were cuboidal in NTX-LID and NTX-ND recipients, whereas they were columnar in TX-LID and TX-ND hosts. Scalloping of the lumenal colloid, which appeared granular in recipients maintained on low iodine diets and glassy in animals maintained on normal diets, was evident in all conditions. Nuclear characteristics were similar in all groups; mitoses were frequently observed. PAS+ granules occurred in the cytoplasm of some follicular cells growing in TX-ND recipients.

Differences in cell size and follicular diameter were the major morphological variations observed among the various experimental groups by light microscopy 28 days after transplantation (Table I, Figs. 6–9). In addition, large cells filled with spherical PAS+ granules were numerous in nearly every follicle growing in TX-ND recipients (Fig. 8). Occasionally the nuclei of these cells appeared pyknotic. The follicular lumen often contained numerous degenerating granulated cells.

Ninety-eight random sections of tissue from six different transplant sites were stained by the immunoperoxidase technique and examined for the presence of calcitonin containing C cells. Immunoreactive cells (Fig. 5) were found in at least one section from five out of the six transplant sites. A total of only 22 positive reacting C cells were found in the 98 sections examined. C cells have since been identified within the follicular basement membrane by electron microscopy.

The specificity of the immunohistochemical technique was demonstrated by: a gradual decrease in the staining of C cells in control thyroid tissue with increasing dilution of the primary antisera, loss of staining when nonimmune rabbit serum was used in place of the primary antiserum, and similar staining of the same cells in adjacent serial sections. The specificity of the pri-

TABLE I. FOLLICULAR DIAMETER AND EPITHELIAL CELL HEIGHT 28 DAYS POST-TRANSPLANTATION

Recipient condition	Follicular diameter (μm)	Epithelial cell height (μm)
Control	90.4 ± 4.2 (99)[a]	7.7 ± 0.2 (102)
Control (LID, 28 days)	73.9 ± 3.3 (98)	11.4 ± 0.2 (90)
Low iodine diet + thyroidectomized	64.0 ± 2.9 (112)	12.5 ± 0.5 (82)
Low iodine diet + intact thyroid	94.8 ± 4.6 (96)	19.5 ± 0.5 (64)
Normal diet + thyroidectomized	134.9 ± 9.3 (92)	13.7 ± 0.4 (100)
Normal diet + intact thyroid	75.9 ± 6.7 (29)	7.8 ± 0.5 (25)

[a] Mean ± SE (n). Analysis of variance, $P < 0.005$.

mary antiserum for thyrocalcitonin was evaluated by absorbing it with increasing concentrations of synthetic HTCT, and then testing the absorbed antisera in the staining sequence. The staining ability of the antiserum decreased when absorbed with increasing concentrations of HTCT until no staining reaction was observed when more than 1 μg of hormone was incubated with 400 μl of the primary antiserum.

Mast cells, normal residents in white adipose tissue, were plentiful in and around all the graft sites.

Follicular ultrastructure. The epithelial cells of follicles growing in TX-LID recipients were attached by intact junctional complexes. Apical borders were convex and protruded into the lumen, which contained very sparse colloid material. Microvilli appeared to be reduced in number as compared to control thyroids. Occasional pleomorphic blebs protruded into the lumen from the apical surfaces of the follicular cells. The endoplasmic reticulum was markedly dilated and contained fine granular electron-dense material. Although the Golgi apparatus was usually hypertrophied, the number of small apical secretory vesicles was reduced in comparison with control thyroid tissue. Mitochondria were elongated and were frequently branched. A general increase in epithelial cell surface area was evident as complex interdigitations along the lateral and basal cell borders. Cytoplasmic colloid droplets were not detected. With the exception of occasional mitochondrial membrane ruptures, other organelles appeared normal.

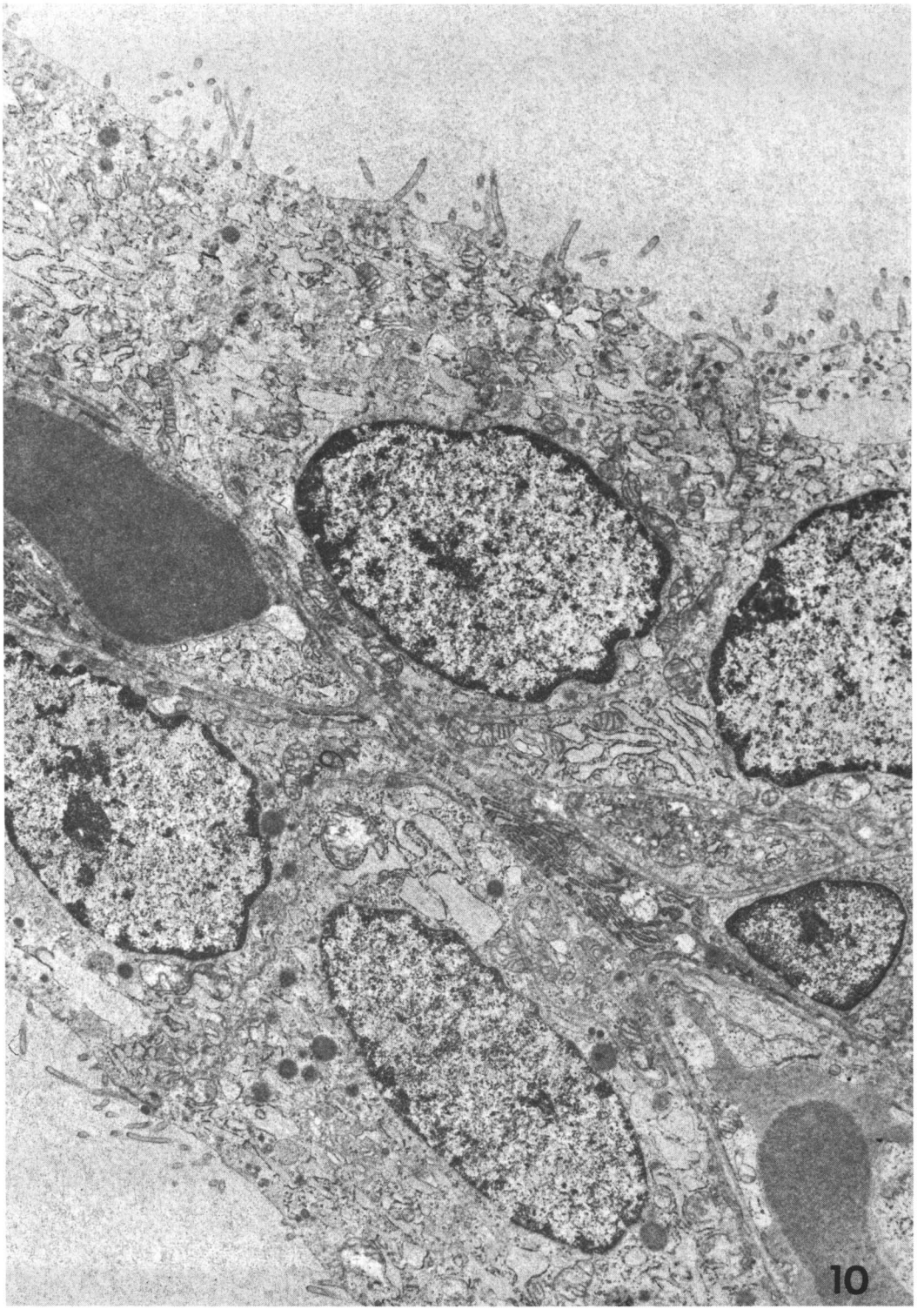
The epithelium of follicular units developing in NTX-LID recipients consisted of a single layer of hyperplastic columnar cells with rounded apices which protruded into the lumen. Microvilli were numerous along the apical border. The endoplasmic reticulum was markedly dilated and contained granular, electron-dense material similar in appearance to the lumenal colloid. The Golgi bodies were hyperplastic and apical secretory vesicles were numerous. Cytoplasmic colloid droplets were frequently observed. There was very little ultrastructural evidence of mitochondrial damage. All other organelles were normal.

Four weeks after inoculation most of the follicular epithelial cells growing in TX-ND recipients were cuboidal or low columnar (Fig. 10), but foci of tall, hyperplastic cells were common. All the follicular cells were connected by intact junctional complexes. The apical borders of the low columnar and cuboidal cells were flat and lined with abundant microvilli (Fig. 10). All the organelles, including the endoplasmic reticulum and mitochondria, appeared normal in

FIG. 10. Electron micrograph through follicular cells of two adjacent follicular units from TX-ND recipients 28 days after transplantation of monodispersed rat thyroid cells. The epithelial cells in both follicular units are closely associated with underlying follicular capillaries. Colloid material completely fills each lumen. ×7480.

FIG. 11. Electron micrograph of a section through the apical portion of a hyperplastic, PAS+ granulated epithelial cell form a TX-ND recipient. The large, membrane-bounded droplets contain material which is similar in appearance to the lumenal colloid. ×16,000.

FIG. 12. Electron micrograph through a PAS+ granulated cell from a TX-ND recipient. Note the increased electron density of the cytoplasm, and the ruptured mitochondria. ×15,400.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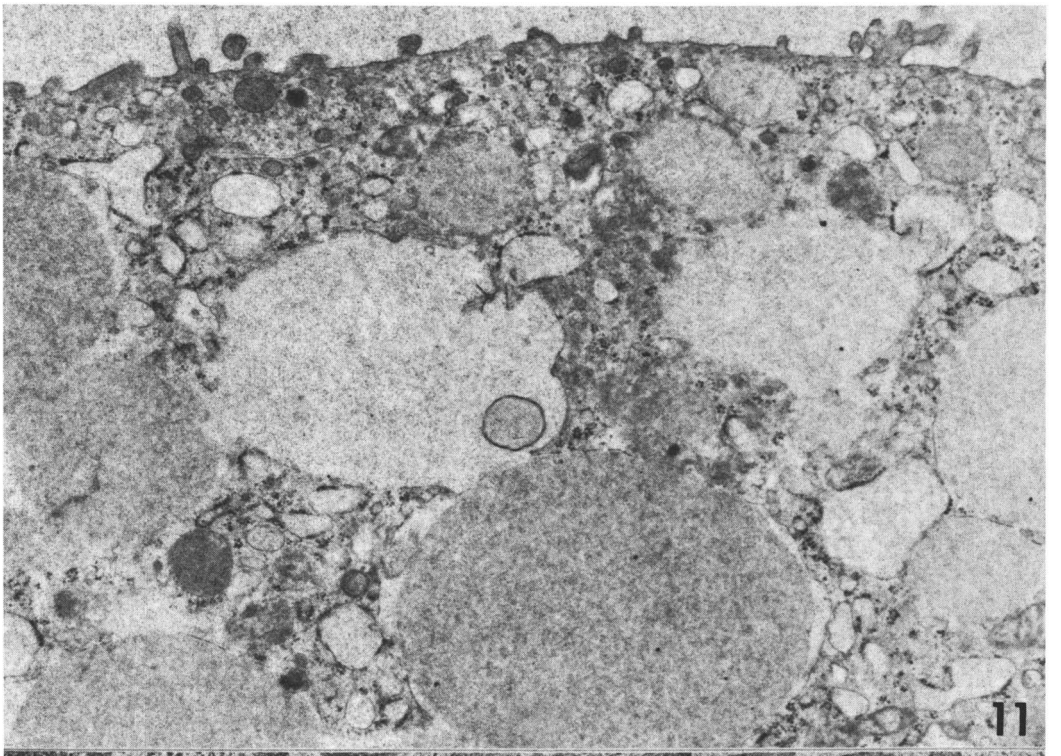

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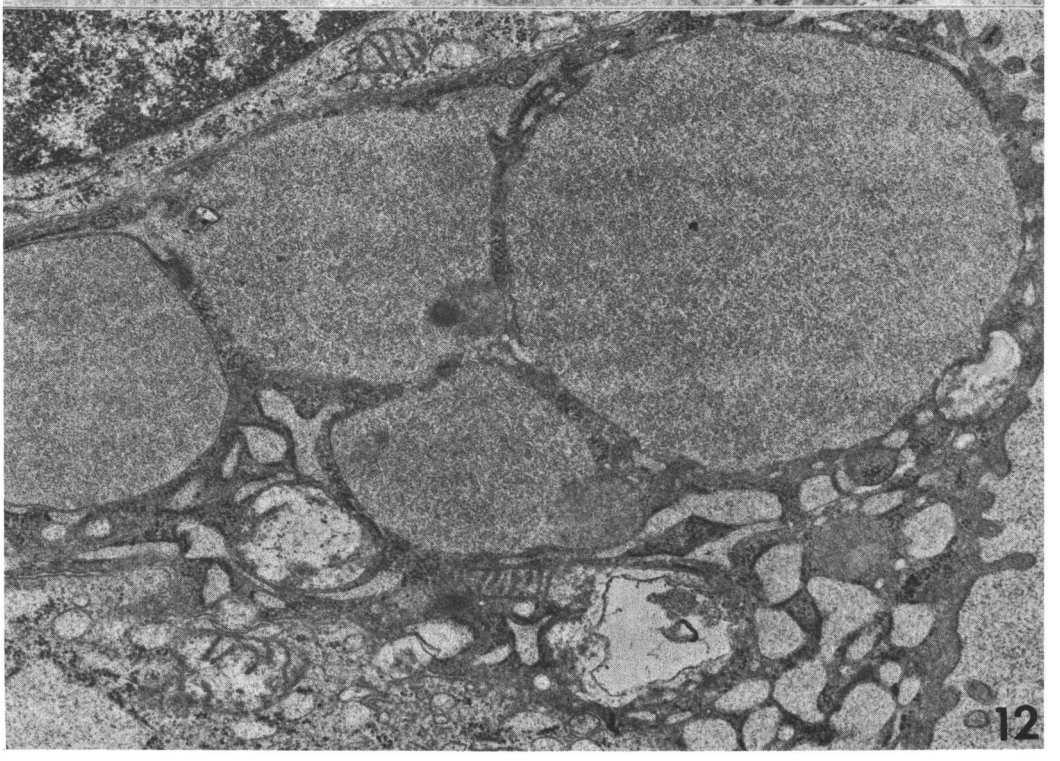

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number, size and morphology. Colloid droplets were frequently observed in the cytoplasm. The follicular colloid had a fine granular appearance and completely filled the lumen.

The ultrastructure of the columnar cells was similar to that of the hyperplastic follicular cells in TX-LID recipients. In addition, however, these cells often contained the large, PAS+ granules observed with the light microscope. These membrane-bounded granules contained a fine granular material similar in appearance to lumenal colloid (Figs. 11 and 12). The cytoplasm of these cells was often more electron-dense than the cytoplasm in adjacent cells. Myelin figures and remnants of disrupted organelles were scattered throughout the cytoplasm (Fig. 12). Focal areas of separation along the lateral membranes between adjacent cells were common.

Occasionally, granulated cells with pyknotic nuclei, pale cytoplasm and disrupted organelles detached from the basement membrane and were shed into the lumen, where degenerating cells and cell fragments accumulated.

The fine structure of the epithelial cells from follicles developing in NTX-ND animals was similar to that of normal rat thyroid cells (12).

Discussion. As we expected, recipient hormonal status had a marked influence on the morphology of follicular structures which develop following the injection of known numbers of monodispersed rat thyroid cells into subcutaneous white fad pads. However, the hormonal status of the recipient apparently didn't have a significant effect on the rate of early follicular unit growth and development as determined by light microscopy from Days 1 to 7. The morphological sequence of development during this period was identical in all the groups and was very similar to the sequence described for alveolar units which develop after transplantation of monodispersed rat mammary cells (2).

The morphological characteristics described for follicles in TX-LID recipients are indistinguishable from those reported for experimental hyperplastic goiters produced *in situ* in rats (9) and golden hamsters (10) and very similar to the pattern observed in pieces of thyroid gland transplanted into the anterior eye chamber of thyroidectomized rats (11). The hyperplastic response of the thyroid tissue in our model, as well as in the others, is secondary to stimulation by elevated thyrotropin (TSH) levels which develop following thyroidectomy, goitrogen administration, or dietary iodine deficiency (10).

The tall columnar epithelial cells and frequent mitoses observed in follicular units growing in NTX-LID recipients are likewise characteristic of thyroid follicular hyperplasia. However, the morphological pattern observed in these hosts 4 weeks after transplantation represents an early stage in the hyperplastic response. Radioimmunoassays of sera from non-thyroidectomized animals maintained on LID revealed that the serum thyroxine levels were not significantly different from control values for the first 4 weeks after initiation of the diet (Mulcahy, Rose, Mitchen, and Clifton, unpublished). In contrast, serum thyroxine levels drop rapidly in thyroidectomized recipients. Stimulation by TSH is therefore more immediate and intense in the latter animals, and results in marked hyperplasia within a short period of time. Since in these experiments autopsies were performed 1 to 28 days after transplantation, follicles from NTX-LID recipients might be expected to appear acutely stimulated whereas those from TX-LID recipients would appear chronically stimulated.

The transplantation of monodispersed thyroid cells into thyroidectomized recipients fed normal diets (TX-ND) resulted in structures at 4 weeks which are morphological equivalents of follicles described in colloid goiter (10). These colloid-filled follicles are significantly ($P < 0.005$) larger than follicles in the other experimental groups and in control thyroid glands.

Colloid goiters have been produced experimentally by feeding animals iodine-deficient diets followed by a return to normal diets (10). This type of goiter is considered to result from compensatory processes

in a previously hyperplastic thyroid gland (9). We believe that the production of large colloid follicles in TX-ND recipients is the result of a similar series of events. Initially, TSH levels are elevated following thyroidectomy. An early phase of cellular hyperplasia is followed by a phase of colloid accumulation as the follicular units produce sufficient thyroid hormones to inhibit pituitary TSH secretion. As a result of the increased number of cells per follicle, a greater quantity of colloid accumulates resulting in greatly increased follicular diameters.

Histochemical and ultrastructural characteristics strongly suggest that the large cytoplasmic granules observed in some follicular cells in TX-ND recipients contain reabsorbed colloid materials. The ultimate degeneration of these granulated cells is presumably a form of exhaustion atrophy.

Thyroid follicles arising in graft sites of intact hosts fed normal diets (NTX-ND) were morphologically identical with follicles from control thyroid glands (12).

Normal architectural and physiological relationships between the developing follicular units and stromal elements evolved in each of the graft sites. Mast cells, which have been shown to play a significant role in thyroid hormone synthesis (13) and release (14), accumulate in close association with the follicular units and perhaps contribute to their functional integrity.

Thyrocalcitonin secreting C cells, identified by immunohistochemistry, make up a smaller percentage of the cells in the thyroid grafts than they do in glands *in situ*, where they comprise ~5% of the parenchymal total (15). This apparent decrease in C cell/follicular cell ratio could result if (i) C cells are migratory, as suggested by Pearse (16), (ii) C cells are more sensitive to the enzymatic dispersion technique, or (iii) C cells are not stimulated to proliferate at the same rate as the follicular epithelial cells.

Summary. We have described an *in vivo* model system for the study of the growth and development of organized thyroid epithelial structures. These epithelial structures respond to various hormonal manipulations in a manner identical to that described for the thyroid gland *in situ*. The light and ultrastructural morphology of the follicular epithelial cells correlate well with that described for thyroid cells in intact glands exposed to similar hormonal manipulations. Therefore, we believe that follicular units which develop after transplantation of monodispersed thyroid cells serve as excellent models for the study of thyroid development and response *in vivo*. This model provides the further advantage of a quantitative approach for the investigation of epithelial cell growth and differentiation, of responses to environmental fluctuations, aspects of cell structure and function, and carcinogenesis.

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