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EFFECTS OF RETINOIC ACID ON EMBRYONIC CHICK SKIN

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The influence of vitamin A on differentiating epithelia was examined in explants of skin from 14-day chick embryos exposed to retinoic acid (RA) in low, moderate, and high doses. The changes observed in RA-treated cultures are both dose- and time-dependent and are reversible when explants are transferred to control medium. The periderm sloughs prematurely and horizontal stratification is lost. Keratinization is inhibited and fewer desmosomes and tonofilaments are seen. Surface epidermal cells develop microvilli, bulge upwards, and detach. Golgi elements, rough endoplasmic reticulum, and polyribosomes are unusually prominent. Mucin granules form and gland-like structures develop with intercellular canaliculi characterized by tight junctions, brush borders, and dense secretory contents.

On the basis of present evidence there are several possible mechanisms by which RA could alter epidermal differentiation. RA-induced gaps in the basal lamina allow direct contact between epidermal basal cells and fibroblasts and collagen fibers which could result in inappropriate dermal signals reaching the epidermis. In younger embryos the entire epidermis, including the mitotically inactive surface cells, appears to respond to RA, and this could imply an epigenetic modulation of cell phenotype. Finally, after the formation of a stratum corneum in older embryos only the relatively undifferentiated basal layer shows a metaplastic response, indicating that RA could be acting directly on the genome.

Vitamin A-induced mucous metaplasia of embryonic chick skin has served for over 20 years as a well-defined model system for the study of altered epithelial differentiation. Since 1953 when Fell and Mellanby [1] initially observed the inhibition of keratinization in chick embryo skin and its subsequent transformation into a mucous secreting structure by vitamin A, many studies have been concerned with the mode of action of vitamin A on a variety of epithelia. Results from these studies indicate that the response to vitamin A varies with species, age, and site of tissue. In addition to chick skin [2-10], excess vitamin A inhibits keratinization in a broad variety of mammalian and avian stratified squamous epithelia [11-13]. Mucin is produced in the chick skin, the cheek pouch, mouse vibrissae, cornea, the esophagus, tongue, and keratoacanthoma. Conversely, vitamin A deficiency leads to squamous metaplasia in calf parotid gland [34], rat bladder [35], rat trachea [36,37], and cornea [38], hamster trachea [39], and mouse prostate [40]. There is a marked reduction in the number of goblet cells in the small intestine of vitamin A-deficient rats, but squamous metaplasia does not occur [41]. In all of these models the initial effects were focal and reversible, selectively altering the differentiation of germinative layers of epithelia. In three tissues, the chick esophagus [30], hamster cheek pouch [11], and embryonic mouse lip vibrissae [17], vitamin A induced intraepithelial mucous gland formation. Hydrocortisone and citral may reverse or inhibit completely the metaplastic effects of vitamin A [42-44].

Most studies of excess vitamin A have used retinol in low to moderate dosage. However, it has become evident that retinoic acid (RA) is more potent than retinol in skin and other extracutaneous tissues [45-47], possibly because of its greater solubility in aqueous media, or possibly because of the presence of specific RA-binding proteins [48-50]. We exposed explants of chick embryonic skin to RA in low, moderate, and high doses and examined them by light microscopic histochemistry, transmission electron microscopy (TEM), and scanning electron microscopy (SEM) in order to more accurately define the early morphological sequence of events.

METHODS

Tissue Culture Techniques

Tarsometatarsal shank skin was taken from white leghorn chick embryos ranging from 13 to 21 days of

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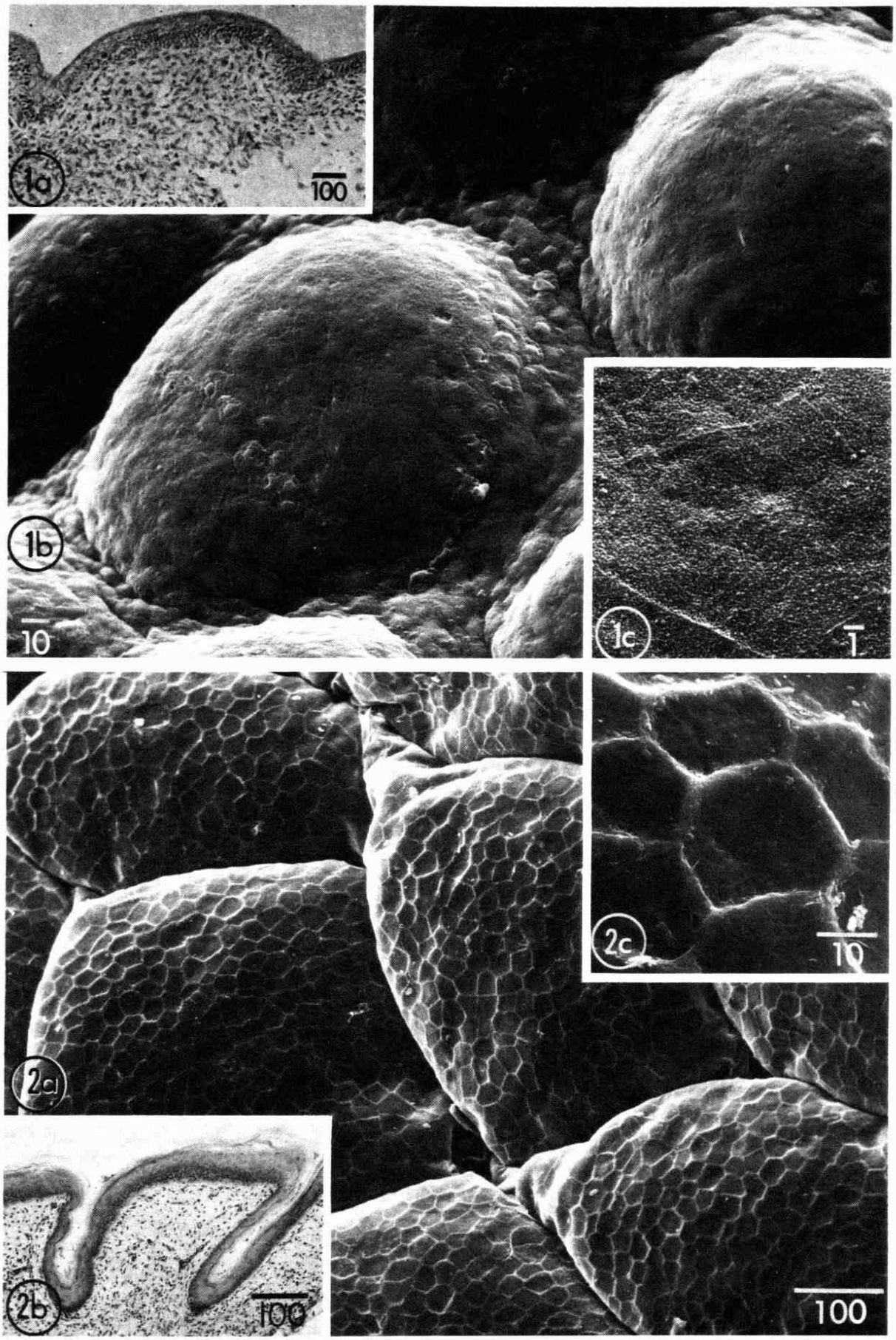
Abbreviations:
BME: Earle's Basal Medium
MEM: Minimal Essential medium
RA: retinoic acid
SEM: scanning electron microscopy
TEM: transmission electron microscopy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gestation, and up to 3 days after hatching. The shanks were immersed in Minimal Essential Medium (MEM) and, after a longitudinal incision, the metatarsal bones, cartilage, and loose areolar connective tissue were gently removed. The skin was cut into 2 × 2 mm pieces and placed on silicone-treated lens paper in Falcon organ culture dishes. The lens paper was supported by a stainless steel grid overlying a center well into which tissue culture media were placed up to the level of the supporting grid (0.6 to 0.7 ml). Several media have produced similar results including MEM, Trowell's medium, Earle's Basal Medium (BME), and Medium 199 with and without added retinol (0.1 mg/liter). In each case, the medium was supplemented with 5% to 15% fetal calf serum, glutamine, and antibiotics (penicillin, streptomycin, and Fungizone). Experiments using serum-free medium failed to produce mucous metaplasia [8,10]. Cultures were kept in a Wedco model #2-17 incubator at 37°C, 100% humidity, under 5% CO_2 in air for periods varying from 3 hours to 8 days. Typically, skin from 14-day chick embryos (stage 40) [51] was cultured for 3 days in Medium 199 with 5% fetal calf serum with and without 20 IU (6.7 μg)/ml RA. Vitamin A acid (all trans retinoic acid, Eastman Chemicals) was dissolved in absolute ethanol with sonication and added to the complete medium immediately prior to use in final concentrations between 0.01 and 100 IU/ml. The final ethanol concentration was 0.03% in all cultures including controls. Specimens were prepared for LM, SEM, and TEM after culture periods between 3 hours and 8 days, and from normal chicks at comparable periods of development in ovo (Fig 1-16: Fig 1, 2 – in ovo; Fig 4, 6, 15a – control; Fig 3, 5, 7-14, 15*b-d*, 16 – vitamin A acid).

Tissue Preparation

For light microscopy tissue was fixed in 10% formalin in tap water with 2% sodium acetate for one hour at room temperature, then dehydrated in a graded series of methanol, embedded in paraffin, and sectioned at 6 μm, then stained with periodic – acid Schiff with and without diastase pretreatment, alcian blue at pH 0.4 and pH 2.4, toluidine blue, colloidal iron, or hematoxylin and eosin. Frozen sections of fixed tissue were stained with Oil Red O.

Specimens for TEM were fixed in 6% glutaraldehyde in 0.1 M phosphate buffer (isosmotic) for 2 hours at 4°C, rinsed in 0.1 M phosphate buffer with 7% sucrose, postfixed in 1% osmium tetroxide for 90 minutes at 4°C, dehydrated through a graded ethanol series into propylene oxide, then infiltrated and embedded in Epon 812. Thin sections, cut with an LKB microtome and a diamond knife, were stained with lead citrate and uranyl acetate and examined in a Siemens 1-A Elmiskop TEM. Specimens for SEM were fixed by adding an equal volume of 6% glutaraldehyde in 0.1 M phosphate buffer to the medium (3% final concentration of glutaraldehyde) for 90 minutes at room temperature. The specimens were washed in distilled water, placed in small stapled packets of lens paper, dehydrated through a graded ethanol series into amyl acetate, and dried by the critical point method with CO_2. They were then attached to aluminum stubs with 3M transfer tape, coated with carbon and gold-palladium, and viewed in an ETEC Autoscan at 20 kv.

RESULTS

Epidermal Differentiation in Control Cultures

Skin from 14-day chick embryos maintained in culture without added retinoic acid for periods up to 8 days shows normal epidermal differentiation as compared with maturation in ovo. The characteristic tarsometatarsal shank skin scales (Fig 1-2) develop normally. In short-term explants (1-2 days) the epidermis consists of stratified squamous epithelium covered by a two-layered periderm. The surface epithelial cells are flat with abundant tonofilaments and junctional complexes, but not yet cornified. Individual keratinocytes contain fine tonofilaments, many free ribosomes but very few polysomes, numerous mitochondria, and scattered profiles of rough endoplasmic reticulum and Golgi elements. No keratohyalin, glycogen, or mucin was observed in the epidermis of these specimens cultured for 2 days. Peridermal cells contained their characteristic granules and some glycogen. By SEM one can appreciate the continuous covering of peridermal cells over the developing skin scales. The peridermal surface appears smooth at low magnification (Fig 4), but on closer examination is uniformly covered with distinctive short microvilli and small anastomosing ridges.

In older explants (3-6 days) from 14-day embryos the epidermis has thickened and cornified, and the periderm is lost. Keratinocytes contain increased numbers of tonofilaments aggregated into tonofibrils, and keratohyalin granules are present (Fig. 6*a,b*). The distribution and appearance of Golgi elements, ribosomes, and mitochondria appear similar to that of earlier specimens. By SEM, some periderm is shed after 3 days in culture, exposing an even continuous layer of flat, smooth-surfaced, polyhedral epidermal cells (Fig 6*c*). Lipid droplets are seen both in the basal layer and the stratum corneum. PAS and other stains for mucopolysaccharides reveal no mucin and little glycogen.

Effects of Retinoic Acid on Epidermal Differentiation

The changes observed in treated cultures are dependent on both the dose of vitamin A acid and the time of exposure to this agent. Thus, with small amounts of RA (0.1 IU/ml) early metaplastic changes occur after 6 days in vitro; with larger doses (20 IU/ml) metaplastic changes occur within 18 hours after exposure. These metaplastic changes are reversible when RA – treated skin is transferred to control medium. However, the mu-

FIG 1. Dome shaped, immature skin scales (1*a*, × 41) (1*b*, × 460); surface of peridermal cells (1*c*, × 2700). Unless otherwise noted, all figures illustrate tarsometatarsal skin removed from 14-day-old chick embryos, and the dose of retinoic acid is 20 IU/ml for three days. Scale markers are given in micrometers.

FIG 2. Mature skin scales at hatching (21 days), (2*a*, × 150) (2*b*, × 41); surface stratum corneum cells (2*c*, × 1100). Scale markers are given in micrometers.


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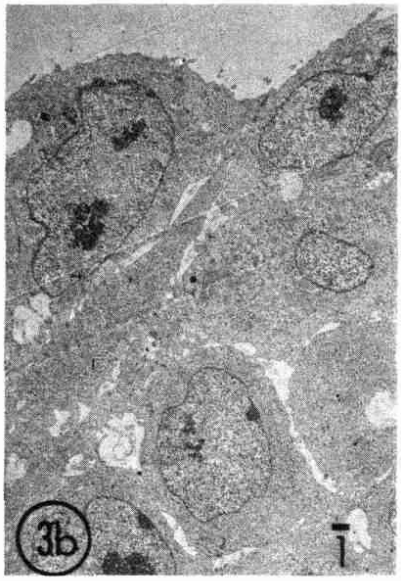
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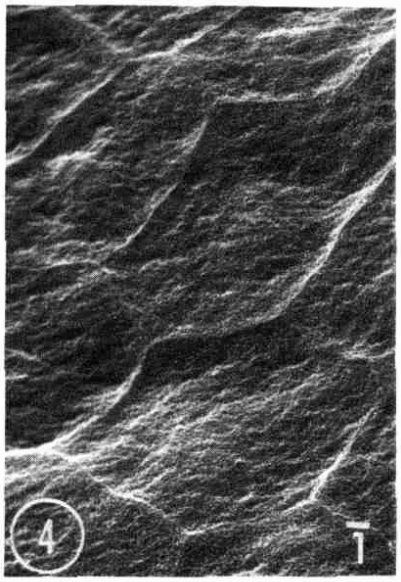
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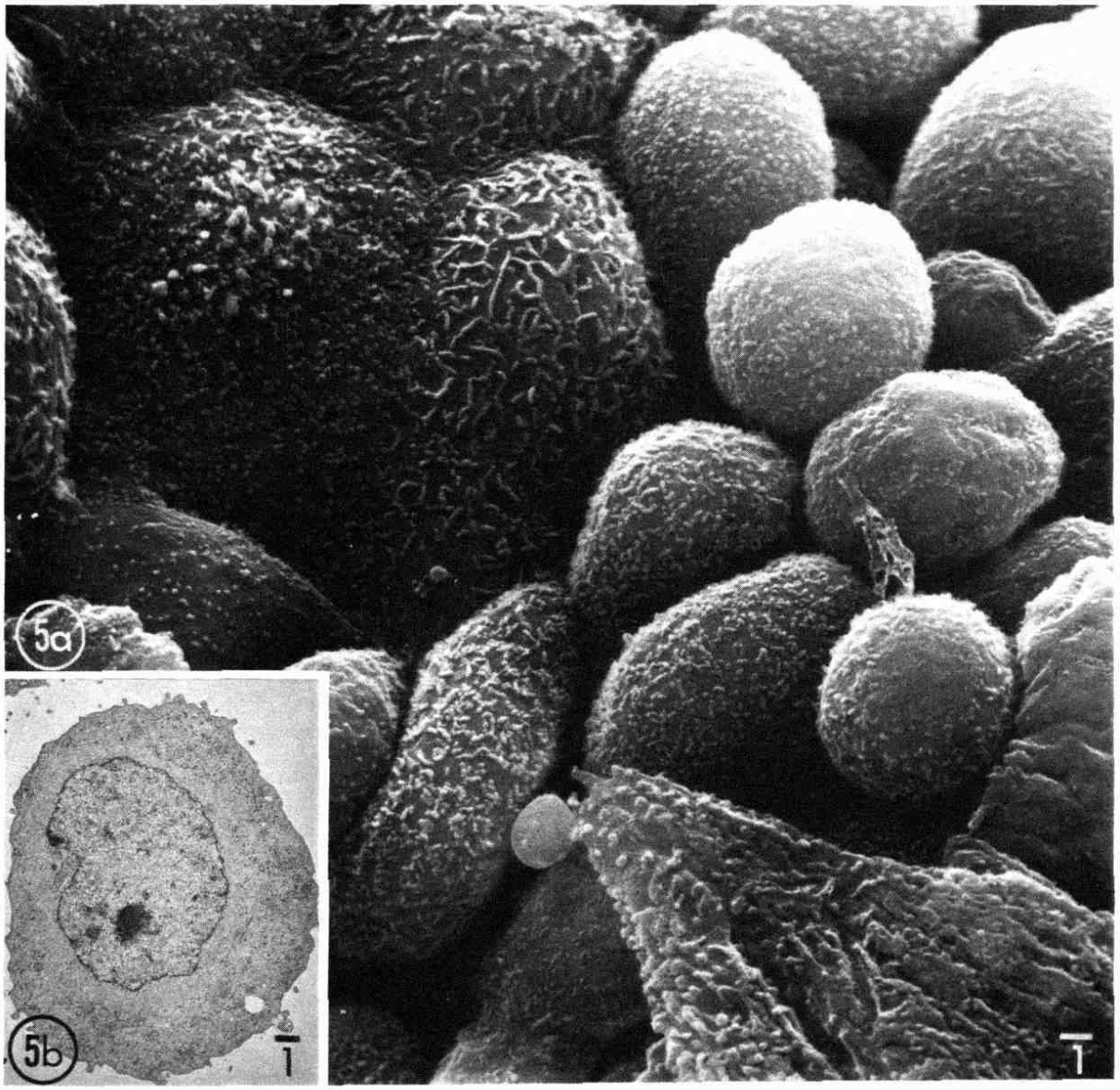
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5b

cin-containing epidermal cells do not redifferentiate to produce keratin. Instead they are gradually replaced by a new population of keratinizing cells (Fig 8).

Focal metaplastic changes are seen with small amounts of RA (e.g., 1 IU/ml for 3 days) or short exposure to larger doses (e.g., 20 IU/ml for 1 day); these foci are surrounded by normal, apparently nonresponding, epidermis (Fig 9*a*). Accentuated metaplasia consistently appears at the cut edge of the explant (Fig 7*c*). With low doses skin scales flatten and at higher doses (20 IU/ml/3 days) the explants decrease considerably in size as dermal elements are lost. By TEM most ribosomes in the keratinocytes appear as polyribosomes (Fig 9*b*) and Golgi elements (Fig 9*d*) increase in number. The epidermis becomes more hyperplastic, leading to acanthosis and loss of horizontal stratification (Fig 3*b*). The periderm begins to slough after only one day of RA treatment and is lost after 2 days. In contrast to the control cultures keratohyalin granules do not form, and cornification does not occur. Even in the superficial layers epidermal cells are spherical rather than flat and may detach (Fig 3*a*, 5*a*,*b*). Individual epidermal cells are variably affected. Numerous microvilli appear on these cells with axial filaments and surface glycocalyx (Fig 10*a*), but they become less prominent in the more rounded cells which appear to be sloughing from the epidermal surface. Cilia do not occur. Desmosomal cleavage (Fig 10*b*) is seen with widened intercellular spaces. Tight junctions (Fig 10*c*) appear between superficial epidermal cells and complex interdigitations occur below such junctions. Elongated gap junctions are seen three to four cells deep into the epidermis. Glycogen appears early in the cytoplasm of superficial epidermal cells (Fig 9*b*,*c*) and, occasionally, within phagocytic vacuoles. Filaments do not disappear completely from epidermal cells, but are finer and fewer in number than in control cultures. Increased numbers of large secondary lysosomes and presumptive lipid droplets are also seen. The basement membrane is discontinuous, and finger-like projections of the basal cells extend through the gaps (Fig 11*a*), occasionally contacting projections of dermal fibroblasts (Fig 11*b*, *arrow*). Collagen fibers sometimes appear above the basement membrane in contact with basal cells (Fig 11*c*).

Prolonged exposure to RA at high doses induces extensive elaboration of both inter- and intracellular mucin (Fig 10*d*,*e*), and in most instances saccular glandlike dilatations develop between epidermal cells (Fig 12, 13). Under these conditions vacuoles of varying sizes appear in the superficial layers and their contents stain intensely with colloidal iron, alcian blue, and PAS (with and without diastase pretreatment). A similarly staining material also fills the glandlike structures and coats the luminal surfaces. The saccular dilatations, possibly representing acini, are surrounded by epithelial cells with tight junctions, and microvilli which occasionally form a brush order (Fig 14) and apical mucin granules (Fig 13). Ductlike structures often extend from these dilatations to the surface. Material similar in density to the mucin granules is seen within these acini, and it coats the epidermal surface.

RA is seen to specifically affect the further development of epidermal germinative cells in skin from older chicks (between 16 and 24 days of gestation; hatching occurs on day 21) (Fig 15). Development initially proceeds normally in the more differentiated cells of the superficial epidermis; they appear to be unaffected by exposure to RA. However, a thickened, metaplastic germinative layer appears beneath the normal stratified squamous epithelium, producing irregular bumps and folds in the surface. After 3 days exposure, however, the superficial epidermal cells rupture (Fig 16), eventually resulting in a cleavage plane, consistently containing acantholytic cells, between the responsive and nonresponsive cell populations (Fig 15*c*).

DISCUSSION

Agents which induce differentiation are thought to affect cell populations capable of DNA synthesis. Holtzer suggests that inductive events occur during the S-phase of special differentiative "quantal" cell cycles, and results may not be seen until after several "proliferative" cycles. Cells already committed to the production of one "luxury" molecule, e.g., keratin, myosin, or mucin, would not likely be induced to dedifferentiate, and new terminal products would thus come from previously uncommitted cells [52].

Most reported effects of vitamin A on epithelia are consistent with this view. It has been shown repeatedly that exposure to excess retinoic acid induces selective hyperplasia of the basal layer of the epidermis [33,54,73]. Vitamin A deficiency also leads to basal cell hyperplasia. Selective effects of RA on basal cells are clearly shown when exposure to RA is discontinued and the tissue is transferred to control medium. In this situation the mucus-containing cells are pushed to the sur-

FIG 3. 14-Day chick skin cultured for one day with retinoic acid. Premature sloughing of peridermal cells exposes rounded, bulging epidermal cells covered with surface microvilli and folds (3*a*, × 2160). Rounded epidermal cells and loss of horizontal stratification (3*b*, × 1400). Scale markers are given in micrometers.

FIG 4. Skin cultured two days in control medium. The periderm is persistent (× 2400). Scale markers are given in micrometers.

FIG 5. Skin cultured for two days in retinoic acid shows markedly rounded, bulging epidermal cells with pronounced surface microvilli and folds (5*a*, × 4200); a sloughing surface cell with persistent cytoplasmic organelles. There is no evidence of keratinization or mucin formation (5*b*, × 3000). Scale markers are given in micrometers.

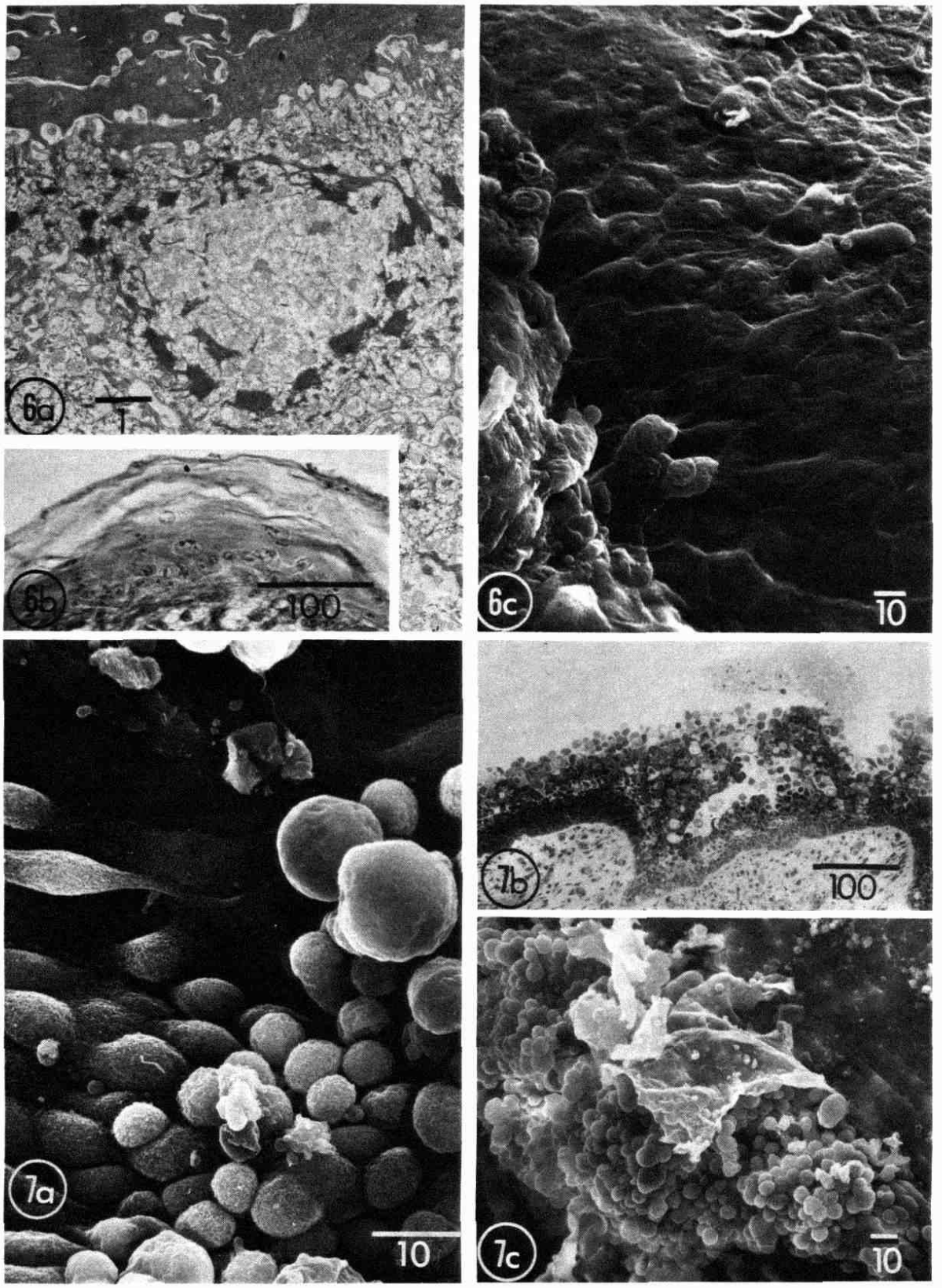
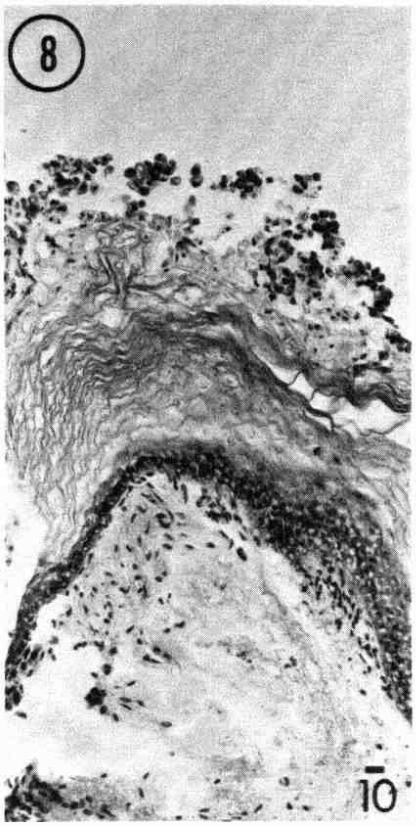


FIG 6. Skin cultured three days in control medium. Keratinization occurs with production of stratum corneum and keratohyalin granules (*6a*, × 8800) (*6b*, × 181). Periderm has sloughed, revealing the mature stratum corneum (*6c*, × 500). Scale markers are given in micrometers.

FIG 7. After exposure to retinoic acid, great numbers of rounded metaplastic surface cells appear (*7a*, × 1350) (*7b*, × 123); the metaplastic effect is most marked at the cut edge of the explant (*7c*, × 418). Scale markers are given in micrometers.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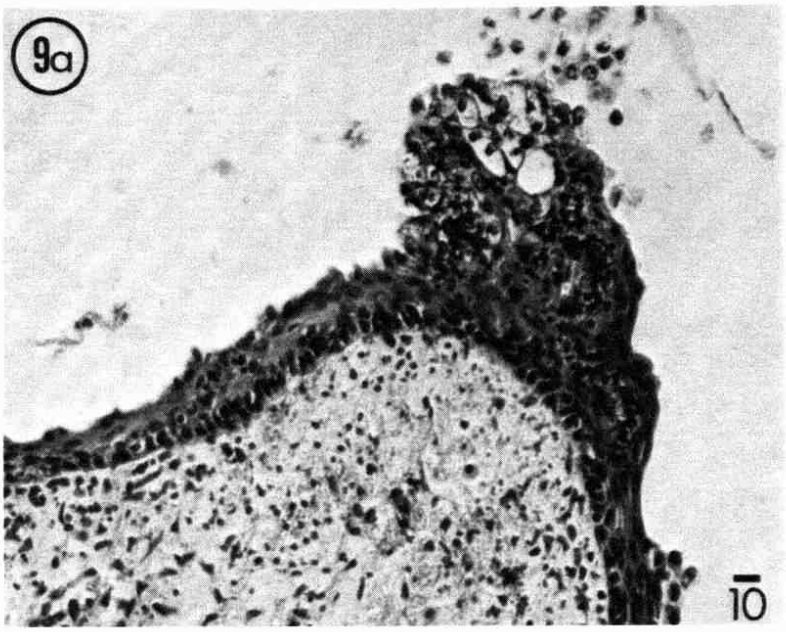
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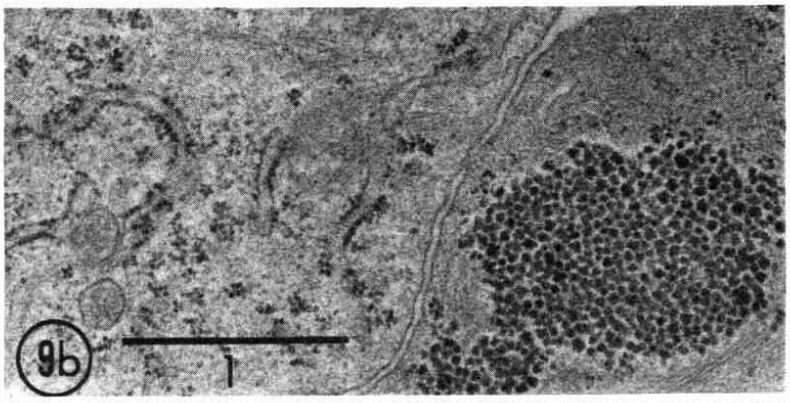
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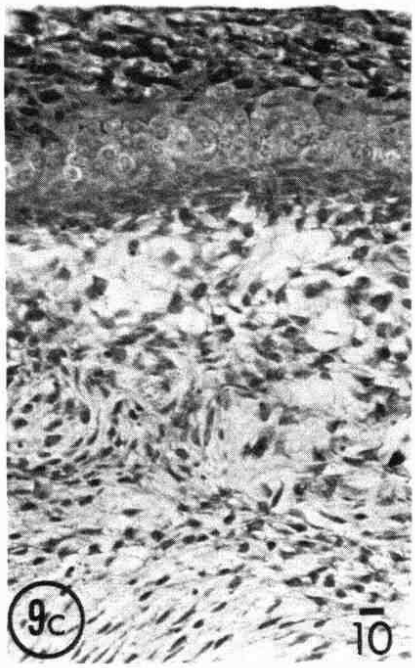
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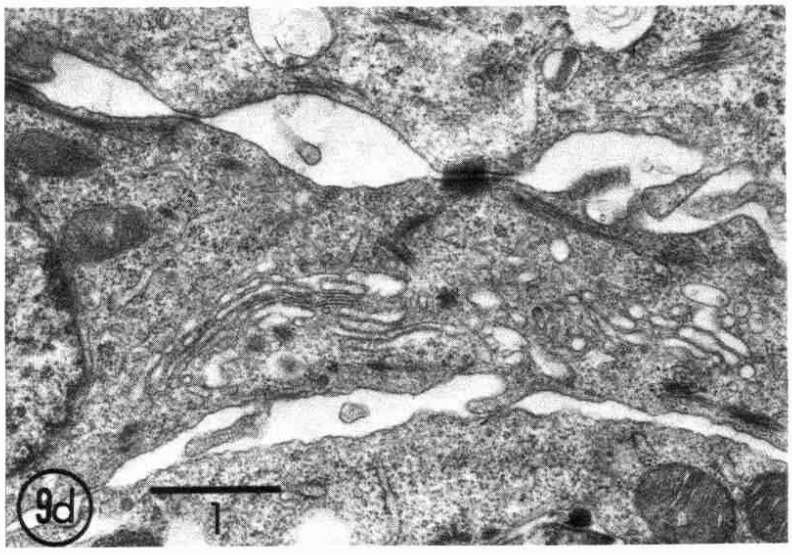
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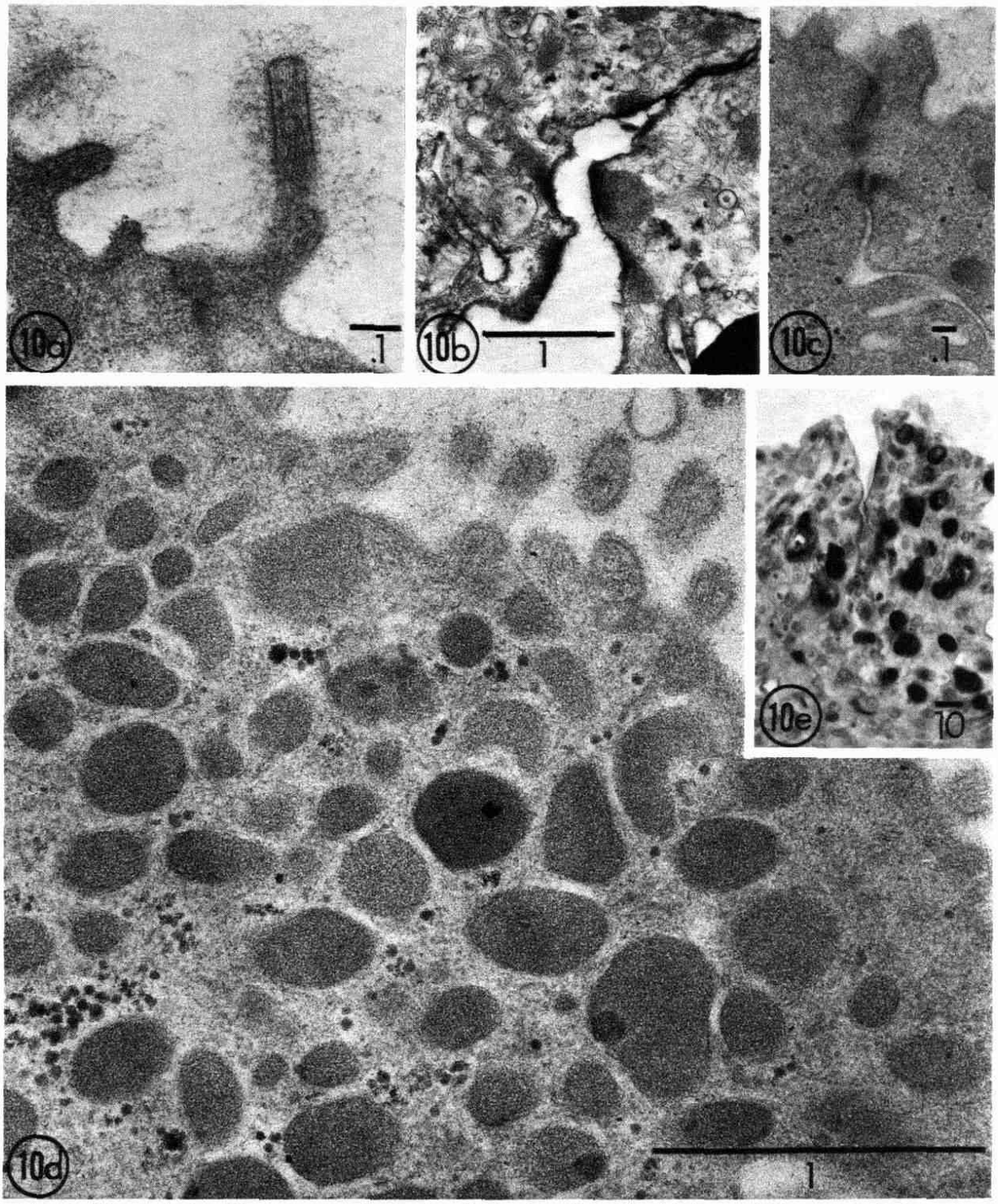

FIG 10. After retinoic acid exposure, metaplastic cells develop microvilli with internal filaments and glycocalyx (10*a*, × 22,000), desmosomal cleavage with widened intercellular space deeper in the epidermis (10*b*, × 18,700), tight junctions (10*c*, × 3400), and apical mucin granules (10*d*, × 50,000). Mucin is demonstrated histochemically (PAS with diastase) (10*e*, × 261). *Scale markers are given in micrometers.*

face by a new generation of keratinizing cells from the basal layer (Fig 8). In addition, metaplasia in skin from older embryos is limited to the basal cells, and the superficial epidermis remains keratinized (Fig 15*c*). The enhanced metaplasia at the cut edges of explants (Fig 7*c*) could be related to the relatively high mitotic and labeling indices in these areas [53]; alternatively, vitamin A acid may simply penetrate more effectively into the tissue at the cut edges. RA stimulates epidermal basal cell DNA synthesis and mitosis, both in vivo and in vitro [24,27,28] and stimulates epidermal turnover in vivo [54]. Epidermal cells in culture actively grow and synthesize DNA and

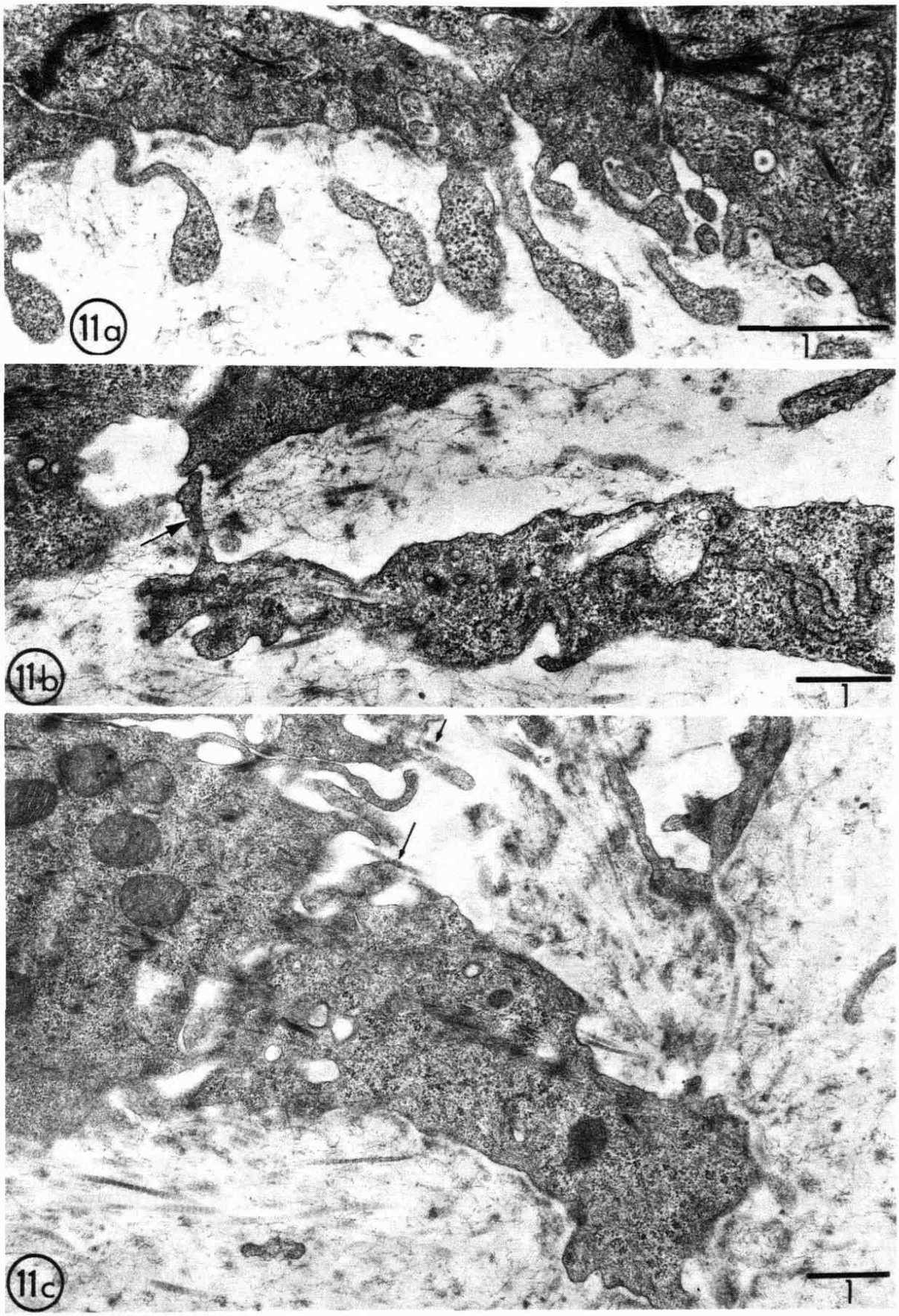

FIG 11. Gaps in the basement membrane and downward extensions of basal cell processes appear after three days exposure to retinoic acid (11*a*, × 24,650); occasionally, direct contacts are observed between a basal cell and a fibroblast projection (11*b*, × 15,600), or collagen fibers (*arrows*) within the epidermis (11*c*, × 13,200). Scale markers are given in micrometers.

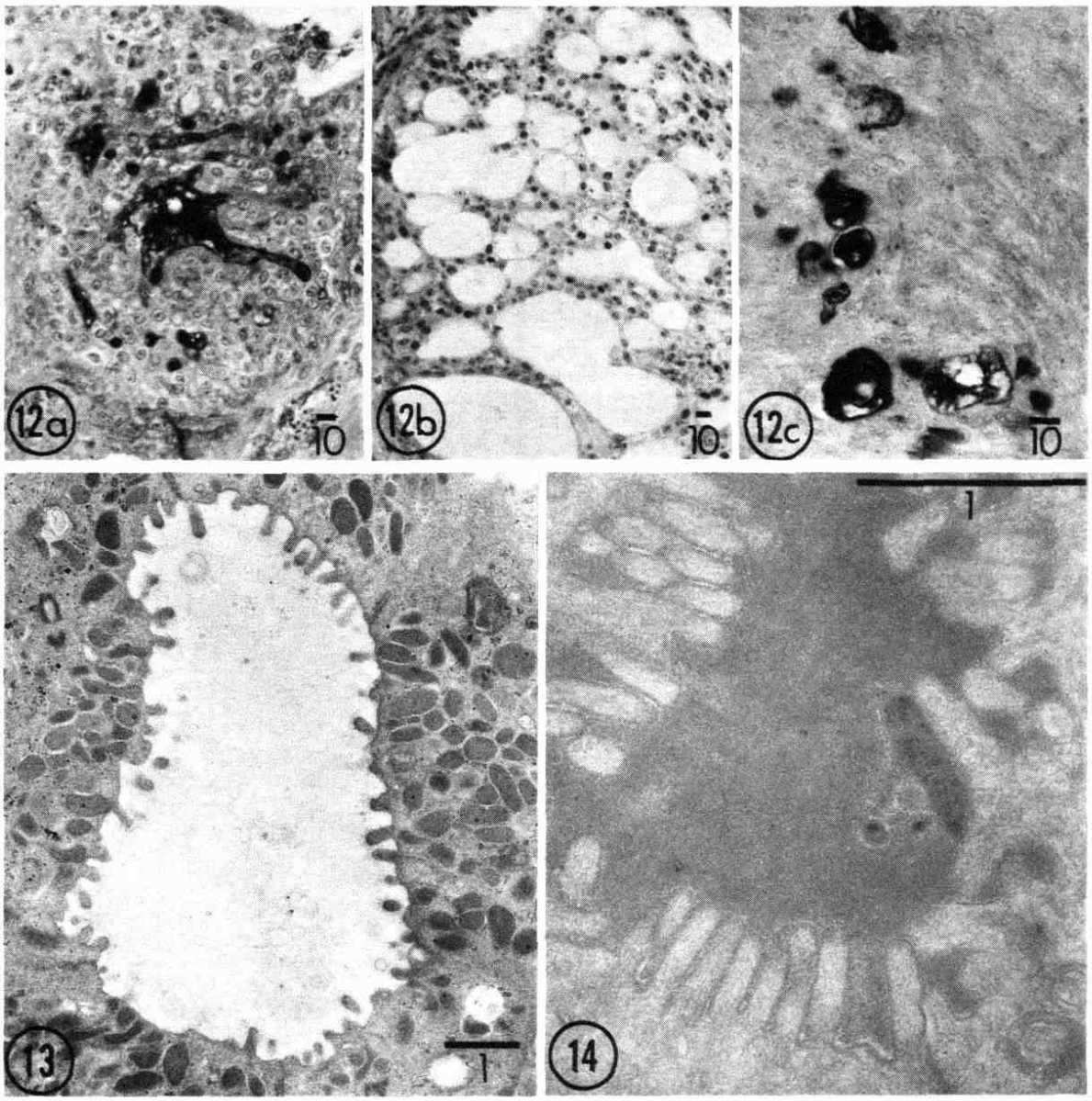

FIG 12. 13-Day chick skin, cultured with 33 IU retinoic acid/ml for 6 days. An intraepidermal mucous gland-like structure with a central lumen, branching ducts, and terminal saccular dilatations (PAS) (12*a*, × 230). Transverse section reveals a tubuloalveolar glandlike structure (12*b*, × 141). Alcian blue staining shows mucin in glandlike lumina (12*c*, × 256). Scale markers are given in micrometers.

FIG 13. Deeper within the epidermis widened intercellular canaliculi similar to acini have developed with microvilli and apical mucin granules (× 10,000). Scale markers are given in micrometers.

FIG 14. With higher doses (33 IU retinoic acid/ml) a brush border of microvilli has formed with electron–dense material in the lumen (× 36,000). Scale markers are given in micrometers.

appear analogous to undifferentiated basal cells. Thus, the effects of RA in each of these systems are consistent with Holtzer's hypothesis.

Several mechanisms have been proposed to account for the action of RA on competent basal epithelia: (1) Lazarus, Hatcher, and Levine suggest that the increased mitotic rate in skin treated with vitamin A may result from labilization of lysosomal membranes with subsequent release of hydrolases, particularly Cathepsin D [55]. Proteases also stimulate mitosis and lead to cellular hyperplasia [56,57]. (2) DeLuca et al suggest that vitamin A may first influence specific glycopeptide biosynthesis then RNA synthesis and cell differentiation [58–61]. (3) Hardy et al speculate that basement membrane gaps induced by excess vitamin A [9,16,31] may allow "inappropriate dermal signals" to stimulate epidermal metaplasia. Such signals could occur via metabolic cooperation [62], transfer of informational macromolecules [63], or via intercellular contacts [64]. A dermal role is also suggested by the demonstration that: (a) gizzard mesenchyme induced mucous metaplasia in overlying isolated chick epidermis [65]; and (b) canine oral mucous membrane keratinizes when transplanted to the ear, but undergoes mucous metaplasia when retransplanted to the trachea [66]. (4) RA binds to a specific cytosol receptor

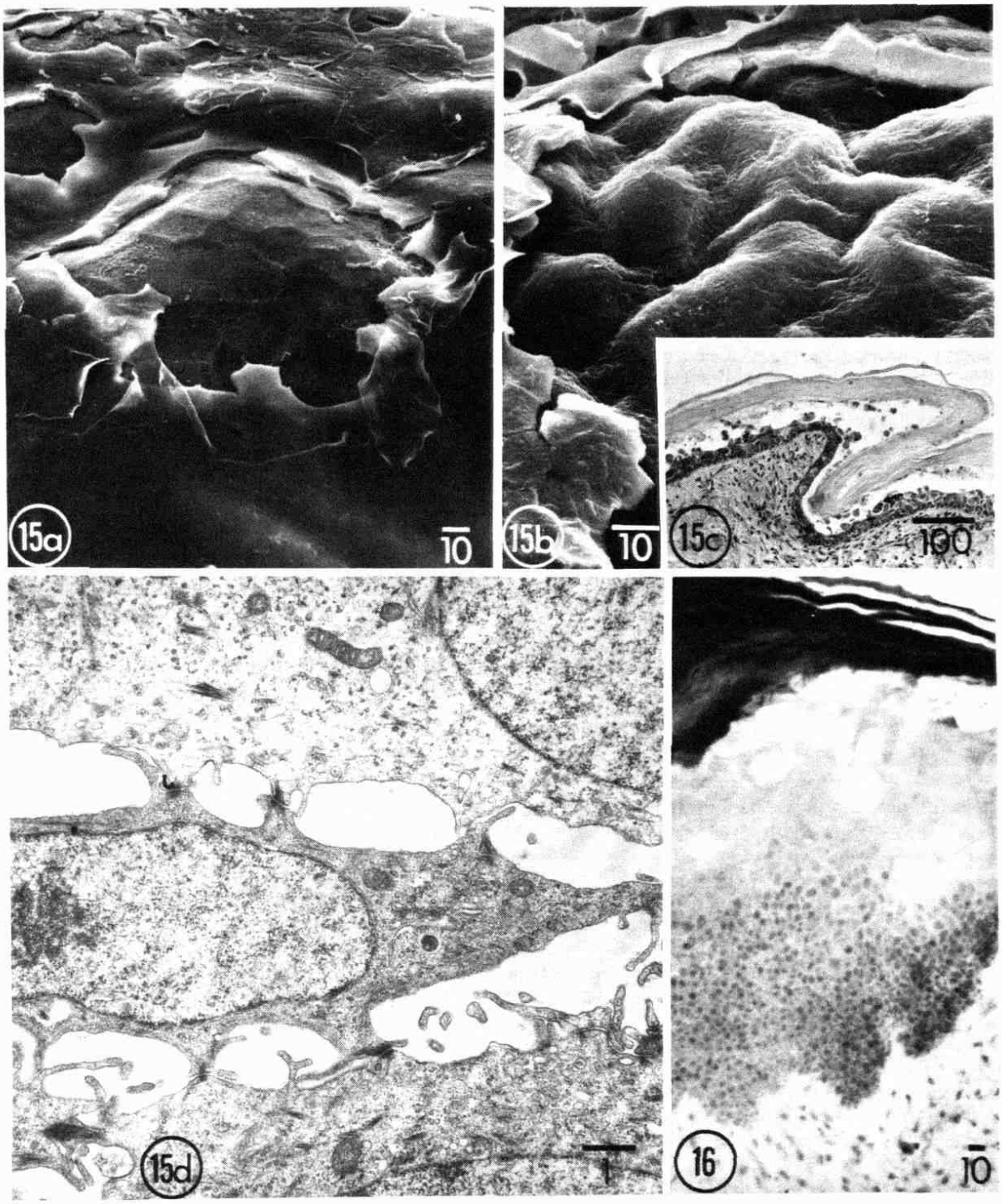

FIG 15. 17-Day chick skin cultured 3 days in control medium. Smooth dome of stratum corneum cells surrounded by sloughing periderm (15*a*, × 400). Pressure from the underlying basal metaplastic epithelium has thrown the nonresponding superficial epidermis into folds. 17-Day chick skin exposed to retinoic acid (15*b*, × 660); cleavage plane between the basal metaplastic and superficial nonresponsive epidermis (15*c*, × 70). TEM at this stage corresponds to the junction of the basal dark cells and the spinous light cells seen in Fig 16 (15*d*, × 8000). Scale markers are given in micrometers.

FIG 16. 21-Day chick skin, exposed to retinoic acid, shows edematous or disrupted spinous cells and increased numbers of small dark basal cells (× 204). Scale markers are given in micrometers.

protein in embryonic chick skin, as well as other embryonic and neoplastic tissues [48–50]. This pattern is reminiscent of the initial binding of steroid hormones to cytosol receptors, and suggests that RA might influence differentiation on a DNA transcriptional level [48].

None of the above mechanisms for the action of RA can be excluded on the basis of the present study. Increased numbers of secondary lysosomes are present after RA treatment, especially at relatively high doses, and early increases in polyribosomes, granular reticulum, and Golgi ele-

ments occurred in retinoic acid-treated basal cells. Thus, increased concentrations of lysosomal proteases, and specific glycopeptides produced in the newly acquired synthetic organelles of the basal cells could both serve in the metaplastic process in organ cultures of chick embryonic skin. In addition, gaps in the basement membrane, allowing direct contact between basal cells and collagen fibers or fibroblasts, are consistently present in RA-treated samples, in this and other systems [9,16,31], and could represent a mechanism for dermal induction of epithelial metaplasia. On the other hand, the observed gaps in the embryonic chick skin system could be the result of nonspecific dermal toxicity in view of the previously mentioned explant shrinkage after retinoic acid exposure. In isolated epidermis and in isolated epidermal cells treated with RA the dermal induction hypothesis would imply the presence of minimal contamination by fibroblasts or other dermal elements to account for any observed metaplasia. In the preparations of isolated epidermal cells frank mucous production (e.g., the presence of mucous granules by TEM) has not been observed [22,24,27,28]. Perhaps the intact dermis is necessary to amplify the metaplastic effect of vitamin A on epidermal cells.

Basement membrane gaps, whenever produced, are not always associated with mucin production. Basement membrane gaps have been observed in a variety of hyperplastic conditions, including carcinoma, keratoses, and psoriasis [39]. Such gaps were observed in carcinogen-induced squamous metaplasia in tracheal epithelium, although vitamin A deficiency-induced squamous metaplasia in the same study did not produce basement membrane discontinuities [39]. In addition, normal columnar orientation and mitotic activity were observed in cultured epidermis lacking basement membrane [67].

Whether collagen itself determines epidermal differentiation (see reviews [68–70]) or whether it only amplifies the vitamin A effect is unclear. For example, collagen could serve as a substrate in glycosyltransferase reactions at epidermal cell surfaces, perhaps involving a retinolglycolipid intermediate [71]. Collagen-cell surface interactions have proven necessary for normal corneal morphogenesis [72].

In addition to the preponderant evidence that vitamin A induces genetic alterations, this agent also affects postmitotic superficial epidermal cells (Fig 7*a*,*b*). In 13-day embryonic chick skin Rothberg has demonstrated that only the basal layer of epidermal cells actively incorporates tritiated thymidine, establishing that the altered superficial cells are postsynthetic [73]. It is well known that vitamin A can affect cell membranes directly [74]. Conceivably, RA may bind to all epidermal cells and change their surface morphology, and then induce genetic changes via membrane messengers [75] to produce new messenger RNA, or glycoproteins, only in those cells capable of DNA synthesis. Cell kinetic studies may be necessary to determine the origin of the surface mucous cells seen after three days of incubation. If these cells derive from superficial postsynthetic epidermal cells, it might indicate that the action of vitamin A occurs at the posttranslational level as suggested by DeLuca and Wolf [58]; vitamin A could serve as a carrier of monosaccharide units and glycosylate preexisting proteins to form the mucin.

Metabolic inhibitors have helped to define the effects of RA in the epidermis. The inhibition of RA-induced mucous metaplasia in rabbit keratoacanthoma by actinomycin D may involve alterations of cell permeability rather than genetic expression since pretreatment but not posttreatment with actinomycin D prevents the cellular uptake of labeled retinoic acid [76]. Continuous applications of puromycin given concurrently with vitamin A acid also prevent mucin formation in the keratoacanthoma and may represent an inhibition of mucin granule development in the Golgi apparatus rather than an inhibition of protein synthesis [77].

Glandular metaplasia has been noted in several stratified squamous epithelia after exposure to vitamin A [11,17,30]. The demonstration that structures resembling branching tubulo-alveolar mucous glands can develop in the epidermis is an indication both of the potential of chick skin to respond to an inductive stimulus and of the strength of the stimulus provided by RA.

Clinically, vitamin A and its analogs have been effective in the prevention and treatment of neoplasia and preneoplasia (reviewed in reference 78). Evidence that vitamin A-induced metaplasia may be linked to its antineoplastic effect is obtained by demonstration that vitamin A acid-treated rabbit keratoacanthomas undergo mucous metaplasia prior to involution [21]. Vitamin A and its analogs have also been used effectively in man in the treatment of acne and disorders of keratinization [45–47,79–80]. The exquisite sensitivity of the embryonic chick skin system may make it useful for further studies of the effects of RA and its analogs [81,82] on normal differentiation, neoplasia, and other diseases.

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