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Immunoreactivity of alpha- and beta-layers in lizard epidermis

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ABSTRACT. Reptilian epidermis may share some mechanisms of keratinization with those of mammalian and avian epidermis. The expression of keratins and proteins associated with the process of keratinization (filaggrin) and formation of the cell corneus envelope (loricrin) has been analysed at the light microscopic and ultrastructural level in the complex normal and regenerating lizard epidermis. The localization of alpha and beta-keratins, and of filaggrin-like and loricrin-like immunoreactivities has confirmed that the epidermis of lizards consists of two different layers, an alpha-layer localizing alpha-keratins and showing weak filaggrin- and loricrin-like immunoreactivities, as in the mammalian epidermis, and a beta-layer that localizes a beta-keratin immunoreactivity, such as in scutate scales and feathers of birds. The present study suggests that the segregation of alpha- from beta-keratin synthesis is correlated with the evolution of an intraepidermal shedding layer that allows the epidermal molt.

KEY WORDS: lizard, epidermis, keratins, loricrin, filaggrin, immunocytochemistry.

INTRODUCTION

The cell renewal of mammalian epidermis, apteric avian epidermis, crocodilian and turtle epidermises is determined by a more or less continuous production of cells from the germinative (basal) layer (ALEXANDER, 1970; FLAXMANN, 1972; MATOLTSY, 1987). Instead, in amphibian anurans (frogs and toads) and in lepidosaurian reptiles (lizards and snakes), a cyclical shedding of the outermost epidermal layers takes place and produces a molt (BUDZ & LARSEN, 1973, 1975; MADERSON, 1985; MADERSON et al., 1998). In relation to the periodic shedding process, epidermal cells progressively differentiate into specific cell layers that constitute cyclically-renewed epidermal generations. The most complex alternance of epidermal generations is present in the lepidosaurian epidermis where a specific and broadly accepted terminology has been established to describe this shedding cycle (MADERSON et al., 1972, 1998; MADERSON, 1985; LANDMANN, 1979; 1986; ALIBARDI, 2002; see Fig. 1).

In particular, an epidermal generation (outer epidermal generation) of lizards morphologically consists of six different layers: oberhautchen, beta-, mesos-, alpha-, lacunar-, and clear layer, the latter forming a shedding complex with the next oberhautchen of the following epidermal generation (Fig. 1A). During their terminal differentiation the lacunar cells, and in particular the cells of the clear layer, accumulate keratohyalin-like granules, which resemble mammalian keratohyalin, and which coalesce with keratin bundles during the terminal differentiation of these cells (ALIBARDI, 1998, 1999, 2000a). Instead, the clear layer of snakes does not accumulate these granules (Fig. 1B).

While the oberhautchen and beta-layer produce a hard form of keratinization (beta-keratin, see BADEN & MADERSON, 1970; BADEN et al., 1974; WYLD & BRUSH, 1979, 1983), the remaining layers produce a softer keratin (alpha-). The hard keratins present in oberhautchen cells form the microornamentation typical of the surfaces of lepidosaurian scales (IRISH et al., 1988) and of the long setae of the climbing pads of some geckos and lizards (MADERSON, 1970; ALIBARDI, 1997; see Fig. 1C). So far, scarce immunocytochemical and biochemical studies on keratin distribution have confirmed the above morphological, ultrastructural and biophysical data (CARVER & SAWYER, 1987; ALIBARDI et al., 2000, 2001). The knowledge of the molecules implicated in the process of keratinization in lower amniotes (reptiles) may give some indications on the evolution of the process of keratiniza-

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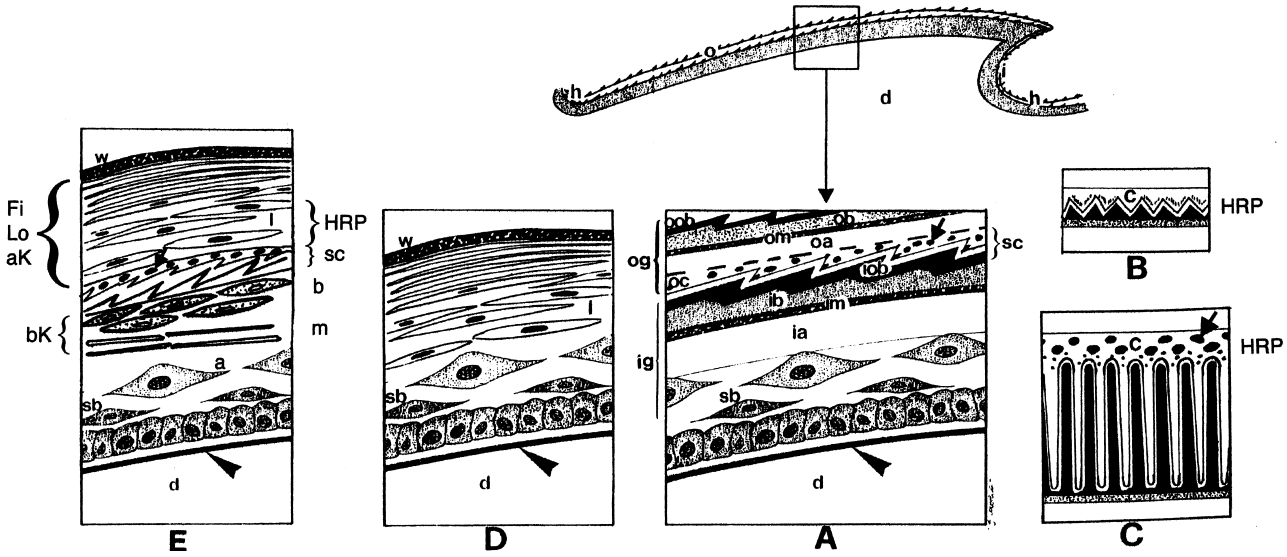

Fig. 1. – Schematic drawing illustrating the histology of lizard and snake scale. **A**, details of the epidermal stratification. **B**, aspect of the shedding complex in snakes (black areas represent the oberhautchen). **C**, aspect of the setae (black rods) of the shedding complex of gecko with keratohyalin-like granules in the clear layer (c, arrow). **D**, aspect of the initial phase of epidermal regeneration. **E**, later phase of epidermal regeneration with formation of the shedding complex and production of beta-keratin over alpha-keratin in oberhautchen and beta-layers. Legends: a, alpha layer; aK, alpha keratin production; b, beta layer; bK, beta keratin production; c, clear layer (containing keratohyalin-like granules, arrow); d, dermis; Fi, filaggrin-like immunoreactivity; h, hinge region; HRP, histidin-rich protein production; i, inner scale surface; ia, inner alpha-layer; ib, inner beta-layer; ig, inner epidermal generation; im, inner mesos layer; iob, inner oberhautchen; l, lacunar tissue; Lo, loricrin-like immunoreactivity; m, mesos layer; o, outer scale surface; oa, outer alpha-layer (incorporating also the lacunar layer); ob, outer beta-layer; oc, outer clear layer (containing keratohyalin-like granules, arrow); og, outer epidermal generation; om, outer mesos layer; oob, outer oberhautchen; sc, shedding complex. sb, suprabasal cells; w, wound epidermis. The arrowhead points to the basement membrane.

tion present in mammals (ALIBARDI, 2002; MADERSON & ALIBARDI, 2002).

Aside from alpha- and beta-keratins, the presence of filaggrin (a histidin-rich protein implicated in keratin aggregation in mammalian keratinocytes, see RESING & DALE, 1991; ISHIDA-YAMAMOTO et al., 2000), and loricrin (a sulphur-rich protein implicated in the formation of the mammalian cell corneus envelope, see MEHREL et al., 1990) has been presently analysed. The study on the expression of the last mentioned proteins was selected according to previous histochemical and autoradiographic studies on sulphur-rich and histidin-rich epidermal layers in lepidosaurian reptiles (SPEARMAN, 1966; BANERJEE & MITTAL, 1978; MITTAL & SINGH, 1987; ALIBARDI, 2001). No comprehensive study is presently available on the molecular characterization of these two types of keratinization (alpha- and beta-) in lizard epidermis. Since the process of epidermal differentiation in both normal and regenerating epidermis of lizards is similar (ALIBARDI, 1998, 2000a,b; ALIBARDI et al., 2000, 2001), both normal and regenerating epidermis can be used to study the process of keratinization.

The present immunohistochemical study attempts to: 1) summarise what is presently known in terms of distribution of some specific proteins in lizard epidermis, 2) address future molecular studies on the modalities of keratinization in reptilian epidermis, 3) compare the modality of keratinization in lizards with those of other vertebrates.

MATERIAL AND METHODS

Ten adult wall lizards (*Podarcis muralis*, Laurentii 1768) with normal and regenerating tails were used in this study. Six geckoes (*Hemidactylus turcicus*, Linnaeus 1758) were used to study the skin of the climbing pads of the arms and feet. Skin pieces, 2-5 mm long, were collected and immediately fixed in Carnoy's fluid (9 parts of ethanol and 1 part of acetic acid) or in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 4-6 hours, dehydrated in ethanol, and embedded in Bioacryl resin under UV light at 0-4°C (SCALA et al., 1992).

Immunocytochemistry was performed on 1-4 μm thick sections to localize keratins, filaggrin and loricrin as previously reported (ALIBARDI, 2000a,b). The beta-1 antibody, produced in rabbit against a chick scale beta-keratin was a kind gift of Dr. RH Sawyer (Biological Science Department, University of South Carolina, Columbia, USA, see SAWYER et al., 2000). The anti-alpha keratin antibodies (AE2, AE3, from mouse hybridoma lines), were purchased from Progen (Heidelberg, Germany): they recognize most keratinization-specific and basic alpha-keratins (SUN et al., 1983; O'GUIN et al., 1987). The anti-filaggrin antibody was characterized and kindly sup-

plied by Dr. B.A. Dale (Department of Oral Biology, University of Washington, Seattle, USA). It is a polyclonal antibody (#466) produced in rabbit and directed against rat filaggrin (DALE B.A., personal communication; RESING & DALE, 1991). It specifically stains keratohyalin-like granules of the granular layer of both human and rat epidermis, and a rat filaggrin band isolated by electrophoresis and immunoblotting (Alibardi & Maurizii, unpublished observations). The anti-loricrin antibody, produced in rabbit, was kindly supplied by Dr. E. Fuchs (Howard Hughes Medical Institute, University of Chicago, USA), and recognises a 15-amino acid sequence toward the C-terminal of mouse loricrin (MEHREL et al., 1990).

Tissues were preincubated for 20 minutes in 5% normal goat serum in 2% BSA in 0.05 M Tris/HCl buffer at pH 7.6, in order to neutralize aspecific antigenic sites on the sections, then incubated overnight at 4°C in the same medium (without the goat serum) containing the primary antibody (dilutions 1: 100-200 for beta-1, 1: 50-100 for alpha-antibodies, 1: 500-1000 for filaggrin, and 1: 200 for loricrin). After prolonged rinsing in the medium, the sections were incubated for 1 hour at room temperature in the same medium containing 1:50 of anti-mouse-IgG (for AE2 and AE3 detection) or anti-rabbit-IgG (for beta-keratin, filaggrin, loricrin detection) FITC conjugated secondary antibodies. After extensive rinsing, sections were mounted in Fluoromount (EM Sciences, USA), and observed under a Zeiss epifluorescence microscope equipped with a fluorescein filter.

For immunoelectron microscopy, 40-90 nm thick sections were collected on nickel grids, and immunostained with the primary antibody as above, but using 1% Cold Water Fish Gelatin in the Tris/HCl buffer to saturate non-specific bindings sites. An anti-mouse or anti-rabbit IgG conjugated to 10 nm large gold particles (Sigma, USA, or Biocell, UK) was used as the secondary antibody. Sections were studied, unstained or lightly stained with uranyl acetate, under a CM-100 Philips electron microscope.

RESULTS

The epidermis in lizards during post-shedding (resting) condition consists of a keratinized pale beta-layer and a darker alpha-layer underneath, followed by suprabasal cells and a basal layer (Fig. 2A). The epidermis of a regenerating tail initially consists of a pluristratified epithelium with a keratinized alpha-layer: the wound epidermis (see Fig. 1D). The AE2 antibody labels essentially the keratinized alpha-layer of normal and regenerating epidermis, a pattern also seen after application of the filaggrin- (Fig. 2C, D), and loricrin-antibodies (Fig. 2E, F). All the other layers (beta and living layers) remain unlabelled with these antibodies.

The ultrastructural analysis shows that while the AE2 antibody specifically labels the external alpha-keratinocytes of both normal and regenerating epidermis, the filaggrin antibody barely, if at all, immunolabels the external keratinocytes (Fig. 2G, H). The keratohyalin-like granules of the clear layer (see Fig. 1A, C) are not labelled with both anti-keratin (AE2 and AE3) and anti-filaggrin antibodies, while they are diffusely but specifically labelled with the anti-loricrin antibody (Fig. 2I). The alpha-keratinocytes of the alpha-layer or of the wound epidermis are diffusely but significantly labelled with the antiloricrin-antibody (Fig. 2J).

The distribution of the beta-1 antibody shows that it recognises antigens exclusively localized in the oberhautchen (weak labelling) and beta-layer (stronger labelling) of both normal and regenerating scales, and over the elongated oberhautchen spinulae (the setae) and the thin beta-layer of the digital pads of gecko (Fig. 3A-F). A weaker labelling is also seen in the first cells of the mesos layer (Fig. 3A) and in the basal-most part of the wound epithelium of the regenerating tail, although the latter labelling was not constantly seen (Fig. 3D). The beta-keratin labelling is substituted by alpha-keratin labelling in the lowermost mesos and alpha-layers (Fig. 3G, H).

The detailed ultrastructural analysis of lizard epidermis shows that, initially the beta-keratin packets of the oberhautchen cells (the small bundles of beta-keratin) are barely, if at all, labelled with the beta-1 antibody (Fig. 3I). Conversely, the compact matrix of the mature beta-layer (Fig. 4A) or beta-keratin bundles within the differentiating beta-cells of the inner beta-layer are intensely labelled with the beta-1 antibody but not with the AE3 antibody (Fig. 4B-D). Keratohyalin-like granules and tonofilament bundles in lacunar and clear cells are also unlabelled with the beta-1 antibody (Fig. 4D). Also the forming setae of the climbing pads of geckos show a specific immunolabelling of the growing and mature setae of the oberhautchen layer while no tonofilaments of the clear cells are labelled (Figs. 3F, 5A-C). At the beginning of setae formation only the pale keratin filaments of the core are labelled while the external fibrous material is not labelled (Fig. 5A). In the growing setae, the more external and denser bundles of beta-keratin are also less labelled than those located more centrally, which are electron-paler (Fig. 5B).

The small bundles of beta-keratin in differentiating beta-cells progressively merge and form larger beta-keratin masses, which are intensely labelled. This contrasts with the unlabelled tonofilaments of the undifferentiated cells present underneath the beta-layer (pre-beta and mesos-cells, Fig. 6A). In early differentiating beta-cells the labelling is also located over few roundish dark granules, sparse among the beta-keratin filaments (Fig. 6B). The latter granules are more frequent in presumptive, undifferentiated mesos-cells underneath the layer of beta-cells, and are located among unlabelled tonofilaments and vacuoles occupied by electron-pale lipid-like material (Fig. 6C). This further indicates that early in their differentiation the first mesos-cells contain some beta-keratin material.

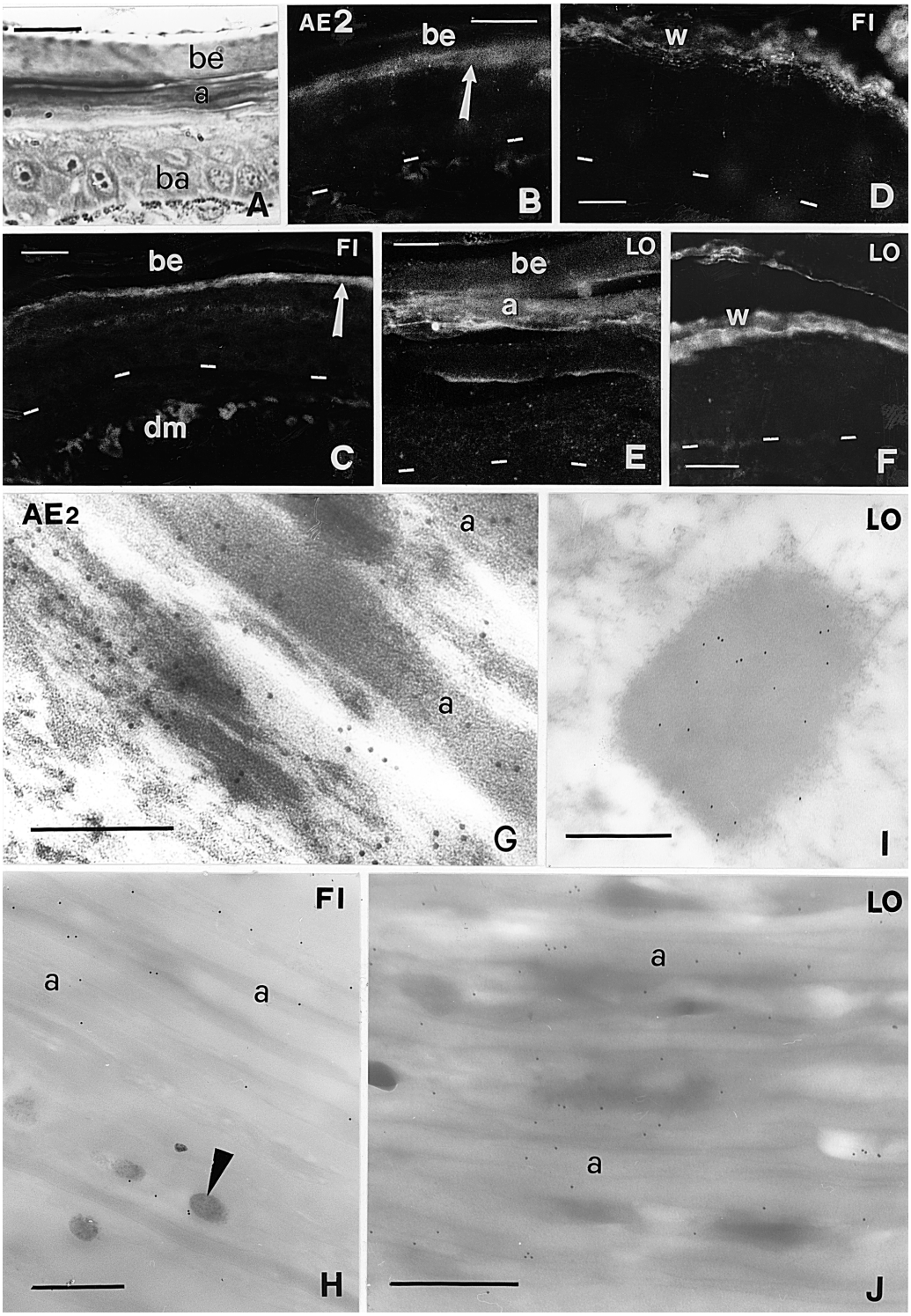

Fig. 2. – **A**, toluidine blue staining of a longitudinal section of normal lizard epidermis. Bar, 20 μm. **B**, AE2 immunofluorescence present in the alpha-layer of normal epidermis only (arrow). Bar, 20 μm. **C**, Filaggrin-like immunofluorescence in the alpha-layer (arrow) of normal epidermis but not in the other tissues. Bar, 20 μm. **D**, filaggrin-like labelled wound epidermis of regenerating epidermis. Bar, 20 μm. **E**, loricrine labelling of the alpha-layer only (a, artifactually detached from the underlying living layers) of the normal epidermis. Bar, 10 μm. **F**, loricrine immunolabelling of the wound epidermis of regenerating epidermis. Bar, 20 μm. **G**, alpha-keratinocytes (a) of the wound epidermis of regenerating epidermis decorated with gold particles after AE2 antibody staining. Bar, 200 nm. **H**, regenerating epidermis. Diffuse labelling for filaggrin of the alpha-keratinocytes (a) of the wound epidermis. The arrowhead points to a melanosome incorporated into the corneus layer. Bar, 500 nm. **I**, loricrine-labelled keratohyaline-like granule in the clear layer of regenerating epidermis. Bar, 500 nm. **J**, regenerating epidermis. Diffuse loricrine labelling of alpha keratinocytes (a) of wound epidermis. Bar,500 nm. **Legends**: a, alpha layer; AE2, AE2 immunolabelling; ba, basal layer; be, beta layer; dm, dermal melanophores (non-specific yellow stain); FI, filaggrine immunolabelling. LO, loricrine immunolabelling; w, wound epidermis; Dashes underline the basal layer.

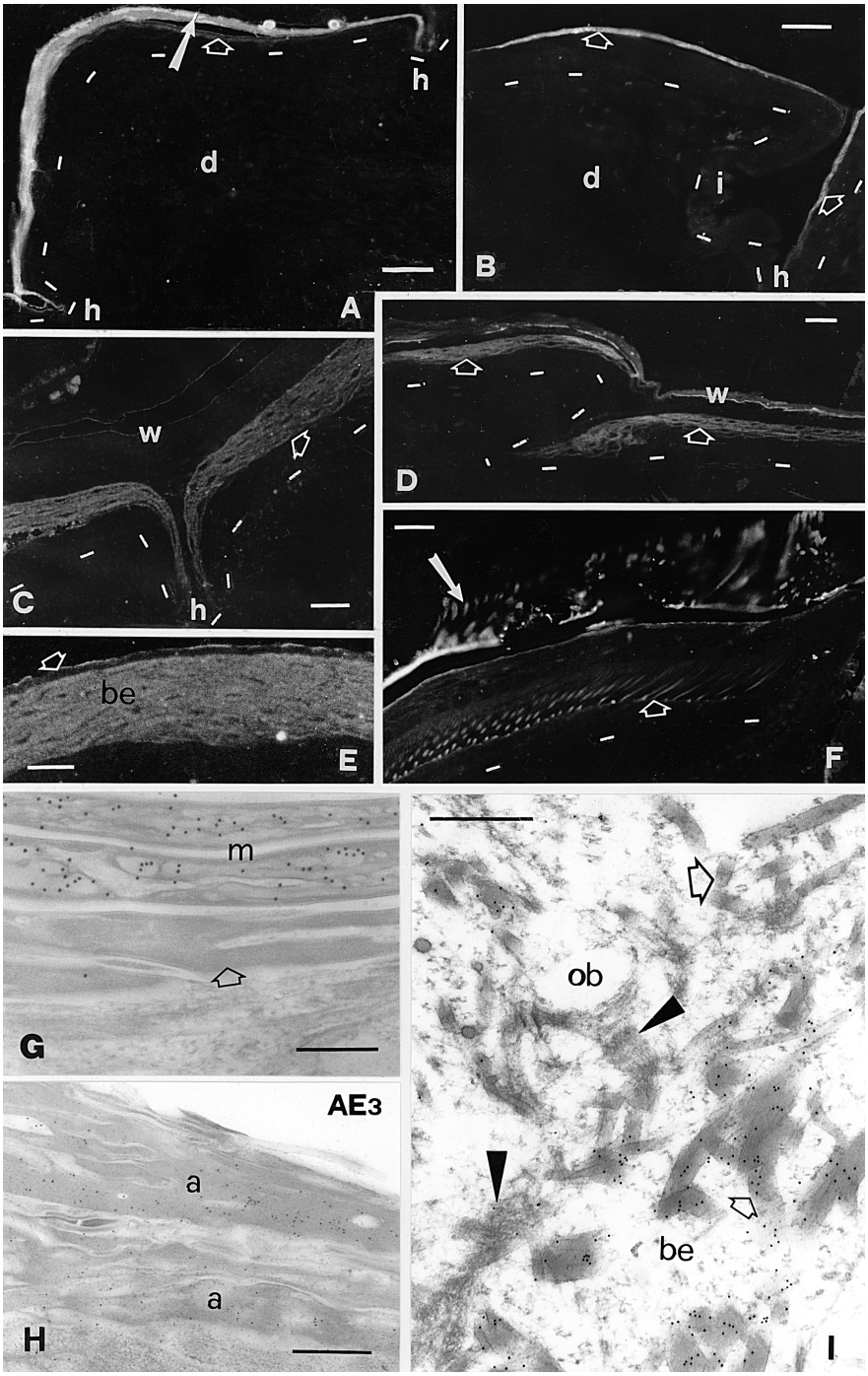

Fig. 3. – **A**, cross section of lizard scale in post-shedding condition (normal epidermis) showing beta-1 labelling in the outer beta-layer (arrow) and less in the mesos-layer (empty arrow). Bar, 20 μm. **B**, longitudinal section of gecko scale in post-shedding condition (normal epidermis) showing beta-1 labelling only in the beta layer of the outer scale surface (empty arrows), but not in the inner scale surface and hinge region. Bar, 20 μm. **C**, cross section of lizard regenerating scale showing beta-1 immunolabelling only in the inner beta-layer (empty arrow). Bar, 20 μm. **D**, longitudinal section of lizard regenerating scale showing beta-1 immunolabelling only in the inner beta layer (empty arrows) and in lowermost wound epidermis (w). Bar, 20 μm. **E**, detail of beta-1 labelling of inner beta layer of regenerating epidermis. The empty arrow points to the weakly positive oberhautchen. Bar, 10 μm. **F**, normal epidermis. Tip of modified scale of gecko climbing pads stained with the beta-1 antibody. Only the setae and the oberhautchen-beta layer of the outer (arrow) and those of the inner (empty arrow) generation are labelled. Bar, 20 μm. **G**, normal epidermis. Ultrastructural detail of mesos-layer of gecko scale showing beta-1-labelling gold particles in the upper mesos cells but not in the lower mesos cells (empty arrow). Bar, 200 nm. **H**, normal epidermis of gecko scale. Other cells of the mesos and alpha-layer (a) are labelled with the AE3 antibody. Bar, 500 nm. **I**, regenerating epidermis of lizard. Intensely beta-1-labelled beta-keratin packets (small empty arrow) in beta cell (be) beneath the cytoplasm of an oberhautchen cell (ob), which contains fibrous material (arrowheads) and unlabelled (large empty arrow) or little-labelled beta-packets. Bar, 500 nm. **Legends**: a, alpha-layer; AE3, AE3 labelling; be, beta cells; d, dermis; h, hinge region; i, inner scale surface; m, mesos layer; ob, oberhautchen cell; w, wound epidermis. Dashes underline the basal layer.

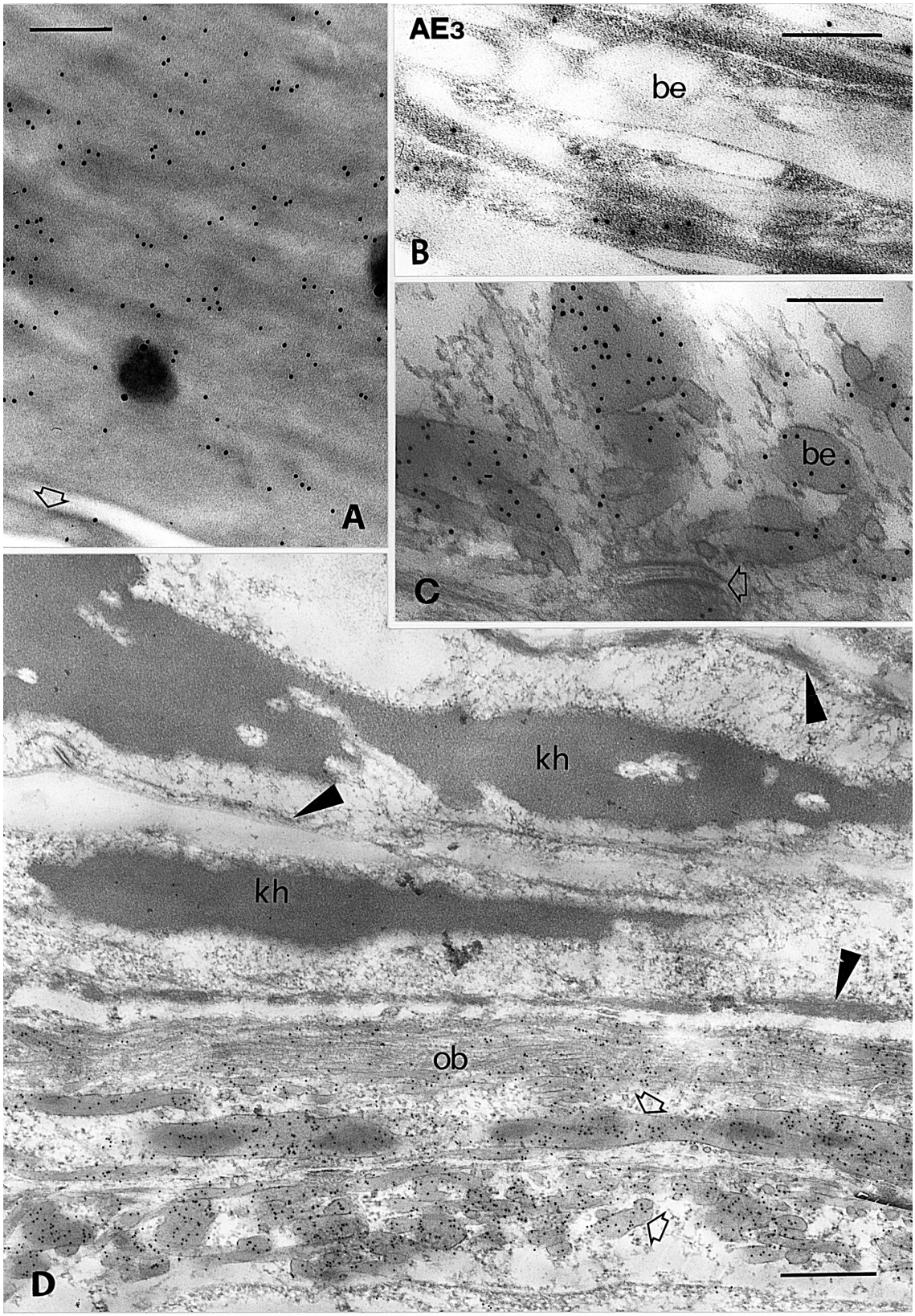

Fig. 4. – **A**, normal lizard epidermis. Uniformly-labelled outer beta-layer with the beta-1 antibody, contacting a narrow mesos cell (empty arrow). Bar, 200 nm. **B**, absence of AE3 immunolabelling over beta-packets (be) of differentiating beta-cell in regenerated scale. Bar, 200 nm. **C**, regenerating epidermis. Beta-1 immunolabelled beta-packets (be) of forming beta-cell, which contacts an unlabelled desmosome (empty arrow). Bar, 200 nm. **D**, regenerating epidermis. Detail of passage region between the clear layer and the underlying oberhautchen (ob) immunostained with the beta-1 antibody. No labelling is present over tonofilaments (arrowheads) and the large keratohyalin-like granules (kh) in the clear layer. Over the oberhautchen cytoplasm is seen a diffuse labelling, which becomes intense over the large beta-packets at the bottom (empty arrows). Bar, 500 nm.

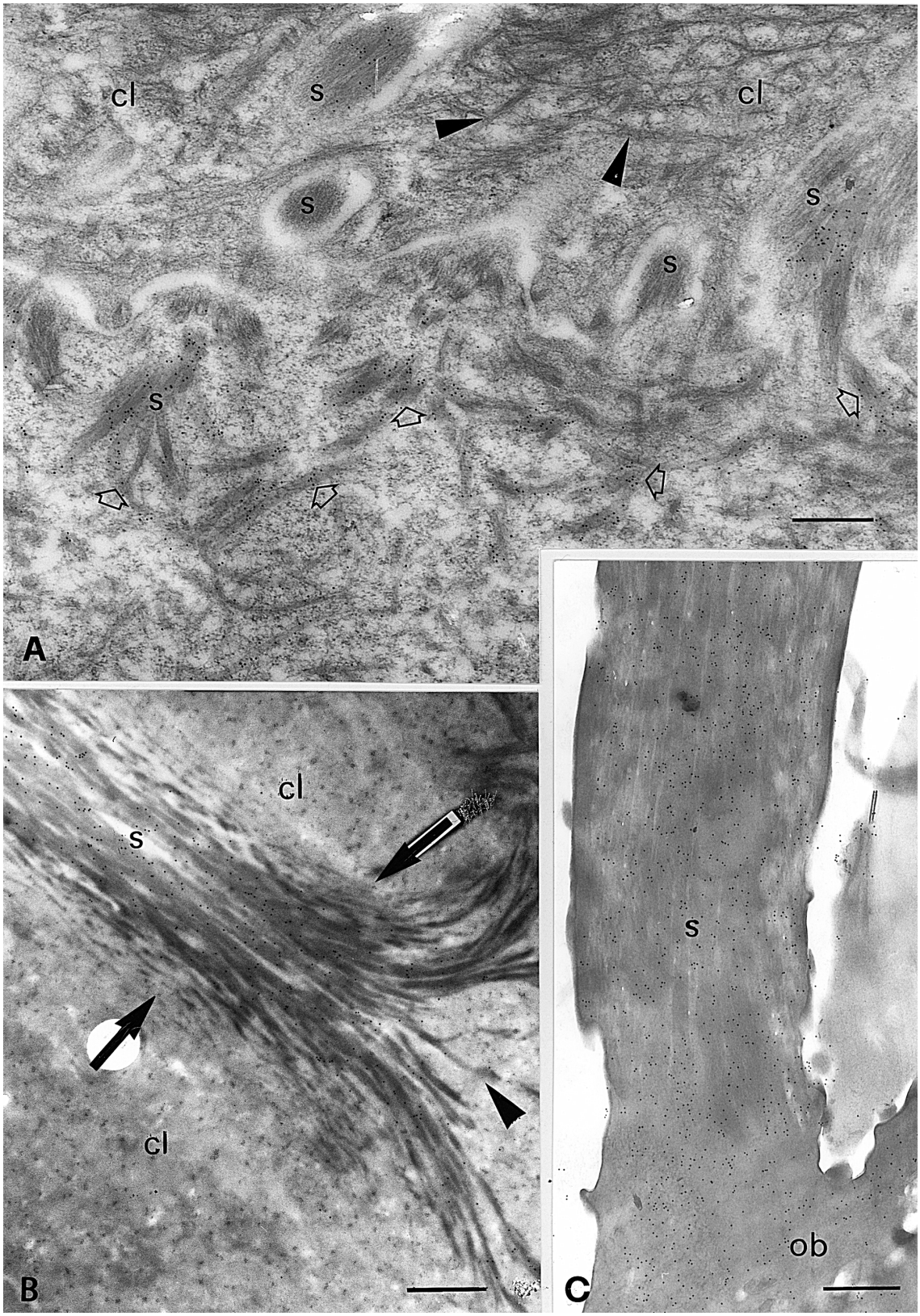

Fig. 5. – **A**, normal epidermis of gecko. Detail of forming setae of gecko; shedding complex stained with beta-1 antibody. Only the beta packets (empty arrows) at the base of the setae (s) are decorated with gold particles but not the tonofilaments (arrowheads) in the cytoplasm of clear cells (cl). Bar, 500 nm. **B**, normal epidermis of gecko. Growing setae of inner generation (s, the large arrows indicate its lateral boundaries, and the arrowhead its base) containing labelled central beta-keratin filaments. No structure is labelled in the cytoplasm of surrounding clear cells (cl). Bar, 500 nm. **C**, normal epidermis of gecko. Mature outer setae (s) in continuity with the oberhautchen (ob), which are homogeneously labelled with the beta-1 antibody. Bar, 500 nm.

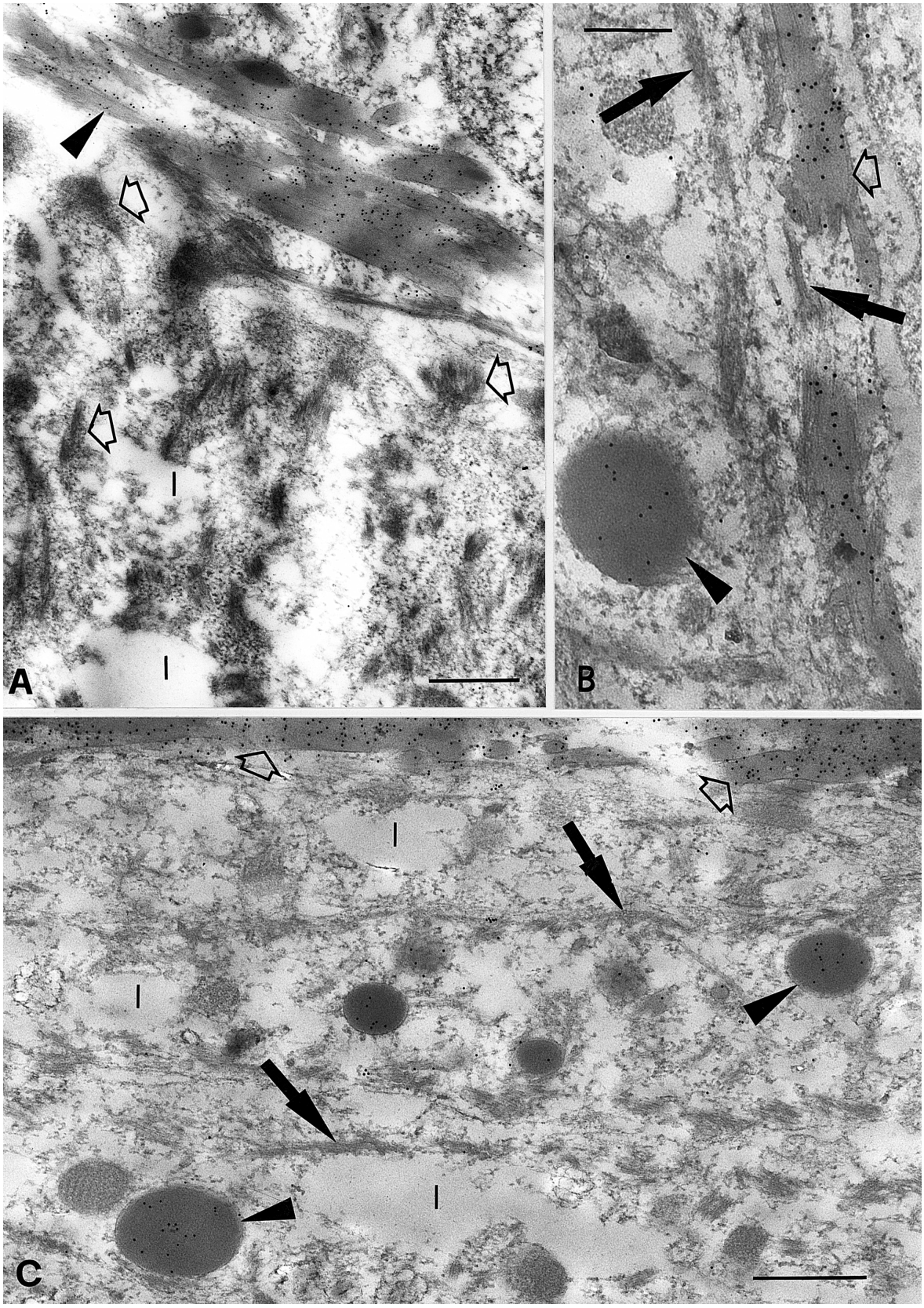

Fig. 6. – **A**, regenerating epidermis of lizard. Detail of beta-1-immunolabelled beta-filaments (arrowheads) in maturing beta cell as compared with the unlabelled tonofilaments (empty arrows) of undifferentiated beta cell at the bottom containing lipidic vacuoles (l). Bar, 500 nm. **B**, regenerating epidermis of lizard. Detail of beta-1-labelled, pale beta-filaments (empty arrows) and of a denser roundish granule (arrowhead) in beta cells. Fibrous tonofilaments are unlabelled (large arrows). Bar, 500 nm. **C**, regenerating epidermis of lizard. Detail of a forming mesos cell beneath the beta-layer (empty arrows), which contains lipid droplets (l) and unlabelled tonofilaments (large arrows). Only few dense granules are labelled (arrowheads). Bar, 500 nm.

TABLE 1

Summary of the immunoreactivities of different epidermal layers (of both normal and regenerating epidermis) to the antibodies utilized in this study (AE2, AE3, Beta-1; Filaggrin; Loricrin). -, negative; -/+, negative or low to diffuse; +, positive. ++, strongly positive. (forming)= early differentiation; (mature)= complete differentiation; one single indication refers to both forming and mature layers

Epidermal Layers	AE2	AE3	Beta-1	Filaggrin	Loricrin
Oberhautchen	-	+ (forming) -/+ (mature)	-/+ (forming) + (mature)	-	-
Beta	-/+	+ (forming) -/+ (mature)	++	-	-
Mesos	-	-/+	-/+	-	-
Alpha	+ or ++	+ or ++	-	-/+ or +	+
Lacunar	- (forming) + (mature)	+	-	-/+ or +	-/+ or +
Clear	- (forming) + (mature)	+	-	-/+ or +	-/+ or +

Table 1 summarises the immunoreactivities of the different epidermal layers of both normal and regenerating epidermis.

DISCUSSION

The present study confirms previous data (ALEXANDER & PARAKKAL, 1969; BADEN & MADERSON, 1970; MADERSON et al., 1972; LANDMANN, 1979), and indicates that lizard epidermis consists of two superimposed, alternating, modalities of keratinization, alpha- that resembles mammalian keratinization, and beta- that resembles avian keratinization.

Also, the observations of the present study complete previous analyses on the distribution of alpha-keratins and keratin-associated proteins in the epidermis of lizards (ALIBARDI, 2000 a,b). Beta-keratin substitutes for alpha-keratin, and is packed into hard and compact beta-cells that merge into a resistant, electron-pale beta-layer containing sparse electron-denser areas. The similarity of some antigenic epitopes of lizard beta-keratin with those of chick beta-keratin is demonstrated by the specific immunolocalization with this antibody produced against chick scale beta-keratin (CARVER & SAWYER, 1987; SHAMES et al., 1988, 1989; SAWYER et al., 2000). The labelling further suggests a phylogenetic affinity between reptilian and avian hard (beta) keratins (SAWYER et al., 2000). This immunoreactivity is well manifested in the compact form of beta-keratin of the mature beta-layer, within the thick beta-packets and in the thick filaments of compacting beta-cells.

The present observations also indicate that the two layers of passage between alpha to beta (oberhautchen, see Fig. 1A) and beta to alpha (mesos), contain small quantities of beta-keratin. While the mesos layer (and the wound epidermis of regenerating tail) rapidly looses beta-keratin as alpha-keratogenic cells are generated underneath, the oberhautchen layer rapidly accumulates beta-keratin as it merges with the underlying beta-cells.

Therefore the epidermis of lizards seems to utilise the mechanism of alpha-keratinization for the production of a softer layer where the extensibility of this keratin may be used for keeping elasticity in the hinge region among scales. The rigid, hard (beta) keratin covering of the outer scale surface also protects the underlying mesos and alpha-layers where the water-barrier is located (MENON et al., 1996; MADERSON et al., 1998). In fact, the mesos layer and also the alpha-layer accumulate lipids that form the barrier, limiting water loss. However, because these layers have a limited extensibility, they must be periodically shed in order to allow somatic growth, and are replaced by a new epidermal generation (MADERSON et al., 1998).

Without biochemical data it remains uncertain whether filaggrin and loricrin molecules are really present in the compact alpha-layer of lizard epidermis (ALIBARDI, 2001). The case of filaggrin in particular remains enigmatic, as this protein shows very poor cross-reactivity even in the granular layer of epidermis of different mammals (RESING & DALE, 1991). Although the observed immunoreactivity may or may not be due to a mammalian-like filaggrin, previous autoradiographic studies have, however, indicated that histidin-rich proteins (HRP) are present in lizard and snake epidermis (ALIBARDI, 2001, 2002, and unpublished observations, see Fig. 1E). As in the epidermis of turtles, crocodilians, and snakes, in lizards weak filaggrin- and loricrin-like immunoreactivities are associated with AE2 immunoreactivity and epidermal regions rich in sulfhydryl groups (BANERJEE & MITTAL, 1978; MITTAL & SING, 1987; ALIBARDI, 2001). It is known that in mammalian epidermis, antibodies that recognise both AE2-positive keratins and filaggrin, seem to recognise a common, uncharacterized, antigenic sequence (DALE & SUN, 1983). It is, therefore, possible that the overlap of immunoreactivity observed in lizard epidermis may be due to the presence of

an antigenic determinant that is recognised by both the AE2 and anti-filaggrin antibody. Histochemical reactions have also indicated that the alpha layer contains more sulfhydrylic groups than the beta-layer, as in the latter most sulphur is probably under a disulfide bond (MITTAL & SINGH, 1987; ALIBARDI, 2001). The association of these groups with non completely polymerized alpha-keratins or with other sulphur-rich proteins may explain the immunoreactivity to loricrin, although the labelling is diffuse within keratinocytes and not linearly associated with the cell corneus membrane as in mammalian keratinocytes (MEHREL et al., 1990; STEVEN et al., 1990; ISHIDA-YAMAMOTO et al., 2000). However, a marginal layer (the thin but dense material associated with the plasmalemma of maturing keratinocytes (LANDMANN, 1979)) is not constantly found in mesos and alpha-keratinocytes of lizard epidermis. Only future biochemical studies will allow clarification of the true identity of the histidin-rich and sulphur-rich molecules present in reptilian epidermis, and comparison of the amino acidic sequence of these putative proteins with those present in mammalian epidermis.

We hypothesise that in lepidosaurian and archosaurian progenitors, cells of the stratum corneum were initially capable of producing alpha-keratin, and later also beta-keratin (MADERSON & ALIBARDI, 2000; SAWYER et al., 2000). Although not constantly seen, the presence of some immunoreactivity for beta-keratin at the base of the wound epidermis suggests that a small quantity of beta-keratin may also be present in this repairing epithelium.

Whatever the case, it has probably been a specific trend in lepidosaurian evolution to segregate cells capable of synthesizing alpha from those capable of producing beta-keratin. This aspect is presently visible in the intermediate region between the alpha- and beta-layer of the epidermis of the living fossil *Sphenodon punctatus* (ALIBARDI, 1999; ALIBARDI & MADERSON, personal observations). In this region beneath the alpha- and beta-layer, cells with alpha- and beta-characteristics are present. It may be speculated that a further cell segregation produced a final alpha-layer (the clear layer) contacting the first beta-layer (the oberhautchen), so that a shedding complex was formed. The different consistency of these two layers, together with the enzymatic process for the degradation of the junctions between clear layer and oberhautchen, produces the detachment of the two layers and the molt (GOSLAR, 1964; LANDMANN, 1979; ALIBARDI, 1998; MADERSON et al., 1998).

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REFERENCES

ALEXANDER, N.J. (1970). Comparison of α and β keratin in reptiles. *Z. Zellforsch*., 110: 153-165.

ALEXANDER, N.J. & P.F. PARAKKAL (1969). Formation of alpha and beta-type keratin in lizard epidermis during the molting cycle. *Z. Zellforsch*., 110: 72-87.

ALIBARDI, L. (1997) Ultrastructural and autoradiographic analysis of setae development in the embryonic pad lamellae of the lizard *Anolis lineatopus*. *Ann. Sci. Nat.* (*Zoologie)*, Paris, 18: 51-61.

ALIBARDI, L. (1998). Presence of acid phosphatase in the epidermis of the regenerating tail of the lizard (*Podarcis muralis*) and its possible role in the process of shedding and keratinization. *J. Zool.*, 246: 379-390.

ALIBARDI, L. (1999). Keratohyalin-like granules and differentiation of the shedding complex in embryonic and regenerating epidermis of lizard and *Sphenodon* (Reptilia, Lepidosauria). *Amph. Rept.,* 20: 11-23.

ALIBARDI, L. (2000a). Ultrastructural localization of alpha-keratins in the regenerating epidermis of the lizard *Podarcis muralis* during formation of the shedding layer. *Tissue & Cell*, 32: 153-162.

ALIBARDI, L. (2000b). Light and electron microscopical localization of filaggrin-like immunoreactivity in normal and regenerating epidermis of the lizard *Podarcis muralis*. *Acta Histochem*., 102: 453-473.

ALIBARDI, L. (2001). Keratohyalin-like granules in lizard epidermis: evidence from cytochemical, autoradiographic, and microanalytical studies. *J. Morphol*., 248: 64-79.

ALIBARDI, L. (2002). Ultrastructure of the embryonic snake skin and putative role of histidine in the differentiation of the shedding complex. *J. Morphol*., 250: in press.

ALIBARDI, L., M.G. MAURIZII & C. TADDEI (2000). Immunocytochemical and electrophoretic distribution of cytokeratins in the regenerating epidermis of the lizard *Podarcis muralis*. *J. Morphol*., 246: 179-191.

ALIBARDI, L., M.G. MAURIZII & C. TADDEI (2001). Immunocytochemical and electrophoretic distribution of cytokeratins in the resting stage epidermis of the lizard *Podarcis sicula*. *J. Exp. Zool.,* 289: 409-418.

BADEN, H.P. & P.F.A. MADERSON (1970). Morphological and biophysical identification of fibrous proteins in the amniote epidermis. *J. Exp. Zool*., 174: 225-232.

BADEN, H.P., S. SVIOKLA & I. ROTH (1974). The structural proteins of reptilian scales. *J. Exp. Zool*., 187: 287-294.

BANERJEE, T.K. & A.K. MITTAL (1978). Histochemistry of the epidermis of the chequered water snake *Natrix piscator* (Colubridae, Squamata). *J. Zool.,* 185: 415-435.

BUDZ, P.E. & L.O. LARSEN (1973). Structure of the toad epidermis during the moulting cycle. I. Light microscopic observations on *Bufo bufo* (L). *Z. Zellforsch*., 144: 353-368.

BUDZ, P.E. & L.O. LARSEN (1975). Structure of the toad epidermis during the moulting cycle. II. Electron microscopic observations on *Bufo bufo* (L). *Z. Zellforsch*., 159: 459-483.

CARVER, W.E. & R.H. SAWYER (1987) Development and keratinization of the epidermis in the common lizard, *Anolis carolinensis*. *J. Exp.Zool*., 243: 435-443.

DALE, B.A. & T.T. SUN (1983). Filaggrin, 56.5 kd and 65-67 kd keratins share an antigenic determinant as defined by the AE2 monoclonal antibody. *J. Cell Biol.* 97: 228a (abstract).

FLAXMANN, B.A. (1972). Cell differentiation and its control in the vertebrate epidermis. *Am. Zool*., 12: 13-25.

Goslar, G.V.H. (1964). Beitrage zum Hautungsvorgang der schlangen. *Acta Histochem*, 17: 1-60.

Irish, F.J., E.E. Williams & E. Seiling (1988). Scanning electron microscopy of changes in epidermal structure occurring during the shedding cycle in squamate reptiles. *J. Morph*., 197: 105-126.

Ishida-Yamamoto, A., H. Takahashi & H. Iizuka (2000). Immunoelectron microscopy links molecules and morphology in the studies of keratinization. *Eur. J. Dermatol*., 10: 429-435.

Landmann, L. (1979). Keratin formation and barrier mechanisms in the epidermis of *Natrix natrix* (Reptilia, Serpentes): an ultrastructural study. *J. Morph*., 162: 93-126.

Landmann, L. (1986). The skin of Reptiles. Epidermis and Dermis. In: Bereither-Hahn J., Matoltsy A.G. & Sylvia-Richards K., (eds), *Biology of the integument*, vol. 2 Vertebrates. Springer-Verlag, Berlin and New York: 150-187.

Maderson, P.F.A. (1970) Lizard glands and lizard hands: models for evolutionary study. *Forma Functio*, 3: 179-204.

Maderson, P.F.A. (1985). Some developmental problems of the reptilian integument. In: Gans C., Billett F. & Maderson P.F.A., editors. *Biology of Reptilia*. Vol. 14B. New York: John Wiley & Sons. p 525-598.

Maderson, P.F.A. & L. Alibardi (2000a). The development of the sauropsid integument: a contribution to the problem of the origin and evolution of feathers. *Am. Zool*., 40: 513-529.

Maderson, P.F.A. & L. Alibardi (2001). Origin and evolution of the mammalian integument. *Amer Zoo*l., 41 (Abstract): 112-113.

Maderson, P.F.A., B.A. Flaxmann, S.I. Roth & G. Szabo (1972). Ultrastructural contribution to the identification of cell types in the lizard epidermal generation. *J. Morph*., 136: 191-210.

Maderson, P.F.A., T. Rabinowitz, B. Tandler & L. Alibardi (1998). Ultrastructural contributions to an understanding of the cellular mechanisms in lizard skin shedding with comments on the function and evolution of a unique lepidosaurian phenomenon. *J. Morph*., 236: 1-24.

Matoltsy, A.G. (1987). Concluding remarks and future directions. In: Sawyer, R.H. (Ed) *The molecular and developmental biology of keratins*. *Curr. Top. Dev. Biol*., 22: 255-264.

Mehrel, T., D. Hohl, J.A. Rothnagel, M.A. Longley, D. Bundman, C. Cheng, U. Lichti, M.E. Bisher, A.C. Steven, P.M. Steinert, S.H. Yuspa & D.R. Roop (1990). Identification of a major keratinocyte cell envelope protein, loricrin. *Cell*, 61: 1103-1112.

Menon, G.K., P.F.A. Maderson, R.C. Drewes, L.F. Baptista, L.F. Price & P.M. Elias (1996). Ultrastructural organization of avian stratum corneum lipids as the basis for facultative cutaneous waterproofing. *J. Morphol.,* 227: 1-13.

Mittal, A.K. & P.N. Singh (1987) Scale epidermis of *Natrix piscator* during its sloughing cycle: structural organization and protein histochemistry (Reptilia: Colubridae). *J. Zool*., 213: 545-568.

o'Guin, M.W., S. Galvin, A. Shermer & T.T. Sun (1987). Patterns of keratin expression define distinct pathways of epithelial development and differentiation. In: Sawyer, R.H. (Ed) *Cell and molecular biology of keratins*. *Curr. Top. Dev. Biol*., 22: 97-125.

Resing, K.A. & B.A. Dale (1991). Proteins of keratohyalin. In: Goldsmith, L.A. (Ed) *Physiology, Biochemistry, and Molecular Biology of the Skin*, vol. 1, Oxford University Press, New York and Oxford: 148-167.

Sawyer, R.H., T. Glenn, J.O. French, B. Mays, R.B. Shames, G.L. Barnes, W. Rhodes & Y. Ishikawa (2000). The expression of beta (β) keratins in the epidermal appendages of reptiles and birds. *Amer. Zool.*, 40: 530-539.

Shames, R.B., W. Knapp, W.E. Carver & R.H. Sawyer (1988). Identification, expression, and localization of beta-keratin gene products during development of avian scutate scales. *Differentiation,* 38: 115-123.

Shames, R.B., L.W. Knapp, W.E. Carver, L.D. Washington & R.H. Sawyer (1989). Keratinization of the outer surface of the avian scale: Interrelationship of alpha and beta keratin filaments in a cornifying tissue. *Cell. Tiss. Res.,* 257: 85-92.

Scala, C., G. Cenacchi, C. Ferrari, G. Pasquinelli, P. Preda & G.C. Manara (1992). A new acrylic resin formulation: a useful tool for histological, ultrastructural, and immunocytochemical investigation. *J. Histoch. Cytoch*., 40: 1799-1804.

Spearmann, R.I.C. (1966). The keratinization of epidermal scales, feathers and hairs. *Biol. Rev*., 41: 59-96.

Sun, T.T., R. Eichner, W.G. Nelson, S.C.G. Tseng, R.A. Weiss, M. Jarvinen & J. Woodcock-Mitchell (1983). Keratin classes: molecular markers for different types of epithelial differentiation. *J. Inv. Dermatol*., 81: 109-115.

Steven, A.C., M.E. Bisher, D.R. Roop & P.M. Steinert (1990). Biosynthetic pathways of filaggrin and loricrin-two major proteins expressed by terminally differentiated epidermal keratinocytes. *J. Struct. Biol*., 104: 150-162.

Wyld, J.A. & A.H. Brush (1979). The molecular heterogeneity in the reptilian epidermis. *J. Mol. Evol.,* 12: 331-347.

Wyld, J.A. & A.H. Brush (1983). Keratin diversity in the reptilian epidermis. *J. Exp. Zool*., 225: 387-396.

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