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Histological and ultrastructural effects of cyclosporin A on normal human skin xenografted on to nude mice

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Summary. Cyclosporin A (CsA) is a potent immunosuppressant with a selective activity on T-helper lymphocytes. However, CsA also exerts biological effects on non-lymphoid cells (fibroblasts, endothelial and epithelial cells). CsA can inhibit in vivo and in vitro DNA synthesis of epidermal keratinocytes (EK) and induces in vivo morphological alterations of kidney epithelial cells. In the present study we investigated the in vivo effects of a short-term CsA treatment (50 mg/kg per day) on DNA synthesis (evaluated through 5-bromo-2′-deoxyuridine incorporation) and on the histological features of normal human skin xenografted (NHSX) on to congenitally athymic nude mice. When compared with control NHSX, CsA induced a statistically significant inhibition of DNA synthesis of NHSX EK. At the light- and electron-microscopic level, apart from a decrease in the thickness of the viable epidermis of NHSX (statistically non-significant), no noticeable differences between treated and control NHSX could be detected. EK, Langerhans cells and melanocytes appeared morphologically unaffected by CsA and no signs of acute toxicity (giant mitochondria, vacuolization, microcalcifications) were seen. These results suggest that CsA exerts a subtle effect on human EK; indeed, despite an unequivocal antiproliferative activity, no significant histological changes related to the acute CsA toxicity seem to be induced on the various epidermal cell types.

Key words: Cyclosporin A – Human skin xenografts – Histology – Electron microscopy

Introduction

Cyclosporin A (CsA), a cyclic undecapeptide of fungal origin, is mainly known as a potent immunosuppressant used with success in the prevention of allograft rejection, by virtue of its T-helper-lymphocyte-directed immunosuppressive activities. However, CsA affects also non-lymphoid cells in various ways. Recent studies have demonstrated that CsA induces a growth inhibition of several normal and neoplastic epithelial cells in vitro (including epidermal keratinocytes) of both human and animal origin (Saydjari et al. 1986a, b; Nickoloff et al. 1988; Fisher et al. 1988; Furue et al. 1988; Ramirez-Bosca et al. 1989; Fairley et al. 1989; Lucia et al. 1986; Andersson et al. 1984). We have recently shown that CsA also exerts in vivo an antiproliferative effect on human epidermal keratinocytes (EK) xenografted onto congenitally athymic, nude mice. This is shown by a decrease in the number of EK in the S-phase of the cell cycle (evaluated by the incorporation of 5-bromo-2′-deoxyuridine, BrdU; Urabe et al. 1989a. Since young nude mice genetically lack a T-cell-mediated immunity, the in vivo effect of CsA on EK is not conveyed via T-cells but is likely due to a direct CsA-EK interaction.

However, it has been observed that CsA treatment induces macroscopically visible changes in human skin, such as hypertrichosis (Wysocki and Daley 1987) or improvement of ichthyosis vulgaris (Velthuis and Jesserun (1985) and psoriasis (Ellis et al. 1986), two diseases in which there is abnormal epidermal differentiation. These facts, strongly suggesting that CsA exerts a direct activity on EK, prompted us to study the possible histopathological effects of CsA treatment on to human skin. The study was carried out using the model of xenografts on nude mice, since this system allows the in vivo long-term maintenance of normal human skin (NHS) and its treatment with high doses of the drug under study.

Materials and methods

Six-week-old congenitally athymic female nude mice (Swiss nu/nu, Iffa Credo, Les Onçis, France), weighing on average 20 g were used. Xenografting of human skin was performed as follows: abdominal human skin of a young female patient undergoing plastic surgery was keratotomized with an electric Castroviejo keratotome at a depth of 0.2–0.4 mm, and circular normal human skin xeno-

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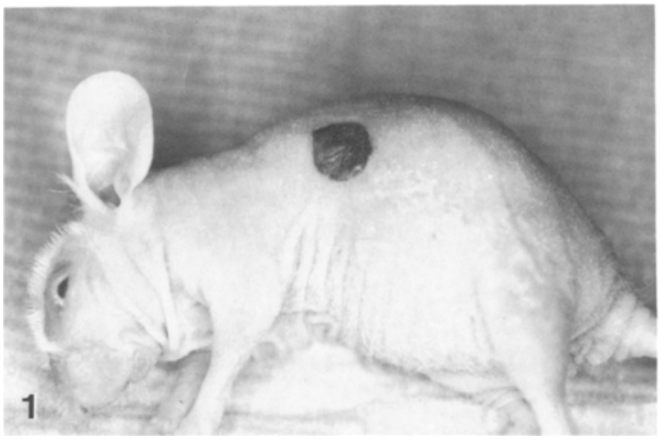

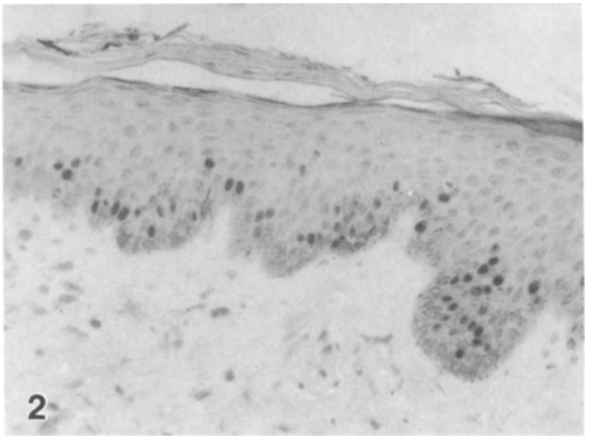

Fig. 1. Normal human skin xenograft (NHSX)-bearing nude mouse 1 month after grafting. Note the pronounced pigmentation of the NHSX

Fig. 2. NHSX immunostained for 5-bromo-2'-deoxyuridine. Note the presence of numerous epidermal keratinocytes (EK) bearing labelled nuclei. Alkaline phosphatase revealed by fast-red TR salt, ×250

grafts (NHSX) of 1 cm in diameter were prepared. These were placed, dermal side down, on a circular graft bed of a slightly smaller size, prepared under intraperitoneal pentobarbital anaesthesia on the right upper dorsal region of each mouse. The grafts were fixed in position with Transpore surgical tape and dressed with self-adhesive sticking plaster. Ten days after grafting the dressings were removed. Mice bearing healthy-looking NHSX were randomly divided in two groups of six members each.

CsA-treated mice received in the back area over a 3-week period a daily subcutaneous injection of 50 mg/kg CsA (Sandoz, Basel, Switzerland) diluted in olive oil. Control mice received a daily injection of an equivalent volume of olive oil. During the last 5 days of the experiment, all mice received three daily intraperitoneal injections of 100 mg/kg BrdU diluted in normal saline. At the end of the study CsA-treated mice were killed and their blood collected for measuring CsA blood levels.

At the end of the study, the NHSX were excised and divided in two parts. One of them was fixed for 4 h in buffered formalin (10%) and embedded in paraffin. Sections (3 μm) were then cut, stained with haematoxylin and eosin and examined under a Zeiss light microscope coupled to a semi-automatic image analyser (Videoplan/Kontron, Munich, FRG). On each specimen, the mean epidermal thickness (excluding the stratum corneum) was calculated from the ratio: epidermal section surface/length of interface between the granular and horny layers (which was more or less straight). The degree of papillomatosis was calculated as the ratio: length of dermal-epidermal junction/length of the corresponding granular-horny layer interface. The visualization of BrdU-incorporating (S-phase) EK was carried out through an avidin-biotin-alkaline phosphatase technique, using a monoclonal antibody to BrdU (Beckton-Dickinson, Mountain View, Calif.), as described in detail elsewhere (Kanitakis et al. 1988). The results were expressed as numbers of BrdU+ cells/mm length of epidermal basement membrane.

For electron microscopy, the second fragment of NHSX was fixed in 2% glutaraldehyde, post-fixed in 1% osmium tetroxide and embedded in Epon. Ultra-thin sections were counterstained with lead citrate and uranyl acetate and examined under a Jeol 1200 EX electron microscope (Centre de Microscopie Electronique appliquée à la Biologie, Villeurbanne, France).

Results

On macroscopic examination, NHSX were more pigmented and had a harder consistency on palpation than the surrounding mouse skin (Fig. 1). During the period of the experiment, no obvious macroscopic change of the NHSX was noted. A noticeable phenomenon, observed during the last 2 weeks of CsA administration, was the growth of white hair, mainly over the dorsal region and the cephalic extremity of treated mice. This observation provided firm evidence that CsA had effectively reached the skin and exerted on it its best-known biological activity (hypertrichosis).

Trough blood levels of CsA (and its metabolites) were measured by a radioimmunological assay. The mean value (±SD) was 1024±644 ng/ml.

BrdU+ EK were seen as cells containing a nucleus with a red reaction product (Fig. 2). The mean number (±SD) of BrdU+ EK per millimetre length of epidermal basement membrane in control and CsA-treated NHSX was 464±269 and 164±113 respectively (Table 1). The difference, evaluated by the Student's *t*-test, was statistically significant ($p<0.05$).

Control animals maintained the main histological features of NHS and were therefore clearly distinguishable from the surrounding mouse skin. The latter comprises a thin epidermis, consisting of three or four cellular layers having a mean thickness of 20 μm; the underlying dermis contains abundant small, resting hair follicles with sebaceous glands. The human epidermis had blended, at both extremities of the NHSX with the

Table 1. Histological and proliferation characteristics of control and cyclosporin-A-treated normal human skin xenografts (mean ± SD)

	Control	CsA-treated
S-phase EK[a]	464±269	164±113*
Thickness (μm)	99±28	85.5±12**
Papillomatosis	1.56±0.26	1.51±0.28**

* $p<0.05$; ** Not significant

[a] Per millimetre length of epidermal-dermal junction

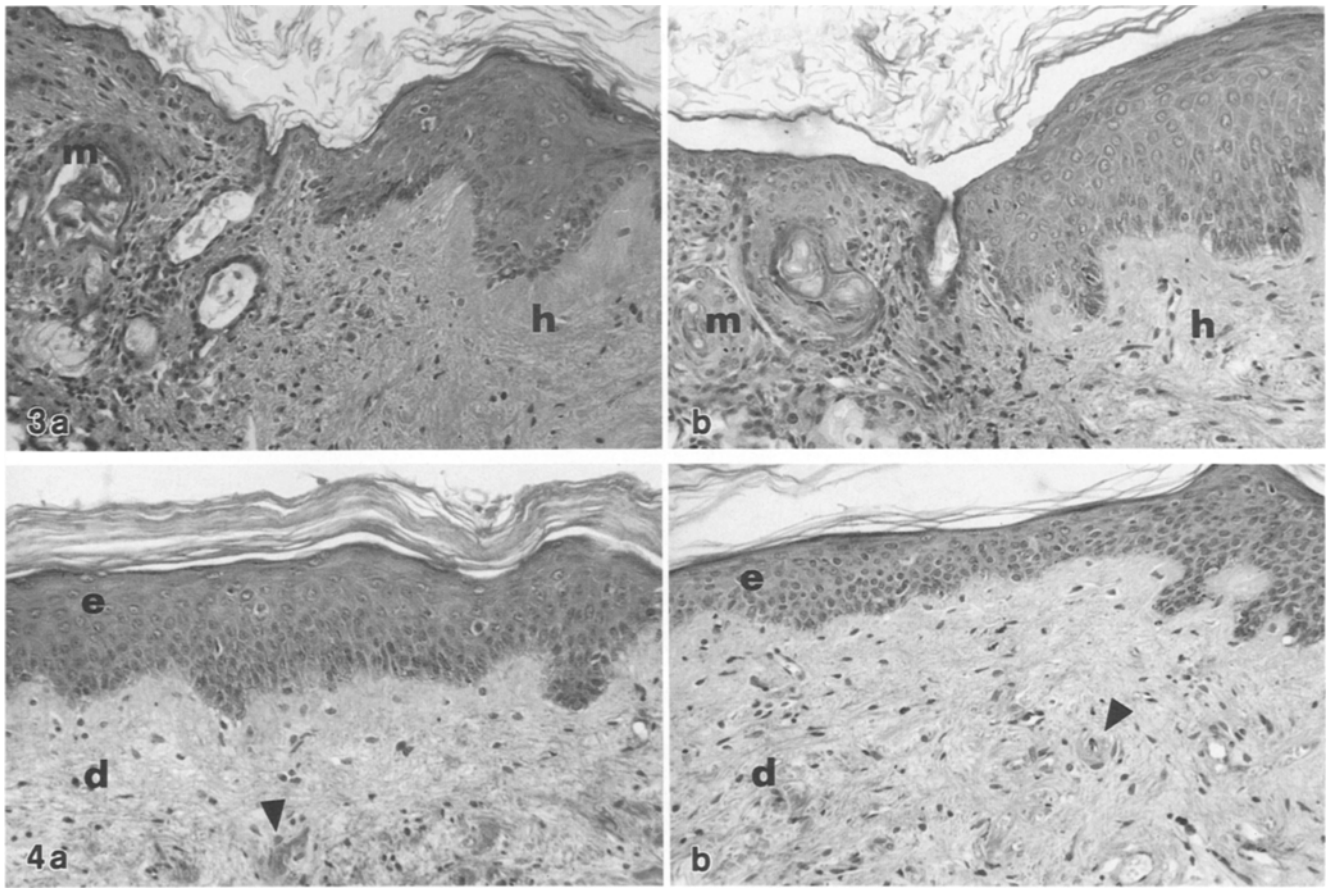

Fig. 3a, b. Light-microscopic aspect of control (**a**) and CsA-treated (**b**) NHSX at the end of the study. Mouse skin (*m*) consists of a thin epidermis and a dermis containing numerous small hair follicles with sebaceous glands. The human skin (*h*) consists of a thick epidermis and a dermis containing coarse collagen bundles but virtually no adnexal structures. The basal epidermal layer is rich in melanin. Haematoxylin and eosin (H&E), ×250

Fig. 4a, b. Histology of control (**a**) and CsA-treated (**b**) NHSX. The epidermis (*e*) has an overall normal appearance, although it has a reduced thickness in (**b**). The dermis (*d*) contains a sparse mononuclear-cell infiltrate and perivascular fibrinoid deposits (*arrowheads*). H&E, ×250

mouse one, but the transition between the two was always clearly visible (Fig. 3a). The horny layer of the NHSX was generally thick and orthokeratotic, although small foci of parakeratosis were seen in some specimens. The remaining epidermal layers (granular, spinous and basal) had a normal appearance (Fig. 4a). The lowermost epidermal layers were rich in melanin granules that were also observable inside dermal macrophages. The thickness of the viable epidermis (from the basement membrane up to the limit of the upper stratum granulosum) was evaluated to be 99 ± 28 μm (mean ± SD), whereas the mean degree of papillomatosis (± SD) was 1.56 ± 0.26 (Table 1). The underlying dermis could be recognized as of human origin, since it contained thick collagen bundles but no adnexal structures. It contained abundant capillaries, occasionally surrounded by an eosinophilic fibrinoid material and a loose mononuclear cellular infiltrate.

By electron microscopy, the submicroscopic features of a normal epidermal keratinization were detectable: EK of the stratum granulosum contained typical keratohyalin granules of varying size and lamellar (Odland) bodies that were seen both inside the cytoplasm and extruded in the intercellular space. Tonofilament bundles and desmosomes had a normal appearance throughout the epidermis. Basal EK were occasionally seen in mitosis. Melanocytes located in the basal layer were dendritic and usually over active, containing abundant, mature melanosomes (Fig. 6a). The latter were also observed inside EK, sometimes as high up as the stratum granulosum (Fig. 5a). Langerhans cells (LC) were observed in all NHSX; they presented a dendritic shape and were present in seemingly normal numbers (Fig. 7a). They always contained typical Birbeck granules, usually in increased numbers, and frequently disclosed lipid droplets, melanosomes, visible rough endoplasmic reticulum and centrioles (Fig. 8a). Similar lipid droplets were also observed in EK (even of the stratum corneum), in basal melanocytes and occasionally in an extracellular location in the dermis.

The epidermal-dermal junction had a normal appearance, comprising a lamina lucida, a lamina densa and anchoring fibrils. The superficial dermis frequently contained melanin-laden macrophages and fibroblasts that

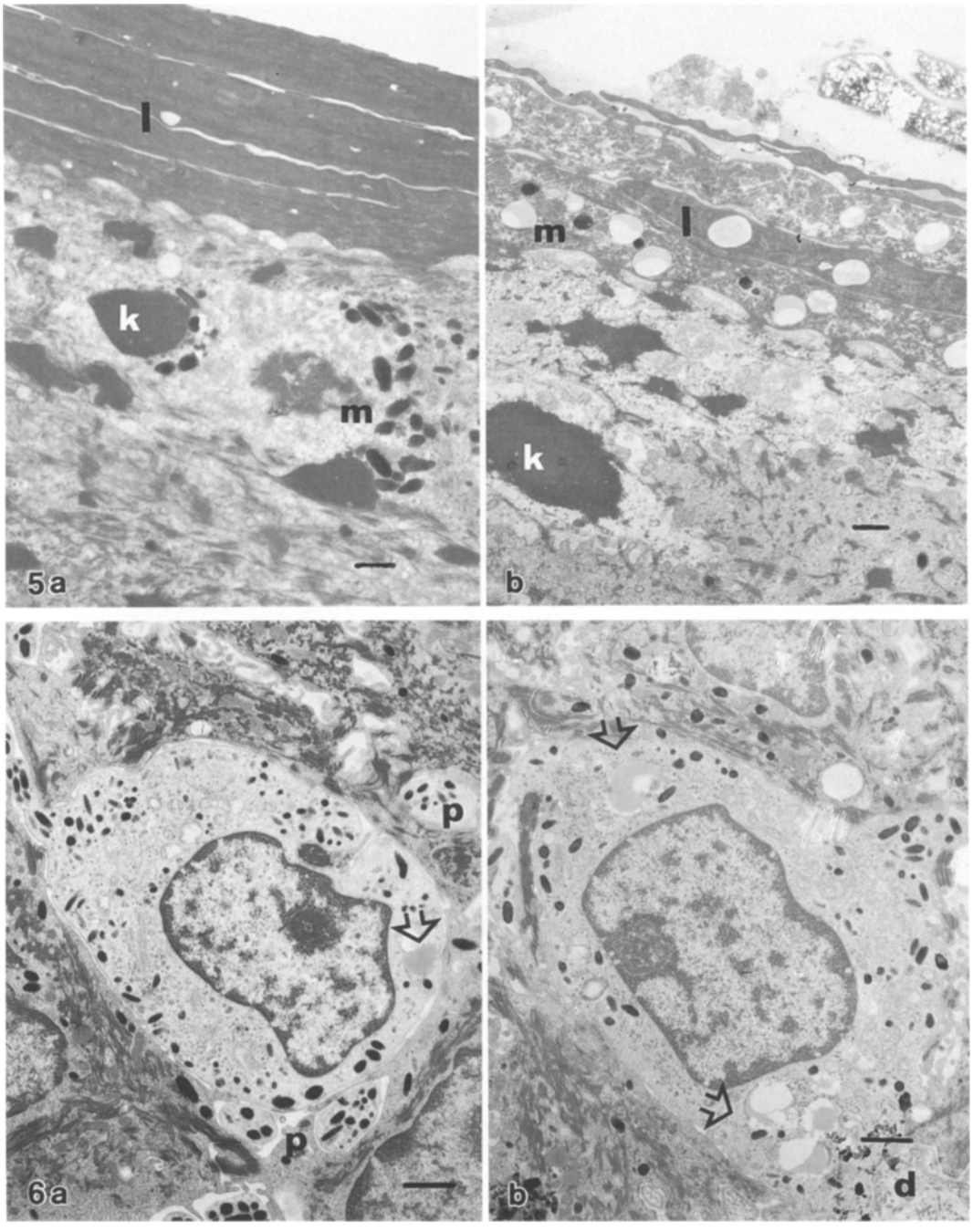

Fig. 5a, b. Ultrastructural aspect of the uppermost epidermal layers of control (**a**) and CsA-treated (**b**) NHSX. Note the abundance of melanosomes (*m*) within EK. *k*, Keratohyalin granules; *l*, lipid droplets. *Scale bar*: 500 nm

Fig. 6a, b. Basal-layer melanocytes of control (**a**) and CsA-treated (**b**) NHSX contain increased amounts of mature melanosomes, observed also within the cytoplasm of neighbouring EK. *d*, Dermis; *p*, dendritic processes; *arrows*, lipid droplets. *Scale bar*: 1 μm

occasionally disclosed a highly dilated rough endoplasmic reticulum. The amorphous eosinophilic perivascular deposits observed in some specimens could be identified as osmiophilic, finely fibrillar fibrinoid material.

CsA-treated NHSX presented similar light- and electron-microscopic features to controls. By light microscopy orthokeratotic hyperkeratosis, increased melanin production and perivascular fibrinoid deposits in the dermis were also present (Figs. 3b, 4b). The degree of papillomatosis was almost identical (1.51 ± 0.28) to that of control NHSX. The mean epidermal thickness of the viable epidermis (85.5 ± 12 μm) was lower than the control NHSX, but the difference did not reach statistical significance (Table 1). In one specimen, the dermis contained dilated cystic epithelial structures; these consisted of a wall made up of pluristratified epithelium reminiscent of the infundibular segment of a hair follicle and contained orthokeratotic keratinous material.

By electron microscopy, CsA-treated NHSX presented similar features to control NHSX: melanosomes

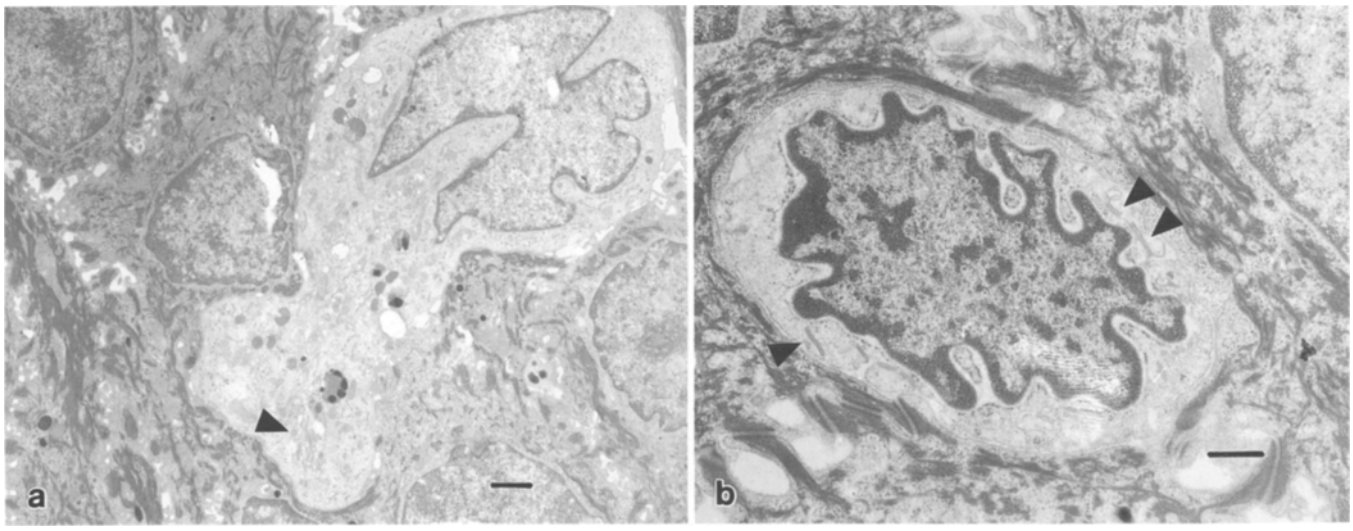

Fig. 7a, b. Epidermal Langerhans cells in control (**a**) and CsA-treated (**b**) NHSX. *Arrowheads* show Birbeck granules. *Scale bars:* **a** 1 μm; **b** 0.5 μm

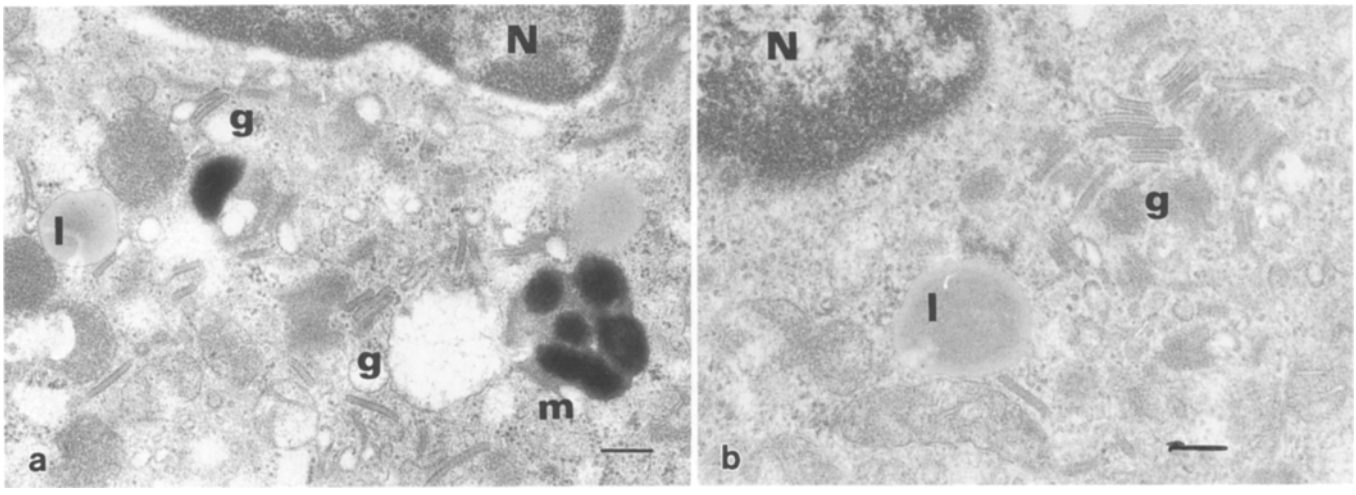

Fig. 8a, b. Cytoplasmic features of epidermal Langerhans cells in control (**a**) and CsA-treated (**b**) NHSX. Note the abundance of Birbeck granules (*g*). *N*, Nucleus; *m*, melanosomes; l, lipid droplets. *Scale bar*: 200 nm

were abundant mainly in the lower epidermal layers but were also occasionally observed inside cells of the strata granulosum and corneum (Figs. 5b, 6b). LC were seen in all specimens and contained abundant typical Birbeck granules, lysosomes, lipid droplets, centrioles and occasionally a dilated rough endoplasmic reticulum (Figs. 7b, 8b). The latter feature was also frequently observed inside dermal fibroblasts. Lipid droplets of medium electron density were observed, apart from LC, also in keratinocytes, basal melanocytes and dermal macrophages. Perivascular fibrinoid deposits were seen in some specimens. Neither giant mitochondria nor isometric vacuolization or calcium deposits were seen in any of the specimens studied.

Discussion

Athymic animals are an elegant model for studying the in vivo biological behaviour of both normal and pathological xenografted tissues. Many studies have been devoted to the study of human tumours xenografted on to congenitally athymic, nude mice (Mattern et al. 1988). Similarly, the same model can be used successfully for the study of NHS maintained in vivo over prolonged periods of time. As previous studies (Reed and Manning 1973; Krueger and Shelby 1981; Haftek et al. 1981) and the present one suggest, NHS xenografted onto nude mice is not only not rejected -due to a deficiency of T-cell-mediated immunity, but retains its main histological and proliferation characteristics. Furthermore, the present study shows for the first time that the normal submicroscopic features of epidermal cells (EK, LC, melanocytes) are present at least 5 weeks post-grafting. The fact that the cells under study are of human origin was demonstrated by immunohistochemical methods, using specific monoclonal antibodies: EK of NHSX express the human-specific β_2-microglobulin (Kanitakis et al. 1989) whereas LC express the human-specific T6 (CD1a) and HLA-DR antigens (Urabe et al. 1989b; Haftek et al.

1989). These data show unequivocally that, at least in a middle-term period of 5 weeks, the human epidermis of NHSX is not replaced by murine cells and is capable of self-renewal, as corroborated by the observation of basal EK in mitosis. To date, we have performed NHSX that have been maintained macroscopically for over 1 year. Detailed immunohistochemical investigations will show to what extent these long-term NHSX still maintain their human-specific histological and immunohistochemical characteristics.

In the present study, minor light and electron microscopic alterations of NHSX were observed when compared with NHS. The most consistently observed changes comprised: a moderate increase in the thickness of the horny layer, an excess of melanin production due to increased melanocyte activity and melanosome production, the presence of lipid droplets inside epidermal and dermal cells and the presence in some specimens of fibrinoid perivascular material that proved to contain immunoglobulin deposits on immunofluorescence examination (data not shown). The reasons for these morphological changes are not known with certainty; however, it can be speculated that circulating molecules (cytokines) released locally by the mouse skin as a response to the injury of the grafting procedure may be responsible for at least some of the alterations observed. Increased IL1 release, for instance, could account for the enhanced melanocyte activity (Kupper 1989), with a resulting accumulation of melanosomes in melanocytes, EK and LC. The presence of lipid droplets inside EK has been observed in vivo in the course of diseases characterized by abnormal keratinization (Anton-Lamprecht 1972) and in vitro in the cytoplasm of CsA-treated lymphocytes (Koponen and Loor 1983). In our study, however, lipid droplets of the same appearance as the ones observed in control NHSX were also observed in the dermis, and they therefore most likely represent the injected CsA-containing olive oil. As far as the perivascular immunoglobulin deposits are concerned, these can be assigned tentatively to host antibodies directed against the NHSX – nude mice, despite their T-cell deficiency, are capable of antibody production.

CsA is a molecule with potent immunosuppressive properties that can also affect epithelial cell proliferation in vitro and in vivo depending on the dosage used (Kanitakis and Thivolet 1990). The results of the present study, concerning DNA synthesis of human EK, confirm those we have previously reported (Urabe et al. 1989a) and demonstrate that CsA affects the proliferative capacity of EK in an apparently T-cell-independent way in vivo. By using an experimental protocol similar to the one of the present study (daily administration of 50 mg/kg CsA for 20 days), Ryffel et al. (1986, 1988) and Mihatsch et al. (1986) investigated the toxicological effects of CsA on rat kidney. They found that after only a 1-week treatment CsA induces morphological changes in epithelial cells of the proximal tubule, consisting of inclusion bodies (corresponding to giant mitochondria), isometric vacuolization and tubular microcalcification. Arteriolar changes observed included fibrinoid necrosis of the media and proliferation of myofibroblasts. Cells with features of myofibroblasts were also observed in humans in CsA-induced gingival hyperplasia, also showing fibroblasts with a dilated rough endoplasmic reticulum (Yamasaki et al. 1987). In our study, light-microscopic examination revealed a decrease in the mean thickness of viable epidermis in CsA-treated compared with control NHSX, that is probably related to the antiproliferative effect of CsA on EK, as evidenced by inhibition of the thymidine-analogue (BrdU) incorporation. Although the difference in the number of BrdU-incorporating EK was statistically significant, the difference in epidermal thickness between control and CsA-treated NHSX did not prove to be. This may well be due to the relatively small number of specimens studied and could be settled by studying a larger number of animals. With regard to the ultrastructural findings, none of the changes attributable to acute CsA renal toxicity (giant mitochondria, microcalcifications and isometric cell vacuolization) were observed in the skin. Furthermore, no consistent differences were observed between control and CsA-treated NHSX. When compared with NHS, CsA-treated NHSX presented similar morphological alterations as control NHSX did. In particular, fibroblasts with a dilated rough endoplasmic reticulum, intracellular lipid droplets and fibrinoid dermal deposits within CsA-treated tissues – that could a priori be assigned to the CsA treatment – were equally observed in control NHSX.

Another issue that merits discussion is the effect of CsA on LC, the dendritic intraepidermal antigen-presenting cells (APC). The effects of CsA on APC and more specifically on LC is controversial; indeed, while some studies have shown an inhibition of the antigen-presenting capacity in vitro of CsA-pretreated LC (Furue and Katz 1988), the major concern of the possibility of drug carry-over from APC to T-cell (Borel 1988) has not been settled. In previous work using the model of NHSX on to nude mice we observed that, in spite of cytostatic activity exerted on LC (Haftek et al. 1989), CsA does not modify either the number or the distribution of these cells (Urabe et al. 1989b) in agreement with a study on the skin of immunosuppressed renal-allograft recipients (Kelly et al. 1987). In the present study we were able to observe that LC under CsA treatment still maintain normal submicroscopic features, including a dendritic morphology and the presence of typical organelles such as Birbeck granules, lysosomes and centrioles. Although the precise origin and function of the most specific organelle, the Birbeck granule is not yet known with certainty, this structure is considered to reflect the endocytotic activity of LC. Indeed, Birbeck granules together with other structures (coated vesicles, endosomes, multivesicular bodies) indicative of a receptor-mediated endocytosis have been shown to increase in number after epicutaneous application of the hapten DNFB (Kolde and Knop 1987; Hanau et al. 1989). Our finding of numerous Birbeck granules in CsA-treated LC suggest that CsA does not hinder the endocytotic activity of LC, a process likely to be involved in the primary phase of antigen presentation (antigen captation).

In conclusion, our results show that CsA, despite an antiproliferative activity on human epidermal cells – shown as inhibition of DNA synthesis – does not in-

duce the acute cytopathic changes observed on epithelial cells of the kidney, nor does it induce noticeable light- and electron-microscopic alterations in comparison with control epithelia – apart from a possible decrease in epidermal thickness that awaits confirmation. It should be remembered that the grafting technique used in this study, employing NHS of small thickness and hence devoid of the deep portion of hair follicles, does not allow the study of the well-known stimulatory effect of CsA on human hair follicles, an effect that can easily be monitored in the mouse skin (Sawada et al. 1987; Urabe et al. 1988). However, the model of NHSX seems perfectly suitable for studies on the activity of various pharmacological molecules on normal human epidermis, since it allows the medium- and possibly the long-term maintenance of living human epidermis that more closely resembles the normal one than do other in vitro systems (EK or skin explant cultures).

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