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Inhibited differentiation of Langerhans cells in the rat epidermis upon systemic treatment with cyclosporin A

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Summary. The immunosuppressant drug cyclosporin A (CsA) is known to cause reduction in number, DNA synthesis and function of Langerhans cells (LC). Since also the differentiation of LC is known to be hampered in conditions of acquired immunodeficiency not due to drugs, we investigated whether this occurs with CsA. Rats were injected subcutaneously with CsA (5, 10 and 50 $mg.kg^{-1}.d^{-1}$) for three weeks; the skin was analyzed by Ia immunohistochemistry and by electron microscopy. Epidermal immunolabeled cells were 15±3.5 (mean ± SEM) per 100 basal keratinocytes in untreated controls and 8.75±1.3, 4.75±1.0 and 1.7±1.2 upon increasing doses of CsA ($p<0.01$). By electron microscopy, monocytoid cells with deep invaginations of the plasma membrane and roundish LC poor in Birbeck granules appeared in the epidermis upon treatment. The results suggest that CsA inhibits the differentiation of LC precursors in the epidermis and that this can in part explain the selective increase in the risk of skin viral disease and cancer in chronically treated patients.

Key words: Differentiation, Electron microscopy, Immunohistochemistry, Monocytes

Introduction

Cyclosporin A (CsA) is a powerful immunosuppressant drug widely used in clinics to prevent undesired cell-mediated immune responses such as rejection of grafted organs and tissues, graft versus host and autoimmune diseases (Weil, 1984; Feutren, 1993).

The use of CsA is by no means devoid of drawbacks. Wide clinical evidence indicates that patients subjected to long term (more than 5 years) treatment with CsA experience increased risk of skin warts and skin malignancies; the latter are the prevalent type of malignancies in these patients. Although the increased risk of cancer is common among all immunosuppressed patients, CsA is burdened by a higher risk of skin, rather than non-skin cancer if compared with other types of immunosuppressant therapy (Gruber et al., 1994; Hepburn et al., 1994; Thiel et al., 1994; London et al., 1995; Barba et al., 1997; Frezza et al., 1997; Dantal et al., 1998). The increase in the risk of cancer upon CsA therapy is dose-dependent (Dantal et al., 1998). A specific effect of CsA on skin immune system might explain the role of this drug in skin carcinogenesis better than its effect on the immune system in general.

The action of CsA depends primarily on selective suppression of T cell activation by inhibiting the transcription of several cytokine genes (Quesniaux, 1993). A secondary mechanism of action proposed for CsA is an interference with the accessory function of antigen-presenting cells, especially epidermal Langerhans cells (LC). Cyclosporin A has been reported to inhibit the lymphocyte proliferation-stimulating capacity of human (Cooper et al., 1990; Dupuy et al., 1991; Teunissen et al., 1991) and murine LC (Furue and Katz, 1988). It has been proposed that this inhibition depends on carryover of the drug at the moment of adding LC to lymphocytes (Péguet-Navarro et al., 1991), but other investigations have shown that CsA has a direct, inhibiting effect on alloantigen presentation by LC (Demidem et al., 1991).

Cyclosporin A treatment influences negatively the number of major histocompatibility complex - class II (MHC-II)-positive LC in human epidermis (Jontell et al., 1988; Gupta et al., 1989; Horrocks et al., 1990; Bergfelt, 1993; Bergfelt et al., 1993) and gingival epithelium (Niimi et al., 1990). One group reported no effect of CsA on the number of LC in the epidermis of human skin grafts onto nude mice (Urabe et al., 1989; Kanitakis et al., 1990), but this same group found decreased synthesis of DNA by LC in the same experimental conditions (Haftek et al., 1991). It cannot be excluded that the results of these experiments depend on metabolic peculiarities of the recipient species, since LC in the mouse epidermis decrease upon topical, but not systemic administration of CsA (Halliday et al., 1986). A hint that CsA possibly affects the proliferation and differentiation

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of cells of Langerhans lineage comes from the efficacy of this drug for the therapy of LC histiocytosis (Mahmoud et al., 1991; Aricò et al., 1995; Körholz et al., 1997).

Reduced cell-mediated immune responses in human oral mucosa upon HIV infection (Riccardi et al., 1990; Romagnoli et al., 1997) and in mouse skin upon ultraviolet B irradiation (Bacci et al., 1998) are associated with inhibited differentiation of LC in situ; however, despite the above cited reports that chronic administration of CsA causes reduction in number of MHC-II-positive LC, the effects of this drug on the differentiation of LC in vivo have not yet been explored. To address this issue, we have studied the morphological and immunophenotypical differentiative responses of rat epidermal LC to protracted treatment with CsA at therapeutic and toxic doses.

Materials and methods

Animals

Fifteen male Wistar rats weighting 250-300 g were used in this study in agreement with the Italian law and the international standards of laboratory animal care. Before entering the experiments the animals were accustomed to the laboratory for 5-7 days; they were kept at 22-25 °C with natural illumination, on a standard diet with free access to water.

Treatment

Three groups of 4 rats each were treated daily for 3 weeks with subcutaneous injections of CsA (generously provided by Sandoz, Basel, Switzerland) at doses of 5, 10 and 50 mg per kg body weight respectively. The drug was dissolved in cremophor and suspended in saline before use. A further group of 3 rats was injected with saline alone, as control. All rats were weighted and sacrified by cervical dislocation after the last injection and the abdominal skin was harvested for microscopical analyses.

Assay of CsA plasma concentrations

Blood samples were collected from the tail vein of treated rats into heparinized glass tubes before sacrifice. Upon centrifugation to separate plasma, CsA concentration was determined by radioimmunoassay using a commercial kit (Sandoz).

Light and electron microscopy

Skin fragments from each rat were fixed in 4% glutaraldehyde in 0.1 mol/liter cacodylate buffer, pH 7.4, osmicated and embedded in Epon 812. Semithin sections (~1μm) perpendicular to epidermal surface were stained with alkaline toluidine blue and used for morphometry as follows. The thickness of epidermis was measured with a calibrated ruler 5 to 10 times per section, at regularly spaced intervals, on one section per animal, and the data were averaged to obtain the epidermal thickness of each animal. Thin (~70 nm) sections were stained with uranyl acetate and alkaline bismuth subnitrate (Riva, 1974) and examined in a Siemens Elmiskop 102 electron microscope at 80 kV.

Immunohistochemistry

Skin fragments of each rat were embedded in Tissue Tek (Bio-Optica, Milan, Italy) and snap frozen. Cryosections were fixed in cold acetone and stained with monoclonal antibody OX4 (Seralab, Crawley Down, Sussex), that labels Ia molecules of all rat strains, followed by indirect alkaline phosphatase-anti alkaline phosphatase complex (APAAP, Dako, Milan, Italy) which was revealed using new fuchsin (Sigma, Milan, Italy) as chromogen. The specificity of the immunostaining was checked by omitting the first antibody or substituting it with an irrelevant one, which always gave unstained slides. For quantitative analysis, the stained cell bodies, containing a nucleus and overlying 100 basal cells were counted in five consecutive fields at x250.

Statistics

Each rat was assumed as a sample unit for statistics. Data are given as means ± standard error; they were subjected to analysis of variance and Student's *t* test for unpaired values with two tails, assuming $p<0.01$ as significant.

Results

Cyclosporin A plasma concentrations

The serum concentrations of CsA were 170±5 ng/mL in rats treated with 5 $mg.kg^{-1}.day^{-1}$, 380±10 ng/mL in rats treated with 10 $mg.kg^{-1}.day^{-1}$ and 3204±518 ng/mL in rats treated with 50 $mg.kg^{-1}.day^{-1}$ ($p<0.01$). Rats treated with CsA at any dose showed no gain in body weight, on the contrary to controls which underwent substantial increase in body weight in the same period of time. At the end of the experiments, rat weights were as follow: controls, 343±9 g; 5 $mg.kg^{-1}.day^{-1}$ CsA treated, 288±16 g; 10 $mg.kg^{-1}.day^{-1}$ CsA treated, 273±13 g; 50 $mg.kg^{-1}.day^{-1}$ CsA treated, 209±10 g ($p< 0.01$).

Light microscopy

In controls, dendritic, Ia-positive cells were 15 ± 3.5 per 100 basal keratinocytes and were located in the basal and suprabasal epidermal layers. These cells underwent progressive reduction in number with increasing doses of CsA, becoming 8.75±1.3 per 100 basal keratinocytes upon 5 $mg.kg^{-1}.day^{-1}$ CsA, 4.75±1.0 upon 10 $mg.kg^{-1}.day^{-1}$ ($p<0.01$ versus controls) and 1.7±1.2 upon 50

$mg.kg^{-1}.day^{-1}$ ($p<0.01$ versus controls and versus 5 $mg.kg^{-1}.day^{-1}$). The thickness of epidermis was as follows: controls, 121±20 μm; 5 $mg.Kg^{-1}.day^{-1}$ CsA treated, 111±11 μm; 10 $mg.Kg^{-1}.day^{-1}$ CsA treated, 88±25 μm; 50 $mg.Kg^{-1}.day^{-1}$ CsA treated, 92±14 μm (p n.s.).

Electron microscopy

Most LC of controls were well differentiated morphologically (Fig. 1); a few cells, recognized as LC because they contained rare Birbeck granules, were apparently devoid of dendrites and had narrow invaginations of the plasma membrane into the superficial cytoplasm (Fig. 2). Very few roundish, monocyte-like cells were sometimes found interspersed among keratinocytes of the basal layers.

Very few LC were well differentiated morphologically in the epidermis of all treated rats. Conversely, many LC were roundish in shape, had few membrane-bound organelles, several lysosomes and numerous invaginations of the plasma membrane in the superficial cytoplasm (Fig. 3). Moreover, the basal epidermal layer of treated rats at any dose hosted numerous monocyte-like cells with narrow, deep invaginations of the plasma membrane in the superficial cytoplasm (Fig. 4); some of these cells were in contact with lymphocytes (Fig. 5).

Discussion

In this study we found that systemic treatment with CsA induces in the rat skin a dose-dependent reduction in the number of MHC-II-positive, dendritic LC and conversely the appearance of monocytoid cells with deep invaginations of the plasma membrane.

We found LC in control rats troughout basal and suprabasal nucleated cell layers, as is known for mice (Baker et al., 1983; Mackenzie and Bickenbach, 1985; Bacci et al., 1998), cattle (Khalil et al., 1982), cats (Marchal et al., 1997), sheep (Townsend et al., 1997) and humans (see, e.g., Murphy et al., 1985; Régnier et al., 1997).

Immunohistochemical data are compatible with a reduction in number of LC and with a loss of Ia molecules by preserved LC as well. It is reasonable to assume that both mechanisms were at work, since the finding of Birbeck granules containing LC was less easy, but not exceptional at electron microscopy even upon the highest doses of CsA, on the contrary to a reduction of around 90% of Ia-labeled cells in the same conditions. These results are in line with the data reported by De Waal et al. (1992, 1996) on interdigitating, dendritic

Fig. 1. A Langerhans cell of a control rat is dendritic in shape and rich in organelles. The arrows point to Birbeck granules. Electron microscopy, x 10,000

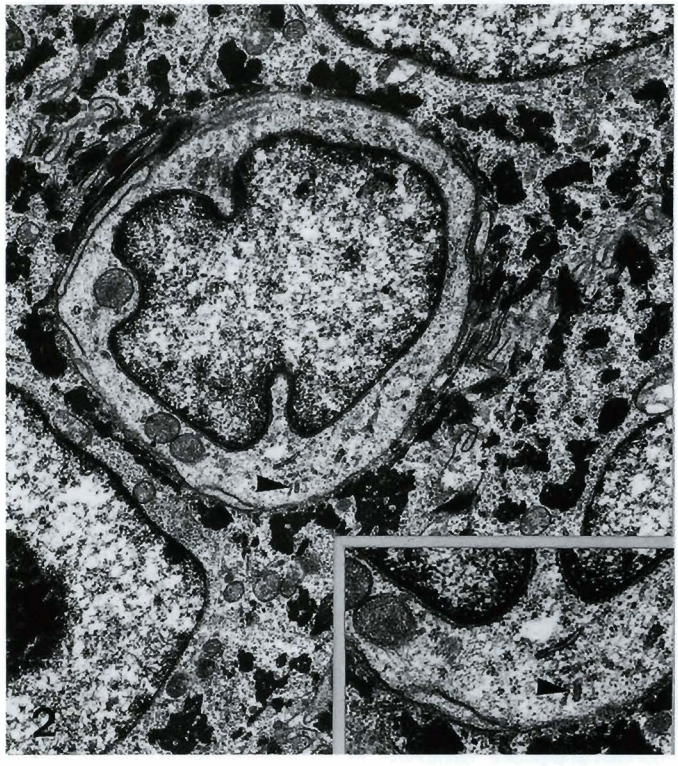

Fig. 2. Langerhans cell of a control rat, roundish in shape with rare granules (arrowead) and many infoldings of the plasma membrane. x 12,000. Inset: detail of Birbeck granule (arrowead). x 21,600

cells in the CsA-treated rat thymus.

Most LC upon high doses of CsA had a roundish cell body. Since they had no increase in organelle content and MHC-II expression they cannot be interpreted as cells moving away from the epidermis during final differentiation. On the other hand, they showed no sign of alteration, such as cytoplasmic vacuolization, mitochondrial swelling or disruption of membrane-bound organelles, so they did not appear as degenerating. Rather, the similarity of these cells to those found in humans in conditions of hampered differentiation of LC within epidermis (Riccardi et al., 1990; Mori et al., 1994) and in mice undergoing recovery of LC within epidermis upon ultraviolet irradiation (Bacci et al., 1998) suggests that all these cells are immature elements along the line of differentiation to LC (Bani et al., 1988; Bani and Giannotti, 1989). The deep, narrow invaginations of the plasma membrane in several LC and in monocytoid cells without Birbeck granules are suggestive of internalization of plasma membrane eventually leading to the formation of Birbeck granules as reported in previous studies on mycosis fungoides and hystiocytosis X (Hashimoto and Tarnowsky, 1968; Bani et al., 1988). The cells found in the epidermis upon CsA have no features of macrophages, therefore cannot be interpreted as corresponding to the macrophages expressing CD36 and MHC-II molecules, described in human skin after ultraviolet-irradiation (Cooper et al., 1985, 1986).

The concomitance of numerous immature cells of monocyte-Langerhans lineage and few mature, Ia-expressing LC in the epidermis upon CsA suggests that LC precursors are still allowed to reach the epidermis but are prevented from achieving full differentiation. Since LC are subjected to continuous turnover (Ghaznawie et al., 1999), the decrease in number of mature LC would follow the lack of sufficient replacement and normal - or perhaps even enhanced - clearance of mature cells during the three weeks of treatment.

We should like to conclude that CsA impairs primarily the maturation of cells of Langerhans lineage in the rat epidermis and only secondarily may affect the expression of MHC-II molecules by LC pre-existing in the epidermis. These effects might be either direct or mediated by alterations of the epidermal micro-environment, such as keratinocyte atrophy (Mori et al., 1994), or both. The alterations reported here upon CsA are in part similar to those observed in the oral mucosa of humans with HIV infection (Riccardi et al., 1990; Romagnoli et al., 1997) and in the mouse epidermis upon agents damaging mature LC (Bacci et al., 1998).

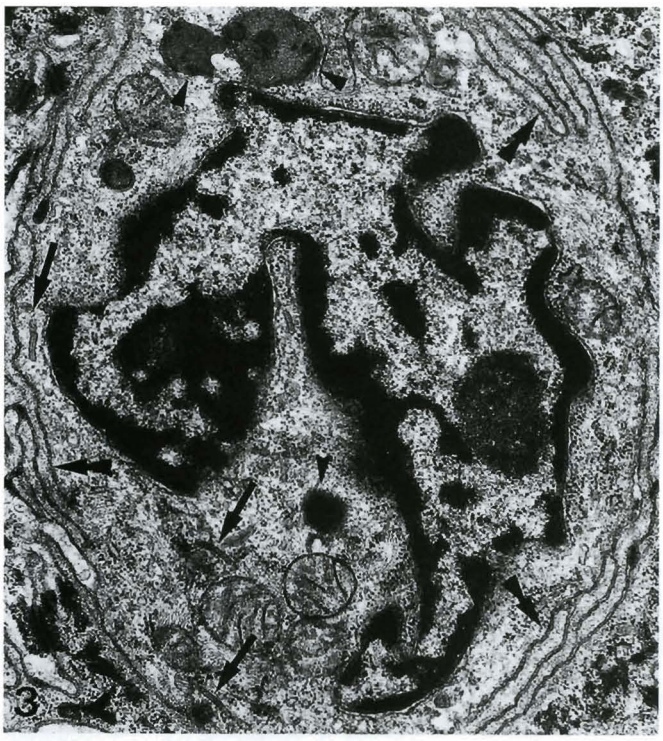

Fig. 3. A Langerhans cell of a rat treated with 10 mg.kg-1 CsA is roundish and poor in membrane bound organelles but contains several lysosomes (arrowheads) and extensive infoldings of the plasma membrane (double arrowheads). The arrows point to Birbeck granules. Electron microscopy, x 20,000

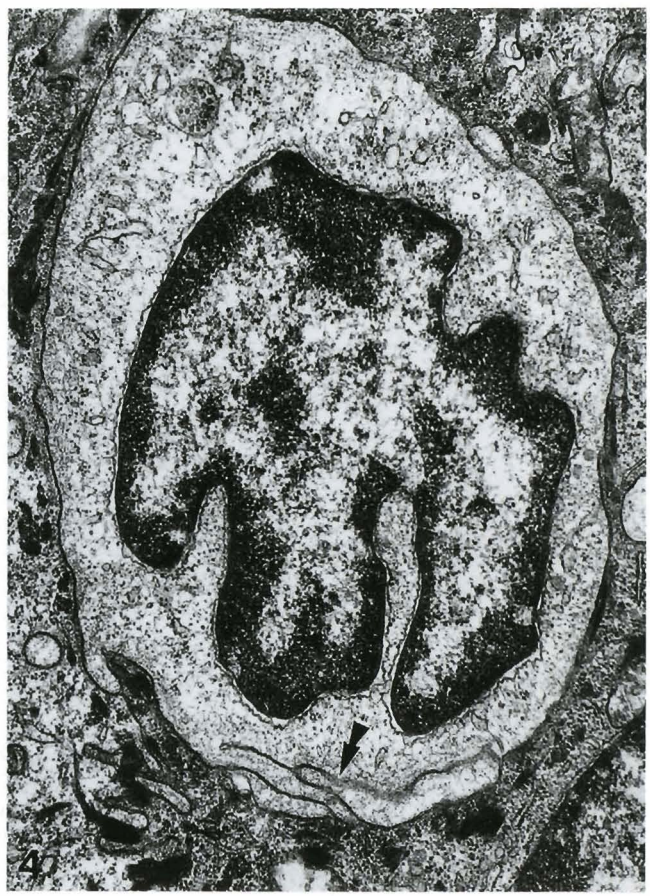

Fig. 4. A monocyte-like cell in the epidermis of a rat treated with 10 mg.kg-1 CsA is poor in organelles and has no Birbeck granules, but contains infoldings of the plasma membrane (double arrowhead). Electron microscopy, x 13,000

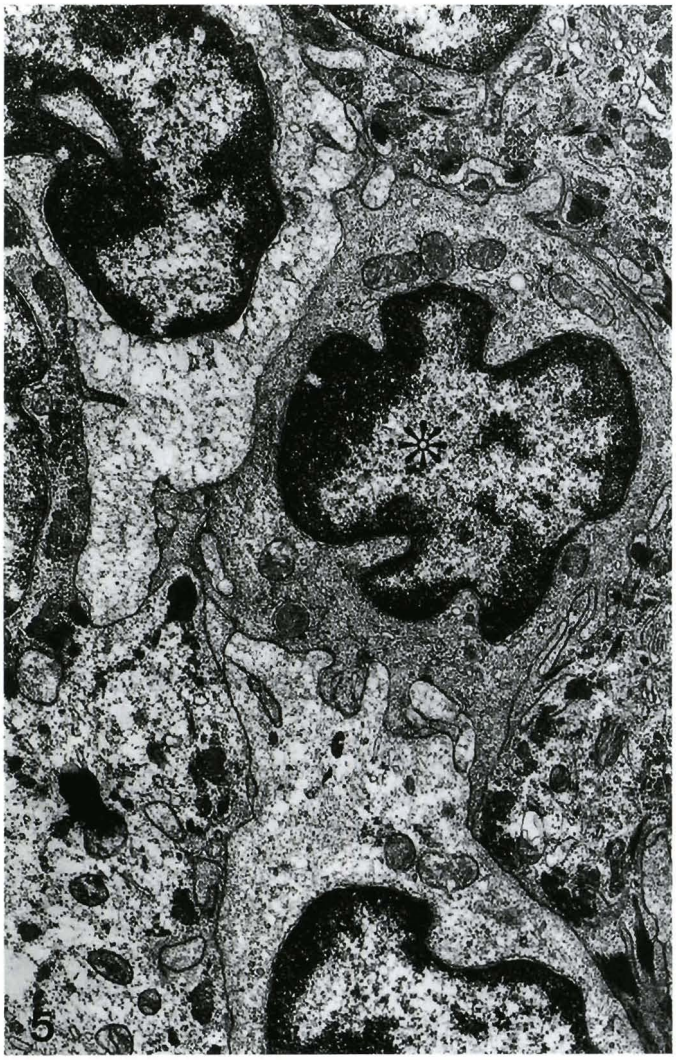

Fig. 5. Two monocyte-like cells in the epidermis of a rat treated with 10 mg.kg-1 CsA contain infoldings of the plasma membrane and are in contact with a lymphocyte (asterisk). Electron microscopy, x 11,000

The block of expression of MHC-II molecules is especially worth noting, since these molecules are necessary for the presentation of antigens to CD4-positive lymphocytes. It seems therefore reasonable that the reduction in the number of well differentiated LC upon CsA treatment leads to depression of local immune responses, such as those against virus-infected or neoplastic epidermal cells. This would explain the selective sensitivity of the skin to virus infection and neoplasia that is observed dose-dependently in humans subjected to chronic CsA treatment (Dantal et al., 1998).

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