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CYCLOSPORIN A SUPPRESSES PROLIFERATION OF RENAL MESANGIAL CELLS IN CULTURE

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Abstract—Rat glomerular mesangial cells were isolated and grown in culture for a prolonged period of time. The proliferation of these cells was suppressed by the immunosuppressivum Cyclosporin A (CSA) up to 75%, as measured by the incorporation of radiolabeled thymidine. This was dependent on the concentration of CSA used, being in the range of 125–2000 ng/ml. This effect was specific for CSA and other immunosuppressive derivatives thereof, while a non-immunosuppressive cyclosporin was ineffective. Neither the density of the cultures, nor the time of application had any influence on the susceptibility of the cells to CSA. The suppression of MC proliferation was reversible after removal of CSA.

These studies demonstrate a suppressive effect of Cyclosporin A on the proliferation of non-lymphocytic cells, the glomerular mesangial cells. This observation may help to explain the beneficial results reported with CSA in the treatment of some kidney diseases.

The cyclic endecapeptide, Cyclosporin A, is used as a potent immunosuppressive drug in human beings in transplantation surgery. Its function has been ascribed to a specific effect on the cells of the immune system, particularly the T-lymphocytes [1]. However, recently other cells, i.e. macrophages, have been reported to be sensitive to CSA† [2, 3].

Cyclosporin A has been used experimentally in the treatment of a variety of diseases in which the immune system is involved such as in autoimmune diseases [4]. One of the organs affected by CSA action is the kidney. This has been known for a long time from accidental overdosage of CSA [5] leading to damage of the kidney by a yet unknown mechanism. These nephrotoxic effects are caused by high concentrations of CSA. Beneficial effects of therapeutic concentrations of this drug have been achieved in patients with chronic kidney diseases, such as proliferative glomerulonephritis [6, 7]. Here the role of T-lymphocytes has not been established definitely, and thus it appears possible that other cells may be a target for CSA action. The glomerular mesangial cell is one of the cell types of the kidney centrally involved in acute and chronic inflammation. Upon stimulation of MC by a variety of substances, e.g. Interleukin-1 (IL-1) [8], lipopolysaccharide [9] or complement factors of the terminal membrane attack complex [10], they start to proliferate more rapidly and produce mediators themselves, e.g. prostaglandins [11]. Recently it was reported that MC produce low levels of IL-1 [12], which is enhanced in immune complex nephritis [13]. MC can be stimulated by IL-1 to synthesize and liberate neutral proteases [14], which may be involved in the metabolism and destruction of the basement membrane. Thus a participation of mesangial cells in the development and persistence of local inflammatory diseases in the kidney is becoming more appreciated.

Mesangial cells can be brought into culture, where they proliferate in the presence of serum factors. They can be maintained under selective culture conditions for a prolonged period of time, still exhibiting biological and biochemical activities of freshly isolated cells [15]. This enabled us to investigate the effects of CSA on mesangial cells in culture.

Here we report that CSA negatively affected the proliferation of cultured rat mesangial cells, demonstrating a suppressive effect of CSA on non-lymphocytic cells in concentration ranges comparable to those effective in lymphocytes. The results suggest that the beneficial effect of CSA administration in some patients with chronic nephrotic diseases may be explained by the suppressive effect of CSA on the pathologically enhanced proliferation of glomerular mesangial cells.

MATERIALS AND METHODS

Cyclosporins

Cyclosporin A (CSA) and the derivatives Cyclosporin G, dihydro-cyclosporin C, and C_9-*O*-acetyl-CSA were kind gifts of Dr J. Borel from Sandoz AG, Basel. They were dissolved in dimethyl-sulfoxide (DMSO) at a concentration of 2 mg/ml. The stock solutions were kept in the dark at 4°. Further dilutions were made with the culture medium. The maximum concentration of DMSO in the assays was 0.1% (v/v). The concentration of CSA in the final dilutions was kindly assessed by HPLC by Drs

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† Abbreviations used: CSA, Cyclosporin A; DMSO, dimethylsulfoxide; MC, mesangial cells; FCS, fetal calf serum.

Zimmer and Christians (Pharmacology, Medical School Hannover) according to the method described [16].

Preparation of mesangial cells

Primary cultures of mesangial cells (MC) were obtained from glomerular outgrowth of male rats (about 6 weeks old) of two strains (Sprague–Dawley and Lewis rats). The preparation and characterization were carried out as described in detail by Lovett *et al.* [17]. The collagenase-treated and washed glomeruli were plated in tissue culture flasks and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM 1-glutamine, 100/ml units penicillin, 100 μg/ml streptomycin, non-essential amino acids and 20% heat-inactivated fetal calf serum (FCS, Gibco, Wiesbaden). The glomeruli attached to the surface of the culture flasks and cells started to grow out of the remnants. After a total of about 14 days regions of stellate, fusiformed cells growing in interwoven bundles were found where the glomeruli had been. These cells were strongly adherent to the surface and rather spread out. Mesangial cells were the only cells to be observed in the culture after 2–4 weeks, if cultured in the presence of 20% FCS. The MC were used for experiments not before the fifth passage, when cultures were homogeneous as tested by different criteria such as morphology in phase contrast microscope, prominent staining for intracellular myosin filaments with an antibody against smooth-muscle myosin (from U. Gröschel-Stewart, Darmstadt), growth in D-valine containing medium, treatment with the aminonucleoside puromycin for 24 hr, etc. These cells formed structures described as hillocks by Sterzel *et al.* [18] after a prolonged period of culture without passage.

Experiments were routinely carried out with one cell preparation, if not stated otherwise. This was named RMC 85/1. It was prepared from Sprague–Dawley rats as described above and initially cultured in the presence of 20% FCS. After about six months with passages every other week, these cells started to proliferate more quickly. They showed a decreased necessity for FCS, i.e. growth factors, with an optimum of 5% FCS, which was normally used in the assays. This preparation was in culture for more than two years. We consider RMC 85/1 as an established cell line. There was no difference in response to CSA from RMC 85/1 to other preparations. However, the other cultures needed high concentrations of FCS throughout the cultivation period and they ceased to proliferate after 9–12 months.

Experimental details

Measurement of MC proliferation. RMC 85/1 were passaged onto 96 well flat bottom microtiter plates (NUNC, Wiesbaden, F.R.G.) and seeded with a density of 500 cells/200 μl and well. After an adherence phase of at least 24 hr CSA was added for different periods of time (see below). Proliferation was measured as the incorporation of (^{3}H)-thymidine into DNA by pulsing with 0.5 μCi/well for the last 24 hr of the experiments. (In some tests 1 μCi (^{3}H)-uridine was added for 24 hr to measure RNA synthesis.) Cultures were terminated by deep-freezing. The plates were thawed and sonified in a bath, before the cells were harvested onto glass fiber mats with an automatic cell harvester. Radioactivity was measured by liquid scintillation counting.

Correlation of thymidine incorporation with absolute amount of DNA or protein. RMC 85/1 were passaged onto Petri-dishes (60 × 15 mm, Primaria, Becton-Dickinson, Heidelberg, F.R.G.). After 48 hr the cells were treated with 0.1% DMSO as solvent control or 2 μg/ml CSA for 4 days. Then the cells were washed and removed by scraping with a rubber policeman. They were homogenized in 2 ml phosphate-buffered-saline, pH 7.4 in an ultra-turrax. Protein was determined by the method of Oyama and Eagle [19] with bovine serum albumin as standard. DNA was measured fluorimetrically according to Thomas and Farquhar [20] with DNA from salmon as standard. Cells from the same test were removed under sterile conditions from the dishes and seeded into 96 well microtiter wells in a density of 500 cells/well and immediately pulsed with 0.5 μCi [^{3}H]-thymidine for 24 hr.

Effect of different cyclosporins on the proliferation of MC. The experiment described here was performed with MC from Lewis rats, passaged about 10 times before the onset of the experiments. MC were seeded with a density of 2000 cells/well in 100 μl of culture medium containing 20% FCS. After 24 hr the cyclosporins were added to a total volume of 200 μl/well and left on the MC for 24 hr, before 0.5 μCi radiolabeled thymidine was added for additional 48 hr.

Effect of CSA on cultures with different cell densities. RMC 85/1 were plated at a density of 500 cells/well in 100 μl culture medium. CSA was added to a total volume of 200 μl/well at days 2, 4, 5 and 10, respectively, for a constant period of 72 hr. The last 24 hr cells were pulsed.

Effect of length of CSA administration. CSA was added to RMC 85/1 in plates 24 hr after seeding (500 cells/well). The cells were incubated with the drug for 3, 4, 5 or 6 days, including the 24 hr pulse.

Reversibility of the CSA effect on the proliferation of MC. After an adherence phase of 24 hr, RMC 85/1 were treated with CSA for 3 days. Then the medium was removed from one half of the plate completely. MC received fresh medium with 20% FCS. This washing procedure was repeated three times in a period of 6 hr in order to remove CSA. Afterwards all cells were incubated for additional 24 hr or 72 hr, respectively. The corresponding halves of the plates remained untouched. Pulse was again for the last 24 hr of incubation.

Inhibition of the proliferation of mitogen activated rat lymphocytes. In order to test the immunosuppressive potency of CSA in Sprague–Dawley rats, lymphocytes from mesenteric lymph nodes were prepared by the method described elsewhere in detail [21]. Mitogen stimulation, i.e. with Concanavalin A (2.5 μg/ml, Pharmacia, Freiburg) and inhibition of proliferation of lymphocytes was tested by the simultaneous addition of CSA as described for MC. Incorporation of radiolabeled nucleotides into RNA or DNA was measured after addition of 1 μCi [^{3}H]-uridine after 20 hr or 0.5 μCi [^{3}H]-thymidine after 44 hr for 4 hr, respectively. Cultures were terminated

Table 1. Proliferation of rat mesangial cells in microtiter wells

Days in culture	[^{3}H]-Uridine incorporated (cpm)	[^{3}H]-Thymidine incorporated (cpm)
5	3694 ± 769	22,141 ± 2327
7	9724 ± 812	72,384 ± 11,109
8	11,783 ± 2078	80,608 ± 11,183
13	24,467 ± 3999	105,096 ± 6922

RMC 85/1 proliferated for 5, 7, 8 or 13 days in the presence of 5% FCS. Incorporation of [^{3}H]-thymidine (0.5 μCi/well) was measured for the last 24 hr as described in Materials and Methods. Results are means of triplicates ±SD of one experiment of two identical ones.

by harvesting as described above; however, here a freeze and thaw cycle was not necessary. The mitogen stimulated proliferation of rat lymphocytes was inhibited nearly to 99% by CSA, beginning at a concentration of 125 ng CSA/ml (data not shown). This result was in accordance with previous reports [22].

RESULTS

Proliferation of mesangial cells

MC proliferated actively in the presence of fetal calf serum. The cells could be seeded in 96 well microtiter plates and grew there for a period of at least 14 days. Incorporation of radiolabeled precursors into RNA or DNA could be measured for the whole period (Table 1).

Suppression of MC proliferation

The proliferation of MC, as measured by thymidine incorporation, could be suppressed by CSA in a concentration-dependent manner. This effect was detectable at a concentration of about 125 ng CSA/ml. It slowly increased and reached a maximum effect at 2000 ng CSA/ml. The effect was highly reproducible and observed in all preparations of mesangial cells. It was not strain specific. MC from Lewis or Sprague–Dawley rats showed the same sensitivity to CSA. MC from the established cell line RMC 85/1 responded equally well compared to MC from preparations with low passage numbers (Fig. 1). Therefore, RMC 85/1 was used in the experiments, if not stated otherwise, because of the relative ease to obtain enough cells to perform studies in a large scale. The viability of MC was not affected by CSA directly. This was tested by the Trypan blue exclusion tests and staining with acridine-orange. The adherence of the cells was not changed by CSA (data not shown).

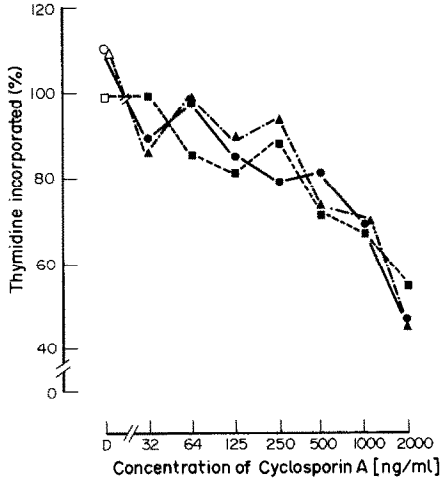

Fig. 1. Suppression of the proliferation of three preparations of MC by CSA. The established cell line RMC 85/1 (-●-) and a preparation with a low number of passages (-■-), both from Sprague–Dawley rats, were seeded with a density of 500 cells/well (5% FCS) and 1000 cells/well (10% FCS), respectively. MC from Lewis rats (-·▲·-) were plated with a density of 2000 cells/well (20% FCS). Incubation was for three days with CSA. Puls (0.5 μCi/well) was for the last 24 hours for Sprague–Dawley cells or for the last 48 hr for Lewis cells. Results are per cent of incorporation of [^{3}H]-thymidine into control values.

Correlation of [^{3}H]-thymidine uptake to absolute protein/DNA content after CSA treatment

The incorporation of radiolabeled thymidine into MC in microwells correlated to the absolute amounts of protein or DNA measured in Petri-dishes after the treatment with CSA or the solvent control of 0.1% DMSO (Table 2).

Effects of different cyclosporins

Derivatives of CSA were administered to Lewis MC in order to test their potency of suppressing the proliferation of the cells. The results are depicted in Fig. 2. Cyclosporin G was slightly more potent than the mother substance CSA. Dihydro-CSC was about as potent as CSA in this experiment, whereas C_9-*O*-Acetyl-CSA was ineffective up to the 2 μg/ml tested. The incorporation of the solvent control (0.1% DMSO) was slightly higher than in the cells which received only culture medium. The reason for this is not known.

Effects of CSA on cultures of different densities

The influence of density or "age" of MC in microtiter wells was investigated by varying the period of cultivation prior to the addition of CSA. The results are shown in Fig. 3. The extent of suppression achieved was the same and independent of the den-

Table 2. Correlation of [^{3}H]-thymidine incorporation with absolute amount of protein or DNA

	Protein (μg/dish)	DNA (μg/dish)	[^{3}H]-Thymidine incorporated (cpm/24 hr)
0.1% DMSO	318 ± 9	18.5 ± 1.6	19,695 ± 5961
2 μg CSA/ml	210 ± 22	11.5 ± 1.5	10,992 ± 1880

RMC 85/1 were treated with 0.1% DMSO or 2 μg CSA/ml for 4 days. Then protein and DNA was measured in homogenates of the cells (2 ml/dish). (^{3}H)-thymidine incorporation into cells transferred to microtiter wells was measured for 24 hr with fresh medium. Results are means of triplicates ± SD for protein and DNA values or means ± SD of ten wells from one experiment of three similar ones.

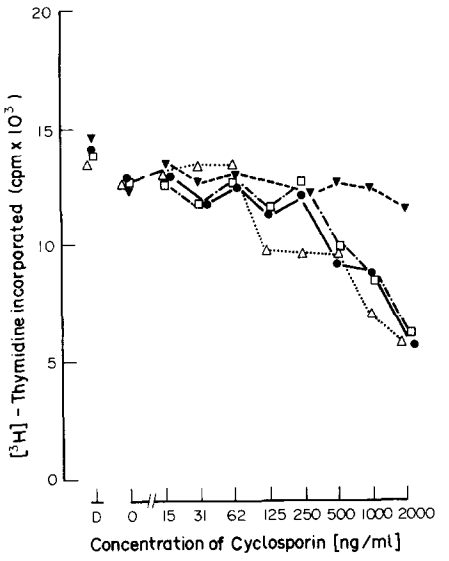

Fig. 2. Effect of different cyclosporins on MC proliferation. Lewis MC were incubated with different cyclosporins for 3 days. Incorporation of radiolabeled thymidine was measured as described in Materials and Methods: —●—, CSA; ···△···, CSG, -·□·-, dihydro-CSC; -▼-, C_9-*O*-acetyl-CSA. Results are means of triplicates of one experiment of a series of three identical experiments. D = 0.1% DMSO, O = medium control.

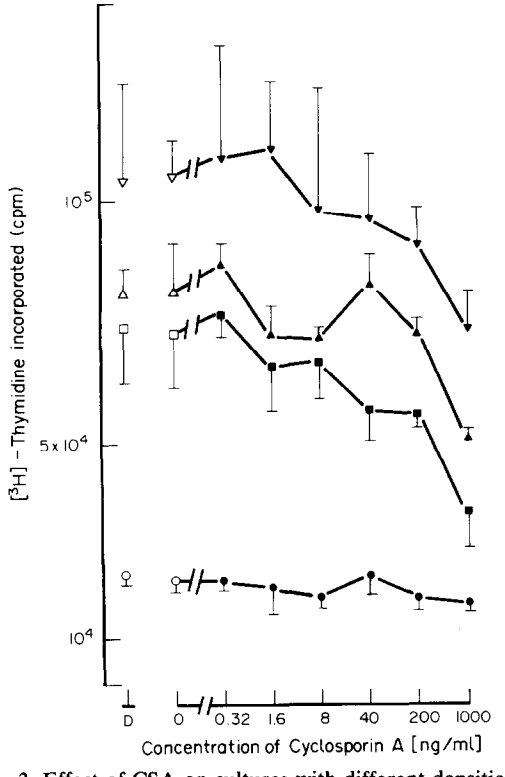

Fig. 3. Effect of CSA on cultures with different densities. CSA was added to RMC 85/1 at day 2 (●), 4 (■), 5 (▲), and 10 (▼) for a constant period of three days. Incorporation and determination of radioactivity was as described in Materials and Methods. Results are means of triplicates ±SD of one experiment of two identical ones. D = 0.1% DMSO, O = medium control.

sities of the cultures. The 2-days-old cultures were inhibited up to 20% with 2 μg CSA/ml. The other curves are practically parallel. Cells cultured in the vessels for a prolonged period of time developed hillocks.

Effect of length of CSA administration

CSA was given to MC for different periods of time. The proliferation of the cells was suppressed by the drug if it was given for 3, 4, 5 or 6 days. The control cells proliferated actively during this period (Fig. 4a). The extent of CSA suppression was the same, independently from the length of administration. This is demonstrated by the percentual expression of the data of Fig. 4a in Fig. 4b.

Reversibility of the CSA effect

The suppressive effect of CSA on the proliferation of MC was reversible (Fig. 5). This is the result of experiments in which CSA was removed after 3 days from the cells. The cells were washed in the presence of high concentrations of serum, which was supposed to remove CSA from the cells. Already 24 hr after removal of CSA the total incorporation in all samples was higher than in the cells which received CSA for the total incubation time. After 72 hr the CSA concentration dependent suppression was completely reversed. The cultures originally treated with CSA proliferated as well as the control cells, which had never come into touch with CSA.

DISCUSSION

In this paper we describe the suppressive effect of Cyclosporin A on rat mesangial cells in culture. The exquisite localization of the mesangial cells in the intracapillary lumen of the glomerulum next to the basement membrane, and its ability to react to stimuli present during inflammation by change of their proliferative state and their biosynthetic profile, emphasize the importance of these cells for the maintenance of glomerular functions. Once proliferating, MC produce immunoregulatory mediators. This raises the question whether these cells can interact with lymphocytes or macrophages and thus participate in inflammation. In the normal, i.e. non-inflamed glomerulum, MC proliferate extremely slowly. This is dramatically altered in the situation where a pathogenic challenge activates the immune system or directly the mesangial cell. Therefore it is of great value to find possibilities to interfere with the pathologically elevated proliferation of MC in such disorders.

Cyclosporin A is normally used in transplantation surgery to suppress the immune system, especially the activation of T-lymphocytes in order to avoid the rejection of the transplanted graft. Here we show that CSA also has a suppressive effect on the proliferation of mesangial cells in culture. This system was chosen, as it closely resembles the pathologic situation of proliferative glomerulonephritis, within the limitations of cell cultures. The suppression of MC proliferation was highly reproducible and observed in two strains of rats. There was no difference in susceptibility of MC to CSA in preparation passaged for a long time, e.g. the established cell

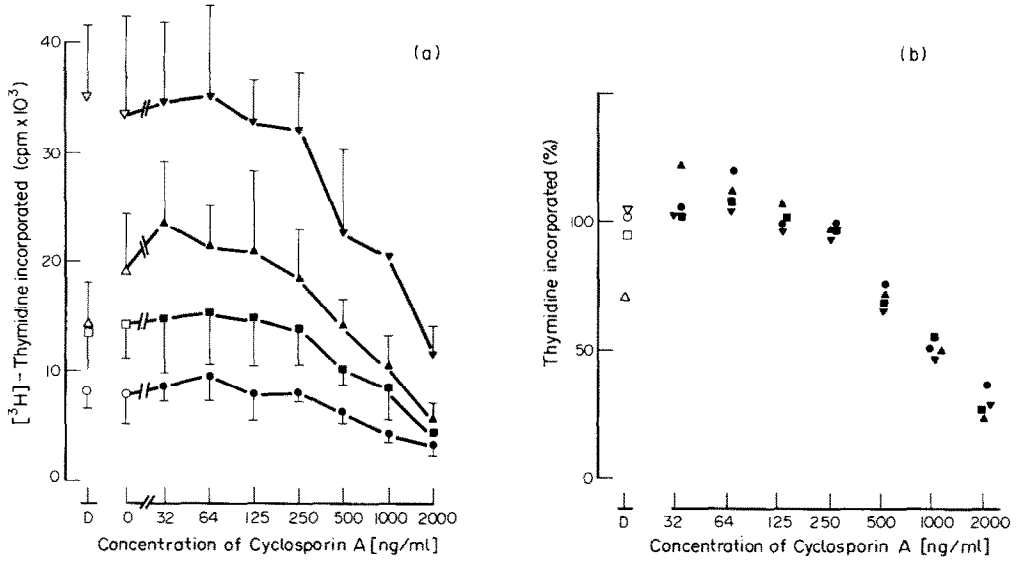

Fig. 4. Effect of length of CSA administration. RMC 85/1 were plated at the same time and incubated for 3 (●), 4 (■), 5 (▲) or 6 (▼) days with CSA, including the 24 hr pulse with radiolabeled thymidine. D = 0.1% DMSO, O = medium control. (a) Incorporated counts per min. Results are means of triplicates ±SD of one experiment of two identical experiments. (b) Per cent of counts incorporated per min of control values for the same experiment.

line RMC 85/1, or preparations with low passage numbers and a high demand of exogenously provided growth factors. The CSA effect was detectable at a concentration of 125 ng CSA/ml, slowly increasing to reach a maximum at 2 μg CSA/ml. The concentrations of CSA used in these experiments were restricted to 2 μg/ml. The *in vivo* existing concentrations of CSA in the mesangial matrix of the kidneys of patients treated with the immunosuppressivum are not known. Studies in rats have shown, that after a single or multiple administration of CSA highest concentrations were found in the liver, in renal fat, and in kidney [23]. We did not exceed 2 μg CSA/ml, being still in the range of the most probable blood level. There have been studies investigating the effect of CSA on the ability of macrophages/monocytes to produce prostanoids, however, using significantly higher concentrations of CSA [2, 3]. In our experiments the concentrations were in the same order of magnitude used to inhibit mitogen activation of lymphocytes (data not shown).

Furthermore, only immunosuppressive cyclosporins negatively affected the proliferation of MC. The derivative C_9-*O*-acetyl-CSA was unable to suppress MC proliferation. This substance was reported to be non-immunosuppressive [24, 25]. CSG was slightly more effective than CSA, as its dose–response curve was shifted to lower concentrations. Thus, the same portion of the molecule in cyclosporins seems to be responsible for the action on mitogen-stimulated lymphocytes and on proliferating mesangial cells. However, in contrast to mitogen stimulated lymphocytes, the suppression never reached 100% in MC. The reason for this is not known. It appears unlikely that only a portion of MC was affected by the drug, as the cultures were homogeneous and consisted of a single cell type.

The effect of CSA on the MC proliferation was not dependent on the age or density of the cultures. This experimental setup was chosen to imitate the different steps in the progress of inflammation. In this respect, it is noteworthy that the cells showed the typical morphology of mesangial cells cultured for a prolonged period of time, i.e. the development of structures, described as hillocks by Sterzel *et al.* [18]. In these hillocks materials are deposited which are synthesized and released by MC. This possibly resembles an *in vitro* model for sclerosis of the glomerulum in proliferative kidney diseases [15, 18].

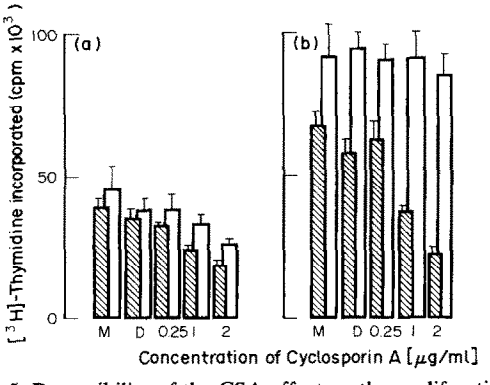

Fig. 5. Reversibility of the CSA effect on the proliferation of MC. RMC 85/1 were treated with 0.25, 1 or 2 μg CSA/ml for 3 days. One half of the samples (hatched bars) was treated with CSA further on for 24 (a) or 72 (b) hours. In the other half, CSA was removed by four washes with 20% FCS and cells received fresh medium for 24 or 72 hr (open bars). Cells were pulsed with thymidine for the last 24 hr. M = medium, D = 0.1% DMSO. Results are means of six wells ±SD from one experiment of three identical ones.

CSA had to be administered for a minimum time in order to achieve a measurable decrease of proliferation. A reliable effect could be observed after 2 days by measuring the incorporation of radiolabeled precursors into RNA or DNA. After 3 days the effect was fully developed and the proliferation suppressed. This was independent of the length of CSA treatment. By extending the incubation period with the drug, the suppressive effect could not be augmented. The experimental setup in the microtiter wells, however, limited the time of observation to about 14 days. Afterwards the control cells were densely grown in the wells and started to detach from the surface. (The apparently stronger decrease after longer treatment (Fig. 4a) is a pitfall of the higher counts incorporated after prolonged cultures. The same data expressed in per cent (Fig. 4b) show that all cultures were equally suppressed by CSA, independent of the length of treatment.)

We conclude from these data that already a short period of CSA treatment is sufficient to achieve the changes in the mesangial cells resulting in the reduced proliferation. This state is reached, independently of the parameters, e.g. cell density, age etc. Proliferating MC in culture seemed to be susceptible to CSA all the time. The effect of CSA on the proliferation of MC was reversible. Similar results have been reported for T cell clones treated with CSA [26]. One problem of such experiments is the hydrophobicity of CSA. Thus four wash cycles with high concentrations of FCS had to be performed in order to remove CSA from the cells. After one day MC started to proliferate better than cells treated for the whole time with CSA. Three days after removal of CSA the suppressive effect was reversed. This points towards a reversible interference of CSA in the regulation of mesangial cell proliferation, which can be overcome quickly after removal of CSA.

The molecular mechanism of CSA action is not clearly established. Recently, we have shown that immunosuppressive cyclosporins interfere with enzymes in the plasma membrane of lymphocytes resulting in a "decoupling" of the activation signal [25, 27]. Maybe a similar mechanism applies here, concerning a putative mitogenic signal, which stimulates the mesangial cells to proliferate in culture or inflammation, e.g. IL-1. CSA may interfere directly with the production of such a factor, and down-regulate the proliferation of MC. Thus CSA may help to restore the non-proliferative state of mesangial cells in the healthy glomerulum. Whether CSA is the only cause *in vivo* or other cells and factors are actively involved in this process of "normalization" of the proliferation rate of the mesangial cells in the inflamed glomerulum remains to be elucidated.

For the first time we could show a direct effect of CSA on isolated cultured mesangial cells and thus bring to attention a novel observation, which may lead us to a better understanding of CSA action on the one side and on the other side of the control of mesangial cell proliferation in the inflamed kidney.

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