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Cyclosporin A protects pancreatic islet cells from nitric oxide-dependent macrophage cytotoxicity

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It has been shown earlier in an in-vitro model of inflammatory islet cell death that activated macrophages lyse islet cells via the release of nitric oxide. Here we report that cyclosporin A suppresses macrophage cytotoxicity. Control experiments showed that the immunosuppressive drug does not improve the defences of islet cells against nitric oxide but inhibits the release of nitric oxide from LPS-stimulated macrophages. This property of cyclosporin A may contribute to the preservation of β cell function seen in cyclosporin A-treated patients with recent onset type I diabetes.

Cyclosporin A; Type I diabetes; Islet cell; Macrophage; Nitric oxide

1. INTRODUCTION

The progressive destruction of insulin-producing β cells during the development of type I diabetes has been discussed to result from the attack of cytotoxic T-cells as well as from toxic products of inflammatory macrophages such as interleukin 1, oxygen radicals and nitric oxide [1–4]. The largely T-cell specific immunosuppressant cyclosporin A was found to extend the remission phase [5,6] and to preserve β cell function [5] in patients with recent onset diabetes.

In the present study we tested the hypothesis that cyclosporin A may not only interfere with T-cell reactions but also act on islet-toxic macrophages. For this purpose we used an in-vitro model of islet cell lysis by activated macrophages [7]. In this model islet cell death is due to the release of nitric oxide by macrophages [4]. We describe here that the addition of cyclosporin A to the incubation system inhibits macrophage cytotoxicity towards pancreatic islet cells.

2. MATERIALS AND METHODS

2.1 *Islet cells*

Pancreatic islets were isolated from 3- to 4-months-old Wistar rats of our own colony by ductal injection of collagenase (Serva, Heidelberg, Germany), density gradient centrifugation and handpicking as described previously [7]. Single cell suspensions were prepared by trypsin treatment followed by 18 h cultured at 37°C/5% CO_2 for recovery in enriched RPMI 1640 containing 4 mM glucose, ampicillin 25 mg/l, penicillin 120 mg/l, streptomycin 270 mg/l, sodium pyruvate 1 mM, L-glutamine 2 mM, non-essential amino acids 1×, $NaHCO_3$ 2 g/l, HEPES 2.38 g/l and 10% FCS (Gibco BRL, Heidelberg) [7].

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2.2 *Macrophages*

Peritoneal macrophages were activated by injection of *Corynebacterium parvum* suspension (Wellcome, Burgwedel, Germany) i.p. 5 days prior to isolation [7]. Macrophages were enriched by adherence to FCS-coated Petri dishes, washes and detachment by Ca^{2+}/Mg^{2+}-free HBSS at 4°C. As determined earlier by immunocytochemistry and electron microscopy, between 92–95% of the adherent cells could be positively identified as macrophages [7,8]. For the analysis of inducible NO production 10^5 macrophages were seeded in 100 μl per well in round-bottom 96-well microtiter plates. Serial dilutions of cyclosporin A (50 μl, Sandimmun, Sandoz, Basel, Switzerland) were added. In a set of samples macrophages were stimulated with 50 μl lipopolysaccharide solution (LPS from *Escherichia coli* 026:B6, Sigma, final concentration 1 ng/ml). After 20 h at 37°C/5% CO_2, 150 μl of the supernatants were collected and the accumulated nitrite determined by the Griess reaction [9]. The resulting extinctions were converted to NO_2^- concentrations using a standard curve obtained with $NaNO_2$.

2.3. *Cytotoxicity assays*

For macrophage-islet cell co-culture experiments islet cells were labelled by [^{3}H]leucine for 20 h as described [7]. Radiolabelled islet cells (5×10^3 cells in 100 μl) were mixed with 100 μl of macrophages ($5 \times 10^4 - 2 \times 10^5$ cells) and incubated in round-bottom 96-well microtiter plates for 16 h at 37°C/5% CO_2. The radiolabel was determined in 100 μl of supernatant. The spontaneous release of ^{3}H was less than 30% and subtracted from all experimental values. As shown before, the release of radiolabel correlates closely with lysis of islet cells, as determined by electron microscopy [7].

For the analysis of NO toxicity islet cells were seeded in half area round bottom microtiter plates at 2×10^4 cells/120 μl per well. After preincubation for 3 h the NO-donor nitroprusside (Merck, Darmstadt, Germany) or *S*-nitroso-*N*-acetyl-penicillamine (SNAP) (kind gift of Prof. E. Noack, University of Düsseldorf, Düsseldorf, Germany) were added. Cyanide ions released by nitroprusside were scavenged by the addition of 8 U rhodanese (Sigma, Steinheim, Germany) and 5 mM $Na_2S_2O_3$. After incubation for 16 h at 37°C/5% CO_2 cell viability determined by Trypan blue exclusion. At least 200 cells per well were counted [10].

2.4. *Statistics*

Differences between mean values were analysed by Student's *t*-test, two sided.

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3. RESULTS

The lysis of islet cells seen after co-culture with activated macrophages was dependent on the target:effector cell ratio yielding 54% lysis at a T:E ratio of 20 (Fig. 1). The addition of cyclosporin A to the incubation mixture inhibited islet cell lysis. A concentration of 1 μg/ml was effective at all T:E ratios while a dose of 0.2 μg/ml was only inhibitory at the T:E ratios of 1:10 and 1:20 (Fig. 1). The addition of the NO synthase antagonist N^G-monomethyl-L-arginine completely inhibited islet cell lysis, demonstrating that macrophage toxicity towards islet cells depends on the arginine–nitric oxide pathway. In controls no effect of cyclosporin A on the viability of the macrophages could be detected. Even at a concentration of 5 μg/ml cyclosporin A the Trypan blue exclusion assay revealed 97.7 ± 0.2% (mean ± S.D. from three experiments) living cells after 16 h of incubation. This corresponds to the survival rate of untreated cells (98.4 ± 1.4%).

Next we tested whether the protective action of cyclosporin A occurs on the level of the islet cell. Islet cells were preincubated for 3 h with cyclosporin A and subsequently exposed to chemical NO-donors. No protection from NO-toxicity was noted in the presence of cyclosporin A even at high concentrations (Fig. 2).

In a third series of experiments it was determined whether cyclosporin A interferes with the arginine dependent cytotoxicity pathway of macrophages. As shown in Fig. 3 incubation of peritoneal macrophages with LPS for 20 h stimulated the release of nitrite which was inhibitable by cyclosporin A and by N^G-monomethyl-L-arginine.

As shown in Figs. 1 and 2 the concentrations of the NO-donors nitroprusside and SNAP were chosen to yield similar amounts of lysis as obtained from macrophages. However, the nitrite concentration in the supernatant of macrophages cannot be directly compared with the concentration of chemical NO-donors. Our

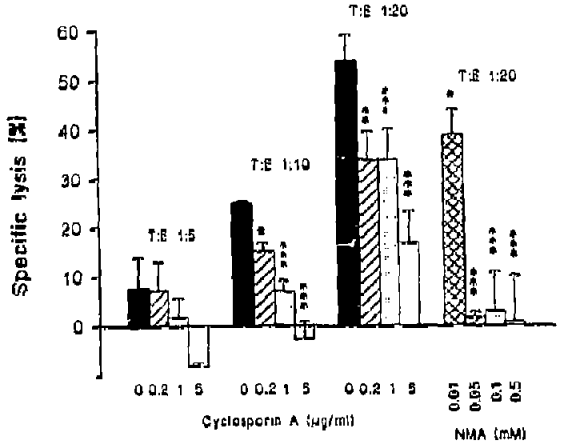

Fig. 1. Inhibition of macrophage cytotoxicity towards islet cells by cyclosporin A or *N*-methyl-arginine. Shown are mean values ± S.D. (n = 4). Statistical differences between islet cell lysis in the absence or presence of pharmacological inhibitors are indicated as $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

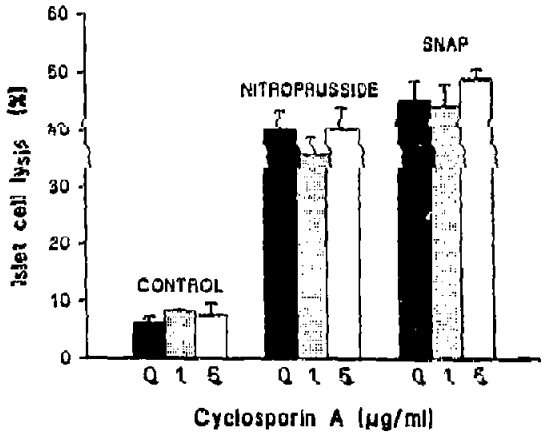

Fig. 2. Cyclosporin A does not inhibit NO toxicity in islet cells. Islet cells were incubated for 16 h with medium (control), 0.5 mM nitroprusside or 0.4 mM SNAP. Shown are mean values ± S.D. (n = 4).

own unpublished observations and results from Feelisch et al. [11] showed that the concentration of an NO-donor may not correlate with its NO-releasing capacity. Furthermore, depending on the NO-generating substance, highly variable amounts of NO may be oxidized to nitrite. On the other hand, macrophage products may influence the formation of nitrite from NO.

4. DISCUSSION

The immunosuppressive drug cyclosporin A has been shown earlier to interfere with a calcium-sensitive T-cell signal transduction pathway involving calcineurin [12,13]. In addition, cyclosporin A inhibits macrophage accessory function which also may suppress T-cell activation [14].

The data presented here indicate that cyclosporin A at pharmacological doses interferes with the arginine-dependent cytotoxicity pathway of macrophages. This conclusion is based on two different observations. First, cyclosporin A suppresses the cytotoxic action of activated macrophages against pancreatic islet cells. The arginine-dependence of the lytic event has been previously demonstrated [4] and is confirmed here by the protection of islet cell lysis in the presence of the NO synthase inhibitor N^G-monomethyl-L-arginine. Second the accumulation of nitrite in the supernatant of LPS-stimulated macrophages is reduced in the presence of cyclosporin A, indicating the suppression of arginine-dependent NO formation. On the other hand, cyclosporin A failed to prevent islet cell lysis induced by chemical NO donors in the absence of effector cells. This finding also indicates, that the drug does not act by protecting islet cells from NO toxicity but suppresses the NO mediated cytotoxic activity of macrophages. Since the macrophage cell suspension contained a negligible quantity of T-cells it is reasonable to assume that

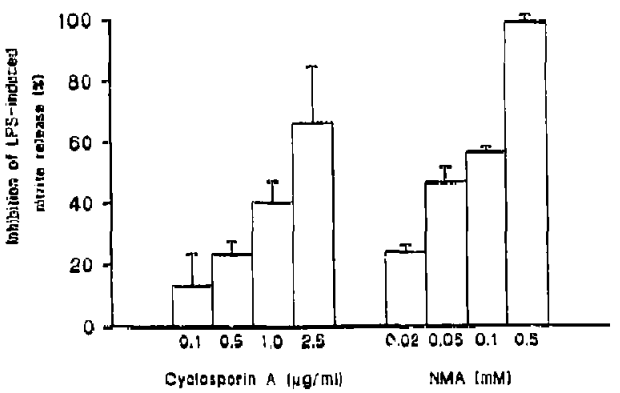

Fig. 3. Inhibition of nitrite release from activated macrophages by cyclosporin A or *N*-methyl-arginine. The incubation with LPS induced 3.22 nmol ± 0.33 of nitrite per 10^5 macrophages per well ($n = 4$) (background nitrite levels subtracted) in the experiments with cyclosporin A and 7.47 nmol ± 3.28 in the experiments with N^G-monomethyl-L-arginine (NMA). Shown are mean values ± S.D. ($n = 4$).

the suppressive effect of cyclosporin A is due to direct interaction with the macrophage.

Interestingly, FK506, an immunosuppressive agent with similar mechanisms of action as cyclosporin A [15,16], was found to exert comparable effects in a model of allograft rejection. FK506 prolonged the survival of allogeneic grafts [17] obviously by inhibiting NO-formation from macrophages infiltrating the graft [17,18].

The production of NO by macrophages requires prior activation by appropriate stimuli such as LPS to cause synthesis of the inducible NO synthase [19]. Similar as in T-cells, cyclosporin A may prevent the activation of specific transcription factors. Another stimulant induced response of macrophages, the phorbol ester-dependent respiratory burst also was found reduced in the presence of cyclosporin A [20]. Furthermore, cyclosporin A was found to inhibit the acquisition of tumoricidal properties by macrophages activated with either macrophage activation factor in combination with lipopolysaccharide or the calcium ionophore A23187 [21]. A recent report described the inhibition by cyclosporin A of stimulant induced superoxide production from polymorphonuclear leukocytes [22]. Taken together, these data imply that cyclosporin A may interfere with signal transduction pathway also in other leukocytes than T-cells.

The suppression of arginine-dependent macrophage cytotoxicity may account in part for the preservation of beta cell function seen in patients with acute type I diabetes receiving cyclosporin A [5]. Islet cells have been shown previously to have a poor defence against free radicals [23] and to be highly susceptible to the arginine-dependent lytic activity of inflammatory macrophages [4,7]. Interference by cyclosporin A with NO and oxygen radical production from islet infiltrating inflammatory macrophages can be expected to support β cell survival.

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