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Cyclosporin A inhibits phorbol ester-induced activation of superoxide production in resident mouse peritoneal macrophages

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Peritoneal resident macrophages from mice are sensitive to inhibition by cyclosporin A (CsA) of phorbol 12-myristate 13-acetate (PMA)-stimulated oxidative burst. Inhibition was assessed in terms of superoxide anion ($O_2^{\cdot -}$) and H_2O_2 production. Key findings were as follows. (*a*) CsA inhibited in a dose-dependent manner the production of $O_2^{\cdot -}$ when cells were stimulated with PMA. CsA did not alter the respiratory burst induced by other stimuli (zymosan, concanavalin A and fMet-Leu-Phe). It was verified that CsA itself had no scavenger effect. (*b*) A concomitant decrease in H_2O_2 liberation following CsA exposure was found. This inhibition was observed both in the initial rate of synthesis and in the accumulation after 15 min of incubation. (*c*) NADPH oxidase activity in the crude supernatant was unaffected by the previous incubation of macrophages with CsA. CsA does not inhibit glucose transport measured as $^{14}CO_2$ production. (*d*) The production of $O_2^{\cdot -}$ was strongly dependent on the glucose concentration. Sodium oleate also stimulated $O_2^{\cdot -}$ production in resident macrophages. These data might be correlated with the inhibitory effect of CsA upon other functions of macrophages.

INTRODUCTION

Production of superoxide anions and hydrogen peroxide in macrophages and neutrophils has been demonstrated to be one of the main functional responses by which these cells kill invading microbes or tumour cells [1–3]. This complex of reactions is usually referred to as the respiratory burst. This response can be induced in cells either by phagocytosis or by the addition of agents such as phorbol esters [4,5]. The components of the superoxide-generating system are not completely established, but kinetic studies in neutrophils suggest that these components are arranged in the membrane so that the electrons flow from NADPH to O_2 via FAD and cytochrome *b* [6]. This system is less well characterized in macrophages, but several lines of evidence suggest that the molecular components are similar in both types of cells [7,8].

Cyclosporin (CsA), a lipophilic cyclic undecapeptide of fungal origin first isolated by Borel *et al.* [9], is now recognized as a potent immunosuppressive drug. It has found widespread application in organ transplantation because of its unique action on the T-cell-mediated immune reaction without myelosuppression [9,10]. Despite the considerable interest in this drug, its mechanism of action remains to be elucidated. Recent studies have indicated that the immunosuppressive properties of CsA are mostly limited to T-cell-dependent immune responses [11]. CsA acts by blocking both production of and responsiveness to interleukin 2 (IL-2) [12–14], a T-cell lymphokine. Also, CsA interacts with monocyte functions, and, as a result, IL-1 production by monocytes is inhibited [15].

In this paper we have investigated the influence of CsA on the oxidative burst in macrophages, determining whether $O_2^{\cdot -}$ and H_2O_2 generation are altered. It is well established that NADPH oxidase is sensitive to several stimuli in macrophages [5]. Here we report the inhibition of the PMA-stimulated oxidative burst in macrophages by CsA in a dose-dependent manner. These data suggest that, in addition to the well-established direct effect of CsA on T-cells, other mechanisms, which would implicate the collaborative interaction between T-cells and macrophages, could be involved in the immunosuppressive action of CsA.

MATERIALS AND METHODS

Materials

Chemicals were of analytical grade and were obtained from Merck, Darmstadt, Germany. Biochemical reagents and enzymes were from Boehringer, Mannheim, Germany, or Sigma Chemical Co., St. Louis, MO, U.S.A. CsA was kindly provided by Dr. J. F. Borel, Sandoz Ltd., Basel, Switzerland. Unless otherwise specified, CsA (1 mg) was dissolved in 10 ml of 0.9% (w/v) NaCl/0.5% (v/v) ethanol/0.2% (v/v) olive oil. Opsonized zymosan was prepared by the standard method [16].

Preparation and incubation of macrophages

Resting macrophages were obtained from peritoneal exudate cells of mice as described in [17]. Briefly, adherent cells were collected after incubation in glass Petri dishes at 37 °C in Krebs–Ringer bicarbonate (KRB) buffer [18] containing glucose (10 mM) and bovine serum albumin (2%, w/v) under air/CO_2 (19:1) for 3 h. Non-adherent cells were removed by three rinses with cold KRB buffer. Adherent cells were detached using a rubber policeman, washed twice and suspended in KRB without albumin but containing 10 mM-glucose. The viability of recovered

Abbreviations used: CsA, cyclosporin A; IL-1 and IL-2, interleukins 1 and 2; PMA, phorbol 12-myristate 13-acetate.

cells was estimated from their ability to exclude Trypan Blue.

Assay of $O_2^{\cdot-}$ and H_2O_2

$O_2^{\cdot-}$ was measured by following the superoxide dismutase-inhibitable reduction of 80 μM-cytochrome *c* at 550 nm at 37 °C as described in [19], using a spectrophotometer equipped with an automatic cell changer. Macrophages [$(1–1.5) \times 10^6$ cells/ml] were prewarmed at 37 °C with 10 mM-glucose and 80 μM-cytochrome *c* for 5 min before the addition of phorbol 12-myristate 13-acetate (PMA) and CsA. Control cuvettes received the vehicle used to dissolve CsA. The reduction of cytochrome *c* was recorded continuously in a Uvikon spectrophotometer. $O_2^{\cdot-}$ generation was calculated by using a molar absorption coefficient of 21.1 $mM^{-1} \cdot cm^{-1}$.

Synthesis of H_2O_2 was measured using the scopoletin assay [20]. The oxidation of scopoletin by H_2O_2 was followed fluorimetrically at $\lambda_{excitation}$ 366 nm and $\lambda_{emission}$ 460 nm at 37 °C. The reaction mixture contained 2 μM-scopoletin, 1 mM-NaN_3, 45 units of horseradish peroxidase/ml, 10 mM-glucose, 50 nM-PMA and $(1–1.5) \times 10^6$ cells/ml. Where indicated, either CsA or its solvent was also present. $O_2^{\cdot-}$ was generated enzymically by xanthine and xanthine oxidase. Xanthine was prepared as a 5 mM stock solution in 1 mM-NaOH prior to addition to the assay [21].

Assay of NADPH oxidase

NADPH oxidase was quantified as $O_2^{\cdot-}$ production by measuring the superoxide dismutase inhibition of the reduction of cytochrome *c* at 25 °C as in [22]. Reaction mixture (1 ml) contained 10 mM-phosphate buffer, pH 7.2/100 mM-NaCl/1 mM-$MgCl_2$/80 μM-cytochrome *c* and the postnuclear supernatant. The reaction was started by the addition of 200 μM (maximal activity) or 50 μM (submaximal activity)-NADPH (final concns.), and the absorbance change at 550 nm was followed.

Other analytical procedures

Oxidation of [U-^{14}C]glucose was measured after collecting $^{14}CO_2$ in 0.2 ml of Hyamine impregnated in Whatman filter papers placed in the centre of an Erlenmeyer flask as in [23,24]. Macrophages were incubated at 37 °C in Krebs–Ringer bicarbonate with 1 mM labelled glucose (0.33 μCi/ml)/50 μM-phenazine methosulphate/20 mM-NaF for 2 h. Where indicated, 100 nM-PMA and 1 μg of CsA/ml were added. The radioactivity recovered in Hyamine was counted with Biofluor as scintillant in a β-counter. Protein concentration was determined by the modified Lowry method [25] with bovine serum albumin as standard.

RESULTS

Effects of solvents of CsA on $O_2^{\cdot-}$ production

Due to the lipophilic nature of CsA, in previous studies the drug has been dissolved in ethanol alone. Other solvents previously used include dimethyl sulphoxide [26], acetone [27], and ethanol mixed with either Tween-80 [28] or oleic oil [29]. In our work, preliminary experiments analysed the effects of some of these solvents on the activation of $O_2^{\cdot-}$ production induced by 100 nM-PMA. Ethanol (0.05–0.5%, v/v), dimethyl sulphoxide (greater than 0.01%, v/v) and acetone (0.006%, v/v) produced 20–25% decrease of $O_2^{\cdot-}$ liberation (control value: 1.02 ± 0.01 nmol of $O_2^{\cdot-}$ released/min per mg of protein). Addition of olive oil to ethanol cancelled out the effect of the ethanol and this mixture was used in the subsequent experiments. These solvents did not modify the production of $O_2^{\cdot-}$ generated enzymically by xanthine and xanthine oxidase [21]. It is suggested that the solvents could act on NADPH oxidase directly, or that they could alter some essential components of the cell membrane.

Effect of CsA on resident macrophages

In order to determine whether CsA alters phorbol ester-induced activation of $O_2^{\cdot-}$ production by macrophages, resident cells were incubated with different doses of CsA (from 0.5 to 5 μg/ml of incubation). The respiratory burst was triggered by the addition of 100 nM-PMA. In previous experiments, it was demonstrated that this concentration of PMA elicits maximal production of $O_2^{\cdot-}$ with a calculated IC_{50} of about 35 nM. Fig. 1 illustrates that, in the absence of CsA, PMA produced an increase in $O_2^{\cdot-}$ production which reached a maximal value at 30 min of incubation. This kinetic pattern agrees with previous studies in phagocytic cells [30]. The simultaneous presence of CsA in the incubation medium produced a decrease in the liberation of $O_2^{\cdot-}$, which was dependent upon the concentration of CsA. Under the conditions used, a maximal inhibitory effect was found at 5 μg of CsA/ml. Fig. 1 also reveals that the presence of 5 μg of CsA/ml shifts the hyperbolic profile observed in control cells to a sigmoid form, indicating a delay in the initiation of response to PMA. When the effect of 2 μg of CsA/ml plus different doses of PMA was studied (Fig. 2), it was observed that the degree of

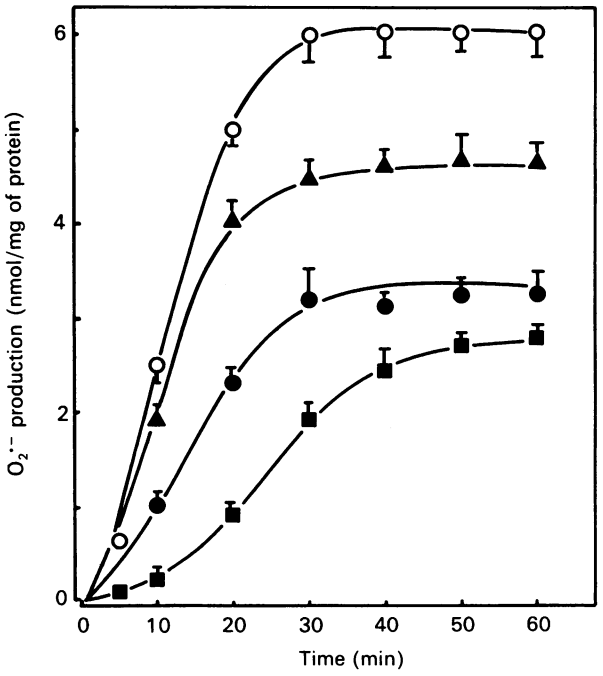

Fig. 1. Effect of CsA on $O_2^{\cdot-}$ production at different times of incubation

Resident macrophages were incubated with 100 nM-PMA in the presence of the following concentrations of CsA: 0 (control) (○), 0.5 μg/ml (▲), 1 μg/ml (●) and 5 μg/ml (■). Means ± S.E.M. from five experiments are presented.

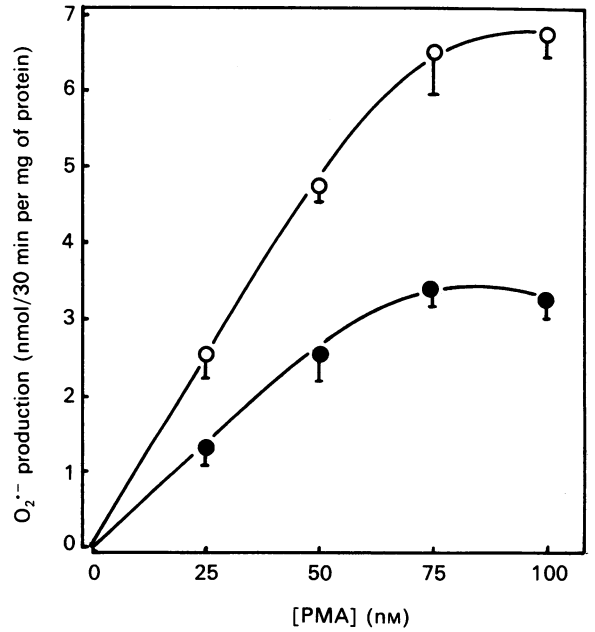

Fig. 2. Effect of CsA on $O_2^{\cdot-}$ production by resident macrophages exposed to different concentrations of PMA

Cells were incubated in the presence of 2 μg of CsA/ml (●) or in its absence (○). Data are means ± S.E.M. of three experiments performed in duplicate.

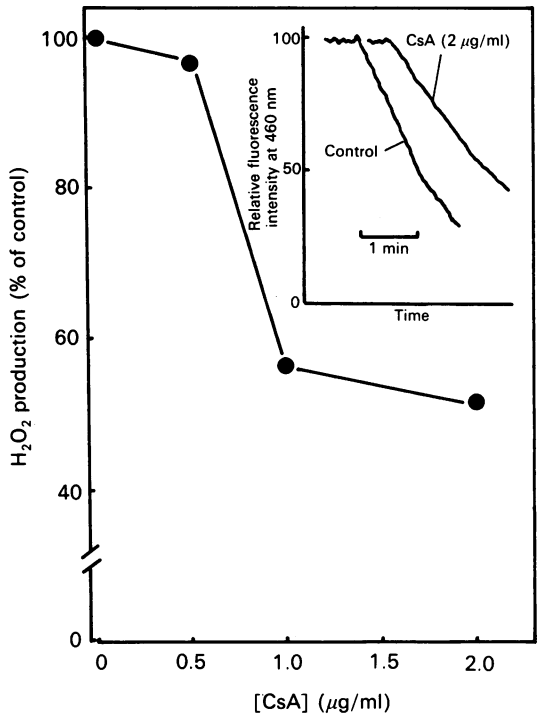

Fig. 3. Effect of CsA on H_2O_2 release by resident macrophages

Cells were incubated with 50 nM-PMA and different doses of CsA for 15 min. After a centrifugation (1 min), 100 μl of supernatant was immediately assayed for H_2O_2 as described in the Materials and methods section. In control conditions, H_2O_2 production was 9.3 ± 0.2 nmol/15 min per mg of protein. This value was normalized to 100%. The inset shows the effect of 2 μg of CsA/ml on the recording of fluorescence during the first min of incubation. The cuvettes contained all components for the incubation of cells plus the reagents for the scopoletin method. The reaction was started with 100 nM-PMA.

inhibition remained constant (about 50% inhibition compared with controls), suggesting that competition between CsA and PMA to occupy some putative receptor(s) is unlikely. Although the absolute values of $O_2^{\cdot-}$ production varied between individual experiments, the degrees of inhibition at different concentrations of CsA were consistent between experiments (results not shown).

Two possibilities may be put forward to explain the decrease in the production of $O_2^{\cdot-}$ due to CsA: first, an enhanced rate of dismutation of $O_2^{\cdot-}$ to H_2O_2, and secondly, defective production of $O_2^{\cdot-}$ caused by an altered NADPH oxidase. The first possibility was tested by measuring both the rate of H_2O_2 production in the first 1 min of incubation, and the total production of H_2O_2 after 15 min of incubation of cells with increased doses of CsA. Fig. 3 shows that CsA produced a concomitant inhibition of H_2O_2 production. The lower rate of H_2O_2 synthesis with CsA may be explained by a decreased access of $O_2^{\cdot-}$ to superoxide dismutase.

To verify the second possibility, i.e. an action of CsA on the $O_2^{\cdot-}$-forming enzyme, a series of experiments was carried out using a crude preparation of NADPH oxidase, as previously described [31]. Table 1 illustrates that NADPH oxidase activity, measured at two concentrations of NADPH, had the same value whether or not cells were incubated with 2 μg of CsA/ml. These results indicate that CsA requires the presence of intact cells. We used the crude extract of cells in order to preserve the original structure, as far as possible, since the enzyme is very labile, and its partial purification involves the use of detergents [31,32]. These data also suggest that the effect of CsA does not involve the irreversible modification in some essential component of the enzyme.

Consistent with our observation of the absolute requirement for glucose for the production of $O_2^{\cdot-}$ (Fig. 4), the reaction of CsA could be interpreted as an inhibition of either the transport or the metabolism of glucose. We used the method described by Taylor *et al.* [23] which gives indirect evidence of glucose transport by the measurement of $^{14}CO_2$ production following metabolism of [U-^{14}C]glucose by the pentose phosphate pathway (inhibiting the glycolytic flux and activating the pentose pathway with phenazine methosulphate). The rate of production of $^{14}CO_2$ from [U-^{14}C]glucose was 2.21 ± 0.03 nmol/h per mg of protein in control cells ($n = 3$), and 2.12 ± 0.04 nmol/h per mg of protein in cells incubated with 1 μg of CsA/ml ($n = 3$). Comparison of these results indicates that CsA did not inhibit energetic flux by altering the transport of glucose. In addition, a putative role for CsA as a scavenger of $O_2^{\cdot-}$ was also studied. $O_2^{\cdot-}$ radicals were produced with a mixture of xanthine and xanthine oxidase. Since the addition of 2 μg of CsA/ml did not modify the liberation of $O_2^{\cdot-}$ (results not shown), the inhibitory effect of CsA on stimulated macrophages cannot be attributed to its putative effect as a scavenger. In another series of experiments, an oxidized-minus-reduced difference spectrum of mouse peritoneal-macrophage homogenate was

Table 1. NADPH oxidase activity in crude extracts of macrophages incubated with CsA

Resting macrophages were incubated with 100 nM-PMA in the absence (control) or in the presence of 2 μg of CsA/ml for 30 min. At this time the cells were harvested and homogenized with a Potter–Elvehjem homogenizer in 10 mM-Tris/0.35 M-sucrose, pH 7.2. The homogenate was centrifuged at 1000 *g* to eliminate debris and unbroken cells. The enzyme activity was assayed in the supernatant (crude extract) with 50 μM- or 200 μM-NADPH. The values are the means of duplicate incubations in each experiment.

Expt.	Addition to cells	[NADPH] (μM)...	NADPH oxidase activity (nmol of $O_2^{\cdot-}$/min per mg of protein) 50	200
1	None		2.55	5.75
	CsA (2 μg/ml)		2.24	5.88
2	None		9.51	20.47
	CsA (2 μg/ml)		9.61	21.02

analysed. It was carried out as designed by Hancock & Jones (Fig. 1*a* in [33]). The absorbance bands observed in the present study resembled those found by these authors. When 100 nM-PMA plus 2 μg of CsA/ml was introduced into the system, it did not modify the pattern found with 100 nM-PMA alone. Finally, a direct effect of CsA on NADPH oxidase activity in the crude supernatant of macrophages was ruled out because the drug did not modify the activity of the enzyme measured at 50 μM- and 200 μM-NADPH (results not shown).

Effect of CsA on induction of $O_2^{\cdot-}$ production by a variety of activators

To determine whether the inhibitory action of CsA is specific for PMA, we have studied the effect of other agents well known as stimulators of the respiratory burst. These stimuli included fMet-Leu-Phe (10^{-6} M), concanavalin A (50 μg/ml) plus cytochalasin B (5 μg/ml), and opsonized zymosan (1 mg/ml). Most of these agents act through specific plasma membrane receptors [34], unlike PMA which binds to intracellular protein kinase C. The interaction with their receptors promotes the liberation of both Ca^{2+} and diacylglycerol [5,34]. Our results indicate that 1 μg of CsA/ml does not alter the effect of these stimuli on either $O_2^{\cdot-}$ production or H_2O_2 synthesis in macrophages (results not shown). These data suggest that the lesion caused by CsA is located at a post-receptor level and that it is specifically related to the binding of PMA and the ability of the phorbol ester to stimulate NADPH oxidase.

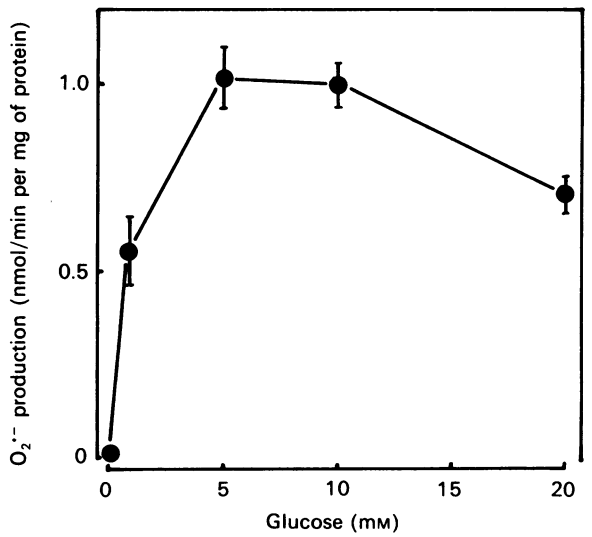

Fig. 4. Effect of glucose concentration on $O_2^{\cdot-}$ production by resident macrophages exposed to 100 nM-PMA

Cells were incubated with the indicated amounts of glucose for 30 min. Data are means ± S.E.M. from three separate experiments.

Effect of glucose and oleate on $O_2^{\cdot-}$ production

Since the reaction forming $O_2^{\cdot-}$ is strongly dependent on the NADPH concentration in a free-cell system [31], we investigated the effect of glucose as a donor of reducing power on $O_2^{\cdot-}$ production in PMA-stimulated macrophages. Fig. 4 shows that synthesis of $O_2^{\cdot-}$ was related to the glucose concentration. When the cells were incubated with 5 mM-deoxyglucose, the liberation of $O_2^{\cdot-}$ was completely inhibited. Previously it has been shown that the use of inhibitors of glycolysis reduces the production of $O_2^{\cdot-}$ in neutrophils [35]. The maximum effect of glucose on macrophage cells occurred at a concentration of 5–10 mM-glucose. However, at 20 mM-glucose, a systematic decrease in $O_2^{\cdot-}$ production was observed.

Macrophages have a high capacity to metabolize glucose, as demonstrated by the stimulation of the glycolytic pathway, measured by the increase of fructose 2,6-biphosphate levels in these cells in response to glucose and to addition of PMA [36]. Moreover, activation of the oxidative burst in phagocytic cells is characterized by an abrupt increase in oxygen uptake and by a large increase in the oxidation of glucose via the pentose phosphate shunt [2]. This latter observation may be related to the requirement of the oxidase for NADPH as a substrate for $O_2^{\cdot-}$ synthesis.

On the other hand, we found that sodium oleate stimulates $O_2^{\cdot-}$ production in resident macrophages at concentrations similar to those previously observed in elicited macrophages [37,38] (results not shown).

DISCUSSION

The present work demonstrates that CsA produces an inhibition of the respiratory burst in mouse resting macrophages, as shown by a decrease in PMA-stimulated $O_2^{\cdot-}$ or H_2O_2 production. Previously, it was shown that CsA inhibits the synthesis of IL-1 by macrophages [15] and other functions of these cells [39], but the action now described has not previously been reported. CsA seems to specifically antagonize the action of PMA because the other studied agonists (f-Met-Leu-Phe, opsonized zymosan and concanavalin A) were not affected by CsA. This may be correlated with the different types of receptors required for these agents. Whereas PMA binds to intracellular protein kinase C, the other agonists have membrane receptors [34].

Several possibilities may be considered in order to explain the inhibition of PMA-stimulated $O_2^{\cdot-}$ synthesis. It is noteworthy that the acute effect of CsA occurs in the

cells without apparent modification of glucose transport, and that the effect of CsA disappeared once the drug was removed from the medium. These data could indicate that CsA did not produce stable changes in essential component(s) of NADPH oxidase, and that its effect might be related to the disturbance of some step(s) in the interaction between PMA and NADPH oxidase [40]. The primary site of action of PMA is to bind to, and activate, protein kinase C [41]. Several reports have provided evidence that the activation of the NADPH oxidase involves the phosphorylation of a component(s) of the system [42]. Thus, cytochrome b_{245} is phosphorylated upon stimulation with PMA [43]. As CsA did not act on NADPH oxidase directly, this could suggest that protein kinase C is a target enzyme of CsA. Bearing in mind the lipophilic nature of CsA [9,10], the interaction of CsA with some lipid components of the cell might also be of relevance. The activation of NADPH oxidase in macrophages by unsaturated fatty acid has been observed [37,38]. One more possible mechanism could be related to the recent finding concerning the electrogenic nature of NADPH oxidase and to the respiratory control exhibited by the enzyme [44,45]. CsA could produce rapid changes in membrane potential by the translocation of some constituents of the macrophage membrane. In connection with this, correlation between membrane potential changes and PMA-induced $O_2^{\cdot-}$ production in human granulocytes has been described [46].

On the other hand, specific membrane receptors for CsA have been demonstrated in peritoneal macrophages [47]. The concentrations of CsA used in our study correspond to those which appear in the blood of patients treated with CsA (0.8–1.2 μg/ml) [48], and are of the same order as those previously described as causing inhibition of IL-1 production in macrophages [15] and those utilized in studies with lymphocytes [28], macrophages [49] and hepatocytes [50]. In any case, the requirement for a higher concentration of CsA in macrophages compared with lymphocytes correlates well with the binding capacity for CsA in the two cell types [47].

In summary, the present results suggest that mechanisms which are involved in the collaborative interaction between lymphocytes and macrophages could also contribute to the suppression of the proliferative response of lymphocytes by CsA. The central role of macrophages in the induction of the mitogenic response of lymphocytes is well recognized [51].

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