

IN addition to its well-established effect on T cells, cyclosporin A (CsA) also inhibits inflammatory cytokine production by macrophages. However, little is known about the mechanism of action of CsA on macrophage cytokine production. We measured the effect of CsA on basal and phorbol-myristate-acetate (PMA)-stimulated production of interleukin-6 using the human monocyte cell line U937 differentiated with dimethylsulfoxide (DMSO). Interleukin-6 levels were measured in supernatant and cell lysates using specific enzyme-linked immunosorbent assays. We found that CsA decreases not only IL-6 release but also cytokine synthesis. The concentration of CsA used did not affect either cell viability or proliferation. Three possibilities may be advanced to explain the CsA-due decrease in IL-6 production by macrophages: (a) inhibition of the synthesis of an early common regulatory protein, (b) inhibition of cytokine gene transcription, or (c) modulation of post-transcriptional events. The first possibility was tested by measuring the effect of cycloheximide on the experimental system during the first 3 hours of culture. Although cycloheximide decreased total cytokine synthesis, the pattern of cytokine modulation by CsA persisted. These data suggest that CsA-mediated macrophage cytokine inhibition is not mediated by an early common regulatory protein. To further explore the inhibition mechanism, we measured IL-6 mRNA levels by Northern blot. IL-6 mRNA levels were unaffected by CsA both in resting and PMA-stimulated cells. We conclude that in human macrophages CsA diminishes IL-6 production at post-transcriptional level.

**Key words:** Cyclosporin A, Interleukin-6, Macrophage, U937.

## Cyclosporin A decreases human macrophage interleukin-6 synthesis at post-transcriptional level

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## Introduction

Cyclosporin A (CsA) is an immunomodulatory substance used in the prevention of transplant rejection and in the treatment of autoimmune disease.<sup>1</sup> Classically, CsA has been reported to exert its immunomodulatory action through its effect on T lymphocytes, mainly helper T lymphocytes, by inhibiting calcineurin dependent interleukin-2 synthesis.<sup>2,3</sup> Recently, however, it has been suggested that the inhibition of T lymphocyte activation by CsA does not appropriately account for all the effects observed following *in vivo* administration of the drug.<sup>4</sup> Accordingly, despite widespread use of the drug, the specific mechanism of action of CsA remains to be fully elucidated.<sup>5</sup> It is also possible that its effects could be due in part to the action of CsA on other cells.<sup>6</sup> In this sense, it should be noted that CsA binds specifically, reversibly and in a time- and temperature-dependent fashion to all human leucocytes. However, granulocytes and cells of the mononuclear phagocyte system

(MPS) sometimes bind more CsA than lymphocytes, probably because the drug is rapidly internalized.<sup>2,7</sup>

Cells of the MPS offer an attractive possibility to study the effects of this drug because they participate in a crucial manner in antigen presentation and, in many cases, are the final effectors of the immune system. Many references in the literature report the capacity of CsA to alter the activities of MPS cells. In general, the capacities, functions and actions of macrophages related to non-specific defense such as chemotaxis, phagocytosis, enzyme release and respiratory burst, can be said to be more resistant to CsA.<sup>8,9</sup> By contrast, those related to immunoregulation or accessory functions, such as monokine production, the expression of histocompatibility antigens and antigen presentation, are more susceptible to the action of CsA.<sup>8,9</sup>

Macrophages synthesize and secrete IL-1 $\beta$ , TNF $\alpha$ , IL-6 and IL-8.<sup>10</sup> These monokines are known mediators of the inflammatory response<sup>10</sup> and are therefore also known as inflammatory cytokines. It has been

reported that CsA inhibits the production of IL-1 $\beta$ , TNF- $\alpha$  and IL-6 in different cells of the MPS.<sup>11,12</sup> However, the mechanism through which this occurs is not well known.<sup>13</sup>

In the light of the foregoing, the aim of the present study was to evaluate and determine the mechanism of action of CsA on the production of interleukin-6 by macrophages. To do so, the human differentiated U937 monocytic line was employed.

## Methods

### Cell line

U937 cells were kindly supplied by Dr J. Olmos of the Hospital Universitario Marqués de Valdecilla, Santander (Spain). The cell line was kept at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> in culture with complete medium containing RPMI 1640 (Sigma®), 100 U/mL of penicillin (Sigma®), 100 U/mL of streptomycin (Sigma®), 2 mM L-glutamine (Sigma®) and 10% fetal calf serum (FCS) (Gibco®) in sterile culture flasks (Nunc®) at a concentration between  $0.75 \times 10^5$  and  $5 \times 10^5$  cells/mL. Cell viability was greater than 90% and the duplication time was between 24 and 48 hours. Cells were regularly screened for lipopolysaccharide (LPS), bacteria, mycoplasma and fungal contamination and found to be negative. Cells were differentiated by culture over 4 days in complete medium containing 1.3% dimethylsulfoxide (DMSO).

### Cell cultures

$5 \times 10^5$  cells together with different stimuli and/or CsA at the following concentration were added to each well of the culture dishes (Costar®) with complete medium: Lipopolysaccharide from *E. coli* (Sigma®) at 100  $\mu$ g/mL and 10  $\mu$ g/mL; phorbol-12-myristate-13-acetate (PMA) (Sigma®) at  $10^{-4}$ M and  $10^{-5}$ M; human gamma-interferon (Sigma®) at 1000 U/mL and 100 U/mL and CsA (Sandoz®) at three non-toxic concentrations *in vivo*: 200, 20 and 2 ng/mL.

Cells were kept at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> for 18 hours, after which the supernatants were centrifuged at 500g for 10 min to sediment cells in suspension and then stored at -70°C until later determination.

After the supernatant had been removed, 400  $\mu$ L of a 0.25 M sucrose-0.02 EDTA solution was added to each well. After the bottom of each well had been scraped vigorously, the content was aspirated and added to the previous cell pellet, thus recovering all the cells. The cells were then subjected to heat disintegration by repeated freezing-thawing (6 cycles of 10 min freezing and 15 min thawing) and were stored at -70°C until determination.

To study the inhibition of early protein synthesis, cycloheximide (Sigma®) at a concentration of 1  $\mu$ g/mL was added during the first 3 hours of cell culture.

### Cytokine determination

The inflammatory cytokine IL-6 was determined by direct double-sandwich enzyme-linked immunosorbent assay (ELISA) of antibodies in the supernatants and cell lysates. BIOTRAK® (Amersham, UK) commercial kit specific for human IL-6 was used; this showed no cross-reactivity with other cytokines. It had a coefficient of variation less than 10% and a sensitivity limit of 0.35 pg/mL. All samples were measured in duplicate. Results were expressed in picograms/10<sup>6</sup> cells. It should be noted that in ELISA assays none of the components of the culture medium (RPMI 1640, antibiotics, glutamine or fetal calf serum) showed immunoreactivity with the cytokine studied.

### IL-6 mRNA expression

Transcription of the IL-6 gene was studied by Northern blot. Differentiated U937 cytoplasmic RNA was isolated by 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 10 mM Tris-HCl pH 7.4, 0.5% Nonidet P40 and 250–1000 U/mL RNAsing (Promega®) lysis, followed by two successive ultracentrifugations with Tris-buffered phenol and 10% SDS and phenol, respectively. RNA was precipitated overnight with cold ethanol and 3M sodium acetate pH 7.2, and redissolved in Tris-EDTA pH 7 buffer. RNA was quantified by determining optical density at a wavelength of 260 nm. The quality of the RNA was checked by the demonstration of two bands corresponding to ribosomal RNA (18s and 28s) and the absence of degradation of the RNA by staining with ethidium bromide in the electrophoresis of a 1% agarose minigel loaded with 1.5  $\mu$ g of RNA.

RNA was denatured by incubation for 1 h at 50°C in a solution of 1 M glyoxal, 50% DMSO and 10 mM NaH<sub>2</sub>PO<sub>4</sub>. Following this, 5  $\mu$ g of RNA were mixed with electrophoresis sample buffer (50% glycerol, 49.6% 10 mM NaH<sub>2</sub>PO<sub>4</sub> pH 7.0, 0.4% bromophenol blue). After loading samples, electrophoresis (35V) was carried out in 1% agarose-10 mM NaH<sub>2</sub>PO<sub>4</sub> pH 7 gels using 10 mM NaH<sub>2</sub>PO<sub>4</sub> pH 7 buffer. Following electrophoresis, the RNA was transferred overnight by capillarity from the gel to a nitrocellulose membrane using 20 $\times$  SSC. This membrane was dried with Whatman paper and the RNA was cross-linked to the membrane by exposure to UV light. Then, the membrane was prehybridized for 2 h at 65°C in a solution containing 6.4% dextran, 3 $\times$  SSC, 1 $\times$  Denhart's, 0.1% SDS and 250  $\mu$ g/mL heat-denatured salmon sperm DNA. IL-6 cDNA and G3PDH cDNA probes commercialized by the American Type Culture Collection (ATCC) were labelled with <sup>32</sup>P using

the commercial 'Ready To Go' kit (Pharmacia Biotech®) following the protocol indicated by the manufacturer. Hybridization (18 h, 65°C) was carried out using the same prehybridization solution containing heat-denatured <sup>32</sup>P-labelled probes. Following successive washings in 0.1 SDS-0, 1 SSC at room temperature and 65°C, respectively, membranes were blotted dry and used for autoradiography with XR film (Cronex 105, Dupont®) at -70°C over 6 days. Hybridization with the G3PDH probe was used as an RNA loading control for blots.

## Results

### Differentiation of the U937 line

The data (not shown) confirming that suitable differentiation of the monocyte cell line toward macrophage cells had been achieved were as follows:

- a decrease in the cellular proliferation rate without viability being affected;
- an increase in adherence to the culture supports and among cells;
- an increased cytoplasm, the disappearance of nuclear polylobulation and a decrease in nuclear atypias and the number of nucleoli;
- an increase in the content of macrophage enzymes (non-specific esterases); and
- an increase in cytoplasmic RNA content.

### Determination of type of stimulus and most effective concentration on interleukin-6 secretion

In order to study the most suitable type of stimulus for inducing the secretion of IL-6 and the most effective concentration, U937 cells were used under both differentiated and undifferentiated conditions. Three classic stimuli were studied at two different concentrations: lipopolysaccharide (LPS), phorbol myristate acetate (PMA) and gamma-interferon ( $\gamma$ -IFN). The mean values of the results of the study carried out in quadruplicate are shown in Fig. 1. Overall, the data obtained indicate that the most potent stimulus of cytokine secretion was PMA at a concentration of  $10^{-5}$ M. IL-6 secretion was greater by differentiated U937 cells than by undifferentiated ones.

### Effect of CsA on the synthesis of interleukin-6

The results of the effect of different concentrations of CsA on IL-6 secretion under both basal and stimulated conditions are shown in Table 1. These data show the mean values obtained in four different assays. As may be seen, both in undifferentiated and differentiated U937 cells, at the highest concentration used (200 ng/mL) CsA decreased stimulated secretion of IL-6. CsA can be said to decrease the secretion of this cytokine more than 50%. The effect of CsA on basal IL-6 secretion was much lower.

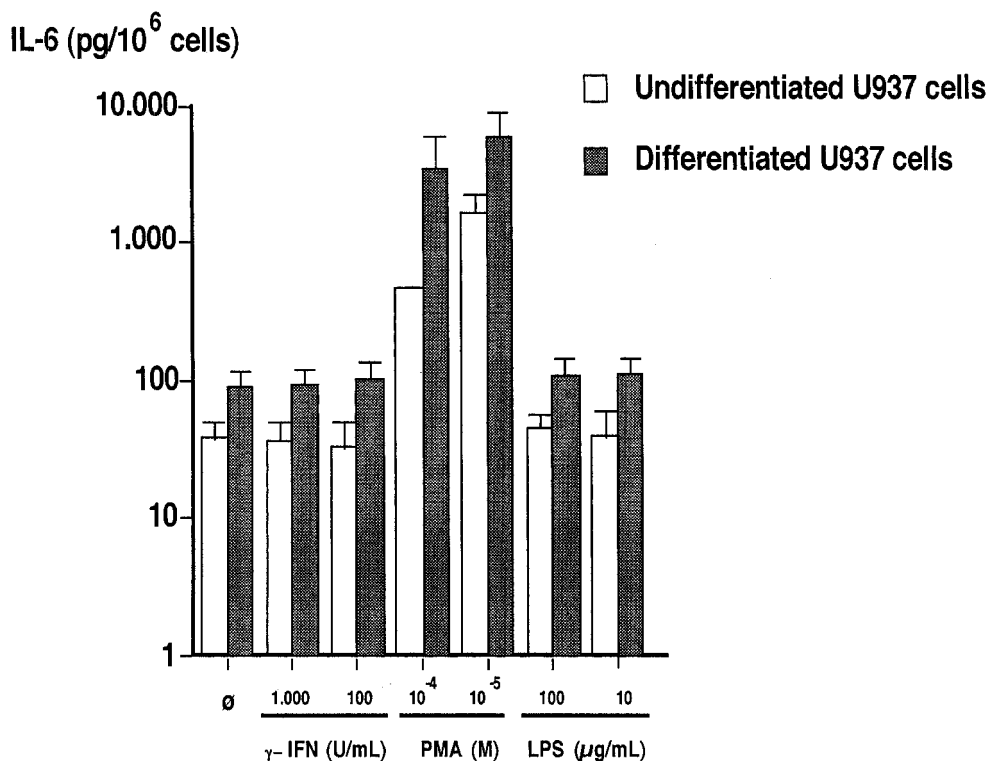


FIG. 1. Effect of different stimuli on IL-6 secretion by U937 cells. The most potent stimulus of cytokine secretion was PMA at a concentration of  $10^{-5}$ M. Definition of abbreviations: IFN, interferon; IL-6, interleukin-6; LPS, lipopolysaccharide; PMA, phorbol myristate acetate. Note the logarithmic scale.

**Table 1.** Effect of CsA on basal and stimulated IL-6 secretion by U937 cells (pg/10<sup>6</sup> cells). Both in undifferentiated and differentiated U937 cells, at the highest concentration used (200 ng/mL) CsA decreased stimulated secretion of IL-6. Definition of abbreviations: CsA, cyclosporin A; PMA, phorbol myristate acetate; SEM, standard error of mean.

	Mean	SEM
<b>Undifferentiated</b>		
Basal	38,4	7,9
+ CsA (2 ng mL <sup>-1</sup> )	37,6	13,1
+ CsA (20 ng mL <sup>-1</sup> )	34,0	12,7
+ CsA (200 ng mL <sup>-1</sup> )	25,2	4,2
+ PMA 10 <sup>-5</sup> M	1705,1	313,5
+ CsA (200 ng mL <sup>-1</sup> )	731,4	66,0
<b>DMSO differentiated</b>		
Basal	90,4	27,6
+ CsA (2 ng mL <sup>-1</sup> )	99,7	33,3
+ CsA (20 ng mL <sup>-1</sup> )	91,4	34,2
+ CsA (200 ng mL <sup>-1</sup> )	67,1	20,6
+ PMA 10 <sup>-5</sup> M	6343,7	3135,6
+ CsA (200 ng mL <sup>-1</sup> )	2035,1	1072,6

Using U937 cells differentiated with 1.3% DMSO, the intracellular content of IL-6 was measured after stimulation with phorbol myristate in two different assays. At 200 ng/mL, CsA decreased the intracellular content of IL-6 from 151,0 ± 97,2 to 100,0 ± 61,3 pg/10<sup>6</sup> cells. So, CsA can be said to decrease the production of cytokines since both the intra- and extracellular contents were affected.

**Effect of CsA on cell viability and cell proliferation capacity**

The first and simplest possibility considered was that CsA would affect cell viability and/or proliferation and hence that the decrease in cytokine production would be due to a decrease in these capacities. Accordingly, a duplicate study was performed in which the proliferation capacity and cell viability of a U937 line subjected to the same stimuli as those used in the study were evaluated. Table 2 shows the results of this study.

As seen, CsA did not affect either viability or proliferation capacity, either in undifferentiated U937

cells or in cells differentiated with DMSO. Therefore the results on cytokine secretion cannot be interpreted in these terms.

In this study, the effects of several stimulatory substances were also assessed. Both PMA and LPS, but not gamma-interferon, were seen to decrease cell viability.

**Effect of cycloheximide on CsA inhibition of IL-6 production**

To study the mechanism by which CsA decreases IL-6 production, three working hypotheses were addressed: that CsA affects the synthesis of an early protein, responsible for the decreased levels of the cytokine; that it exerts its action at transcriptional level, and that its effect is mediated at post-transcriptional level.

The first mechanism proposed was evaluated by treatment with cycloheximide (an inhibitor of protein synthesis) of differentiated U937 cells during the first 3 hours of culture. Prior to this study it was ascertained that cycloheximide does not affect cell viability (data not shown).

The effect of cycloheximide on the secretion of IL-6 is detailed in Fig. 2. As may be seen, although overall this drug decreased both basal and stimulated cytokine secretion in differentiated cells, which was expected, the inhibitory effect of CsA persisted in all cases. It is therefore unlikely that the mechanism of action of CsA would depend on inhibition of the synthesis of an early protein.

**Effect of CsA on expression of the IL-6 gene**

Study of the action of CsA on the expression of IL-6 mRNA by Northern blot was carried out on the differentiated cell line under both basal and stimulated conditions. Two assays were performed and, in each, Northern blot was implemented in duplicate. Figure 3 shows the results of one of the assays. As may be seen, CsA did not affect the levels of IL-6 mRNA under either basal or stimulated conditions.

**Table 2.** Effect of CsA and different stimuli on cell proliferation and viability. In this study we started out with a viable cell concentration of 250,000/mL, obtaining the data after 18 hours of culture. CsA did not affect either the viability or proliferation capacity of either differentiated or undifferentiated cells. Additionally, both LPS and PMA, but not interferon, decreased cell viability. Definition of abbreviations: CsA, cyclosporin A; LPS, lipopolysaccharide; PMA, phorbol myristate acetate

	Undifferentiated U937 cell line		Differentiated U937 cell line	
	Viable cells/mL	Viability (%)	Viable cells/mL	Viability (%)
Basal conditions	370.000	91	317.000	95
PMA 10 <sup>-5</sup> M	240.000	84	128.000	76
LPS (100 µg/mL)	335.000	88	280.000	88
γ-Interferon (1000 U/mL)	400.000	94	285.000	92
CsA (200 ng/mL)	385.000	94	300.000	93
CsA (200 ng/mL) + PMA 10 <sup>-5</sup> M	240.000	77	115.000	81

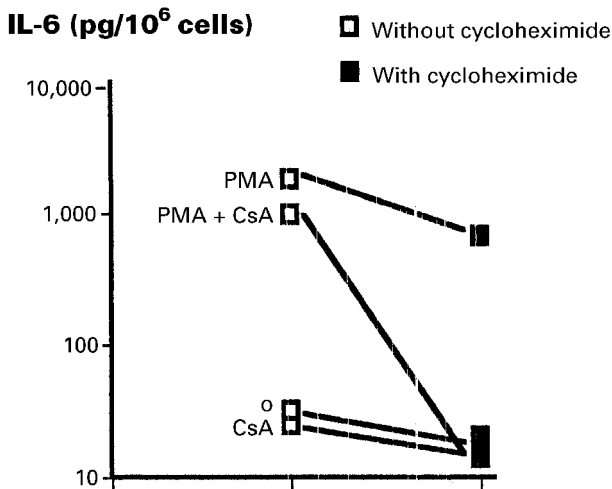


FIG. 2. Effect of cycloheximide on the action of CsA in U937 cells. The effect of cycloheximide was evaluated in differentiated cells. Although overall cycloheximide decreased both basal and stimulated cytokine secretion, the inhibitory effect of CsA persisted in all cases. Definition of abbreviations; CsA, cyclosporin A; IL-6, interleukin-6; PMA, phorbol myristate acetate.

## Discussion

### Differentiation of the U937 cell line

Study of the mechanism of action of CsA on inflammatory cytokine production by human macrophages could have been carried out in tissue macrophages obtained *ex vivo*. However, the limited number of cells available for the different experiments, together

with the difficulty involved in obtaining healthy control cells, within-group variability and, above all, the influence of the action of the drug on other cells – especially lymphocytes – counselled against this. Therefore, to study the mechanism of action of CsA on cytokine production in macrophages and to have available a sufficient number of cells, the possibility of using a monocyte cell line – namely, U937 cells – was implemented. This had the additional advantage of enabling study of a completely pure population; that is, completely excluding the presence of lymphocytes, which might alter the results obtained. The literature contains references to use of the U937 cell line in the evaluation of different macrophage functions.<sup>14</sup> This human cell line grows continuously in suspension and was initially obtained from a pleural effusion in a patient with histiocytic lymphoma.<sup>15</sup> Its phenotype corresponds to immature monocyte cells arrested in a differentiation state close to the myelomonocytic stem cell.<sup>15</sup> It should be recalled that macrophages are highly differentiated cells and therefore have low proliferation capacity while cell lines show a strong duplication rate. Thus, the first step in this part of the work was to differentiate the human U937 monocyte cell line.

In the presence of several substances U937 cells undergo a process of differentiation and therefore acquire morphological and functional characteristics similar to those of macrophages.<sup>16</sup> Since one of the interests in the present work was to study the effect of PMA on cytokine secretion, DMSO was chosen as a

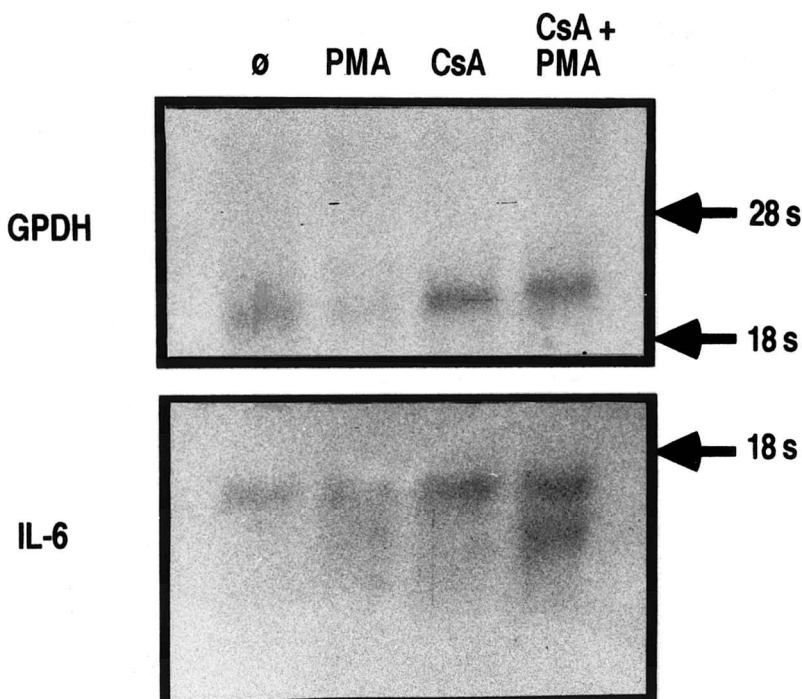


FIG. 3. Effect of CsA on IL-6 mRNA expression. Study of the action of CsA on IL-6 mRNA expression by Northern blot was carried out on the differentiated cell line under both basal and stimulated conditions. As seen, CsA did not affect IL-6 mRNA levels under either basal or stimulated conditions. Definition of abbreviations: CsA, cyclosporin A; GPDH, glyceraldehyde phosphate dehydrogenase; IL-6 interleukin-6; PMA, phorbol myristate acetate.

differentiating agent because it displays differentiating capacity on the U937 cell line.<sup>17</sup> Differentiation was evaluated by kinetic, morphological, cytochemical and molecular techniques. The results obtained, which were similar to those reported by other authors,<sup>15,17</sup> allowed us to conclude that the protocol used is indeed able to differentiate this cell line.

### Choice of cellular stimulus

DMSO-differentiated U937 cells retain their capacity to be stimulated later on with different agents,<sup>17</sup> among them PMA.<sup>18</sup> It should be noted here that differentiation and activation are not incompatible concepts and that it is in fact possible to employ several substances<sup>16</sup> to activate cells differentiated with other agents. Here, we evaluated the effect of three known macrophage stimulating agents and observed that for this purpose the most suitable agent was PMA at a concentration of  $10^{-5}$  M. Although according to the literature lower concentrations of this substance are usually used,<sup>15,17</sup> in each experimental system it is necessary to obtain evidence of the most effective concentration in the induction of a given effect. Therefore in the present study the aforementioned concentration was used. Although at this concentration PMA directly decreases cellular viability, from a quantitative point of view the increase that it induces in cytokine secretion cannot be attributed to the elimination of cytokines by dead cells.

### Production of interleukin-6 by U937 cells

Once the required experimental conditions had been established, IL-6 secretion by U937 cells was studied. The secretion of IL-6 was stronger in differentiated than in undifferentiated cells. Recently it has been reported that the CD4<sup>19</sup> and CD23<sup>20</sup> surface molecules present in differentiated MPS cells are involved in the secretion of IL-6 by U937 cells. Although none of them used a protocol identical to the one used here, different studies have documented the production of different inflammatory cytokines by these cells using bioassays,<sup>21</sup> immunoassays<sup>22-25</sup> or molecular biology techniques.<sup>21,24</sup> Hass *et al.*<sup>22</sup> did not detect IL-6 secretion, possibly due to the stimulation conditions, and the values described by Jiang *et al.*<sup>23</sup> for IL-6 are lower than our own, although they used different protocols.

### Effect of CsA on interleukin-6 production

Both in undifferentiated and differentiated U937 cells, at the highest concentration used (200 ng/mL) CsA decreased stimulated secretion of IL-6, more than 50% a value similar to that reported by Moutbarrik *et al.*<sup>26</sup> In human monocytes at

therapeutic concentrations *in vivo*. Additionally, CsA decreased the intracellular concentration of IL-6. Accordingly, in the differentiated U937 cell line CsA can be said to decrease the production of inflammatory cytokines. The effect of CsA on the U937 cell line is not well documented. In a review of the literature no references to these phenomena were found.

### Mechanism of action of CsA on the production of interleukin-6

From the theoretical point of view, the effect of CsA on the production of interleukin-6 could be due to a decrease in cell viability and proliferation and hence to a decrease in the number of producing cells, to the alteration of an early protein that would regulate the gene expression of cytokines, to a direct action on gene transcription, or to a modification of post-transcriptional events.

The data obtained here allow us to exclude a direct effect of CsA on cell proliferation and viability. We can also exclude its action at early level since incubation with cycloheximide during the first hours of incubation did not affect the inhibitory effect of CsA. The expected decrease in IL-6 production by U937 cells due to the action of cycloheximide is consistent with the observations of other authors<sup>27</sup> who have reported that this drug inhibits stimulation of IL-1 $\beta$  synthesis in the U937 cell line.

Finally, this immunomodulator does not alter IL-6 mRNA expression, allowing us to conclude that the effect of CsA on IL-6 production occurs at post-transcriptional level. Results similar to those obtained by us have been published by Kato *et al.*<sup>28</sup> who observed that CsA scarcely affects the expression of the IL-6 gene in human peripheral blood monocytes. Likewise, in a murine mast cell line Nair *et al.*<sup>29</sup> reported that CsA does not alter IL-6 mRNA expression although it does affect IL-3 mRNA. It would thus appear that in the human MPS CsA has little effect on the transcription of monokine genes since the mRNA levels of IL-1,<sup>30</sup> TNF<sup>30</sup> and IL-8<sup>31</sup> are not affected by CsA either.

The exact mechanism by which CsA exerts its effect on cells of the MPS is not known although it has been suggested that its effect could be related to an inhibition of protein secretion/synthesis.<sup>32,33</sup> If, as commented earlier, it is true that CsA inhibits the secretion of inflammatory cytokines and does not affect the transcription of their genes, its action would appear to occur at post-transcriptional level.

In view of the decrease in immunogenic protein, CsA may act at some step between mRNA and synthesis of the protein. Although this aspect was not studied explicitly in the present work, the idea that CsA might act on intracellular membranes involved in protein synthesis could be entertained. At least three

theoretical possibilities could be advanced to account for this. First, one could be dealing with a non-specific interaction, owing to the lipophilic nature of this immunomodulator. In this sense, it has been demonstrated that other lipophilic substances or lipid emulsions are able to inhibit cytokine production by macrophages through this mechanism.<sup>34</sup> Second, although there is no consensus about the possibility,<sup>35,36</sup> CsA might inhibit protein kinase C activity in macrophages. Thus, it has recently been observed that inhibition of this enzyme decreases TNF- $\alpha$  production in LPS-stimulated macrophages.<sup>37</sup> Finally, another possibility would be that one would be dealing with a specific action derived from the interaction of CsA with cytokines associated with intracellular membranes.<sup>38</sup> Further studies are evidently required to clarify all these aspects.

It should be noted that some authors have situated the site of action of CsA on monokine secretion at the level of release since they observed a decrease in the secretion of TNF- $\alpha$  with normal expression of the gene and normal intracellular cytokine synthesis.<sup>13</sup> However, these studies were carried out in mouse MPS cells while those used here were from a human source.

Accordingly, it may be concluded that at therapeutic and non-toxic concentrations *in vivo*, CsA decreases the production of interleukin-6 by human macrophages at post-transcriptional level.

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