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Article

Preliminary study to characterize differences in potential immunomodulatory effects of cyclosporine A using BALB/c and ICR mouse splenocytes

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Cyclosporine A (CsA) is widely used as an immunosuppressant for the treatment of autoimmune diseases and immune regulation in transplant patients. Due to its wide applicability, studies of unwanted side effects of CsA are imperative. It has been found that not all patients treated with CsA display the same types/patterns of adverse effects. To ascertain the bases for these differential responses, potential differing effects of CsA on B-lymphocytes were analyzed. This entailed an assessment of changes in CsA viability and mitotic activity within splenocyte populations from BALB/c and ICR mice. These particular strains were examined because: (1) in each of them, previously have been shown that differed in the respond to biological response modifiers, such as bacterial agents, and/or immunogens; (2) our own earlier studies showing strain-associated differences in *ex vivo* splenocyte/lymphocyte responses to other drug; and, (3) a potential immunomodulatory effect of any agent should be studied in at least two different strains during a broad toxicological evaluation. Splenocytes from each strain were treated with 200 µg/mL CsA, and $CD4^+$, $CD8^+$, and $CD19^+$ cell viabilities were monitored at various time points during the exposure period. In general, there appeared to be a trend toward greater decreases in viability among BALB/c B-lymphocytes than their ICR counterparts as incubation progressed. Differences related with T-lymphocyte sensitivity to drug associated to strains was not observed, because it was uniformly lethal throughout. With regard to mitotic activity, cells from ICR mice were more susceptible to inhibition of spontaneous cell division at low concentrations of CsA (relative to the rates of blastogenesis by BALB/c counterparts). At higher concentrations of the drug however, there were no differences in the sensitivity of each strain. This work provides new insight into the mechanism of action of CsA and illustrates the need for at least two different strains of mice/rodents for the evaluation of the overall toxicological potential of any test agent.

Uniterms: Immunotoxicity. Cyclosporine A/immunomodulatory effects. Cyclosporine A/ adverse effects. B-lymphocytes. BALB/c ICR. Blastogenesis.

Ciclosporina A (CsA) é amplamente usada como imunossupressor para o tratamento de doenças autoimunes e regulação imune nos pacientes transplantados. Devido à alta aplicabilidade, são imperativos os estudos sobre seus efeitos colaterais indesejáveis. Descobriu-se que nem todos os pacientes tratados com CsA apresentam os mesmos tipos/padrões de efeitos adversos. Para averiguar as bases dessas respostas diferentes, analisaram-se efeitos potenciais diferentes da CsA nos linfócitos B. Isto envolveu a avaliação de alterações na viabilidade da CsA e da atividade mitótica dentro das populações de esplenócitos de camundongos BALB/c e ICR. Essas espécies, em particular, foram examinadas porque: (1) cada uma delas mostrou, previamente, respostas diferentes a modificadores de respostas biológicas, tais como agentes bacterianos e/ou imunogênicos; (2) nossos estudos anteriores mostraram diferenças associadas às espécies em respostas *ex vivo* de esplenócitos/linfócitos a outro fármaco e (3) qualquer efeito imunomodulatório potencial de um agente em teste deveria ser estudado, no mínimo, em duas espécies diferentes durante a avaliação toxicológica ampla. Esplenócitos de cada espécie foram tratados com 200 µg/mL de CSA e a viabilidade das células $CD4^+$, $CD8^+$ e $CD19^+$ foi monitorada em vários tempos

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durante o período de exposição. No geral, parece haver uma tendência em relação a aumentos maiores na viabilidade entre os linfócitos B de BALB/c do que no de ICR, à medida que a incubação progride. Não se observou diferenças na sensibilidade do linfócito T, uma vez que o fármaco foi uniformemente letal. Com relação à atividade mitótica, as células de camundongos ICR se mostraram mais suscetíveis à inibição da divisão celular espontânea em baixas concentrações de CsA (relativamente às taxas de blastogênese de BALB/c). Em concentrações maiores do fármaco, entretanto, não houve diferenças na sensibilidade em cada uma das espécies. Este trabalho propicia nova visão do mecanismo de ação de CsA e ilustra a necessidade de, pelo menos, duas espécies diferentes de camundongos/roedores para a avaliação da toxicidade potencial de qualquer agente em teste.

Uniterms: Imunotoxicidade. Ciclosporina A/efeito imunomodulatório. Ciclosporina A/efeitos adversos. Linfócitos B. BALB/c. Blastogênese.

INTRODUCTION

Cyclosporine A (CsA), a metabolite derived from the fungus *Tolypocladium inflatum gams* (Cohen *et al.,* 1984; Kahan, 1985; Thomas, Gordon, 1986), known as a immunosuppresive agent (Kahan, 1985; Fukuzawa, Shearer, 1989; Flores *et al.,* 2004; Rezzani *et al.,* 2006; Waiser *et al.,* 2006) used to prevent host versus graft responses in patients subjected to heart, liver, kidney, bone marrow, and/or pancreas transplants (Ptachcinski *et al.,* 1985; Thomas, Gordon, 1986). It has also been used for the treatment of autoimmune disorders (Kahan, 1993; Roy *et al.,* 2006) and lymphoproliferative diseases (Foa *et al.,* 1981; Gabor *et al.,* 1996; Sairanen *et al.,* 2005). CsA's wide applicability makes the study of its mechanism of action, pharmacodynamics, and unwanted side effects, imperative. Some of the unwanted adverse effects reported include nephrotoxicity and hepatotoxicity (Canafax, Ascher, 1983), hirsutism, and neurologic complications (Ptachcinski *et al.,* 1985). In general, these toxic effects have been attributed to cell damage/death in the target organs (Roy *et al.,* 2006).

Use of this drug in different immunological studies has also allowed many of the complex inter-cellular interactions involved in immune responses to be elucidated (Hess *et al.,* 1986). At the cellular level, CsA binds to Cyp-M (a cyclophilin-family protein), forming a complex that interacts to inhibit calcineurin phosphatase activity. Currently, many regulatory molecules are believed to be suppressed by CsA, including myc and tyrosine kinases (among others), leading to disruption of the pathways under their respective control (Roy *et al.,* 2006). On the other hand, CsA is able to affect different T-lymphocyte populations selectively. Its activity does not cause myelosuppression (Canafax, Ascher, 1983; Ptachcinski *et al.,* 1985); it does however, notably alter growth factor production (Kahan, 1993). In cell cultures from renal allograft recipients, concentrations of TGF-β1 and its receptors (Type I and II) in supernatants, as well as lymphocyte proliferation, were higher when CsA was included in a patient's immuno-suppression maintenance scheme (Waiser *et al.,* 2006). CsA is able to inhibit the production of interleukin (IL)-2 and suppresses the expression of its receptor (i.e., CD25) in cytotoxic T-lymphocytes (Hess *et al.,* 1986). In addition, CsA blocks T-helper lymphocyte activation, resulting in decreased cytokine production from this critical cell sub-population (Thomas, Gordon, 1986).

CsA is a cytotoxic agent for leukemia cell lines, where it mainly triggers apoptosis. In viability assays, it has been shown that CsA-induced cell death is dependent on the concentration and length of exposure. Secondary effects observed are: lower cell proliferation, an increase in frequency of Anexin V^+ cells, and release of cytochrome c. Although many studies have provided insight about CsA's mechanism of action in $CD4^+$ and $CD8^+$ sub-populations (Diaz-Romero *et al.,* 2001), the effect of the drug on B-lymphocytes ($CD19^+$ cells) has not been precisely determined and only a few studies on this issue are available in the literature (see Healy *et al.,* 2006 or Gary-Gouy *et al.,* 2006).

Based on the fact that mice have been used in immunotoxicology studies due largely to their well-characterized immune systems, in the present study, it was decided to analyze the immunomodulatory potential of CsA in splenocytes from two murine strains (i.e., BALB/c and ICR), with a specific focus on its effects upon $CD19^+$ cells. The first goal of these particular studies was to confirm our earlier hypothesis, which held that in order for investigators to effectively evaluate the potential immunotoxicity of any formulation, at least two different strains of mice are needed, depending on the genetic, phenotypic and functional background of the mice strain used. The second goal was to assess T-cell-independent CsA immunotoxicity in these mice.

MATERIAL AND METHODS

Mice

Nine ICR (Pharmacy Department Biotherium, Universidad Nacional de Colombia) and eleven BALB/c

(Instituto Nacional de Salud Biotherium) 4-week-old female mice were used in this study. Female mice were used as they are less aggressive than males and therefore less prone to cause skin injuries to one another, injuries that would hamper immune function evaluation(Vahter *et al.,* 2007). Animals were kept in groups of three per cage while one cage (group of BALB/c mice) contained five individuals. During the experiments, the mice were provided with a standard pellet diet (Rodentina, Purina S.A., Bogotá, Colombia) and water *ad libitum*. All experiments were approved by the Ethics Committee of the Fundación Instituto de Inmunologia de Colombia. All mice were handled according to the procedures required by the Colombian Ministry of Social Protection (Resolution No. 008430 of 1993).

Obtention of splenocytes

Mice were sacrificed by cervical dislocation, according to the rules issued by the Department of Pharmacy of the Faculty of Science, Universidad Nacional de Colombia. Each mouse spleen was then aseptically removed, placed in a Petri dish containing RPMI 1640 (GIBCO, Scotland, UK) supplemented with 10% fetal bovine serum (FBS), and single cell suspensions were prepared by syringe-mediated disaggregation. Mononuclear splenocytes were then separated via a Ficoll-Hypaque density gradient ($\rho = 1.077$; ICN Biomedical Inc., Aurora, OH).

CsA treatments

Splenocyte cultures were used for the analysis of $CD4^+$ (T-helper lymphocyte), $CD8^+$ (cytotoxic T-lymphocyte), and $CD19^+$ (B-lymphocyte) expression kinetics after CsA exposure. To assess subpopulation mortality, aliquots of cells were divided into four subgroups: one for the analysis of baseline subpopulation frequencies (i.e., markers measured immediately in duplicate samples from each mouse) and the remaining three for the study of the effects on splenocyte marker expression/mortality, after 24, 48, or 72 hr of culture in the presence of the drug. Specifically, 4×10^5 cells/well (pre-counted in Neubauer Chambers in duplicate wells) were placed in 24-well dishes and then received 200 µg of CsA/mL medium (Sandimmune, Novartis Pharma AG, Basel, Switzerland); time-matched control wells received medium alone. Only this CsA level was used because it is the highest dose found previously to induce a specific effect (i.e., inhibition) upon Ca^{2+}-ATPase and nitric oxide synthase (NOS) (Hutcheson *et al.,* 1995).

Flow cytometric analysis of viability

Splenocytes obtained immediately after splenic disaggregation/Ficoll separation (baseline count) and after different incubation times with CsA, were labeled with antibodies to CD4 (anti-mouse CD4-fluorescein isothiocyanate [FITC] conjugate), CD8 (anti-mouse CD8-phycoerythrin-[PE]), or CD19 (anti-mouse CD19-FITC) (DAKO Corp, Carpenteria, CA). To assess viability of each population, 7-amino-actinomycin D (7-AAD, Pharmingen, San Diego, CA) was employed. Expression of these surface markers (and of 7-AAD within each population) was monitored by flow cytometry (FACScan, Becton Dickinson, San José, CA) every 24 hr during the exposures for up to 72 hr. In each analysis, 30,000 events were acquired (Pheng *et al.,* 2000, 2002). Results are expressed as the mean of all replicates from mice of the same strain. CellQuest analyses were used to differentiate among the three lymphocyte populations as well as within each population (i.e., single positives vs. double-positive for marker and 7AAD).

T- and B-lymphocyte spontaneous blastogenesis assay

To assess spontaneous blastogenesis, aliquots of harvested splenocytes (T- and B-lymphocytes) from each mouse were placed in 96-well plates (5×10^5 cells/well) and then exposed (in triplicate) to 400, 200, 100, 50, or 25 µg CsA/mL with no mitogen; control wells received vehicle only. Culture supernatants were collected after 72 hr of incubation, and the cells then pulsed with 1 µCi [^{3}H]-thymidine (Amersham-Pharmacia, Buckinghamshire, UK)/well in fresh (drug-free) medium. The cultures were harvested 16 hr later and incorporated radiation was quantified on an LS6500 scintillation counter (Beckman, Fullerton, CA). Inverse stimulation indexes (ISI) were calculated as the ratio of the mean (of triplicates wells) counts per minute (cpm) of cells not cultured with the drug to the mean cpm of cells that were incubated with the drug. ISI values higher than 2.0 were considered indicative of significant inhibition of spontaneous replication due to the CsA treatment.

Statistical analyses

The results were analysed using the template created with Cell-Quest software (Becton Dickinson). An analysis of variance (ANOVA) and a two-tailed Student's t-test were performed in order to determine inter-treatment differences (results of treated cells vs. those from CsA-

free controls). Multiple comparisons were also performed with the Tukey test. Results were considered statistically significant at a p-value < 0.05.

RESULTS

B-lymphocyte mortality after CsA treatment

In this assay, the activity previously reported for CsA on T-helper ($CD4^+$) and cytotoxic T lymphocytes ($CD8^+$), was experimentally reproduced (Table I). These populations showed mortality greater than 90% by 24 h. This effect was observed in both strains. However, the effect always tended to be more marked in the first 24 h among the ICR cells compared to cells from BALB/c mice. Longer incubations led to the complete loss of any significant inter-strain differences in this parameter.

In the current studies of the B-lymphocyte (i.e., $CD19^+$) sub-population, analyses of the background (i.e., control) survival rates of B-lymphocytes across the time-points assayed showed that there were no inter-strain differences after any given exposure period. However, while it did appear that there was a large (≈ 4-fold), increase in mortality among the medium-treated cells of the BALB/c hosts as a function of time, this change was found not to be statistically significant.

TABLE I - Effects of CsA treatment length on $CD4^+$ and $CD8^+$ lymphocyte viability

Treatment length	Host strain	Medium-only	CsA[a]
24 hr CD4	ICR	97.73 ± 3.51	*3.73 ± 0.59
	BALB/c	97.23 ± 2.62	*9.00 ± 1.11
48 hr CD4	ICR	96.66 ± 2.54	*0.33 ± 0.10
	BALB/c	94.83 ± 4.30	*0.73 ± 0.21
24 hr CD8	ICR	95.67 ± 2.69	*2.93 ± 0.53
	BALB /c	94.68 ± 3.90	*7.67 ± 2.53
48 hr CD8	ICR	95.00 ± 3.69	*0.00 ± 0.00
	BALB /c	94.03 ± 1.13	*1.00 ± 0.03

[a]CsA treatment level = 200 µg/ml. [b]Data corresponding to percentage of cells still alive (mean ± SD) at the indicated timepoint for each given treatment. Results were obtained using the CellQuest Program in the window restricted to indicated cell population. *Value significantly different from time-matched controls.

Viabilities among CsA-treated $CD19^+$ cells from the BALB/c hosts were decreased (compared to levels in control medium-treated autologous counterparts) as the length of incubation with the drug increased (Table II). Specifically, the percentages of dead BALB/c B-lymphocytes due to co-incubation with the CsA rose from 6.60% at 48 h to 18.84% at 72 hr. Considering that a large increase in mortality was likely due to the incubation process itself (see above), it remains to be determined how much of this nearly 3-fold increase in relative mortality is attributable to the CsA itself, and how much of this increase is merely a reflection of building upon the shift in background mortality among the cells, i.e., did the CsA just simply hasten the death of already dying cells or did the drug induce lethality in outright "healthy" cells?

TABLE II - Effects of CsA treatment length on B-lymphocyte viability.

Treatment length	Host strain	Medium-only	CsA[a]
24 hr	ICR	[b]9.61 ± 9.04	[b]12.93 ± 7.71
	Balb/c	1.97 ± 0.78	11.87 ± 11.26
48 hr	ICR	13.93 ± 16.94	5.11 ± 2.31
	Balb/c	1.80 ± 0.47	*6.60 ± 1.85
72 hr	ICR	11.32 ± 8.49	9.68 ± 1.05
	Balb/c	7.55 ± 0.20	*18.84 ± 9.54

[a]CsA treatment used = 200 µg/ml. [b]Data indicate percentages of non-viable cells after treatment for the indicated time period. Results were obtained using CellQuest Program (Flow cytometry) in the window restricted to $CD19^+$ population. *Value significantly different from that of corresponding control (medium-only) cells.

In contrast to the effects seen with the cells from the BALB/c hosts, the effect of CsA on ICR B-lymphocytes did not significantly differ from medium-treated counterparts at any timepoint. Furthermore, unlike with the BALB/c cells, the percentages of dead B-lymphocytes among the ICR splenocyte preparations barely increased from 5.11% to 9.68% at the corresponding 48→72 hr timepoints. Nevertheless, while there were clear strain-related differences in the degree of change in viability due to the CsA treatment (compared to that attributable to the incubation process itself), the results did not indicate that there were strain-related differences in susceptibility to CsA toxicity at any particular timepoint or over the entire incubation period tested.

As several of the inter-strain differences barely missed being considered significant (i.e., p-values < 0.056), these studies have to be repeated with even larger numbers of mice to verify the significance of the observed outcomes. Nonetheless, it is clear from these results that while the relative levels of dead ICR B-lymphocytes due to CsA

treatment remained relatively constant across the timeframe tested, those from BALB/c hosts displayed a trend toward time-related increase. As for now, this suggests a possible strain-dependent difference in B-lymphocyte susceptibility to the effects of CsA.

Spontaneous blastogenesis among CsA-treated splenocytes

Cells were subjected to 72-hr of cell culture with CsA at different concentrations with the aim assessed any drug-induced effect on spontaneous blastogenesis. Using ISI as a measure to reflect this effect, the more likely a cell was to spontaneously proliferate in the presence of the CsA, the lower the ISI value. Assuming, within each strain, a uniform level of spontaneous blastogenesis among the remaining viable non-T-lymphocyte cells (see Table I) from that strain, the numerator in the ISI calculation would remain relatively constant. Thus, any observed shift to lower ISI values at higher CsA concentrations would indicate that the equation denominator was increasing in a concentration-related manner, possibly meaning that the cells (i.e. surviving B-lymphocytes, monocytes, etc.) displayed an increase in spontaneous blastogenic activity.

As shown in Figure 1, the ISI values associated with cells from BALB/c mice remained fairly constant across the range of CsA concentrations tested. When the data were analyzed among all the different tested concentrations for possible differences due to host strain, only the effect from 25 µg CSA/mL (and 12.5 µg/mL; data not shown) was significantly pronounced ($p < 0.05$) among the cells from ICR mice. Reasons for the abrupt shift from CsA acting as inhibitor of spontaneous ICR splenocyte cell division (at the lowest sub-lethal doses tested) to one where spontaneous blastogenesis was stimulated (at higher and even lethal concentrations), remain to be established in our ongoing studies and could be associated with selective hormetic response for ICR cells [similar to that reported for some antitumoral drugs (Wakabayashi *et al.*, 2010)]. It is possible that, for low CsA´s concentrations, processes such as apoptosis have been induced, yet for high concentrations this effect has been inhibited, explaining the increased cellular mortality at lower CsA concentrations,

The effect from the low concentration(s) of CsA upon the BALB/c splenocytes (non-T-lymphocyte) spontaneous blastogenesis was less evident than on ICR counterparts, suggesting inter-strain differences in sensitivity to the drug (discussed below). Further studies are needed to determine more precisely what is occurring among the cells of each strain during incubation with CsA to give rise to this apparent difference in sensitivity.

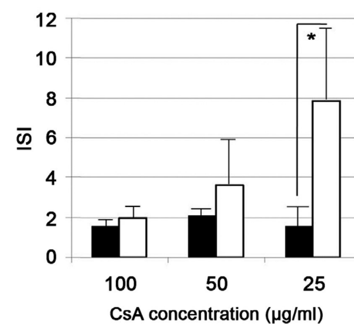

FIGURE 1 - Spontaneous splenocyte blastogenic activity in cultures bearing different CsA concentrations. Values shown are the mean ISI values (± SD; ISI = Inverse Stimulation Index) from ICR (n = 9; open bars) and BALB/c (n = 11; solid bars) mice/ concentration. *Significant differences between BALB/c and ICR strains for CsA effect on cells (at $p < 0.05$).

DISCUSSION

CsA, a drug effective in the prophylaxis and treatment of graft-vs.-host disease after allogeneic transplantation, exhibits a suppressor effect on lymphocyte (particularly B-lymphocyte) function. This could be one of the causes for the low circulating antibody levels that have been documented among CsA-treated patients in some clinical studies (reviewed by Sena *et al.,* 2006). Several researchers have attempted to ascertain the mechanisms by which CsA exerts this effect. Unexpectedly, early studies with lymphoblastoid (Epstein-Barr virus transformed B-lymphocytes) and WEHI-231 (mouse [BALB/c x NZB] F1, lymphoma-lymphoblastic) cell lines showed that CsA - rather than impacting negatively on these lymphocytes - appeared to protect them from Fas receptor-mediated (Beatty *et al.,* 1998) and/or ionomycin-induced cell death (Genestier *et al.,* 1994).

Unlike the results in these previous studies, we report a potential immunosuppressive effect of CsA on B-lymphocytes when present among the heterogeneous cell populations associated with the spleen. Specifically, B-lymphocyte mortality was elevated in splenocytes treated *ex vivo* with CsA. While these outcomes initially appeared to occur in a manner seemingly independent of the host strain from which the cells were derived, a general trend developed over the course of the exposure period wherein ICR mouse splenocytes displayed lower sensitivity to the underlying lethal effect of CsA than did their BALB/c counterparts.

These results suggest potential strain-prone B-lymphocyte toxicity due to the greater effect on the viability of this cell population in the spleens of BALB/c mice. It is possible that some aspect of the genetic background/

biology of the ICR mice might make their splenocytes (i.e., their associated immune cell populations) more resistant to any deleterious effects from the CsA. Besides the genetic, phenotypic and functional background of the mice strain used, which were demonstrated in the time in absence of treatment [BALB/c cells increased their spontaneous mortality by approximately 6 %, from 48 to 72 hr (from 1.80 +/-0.47 to 7.55 +/-0.20) which suggests a different kinetic of the apoptosis process], the effect of the CsA was demonstrated. In other words, the CsA treatment increased the effect on the cellular viability in the BALB/c cells, with regard to the ICR (evidenced from 24 hr of culture, still in absence of treatment). Such contrasting responses were not entirely unexpected. Several studies performed *in vivo* or *ex vivo* using isolated cells have indicated that the abilities to respond to biological response modifiers (such as select β-D-glucan priming agents; Ohno *et al.,* 2995), immunogens (such as select plasmid expression vectors and their corresponding coded proteins; Ito *et al.,* 2000), allergens, environmental contaminants (such as dust mite antigen ± diesel exhaust particles; Ichinose *et al.,* 2004), carcinogens (such as azoxymethane + dextran sodium sulfate; Suzuki *et al.,* 2006) and selected bacterial pathogens (such as *Listeria monocytogenes*; Park *et al.,* 2004) or parasites (such as *Plasmodium berghei*; Scheller *et al.,* 1994) differed among different mice strains, often to a very significant degree. Our own recent studies have also reported similar strain-associated differences in *ex vivo* splenocyte/lymphocyte responses to drugs such as pentamidine isethionate and vancomycin (Plaza *et al.,* 2007; Salguero *et al.,* 2009). Based on these documented differences, it was logical for us to use these two strains to determine the basis for the differential types/patterns of adverse effect responses being observed in patients receiving CsA for the treatment of autoimmune diseases/ immune regulation after transplantation.

Regarding the BALB/c viability data, it is not apparent why there should be such a clear difference in outcomes compared to those from the above-noted earlier studies with CsA. One possible explanation is that the approach employed here allowed the drug to simultaneously affect multiple cell populations, an approach more representative of an *in vivo* scenario. As a result, because the splenocytes can constantly interact with one another and with different cell (by-)products, apart from any direct effects of CsA on B-lymphocytes, there could also be secondary effects attributable to the presence of other cell types (i.e., the latter modulate the immunomodulatory potential of the drug against resident B-lymphocytes).

Differential CsA immunomodulatory activity against splenocytes *in situ* were also evidenced by differing responses (reflected by ISI values) of the splenocytes (in particular, associated B-lymphocytes) from the BALB/c and ICR mice. In this case, the effect was mainly observed in ICR mice splenocytes at the lowest (non-lethal) doses of concentrations tested (i.e., ≤ 25 µg/ml CsA; ISI >2, indicative of inhibition of normal "spontaneous" replication). Cells from BALB/c hosts appeared to display no changes in spontaneous blastogenesis regardless of the concentration of CsA applied over the 72 hr period. Studies to assess a concentration-dependency of similar effects in other cell types have demonstrated an increase in the percentage of G_0/G_1 cells among CsA-treated kidney cells (Healy *et al.,* 1998). These authors suggested that CsA was likely inducing some direct effect on cell division rates. Based on the results reported here, a similar effect is likely to occur among the treated splenocytes.

There are two plausible mechanisms to explain the decreases in normal blastogenesis seen here by the ICR cells (at the lower CsA concentration). First, these shifts can simply be explained by the effect of the drug on cell viability (i.e., reduction in the number of live cells available to divide). The interstrain B-lymphocyte viability differences after 72 hr coculture with CsA (≈80% BALB/c vs. ≈90% ICR) can partially explain the observed variability in the ISI values at this timepoint, bearing in mind that the higher the percentage of viable cells, the higher the replicative potential. This suggests that the ability of CsA to somehow affect the ability of ICR B-lymphocytes to undergo spontaneous division seems to be occurring, even among a smaller population of live cells. A second possible mechanism for the observed effect is related to the release of products essential for B-lymphocyte replication. Recalling that at the lower concentrations where inhibition of spontaneous replication (i.e., higher ISI values) was noted, the CsA had little effect on B-lymphocyte viability (data not shown). Unlike the higher concentration scenario, in which T-lymphocyte populations were near-totally eradicated, under the conditions associated with lower CsA concentrations, i.e., one in which the near-full spectrum of splenic immune cells were now likely available (if lethality against T-lymphocytes were concomitantly lower than at 200 µg/ml concentration), CsA might now be able to also affect B-lymphocyte blastogenesis in an indirect manner.

Specifically, it is known that CsA treatment can reduce IL-2 production by various immune cell types, including T-lymphocytes (Hess *et al.,* 1986; Ho *et al.,* 1996; Masri, 2003; Kang *et al.,* 2007). An obvious critical effect from this would be a reduction in normal B-lymphocyte activation as well as in its ability to pro-

liferate. In addition, it has also been shown that among T-lymphocytes (see review by Cohen *et al.,* 1984), effector and regulatory subtypes were affected differently by CsA. In particular, CsA treatment selectively reduced T-effector (specifically T-helper) lymphocyte proliferation/function while concurrently giving rise to an increase in the relative number of regulatory T-lymphocytes (Tregs) and local production of Treg-inducing cytokines. Yates *et al.* (2007), postulated that this could be the consequence of a selective effect of CsA on signaling (either at the level of receptor expression or post-receptor processing) by IL-2 or other factors (i.e., calcineurin, Jun N-terminal kinase/p38) required by Tregs to escape from the spontaneous apoptosis occurring during normal *balanced* immune responses (Naujokat *et al.,* 2003; Boldt *et al.,* 2006). That these cells are known to inhibit B-lymphocyte (LPS-induced) proliferation (reviewed in Curotto de Lafaille and Lafaille, 2002) allows us to suggest that, in response to the lower concentrations of CsA, there was likely a decrease in IL-2 being released by - and a concurrent increase in the relative number (and activities) of Tregs- among the test populations of splenocytes examined here, a functional event perhaps associated with the hormetic phenomena.

However, the fact that IL-2 is a survival factor needed to limit apoptosis among Tregs, and that the CsA is expected to be inducing a decrease in IL-2 levels, presents a challenge to our conclusion. Recent investigations have sought to better define other cytokines (apart from IL-2, including IL-4, -6, -7, and -15) that are protective against apoptosis (see Yates *et al.,* 2007). Future studies in our laboratory will be conducted to investigate whether CsA treatments cause any changes (i.e., decreases) in the production of these other Treg apoptosis-sparing cytokines by splenocytes from each mouse strain.

Because the results observed in the blastogenesis assays reflect the cumulative results of differential CsA activity upon multiple sub-populations in the spleens, the true effects of CsA upon each specific lymphocyte sub-population are likely to be masked. As for now, it remains unclear which (if any) particular lymphocyte type is primarily responsible for the results observed in the current study. Studies using B-lymphocyte mitogen lipopolysaccharide (LPS) are underway to obtain a better understanding of CsA effects on the ability of this cell type to contribute (or otherwise) to the ISI measurements. Furthermore, based on the observations that CsA affected B-lymphocyte viability within the splenic populations tested, we also believe it is important to more precisely determine how the drug affected viability (i.e., by increasing apoptosis or non-apoptotic mechanisms).

CONCLUSIONS

While the specific mechanisms underlying these outcomes are not yet clear, it is hoped the finding that cells within the ICR mouse splenocyte populations were more susceptible to low-concentration CsA than the BALB/c splenocytes, and that BALB/c cells appeared more susceptible to a lethal effect from the drug (first goal), will ultimately help to explain why not all patients treated with CsA uniformly display the same types/patterns of adverse effects. This study has provided preliminary evidence related with the *ex vivo* immunomodulatory activity of CsA on critical lymphocyte sub-populations that normally reside in the spleen. Moreover, the study has documented that CsA exert a cytotoxic effect on splenic $CD19^+$ (B-lymphocyte) sub-populations, a result previously unreported (B-lymphocytes from BALB/c are more susceptible to CsA effect than the ICR CD19+ splenocytes, supporting the solving of the second goal). Future studies should assess the effect of CsA on cellular mitosis, including that after stimulation of T- and B-lymphocyte mitogens.

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