Immunosuppressive activity of cyclosporine A formulations in mononuclear cells

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Abstract

Objective
To compare the immunosuppressive activity of two commercial formulations of cyclosporine A (Cyclosporin Microral®, Sigma Pharma, Brazil, and Sandimmun Neoral®, Novartis, Switzerland), in mononuclear cells from healthy volunteers as well as from chronic renal patients.

Methods
Both formulations, in their commercial packages, were requested from the hospital pharmacy, transferred to identical vials and labeled by an independent researcher as cyclosporine 1 and 2. Concentration curves were established for the two preparations and their inhibitory capacity on PHA-induced mononuclear cell proliferation was measured in cultured peripheral blood lymphocytes and whole blood obtained from 12 healthy volunteers as well as from 11 hemodialysis patients.

Results
The study showed that both commercial formulations are immunosuppressive with similar but statistically different inhibition curves for PHA-induced mononuclear cell proliferation, in peripheral blood lymphocytes and in whole blood from healthy volunteers, and statistically not different in whole blood from hemodialysis patients at the concentrations tested.

Conclusion
The two commercial cyclosporine formulations studied showed equivalent immunosuppressive activity.

Introduction
The use of generic drugs can markedly reduce healthcare costs during the post-transplantation period. Efforts to develop such drugs have been internationally encouraged by Health Regulatory Agencies. The methodology and requirements for approving generic drugs are thorough, involving controlled bio-equiva-
lence studies, whose ultimate aim is to determine if the generic drug is absorbed keeping similar blood levels compared to the reference standard. Biological activity tests are, however, not required. In practice, it is assumed that the new formulations, once absorbed and having reached adequate blood concentrations, will present the same biological activity originally described as therapeutic. In the particular case of cyclosporine, its biological activity as an immunosuppressive agent is vital for the success of transplantations. The objective of this study was to assess and compare the immunosuppressive biological activity of two cyclosporine microemulsion preparations existing in the Brazilian market (Signasporin Microral® and Sandimmun Neoral®), in order to offer a scientific contribution to the decision-making process of the transplant community.

**Methods**

**Drugs and reagents**

Signasporin Microral® (SigmaPharma / Nature’s Plus, Brazil) and Sandimmun Neoral® (Novartis, Switzerland) were obtained from the pharmacy of “Hospital Universitário Antonio Pedro”, Niterói, RJ, Brazil, as oral solutions at 100 mg/ml. The two preparations were diluted in RPMI-1640 culture medium (Sigma, St. Louis, USA), and placed in identical, amber-colored sterile vials, light-protected, and randomly identified as Cyclosporine 1 and Cyclosporine 2 by an independent researcher who only provided the identification code after the results had been obtained. The following reagents were also used in the cell cultures: RPMI-1640 (Sigma, St. Louis, USA), fetal bovine serum (Fazenda Pigue, Brazil), PHA (Sigma, St. Louis, USA).

**Cell preparations**

Heparinized venous blood from healthy volunteers as well as from hemodialysis patients was collected and processed to obtain two different cell preparations: for the whole blood preparation, it was diluted in RPMI-1640 (1:5); for the preparation of peripheral blood mononuclear cells, it was fractioned by centrifugation in Ficoll-Hypaque density gradient (Sigma, St. Louis, USA); the mononuclear fraction obtained was washed twice and suspended in RPMI with 5% fetal bovine serum being cell number adjusted to 1x10^6 cells/ml.

**Proliferation assay**

The mononuclear cell proliferation measurements were made determining the incorporation of H-thymidine by the cell DNA. The two preparations were transferred onto microculture dishes with 96 wells, to which 5 µg/ml phytohemagglutinin (PHA) was added, in the presence or absence of a cyclosporine 1 and 2 concentration curve (20 ng/ml to 200 µg/ml), freshly diluted, and incubulated at 37°C, in a sterile atmosphere, with 5% CO₂, for 72 hours (peripheral lymphocytes) or 120 hours (whole blood). Six hours prior to the end of the incubation period, the cell cultures were pulsed with 0.5 µCi H-thymidine, and, at the end of this period, the cells were harvested, using a liquid scintillation counter to determine its radioactivity.

The sample processing procedure is showed in Figure 1.

**Figure 1 - Phytohemagglutinin (PHA)-induced mononuclear cell proliferation assay in whole blood (left) and in mononuclear cells from peripheral blood (right).**

**Statistical analysis**

Results are presented as means only (figures) or means and standard deviations (text). The results of the groups belonging to each one of the three experimental situations (volunteers isolated cells, volunteers whole blood and hemodialysis patients whole blood) were statistically treated in an independent manner and evaluated by one-way ANOVA, using the
EPISTAT program. A significance level of p<0.05 was established.

**Results**

The characteristics of study groups (healthy volunteers and hemodialysis patients) are summarized in Table 1.

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*Mean ± Standard deviation

**Healthy volunteers**

The results obtained from the two cyclosporine preparations, comparing their inhibition capacity on the PHA-induced proliferation of mononuclear cells are shown in Figure 2. The observed values reflect the proliferation inhibition mean of all the tested volunteers (n=12). In this preparation, obtained from peripheral mononuclear cells, the curves for both cyclosporine formulations are similar. A small, yet statistically significant difference was observed only at the 20ng/ml concentration, where the proliferation inhibition by CsA1 (23.6±2.4%) was superior to the one by CsA2 (20.2±1.1%), p<0.01.

The inhibitory effect obtained in whole blood cultures (Figure 3) of the 2 preparations was statistically different at the 200 ng/ml and 2 ug/ml concentrations, this time in favor of CsA2 (50.5±2.2% vs. 56.5±3.6%, and 70.3±1.9% vs. 74.4±2.9%, for the 200 ng/ml and 2 ug/ml concentrations, respectively; p<0.001 in both situations).

**Hemodialysis patients**

This group consisted of 13 adult patients with chronic renal failure under hemodialysis treatment, whose blood was collected immediately before the dialysis procedure. Only 11 patients were deemed suitable for analysis as the samples from two patients showed no proliferation response. The results, limited to the whole blood proliferation assay, are presented in Figure 4. In this group, the results obtained with the two cyclosporine preparations showed no statistically significant differences.

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Disclosure of blindness

The disclosure of the identification code of the cyclosporine preparations studied by an independent researcher, revealed that the preparation identified as CsA 1 corresponded to Sandimmun Neoral®, while the one identified as CsA 2 corresponded to Sigmasporn Microral®.

Discussion

The objective of this study was to investigate the inhibition capacity of the formulations on the phytohemagglutinin (PHA)-induced proliferation of mononuclear cells. This classical mononuclear cell proliferation assay, that served as a model for Borel’s original description of cyclosporine, is widely used for the screening of drugs with immunosuppressive potential.³ Phytohemagglutinin is a mitogenic agent for human lymphocytes, which acts basically on T-lymphocytes.⁵ ⁶ Two kinds of cell preparations were used: a preparation of peripheral mononuclear cells and a whole blood preparation. The whole blood preparation, validated by the international literature and adapted in our laboratory,⁷ ⁸ represents an interesting biological model, the presence of the other blood cells during the culture process reflects the situation found in vivo. The two cell preparations were then cultivated, with or without PHA stimulation, and in presence or absence of two cyclosporine preparations at different, increasing doses. During the assay the researcher was not allowed access to the identity of the drugs tested.

The statistical difference observed between the two commercial preparations in the assays using purified mononuclear cell preparations from healthy volunteers was limited to a single cyclosporine concentration (20 ng/ml). The analysis of the results obtained by the whole blood technique, a simple and low cost procedure also revealed a progressive proliferation inhibition by increasing cyclosporine concentrations, showing differences between the two commercial formulations (at the 200 ng/ml and 2 ng/ml concentrations). Based on these results, we chose to perform only whole blood proliferation assays from the hemodialysis patient’s samples. This strategy had the additional advantage of allowing evaluation of cyclosporine action in the presence of plasmatic factors which are potentially capable of interfering with the immune response.⁹ ¹⁰ No difference was observed between the inhibitory capability of the two commercial formulations at the different concentrations tested. In the hemodialysis patients, however, there seemed to be a greater sensitivity of the mononuclear cells to the antiproliferative action of cyclosporine, reflected by a much more pronounced inclination of the inhibition curve, as compared to the one obtained from the healthy volunteers. This greater sensitivity may result from the inhibitory action of uremic toxins, present in the whole blood assay. This possibility is currently being investigated in our laboratory.

Conclusion

Both preparations showed equivalent immunosuppressive activity with an inhibitory effect on the proliferation of mononuclear cells, both in peripheral blood and whole blood, at concentrations which are concordant with the literature.¹¹ ¹³ A slight difference was observed between the two commercial formulations under study, showing a tendency towards a better performance of the preparation identified as cyclosporine 2 in the whole blood preparation of healthy volunteers.¹⁴

References


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