Surg. Res. Comm., 1989, Vol. 6, pp. 43-49
Reprints available directly from the publisher
Photocopying permitted by license only

© 1989 Harwood Academic Publishers GmbH
Printed in the United Kingdom

CYCLOSPORIN A MODIFIES LIVER REGENERATION FOLLOWING PARTIAL HEPATECTOMY

I. GARCÍA-ALONSO, J. MÉNDEZ* and E. BARBERÁ

Laboratory of Experimental Surgery, Department of Surgery, Basque Country University School of Medicine, Leioa (Vizcaya), Spain

(Accepted for Publication 21 November, 1988)

In order to study the possible changes induced by cyclosporine A (CsA) on hepatic function, we have assessed its effect on the synthesis of DNA in hepatocytes after partial hepatectomy. CsA was administered intraperitoneally to four groups of rats in which the intranuclear DNA of the hepatocytes was quantified by a microspectrophotometric method on slices stained by the Feulgen test. The administration of CsA intraperitoneally provoked an increased in the mean Feulgen-DNA content of the hepatocytes in the normal animals as well as in the ones subjected to a 40% partial hepatectomy, which means an increase in the level of hepatocytic polyploidy. Therefore, it may be concluded that CsA induces the synthesis of DNA in normal hepatocytes and also enhances the synthesis of DNA induced in hepatocytes after 40% hepatectomy.

KEY WORDS: Cyclosporine, Hepatectomy, Liver Regeneration, DNA.

INTRODUCTION

Cyclosporine-A is a fungal metabolite of polypeptidic nature which exerts a selective immunosuppressive activity on lymphocytes. It was introduced in liver transplantation by Calne *et al.*[1] In 1980, Starzl *et al*[2] started using it with prednisone and obtained a remarkable improvement in rejection prophylaxis and therapeutics[3].

As the prospects of survival have improved, the orthotopic hepatic transplantation (OHT) technique has been extended to pediatric patients, and new surgical techniques adapted to their peculiar circumstances are being tested. So, orthotopic transplants are being performed with adult livers reduced to one or part of one of their lobes,[4-6] and relying on the recipient's regulating systems for the postadaptation of the liver size to its own necessities via hepatic regeneration. It is important in these cases that the drugs administered during the postoperative period do not affect that regenerative activity.

Calne *et al.*[7] first reported some cases of CsA hepatotoxicity in patients subjected to renal transplantation. A few years later the potential hepatotoxicity of the drug was made evident by experimental studies in rats.[8-9] More recently other authors have reported biochemical alterations (increase of bilirubin and alkaline phosphatase) in patients subjected to different transplants and treated with CsA.[10-12]

Considering the importance acquired by CsA and the hepatic regeneration in orthotopic liver transplantation in children, we decided to investigate the possible effects

Address correspondence to: Dr. I. García-Alonso, Laboratory of Experimental Surgery, Department of Surgery, Basque Country University School of Medicine, 48940-Leioa (Vizcaya), Spain.

of this drug on the regenerative phenomenon. For this purpose, we have assessed its activity on the intranuclear-DNA synthesis in the hepatocytes in rest and after stimulation by hepatectomy.

MATERIALS AND METHODS

Female *Sprague-Dawley rats* weighing between 200 and 220 g were used. Four groups of ten animals were considered: control, hepatectomy, CsA and Hepatectomy + CsA.

Surgery was performed under Nembutal (Sodium pentobarbital) anaesthesia (30 mg/kg i.p.) between 4:00 p.m. and 6:00 p.m. to standardize for natural diurnal rhythms. Through a midline incision reaching 2 cm posteriorly from the xiphoid process of the sternum the left lateral lobe was easily delivered, securely ligated and then excised. In this way portions of the hepatic parenchyma ranging in extent from 35 to 40 percent of the total liver were removed.

CsA was dissolved in 95% ethanol and diluted with *Intralipid* 10% (kavi-Vitrum) to 20 mg/ml and was administered intraperitoneally at a dose of 20 mg/kg for 3 days, starting the day before operation in partially hepatectomized animals.

Animals were sacrificed 40 h. after hepatectomy or at the third day of CsA-therapy. Under Nembutal anaesthesia (30 mg/kg i.p.) liver was removed and two pieces of 0.5 cm each side were obtained from the median lobe. Samples were fixed in 10% formol and then embedded in paraffin.

DNA quantification was carried out by means of microspectrocytophotometric techniques on 5 μm histological sections stained by the Feulgen test. This reaction has the characteristic of being absolutely specific for DNA, and conforms to a stoichiometric-type reaction (a direct ratio between the number of molecules of colorant in the tissue and the number of DNA molecules).

Five slices from each animal were assessed. In each one of them 100 hepatocyte nuclei and 25 lymphocyte or Kupffer cell nuclei were quantified (the later for internal control). With this, the mean DNA content of the hepatocyte nuclei in each animal under study was calculated.

RESULTS

The mean-Feulgen-DNA content of the nuclei from each experimental series was calculated and the results were as follows: 12.9 units in the static controls; 18.5 in the regenerative ones; in the animals treated with CsA the mean was 15.5 in the static livers and 23.6 in the regenerative ones (Table 1).

The statistical analysis of these results showed that a statistically significant difference exists between the DNA values obtained on the static series and also between the animals subjected to treatment with CsA and their respective control animals ($p < 0.01$).

Administration of CsA to the control animals produced an increase in the intranuclear-DNA content of the hepatocytes. Its administration to the animals subjected to hepatectomy provoked a greater DNA synthesis, which was revealed by two facts: 1) an increase in the mean nuclear-DNA content of the hepatocytes, 2) the appearance of a larger number of hepatocytes with high polyploidy level (Figure 1).

In order to show these differences more clearly, we carried out a study on the dispersion of the obtained Feulgen-DNA values. So, we calculated the arithmetical mean of the end values of each experimental series; that is, the mean of the 1,000 highest and lowest values. The quotient of both mean-end values was taken as index

TABLE I
Feulgen-DNA values of hepatocytes

	normal		regenerating	
	Control	CsA i.p.	control	CsA i.p.
M	12.90	15.54	18.49	23.67
SD	0.94	0.97	1.29	1.37
X	14.21	17.32	19.73	25.55
x	11.98	13.39	16.67	20.87
X-x	2.23	3.93	3.06	4.68

M: mean; SD: standard deviation; X: maximum value x: minimum value; X-x: difference between the maximum and the minimum value of each series.

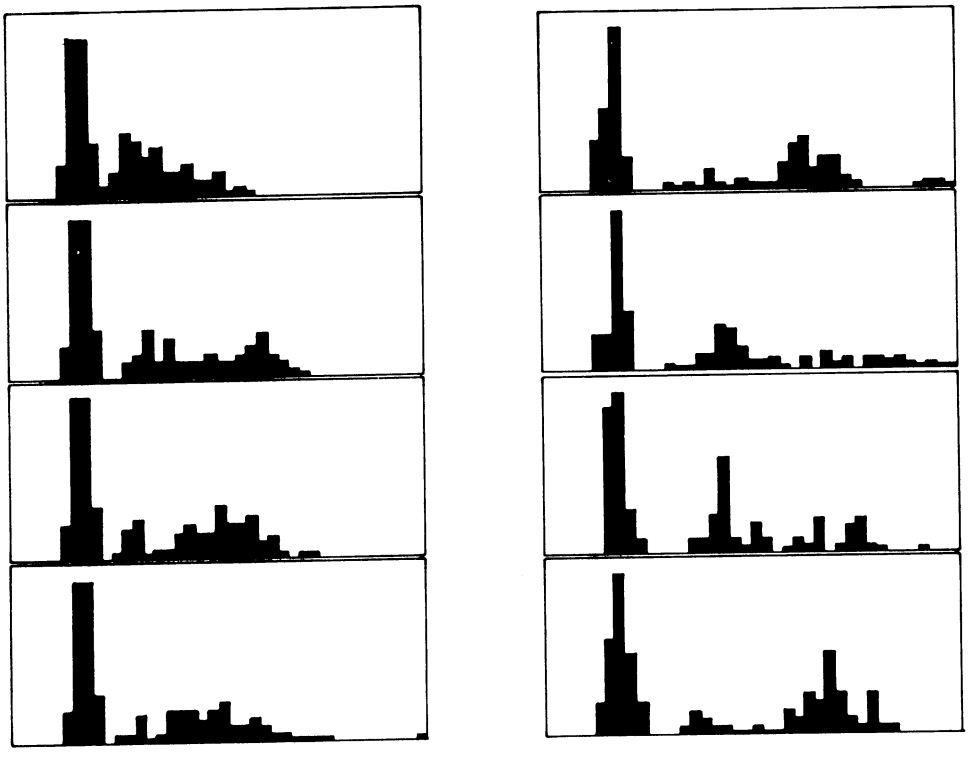

FIGURE 1 Frequency histogram in which the number of cells is shown in the ordinate axis and the DNA content of the abscissae. The figures on the left correspond to hepatocytes from hepatectomized rats. The ones on the right represent hepatocytes from animals treated with CsA and hepatectomy: it may be observed that these histograms are more extended than the others, and indicates a greater DNA-synthesis (more replicating hepatocytes and higher ploidy levels).

TABLE II
Study of the degree of dispersion of the Feulgen-DNA values

	resting liver			regenerating liver		
	M	m	M/m	M	m	M/m
Control	16.27	10.93	1.501	25.85	12.75	2.036
CsA i.p.	19.21	12.25	1.575	33.61	16.46	2.043

M: Mean of the highest values of the series; m: mean of the lowest ones.

of the degree of dispersion of the values on each experimental series (Table 2).

Comparing the values obtained on each series we observed that: 1) The degree of dispersion was not modified by the treatment with CsA but it was modifed by hepatectomy; 2) The mean of the upper end-values was higher ($p < 0.05$) on the animals treated with CsA (with and without hepatectomy); 3) The mean of the lower end-values was not modified significantly by the CsA on the control animals but it was increased on the ones subjected to hepatectomy ($p < 0.05$).

DISCUSSION

DNA Quantification

The sign of regeneration on the hepatic parenchyma is DNA synthesis in the hepatocytes.[13] The existence of replication of hepatocytes can be assessed by two different methods:

Firstly, by using the autoradiographic technique, with which, after inoculating DNA precursors labelled with radioisotopes, the synthesizing cells – as they incorporate a higher index of radioactive product – are detected.

Replication can also be assessed by individual estimation (cell by cell) of the nuclear-DNA content. By this procedure the synthesizing cells are identified for having a DNA content intermediate between two ploidy levels. This procedure is often used when the cells are free,[14] but can also be done by high-precision cytophotometry when the cells are in constituent tissues.[15]

We have used the cytophotometric method to estimate the quantity of DNA in the nuclei, as they contain quantities of DNA diploid or multiples of the diploidy in the hepatocytes of non-regenerating livers, such as 4C, 8C and 16C.[16,17] Estimations of the DNA content in such populations show a very low error, and so the mean of the values obtained by measurements on a wide sample of cells presents a very small deviation. As can be seen in the frequency histogram in which the number of cells is shown in the ordinate axis and the DNA content on the abscissae, the cells with a regular content appear clustered on well-defined peaks.

When a population of cells is in phase of synthesis, they show quantities of DNA intermediate between two ploidy levels.[14,15] This originates a dispersion of the values along the histogram.

This procedure gives higher precision and regularity than the autoradiographic method, as the cells measured are clearly distinguishable histologically and so it is not possible to include in the sample cells other than hepatocytes. Moreover, the sizes of the sample can be enlarged as much as desired. This possibility of working with large samples was specially important for us, because our final aim was to contrast samples subjected to different treatments.

RESULTS IN DNA-QUANTIFICATION

There have been very few studies which relate the immune system and liver regeneration. To show that relation, the influence of different treatments on the regenerative rate has been estimated.[18,19] Therefore, we have also tried to correlate CsA-treatment and the rate of the hepatic DNA-synthesis. According to our results, we have been able to verify that the CsA administered intraperitoneally produces, even without hepatectomy, a global increase in the DNA content of the hepatocytes, which, as previously demonstrated, indicates that an important number of hepatocytes are synthesizing DNA.

The increment, however, is less than the one produced by a single hepatectomy. In other words, the administration of CsA and the hepatectomy produce a similar effect on the synthesis of DNA, but the former to a lesser extent.

The hepatectomized and CsA-intraperitoneally treated animals showed a regenerative response higher than after hepatectomy alone. This seems to indicate that the hepatectomy and the CsA administered intraperitoneally act synergically and provoke a regenerative induction more intense than the one expected after a single hepatectomy.

Considering that CsA had been defined as a non-cycloactive product,[20,21] it should have not been expected that CsA would interfere with liver regeneration.

Moreover, several suggestions found in the literature about the positive role played by the thymocytes in liver regeneration,[18,22–24] and the interference excerted by CsA over the metabolism of these cells[25] were in favour of a negative influence of this product on the regenerative process.

Babaera *et al.*[19] suggested that T lymphocytes might play an important role in the regenerative hepatic process induced by partial hepatectomy. Also in this direction were the experiences of Yokomura *et al.*[26] and Miyahara *et al.*[27] which showed regenerating hepatocytes activate syngeneic lymphocytes (in vitro). As T lymphocytes are selectively suppressed by CsA it could be suspected that this drug would interfere with liver regeneration.

In fact, in our experience CsA has started DNA-synthesis in normal hepatocytes and has enhanced liver regeneration induced by 40% hepatectomy. Though we have not found any references about the former result, the later agrees with the finding recently published by Kym *et al*[28]: after 70% hepatectomy and CsA treatment (4 oral doses of 10 mg/kg/day). They estimated the moist weight of the remaining liver and the mitotic index of hepatocytes and found that CsA enhanced the mitotic levels as 24 hours after surgery, although it was not statistically significant ($0.2 < p < 0.3$).

Perhaps Kym's results are not statistically significant because mitotic index estimation is not such an accurate method as DNA cytophotometry. Another possible explanation could be the greater extent of their hepatectomy; when the stimulus for regeneration is too large the effect of CsA may be concealed.

HYPOTHESIS SUGGESTED

It is possible to establish two hypothesis about the relationship between CsA and hepatic proliferation.

The first one involves the immune system and presumes an indirect effect of CsA on hepatocytes. Thus, the immunosuppresive activity of CsA, perhaps through a

suppresive effect on the monocytemacrophage system,[29] would permit an accentuation of the regenerative response after hepatectomy. Previous studies, although not in full agreement, relate hepatic regeneration to the immune system and support this hypothesis.

Secondly, it could be argued that CsA has a direct effect over hepatocytes, which has not been described so far. Only experience on isolated hepatocytes cultured "in vitro" with CsA could give us some clues about this hypothesis. Confusing results, however, might also be expected,[30] as the hepatocytes isolated and cultured "in vitro" substantially alter their functional characteristics.

References

1. Calne, R.Y., Rolles, K., White, D.J.G., Thiru, S., Evans, D.B., McMaster, P., Dunn, D.C., Craddock, G.N., Henderson, R.G., Azir, S. and Lewis, P. (1979) Cyclosporin A as the only immunosuppressant in 34 recipients of cadaveric organs: 32 kidneys, 2 pancreases, and 2 livers. *The Lancet*, **2**, 1033–1036.
2. Starzl, T.E., Klintmalm, G.B.G., Porter, K.E., Iwatsuki, S., and Schroter, P.J. (1981). Liver transplantation with use of cyclosporin-A and prednisone. *N. Eng. J. Med.*, **305** (5), 266–269.
3. Starzl, T.E., Iwatsuki, S., Van Thiel, D.H., Gartner, J.C., Zitelli, B.J., Malatack, J.J., Schnade, R.R., Hakala, T.R. and Rosenthal, J.T. (1983) Report of Colorado-Pittsburgh liver transplantation studies. *Trans. Proc.* **15** (4) 2582–2585.
4. Bismuth, H. and Houssin, D. (1984) Reduced-sized orthotopic liver graft in hepatic transplantation in children. *Surgery*, **95** (3), 367–372.
5. Rossi, G., Carlis, L. de, Doglia, M., Fassati, L.R., Tarenzi, L. and Galmarini, D. (1987) Orthotopic liver transplantation of partially hepatectomized liver in the pig. *Transplantation*, **43** (3), 362–365.
6. Broelsch, C.E., Edmond, J.C., Thistlewaite, J.C., Rouch, D.A., Whitington, P.F. and Lichtor, J.L. (1988) Liver transplantation with reduced-size donor organs. *Transplantation*, **45** (3), 519–523.
7. Calne, R.Y., White, D.J.G., Thiru, S., Evans, D.B., McMaster, P., Dunn, D.C., Craddock, G.N., Pentlow, B.D. and Rolles, K. (1978) Cyclosporin-A in patients receiving renal allografts from cadaver donors. *The Lancet*, **23**, 1323–1326.
8. Thompson, A.W., Whiting, P.H., Cameron, I.D., Lessels, S.E. and Simpsom, J.G. (1981) A toxicological study in rats receiving immunotherapeutic doses of cyclosporin A. *Transplantation*, **31**, 121–129.
9. Siegel, H. and Ryffel, B. (1982) Effects of cyclosporin on reninangiotensin-aldosterone system. *The Lancet*, **2**, 1274.
10. Ota, B. and Bradley, M. (1983) Side effects of cyclosporin in 100 renal allograft recipients. *Transp. Proc*, **15** (4), 3150–3155.
11. Magreiter, R., Kramar, R., Huber, C., (1984) Transplante renal y hepático combinado. *The Lancet* (Ed. esp.) **5** (3), 221–222.
12. Nogueira, H.J. and Cutler, R.E. (1985) Cyclosporine: part II, nephrotoxicity and other adverse effects. *Dial. Trans. Today*, **3**, 585–586.
13. Higgins, G.M. and Anderson, R.M. (1931) Restoration of the liver of the white rat following partial surgical removal. *Arch. Pathol*, **12**, 186–202.
14. Fukuda, M., Böhm, N. and Fujita, S. (1978) Cytophotometry and its biological application. New York, Ed. Gustav Fischer Verlag.
15. Barberá-Guillem, E. (1972) Anális citofotométrico de los megacariocitos humanos. Tesis Doctoral. Valencia.
16. Ris, H. and Mirsky, A.E. (1949) Quantitative cytochemical determination of desoxyribonucleic acid with the Feulgen reaction. *J. Genet. Physiol.* **33**, 125–146.
17. Swift, H. (1953) Quantitative aspects of nuclear nucleoproteins. International *Rev Cytol*, **2**, 1–76.
18. Kolpashchikova, I.F. (1979) Effect of transplantation of thymus, bone marrow, and spleen cells on the regenerative processes in pathologically changed liver. *Bull. Exp. Biol. Med.* **88**, 477–480.
19. Bavaeba, A.G., Kraskina, A.N. and Iudina, N.V. (1980) Stimulation of the mitotic activity of the hepatocytes and kupffer cells in the liver of nonoperated mice by T and B lymphocytes from partially hepatectomized . . . donor. *Bull. Exp. Biol. Med* **89** (1), 69–70.
20. Dos Reis, G.A. and Shevach, E.M. (1982) Effect of cyclosporin-A on T cell function in vitro: the mechanism of suppression of T cell proliferation. *J. Immunol.*, **129** (6) 2360–2367.
21. Borel, J.F. and Lafferty, K.J. (1983) Cyclosporin: speculation about its mechanism of action. *Trans. Proc.* , **15** (3), 1881–1885.

22. Desser-Wiest, L. and Desser, H. (1980). Inhibition and stimulation of rat thermocytes in vitro by partial hepatectomy. *Exp. Path.*, **18** (2), 114–117.
23. Gieldanowski, J. (1983). Immunomodulatory drugs as the stimulators of proliferative process. *Arch. Immunol. Ther. Exp.*, **31** (3) 345–348.
24. Schaff, Z., Lapis, K. and Szendroi, M. (1984) D-galactosamine-induced liver injury in immunosuppressed mice. *Acta Morphol. Acad. Sci. Hung.*, **32** (1), 67–72.
25. Reem, G.H., Cock, L.A. and Palladino, M.A (1983) Cyclosporin inhibits interleukin-2 and interferon-gamma synthesis by human thymocytes. *Trans. Proc.*, **15** (4), 2387–2389.
26. Yokomuro, K., Miyahara, S., Takahashi, H. and Kimura, Y., (1984) Regeneration and the immune system. II. Suppressor activities of lymphocytes activated in vivo by liver regeneration and their genetic control. *Eur. J. Immunol.*, **13** (11), 883–889.
27. Miyahara, S., Yokomuro, K., Takahashi, H. and Kimura, Y. (1984) Regeneration and the immune system. I. In vitro and in vivo activation of lymphocytes by liver regeneration and the role of kuppfer cells in stimulation. *Eur. J. Immunol.*, **13** (11), 878–883.
28. Kim, Y.I., Salvini, P., Auxilia, F. and Calne, R.Y. (1988) Effect of cyclosporin A on hepatocyte proliferation after partial hepatectomy in rats: comparison with standard immunosuppressive agents. *Am. J. Surg.*, **155**, 245–249.
29. Wright, B., Greig, R. and Poste, G. (1984) Inhibition of macrophage activation and tumor cell cytolysis by cyclosporin A. *Biochem. Biophys. Res. Commun.* **123** (2), 710–715.
30. Francavilla, A., Ove, P., Polimeno, L., Sciascia, C., Coetzee, M., Pellici, R., Todo, S., Kam, I. and Starzl, T. (1987) Different response to epidermal growth factor of hepatocytes in cultures isolated from male or female rat liver. *Gastroenterol.*, **93**, 597–605.