

Possible role of brown adipose tissue as a mediator during cyclosporine-A treatment

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Summary. Cyclosporine-A (CsA) is a potent immunosuppressor used successfully to control rejection in organ transplantation. According to the most recent evidence, this drug modifies the lipid metabolism of the patient, provoking a rise in the blood lipids, constituting an important risk factor for acceleration of the atherogenic process. Taking into account that brown adipose tissue (BAT) constitutes the major storage site for cholesterol and triglycerides in the rat, and given the apparent lack of references about the implications of CsA on this tissue in the literature, we proposed to study the possible morphological changes occurring in BAT following the administration of this drug.

Two groups of female Sprague-Dawley rats were set up, the control group and a treated group in which each animal received subcutaneous injection of 5 mg/kg body weight/day of CsA. After 4, 11, 25 and 34 days of treatment, subgroups of animals were sacrificed and the brown adipose tissue removed was apportioned for subsequent microscopic assessment.

The greatest degree of atypia and activity in the BAT was observed after administration of 11 doses of the drug, at which point there was a marked reduction in the cell size with loss of lipidic coalescence. With subsequent doses, the tissue slowly initiated a process of recovery.

CsA also induced morphological changes in the BAT that, in the early stages of the study, appeared to be correlated with a lipolytic response of the tissue to the drug; thus, the BAT may be acting as a system to eliminate the excess of lipids in the blood provoked by CsA administration, while toward the end of treatment, there was a certain stability between the drug and the activity of the brown adipose tissue, and a tendency to reach a balance between lipolysis and lipogenesis.

Key words: Brown adipose tissue, BAT, Cyclosporine, Lipolysis, Hyperlipemia

Introduction

Since its discovery by Borel in 1970 (Borel et al., 1976), cyclosporine-A (CsA) is being used successfully as an immunosuppressor drug to control rejection both in pediatric and adult organ transplant recipients (Calne et al., 1978). Many small children require transplantation to correct functional disorders (mainly renal and hepatic), and as a result of surgical advances and the application of immunosuppressor drugs such as CsA, the survival of the patient is assured in 70%-90% of cases (Ptachcinski et al., 1986; Shaw et al., 1988a,b). However, the most common drawback in these cases is that very high doses of the drug are needed to maintain therapeutic blood levels (Shaw et al., 1988; Whittington et al., 1990). The primary effect of CsA is to inhibit the development of the second signal essential for T and B cell maturation (Kahan, 1989a). Its increasingly widespread therapeutic use has led to the onset of a series of collateral effects, some with beneficial results, such as in the treatment of certain dermatoses (Thivolet and Kanitakis, 1991) and, especially, in the prevention of post-transplantation graft rejection; others, in contrast, are derived from the inherent toxicity of the drug (Whiting et al., 1985), the major impact of which is observed in kidneys and liver (Dossantos et al., 1991; Ballardie et al., 1992; Wolf and Neilson, 1992). Very recent studies point to CsA as the possible cause of the hyperlipemia found in some transplant recipients (Caltran et al., 1979; Lowry et al., 1987; Stamler et al., 1988). The drug is extremely lipophilic and, consequently, is encountered predominantly in the plasma lipoprotein fractions. The low-density lipoproteins (LDL) serve as vehicles for its distribution and provide high local (cellular) concentrations of the drug.

López-Miranda et al. (1989) observed atherogenic changes in the lipid profile of rats treated with CsA, a drug which raises the plasma total cholesterol level as a result of an increase in the cholesterol transported by LDL and very low-density lipoproteins (VLDL). It also increases the amount of plasma triglycerides transported by the VLDL, suggesting that the drug induces an

atherogenic change in the plasma lipoproteins which could contribute to the pathogenesis of premature atherosclerosis in transplant recipients. Similar effects had been found earlier by other authors (Hess et al., 1987; Jevnikar et al., 1988).

Ferns et al. (1990) observed that CsA has a cytopathic effect on the cells of the macrovascular tissues, among which BAT is included. Other authors have demonstrated that the accumulation of CsA in white adipose tissue between 4 and 24 hours after administration surpasses the serum concentrations reached by the drug (Niederberger et al., 1983).

BAT is widely distributed in mammals, constituting up to 5% of body weight in some cases. It is located in well defined regions which differ little from one group of mammals to another. It is very abundant in the human embryo (Merklin, 1974; Moragas and Toran, 1983), and remains so for some time after birth. In the rat, the greatest deposit is the interscapular BAT, which we have chosen for our study since it is a uniform and well-delimited structure made up of polygonal cells, referred to as *brown adipocytes*. These cells are characterized by their round nucleus, with one or two patent nucleoli, and granular cytoplasm owing to the presence of numerous mitochondria, lipid droplets and other cell organelles. These cells are immersed in a connective matrix, accompanied by fibroblasts and mast cells.

The brown adipose tissue has two main functions: to store triglycerides and to mobilize them to provide heat. The function of the BAT is one of the alternatives to diet-supplied energy and is taken into account in the dietary lipid balance (Herrera, 1977; Savard et al., 1984; Rothwell and Stock, 1985; Williamson, 1986; Holm et al., 1987). Therefore, since CsA appears to provoke changes in lipid metabolism (Caltran et al., 1979; Hess et al., 1987; Lowry et al., 1987; Jevnikar et al., 1988; Stamler et al., 1988; López-Miranda et al., 1989), and in view of the involvement of BAT in this type of interaction (Herrera, 1977; Moragas and Toran, 1983; Savard et al., 1984; Williamson, 1986) and the lack of references to the topic in the literature, we selected this drug to study the effects that its administration, at different doses, would have on the morphology and function of the BAT in the rat, for which purpose we have followed the evolution of this tissue during prolonged CsA treatment.

Materials and methods

Study animals

We used 24 3-month-old female Sprague-Dawley rats weighing approximately 250-300 g. The animals were kept in a module with individual compartments for each, under conditions of constant light and temperature (12 h light/dark and 22 °C), and were fed on a complete maintenance diet for rats (UAR-Panlab, S.L.)[®]. Two groups were established: a control group of 8 animals that received daily subcutaneous saline injection, and a group of 16 treated animals that received a therapeutic

dose of 5 mg/kg body weight/day of CsA (Sandimmun[®], Sandoz) subcutaneously. The animals were sacrificed 4, 11, 25 and 34 days after the start of treatment by means of an anaesthetic overdose of ketamine (Ketolar)[®], after which the BAT was removed by means of an incision along the mediodorsal line. Once the brown adipose tissue was resected, it was divided into portions and processed for the different studies.

Light microscopy

The fragments to be subjected to fat-specific cytological methods were frozen in liquid nitrogen and cut into 5 to 7 µm slices using a Starlet 2212-Bright cryostat, and stained with oil red (isopropyl alcohol). Those that were to undergo general histological assessment were fixed in 10% formaldehyde, alcohol-dehydrated and embedded in paraffin. The blocks were then cut into 5 to 7 µm pieces using a Medin microtome, and the preparations were stained with haematoxylin-eosin and Masson's trichrome (a variant of Goldnes-Gabe).

Electron microscopy

The sections of fat were fixed in 3% glutaraldehyde, with Millonig's buffer for at least 2 hours. Once the fixative was removed, the pieces were submerged in Millonig's solution for washing and conservation, where they were stored until processing by scanning and transmission electron microscopy techniques.

For viewing under the scanning electron microscope, conventional dehydration and metalizing techniques were used. For transmission electron microscopy, the fragments were post-fixed in 1% osmium in Millonig's buffer supplemented with calcium for 1 hour, acetone-dehydrated, contrasted by uranyl acetate and embedded in araldite. Once the blocks were obtained, ultrathin sections were taken and counterstained using lead citrate.

Biometric techniques

For the development of the biometric techniques, 55 light photomicrographs and 134 transmission electron photomicrographs were taken. The light photomicrographs enabled us to quantify the number of cells per photographic field, and the method consisted in determining the profiles of the cell boundaries, after which they were counted manually.

The transmission photomicrographs provided the remainder of the parameters studied. For our measurements, we used a K-90 Placon digital planimeter equipped with a program for data integration, a movable arm and a magnified tracer which, when passed over the cellular and subcellular boundaries appearing in the photographs, provided the total cell area (CA), the lipid area (LA) of the cell and the nuclear area (NA). The number of lipid droplets per adipocyte (D) and the number of mitochondria per adipocyte (M) were

determined manually, and the remaining cytoplasmic area (RA) was calculated using the following arithmetic operation: $RA = CA - (NA + LA)$.

Statistical method

To identify the possible significant differences between study groups, a descriptive statistical method was applied to the results obtained by means of biometrics to determine the arithmetic mean and standard deviation. The method chosen was the Student-Newman-Keuls test for multiple comparisons.

Results

The plasma CsA levels were maintained stable throughout the entire drug administration period, reaching values of 356 mg/ml. It was observed that the concentrations increased at 24 hours and returned to zero 24 hours after the last administration.

The biometric results appear in Graphs 1 and 2, expressed as the arithmetic mean of the values obtained for each parameter.

With respect to the morphology of the adipocytes from rats subjected to acute CsA treatment (4 doses), a marked reduction in cell size was observed, with loss of the polygonal shape and reduced intercellular space, resulting in an increase in the number of cells per photographic field when compared with the controls (Fig. 1a). When the 11 doses had been administered (Fig. 1b), these effects were even more pronounced. However, when the treatment was continued until 34 doses had been given (Fig. 1c), a slow, gradual recovery of the characteristic morphological pattern of the brown adipocyte was observed. The statistically determined differences between the groups were found to be highly significant.

With respect to the number of cells per photographic field ($F = 43.99$; $P < 0.001$) (Graph 1), with the exception of the animals that received 4 doses, all the treated

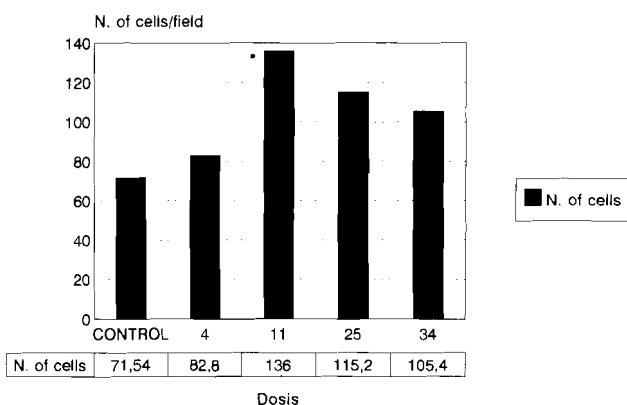
groups showed highly significant increases when compared with controls. When the different treatment groups were compared, highly significant differences were again found in every case except the comparison between 25 and 34 doses.

The differences between groups with respect to the total cell area were always highly significant ($F = 26.26$; $P < 0.001$).

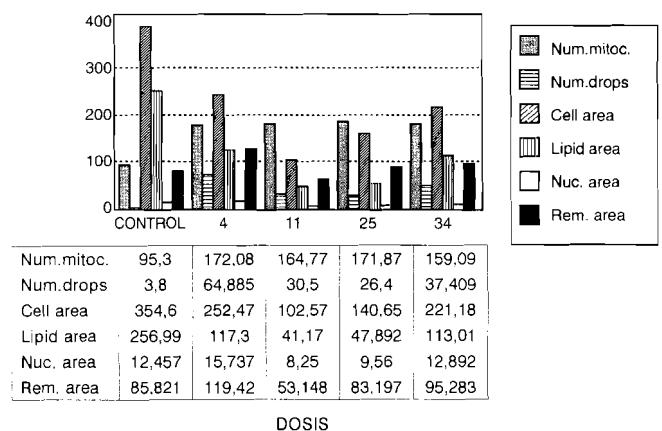
After 4 days of CsA treatment, a considerable reduction in the size of the lipid droplets and an increase in their number was detected in the cell cytoplasm (Fig. 2a). These effects were more marked after 11 days of administration of the drug, stage at which the adipocytes presented abundant pinocytotic vesicles, indicating intense activity on the part of the BAT (Fig. 2b). Nevertheless, when treatment was prolonged to 34 days, a new lipid coalescence was observed, with an increase in the cell lipid area and a decrease in the number of lipid droplets per adipocyte (Fig. 2c). Moreover, at this same point, we found that the endothelial cells of the numerous vessels within the BAT appeared loaded with large fat-filled vesicles within their cytoplasm.

With respect to the numbers of lipid droplets per adipocyte, the Student-Newman-Keuls test disclosed the existence of highly significant differences ($F = 33.60$; $P < 0.001$) between the control group and the treated groups and between the groups that received 4 and 11 CsA doses, while the differences between the groups receiving 11, 25 and 34 doses were not statistically significant. When the lipid areas were compared, the statistical results revealed significant differences ($F = 32.33$, $P < 0.001$) between treated groups and the control group, between 4-dose and 11-dose animals and between 25-dose and 34-dose animals, while the differences between animals receiving 11 doses and those receiving 25 were not all significant.

Other important aspects of the acute phase of treatment were the increase in the number of immune cells, both within and surrounding the capillaries and vessels, as well as the exhaustion of the carbohydrate metabolism, manifested by a decrease in the cytoplasmic



Graph 1. Histogram showing the variation in the number of adipocytes per photographic field with respect to the number of doses administered, ($F = 43.99$; $P < 0.001$).



Graph 2. Comparative study of the evolution of the brown adipocyte throughout the study period. A P value of less than 0.05 was considered indicative of statistical significance.

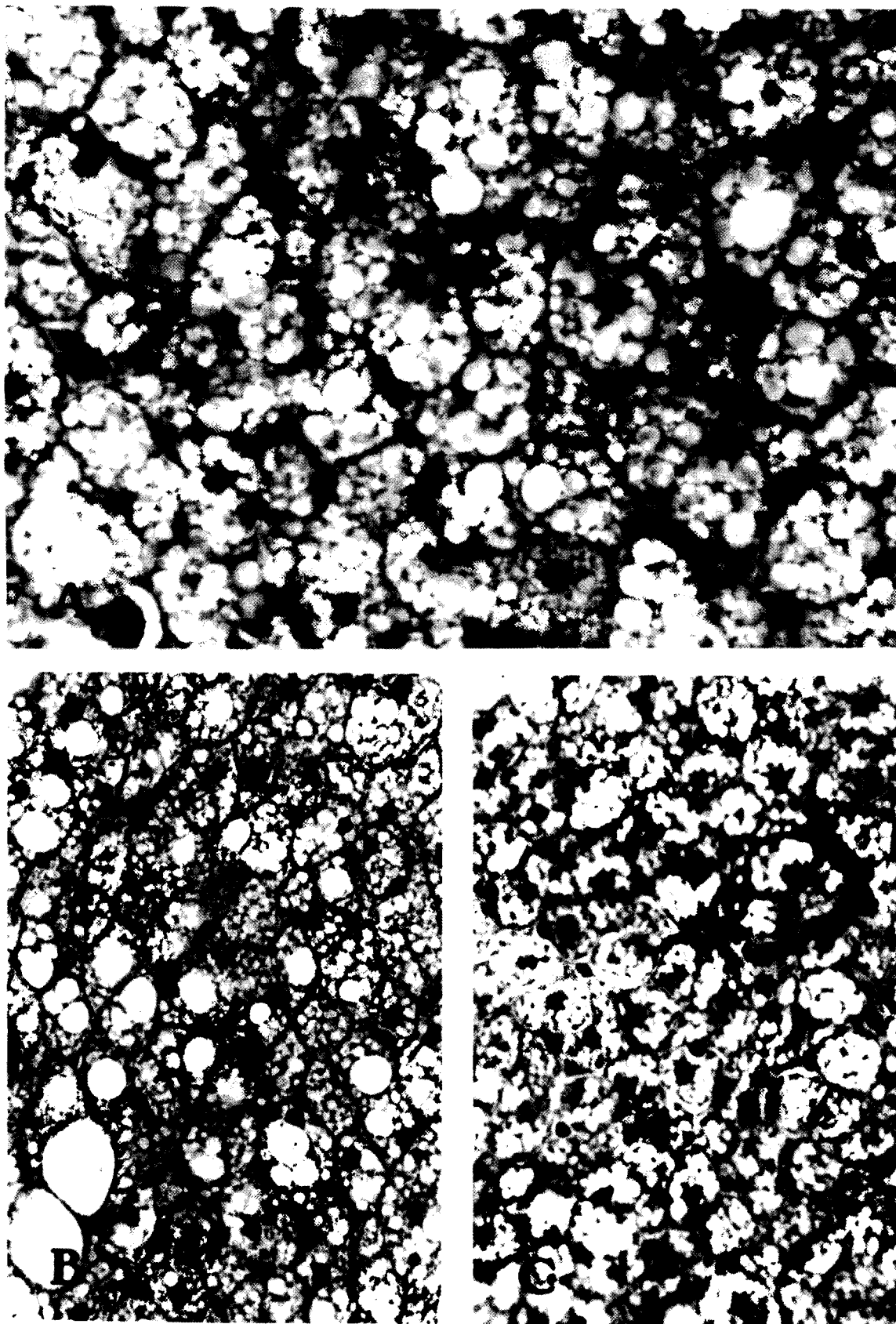


Fig. 1. Changes in the size of the rat brown adipocytes following administration of cyclosporine.
a. control adipocytes.
b. adipocytes after 11 doses.
c. adipocytes after 34 doses. x 400

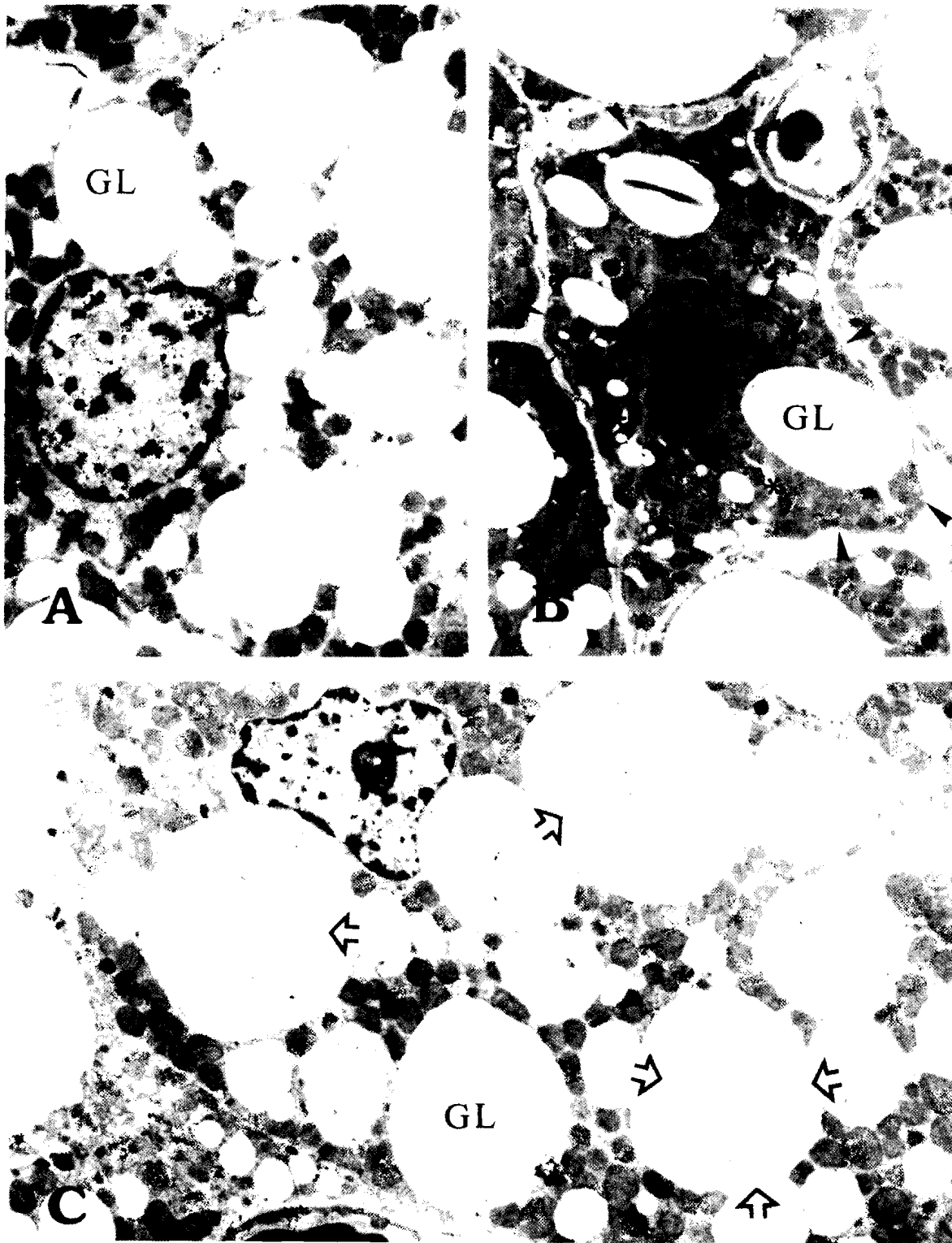


Fig. 2. Changes in the size and number of the lipid droplets (GL) during cyclosporine treatment. **a.** 4 doses: abundant small-sized droplets. **b.** 11 doses: small lipid deposits (*) and numerous pinocytotic vesicles (arrowheads). **c.** lipid coalescence (arrows) after 34 days of treatment. x 3,000

glycogen deposits. These deposits reappeared in the vicinity of the lipid droplets in adipocytes from rats that received 34 CsA doses (Fig. 3).

The presence of lysosomes in brown adipocytes is common; however, they became more numerous when the animal was treated with CsA and, by day 25, they were grouped together, adopting a peripheral position in the cell cytoplasm (Fig. 4).

Macrophages and mast cells were present from the beginning of the study; after 25 days of treatment, the former appeared loaded with lipid vesicles and lysosomes (Fig. 5), while mast cell degranulation increased (Fig. 6).

The number of mitochondria per adipocyte rose slightly with respect to the levels at the start of treatment, and remained more or less stable until the end. This mitochondrial hyperplasia could be related to the increase in the numbers of lipid droplets per adipocyte and the decrease in the cell lipid area, since this organelle is the site of fatty acid oxidation. The small variations found may have been due to errors in counting given the difficulty involved in the measurement of this parameter. The statistical study disclosed significant differences only in the comparison

between control and treated animals ($F = 12.06$; $P < 0.001$).

We found slight variations in the nuclear area which could have been related exclusively to the section obtained upon cutting. In the group treated with 11 CsA doses, this reduction in the nuclear size may have been associated with the decrease in cell size. Our statistical study showed low significance ($F = 6.71$) in the differences between controls and the 11-dose group, between the 4-dose and the 11-dose groups, between 11-dose and 24-dose animals and between the 11 and the 34-dose groups.

The remaining cytoplasmic area increased slightly after 4 days of drug administration, a fact which could be related, to a certain extent, to the decrease in the lipid area. However, after 11 days the remaining area was again reduced, probably as a consequence of the decrease in cell size observed at this stage. The statistical results showed significant differences ($F = 10.63$; $P < 0.05$) between the control group and the groups that received 4 and 11 CsA doses, respectively, as well as between a number of the treated groups: 4 vs 11 doses, 4 vs 25 doses, 11 vs 25 doses, 4 vs 34 doses and 11 vs 34 doses, respectively.

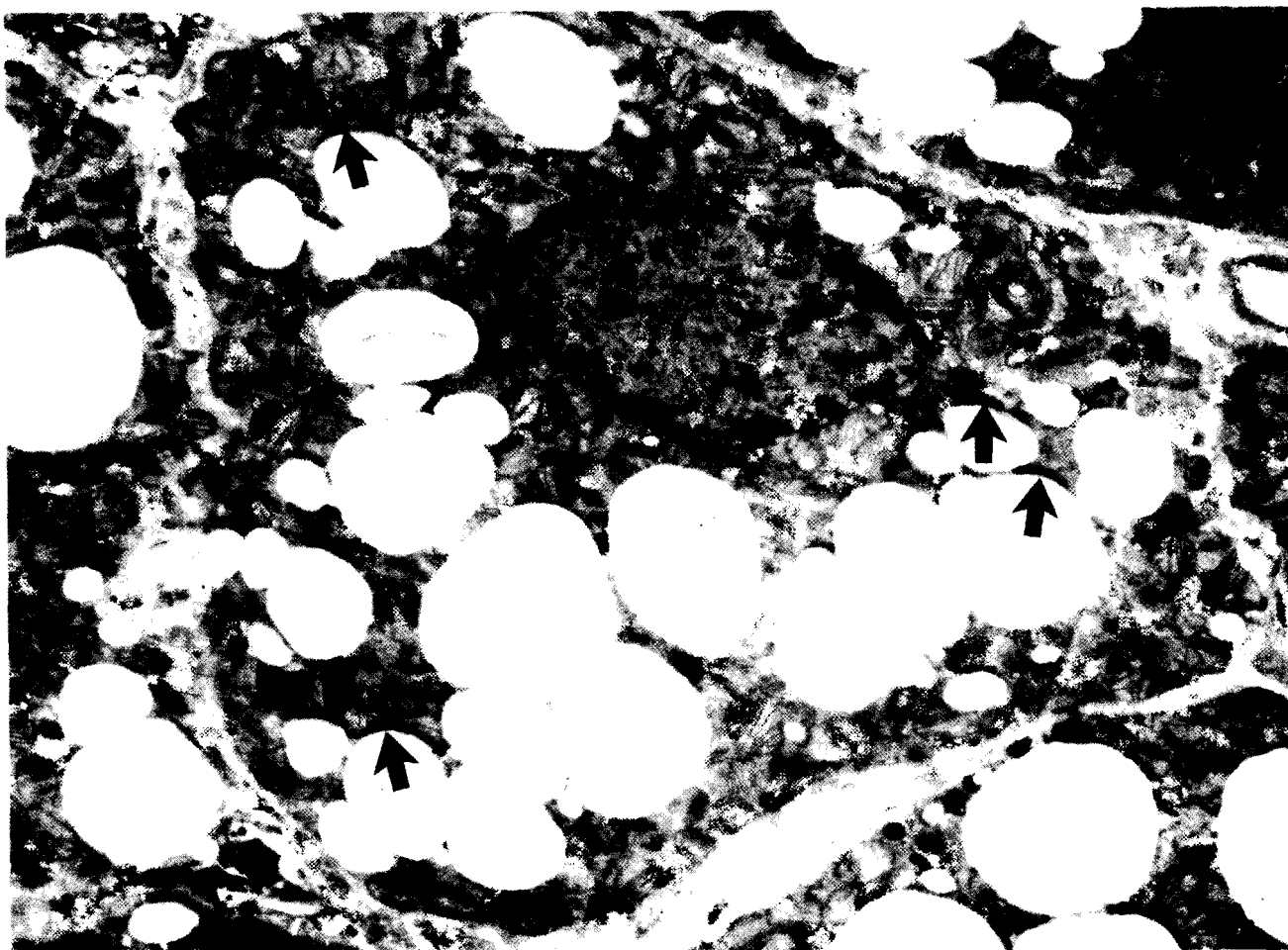


Fig. 3. Glycogen deposits (arrows) in adipocytes from rats that received 34 cyclosporine doses. $\times 3,000$

Graph 2 shows the comparative study of the evolution of the brown adipocyte of the rat throughout the trial period. The values for cell and lipid areas are

clearly displaced to the right, while a balance is maintained on the left by the number of lipid droplets per adipocyte.

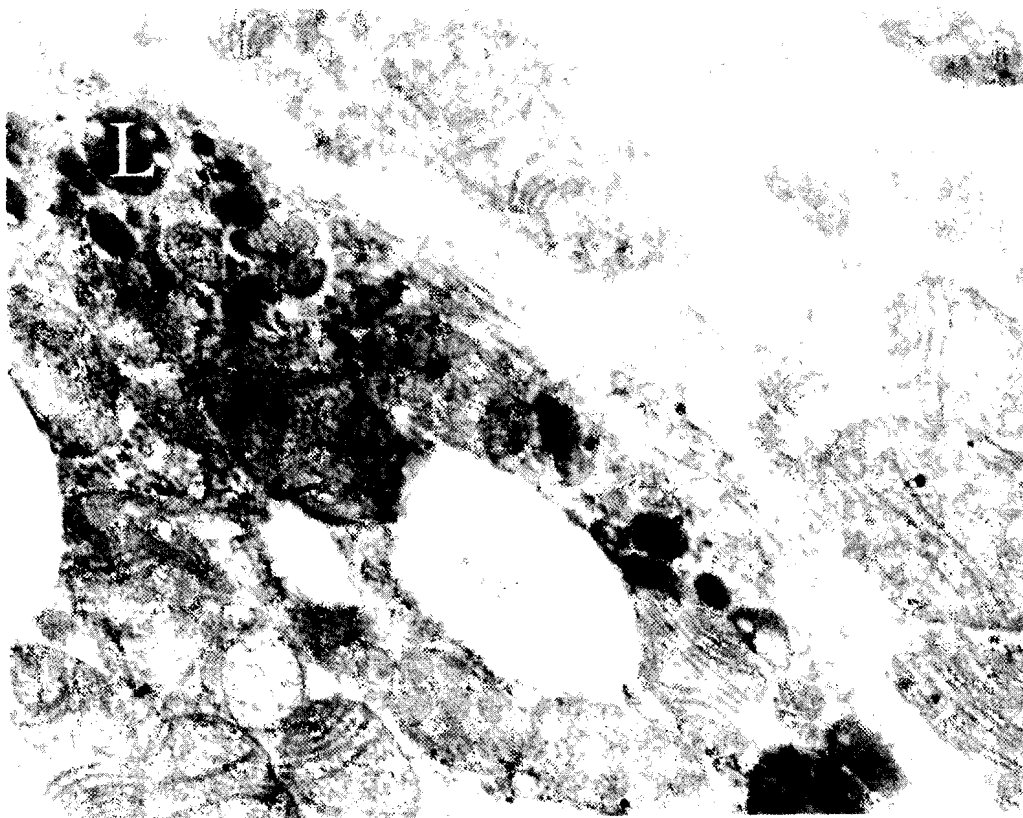


Fig. 4. Brown adipocyte showing numerous peripheral lysosomes (L) after administration of 25 doses of cyclosporine-A. x 12,000

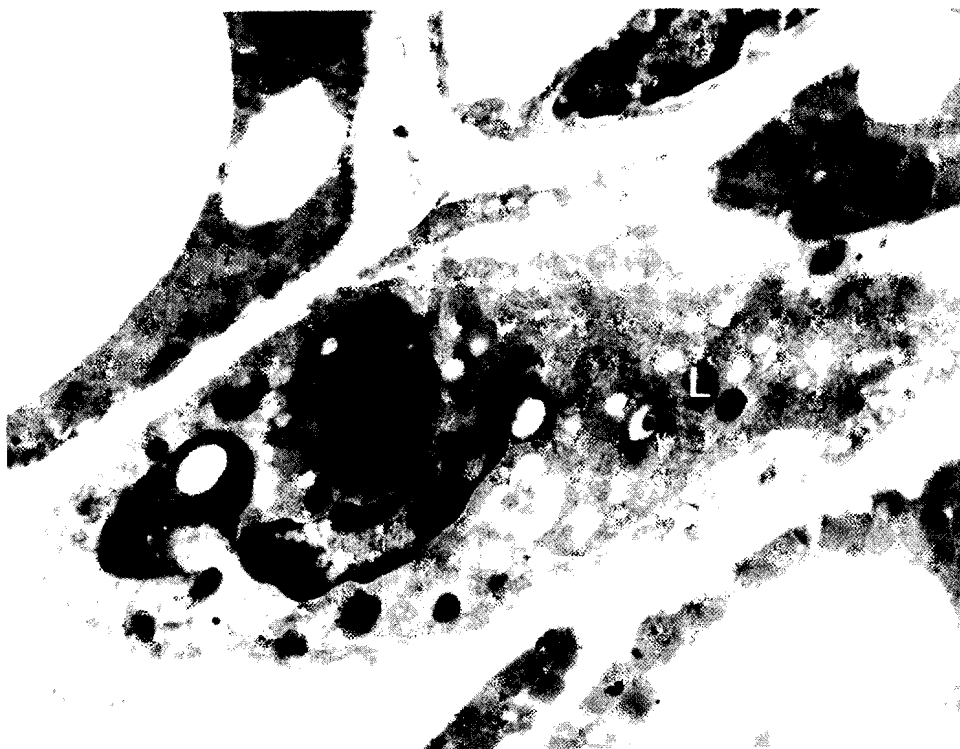


Fig. 5. Presence of macrophages in the brown adipose tissue of animals subjected to continuous cyclosporine-A treatment. Lysosomes (L). x 7,000

Discussion

The primary effect of CsA is to inhibit the maturation of T and B cells (Kahan, 1989a) by inhibiting the activation cascade (Herold et al., 1986) and the synthesis of gamma-interferon, the cytokine that amplifies the signal, activating macrophages and mast cells (Kahan, 1989b). Thus, the therapeutic value of this drug is centred on the prevention of rejection in organ transplantation. Since its earliest use, a number of adverse effects have been known.

Haynes et al. (1985) suggested that both the immunosuppressor role and the toxicity of CsA were related to its hydrophobic property which facilitates its solubilization within the lipid bilayer, diminishing the membrane fluidity. This leads to a reduction in LDL clearance in the hepatocyte, increasing the plasma LDL levels and predisposing the individual to the development of atherosclerosis (Stamler et al., 1988; Ferns et al., 1990).

The mechanism by which the drug increases the lipid levels is unknown, but it may be the result of a deficient liver synthesis of the plasma lipoproteins or of a reduction in the peripheral catabolism (Phillipson et al.,

1985; Simons et al., 1985; Nestel, 1986).

Nor is it known whether it is the original molecule or its metabolites that produce the activity of the drug. This fact, together with its possible interaction with other drugs, its variable absorption and the lack of an ideal vehicle for its administration, complicate the clinical use of CsA.

The pharmaceutical product employed by us, and that most commonly used from the clinical point of view, is Sandimmun®, the vehicle for which cremophor-EL, a mixture of polyethoxylated castor oil and ethanol, is considered by several authors to be responsible for the increase in the blood lipid level (Nestel, 1986; Stamler et al., 1988). Several studies carried out by Jevnikar (1988) using other vehicles demonstrated that CsA potentiated the hyperlipemic effect of the vehicle, suggesting that this modification might be secondary to the liver function disorder.

The toxicity of CsA has been studied in numerous animal models, particularly in rat. Although its therapeutic efficacy and safety are not clearly related to the dose administered, it appears to be that the route of administration and the dose influence the pharmacokinetic model of the drug (Wassef et al., 1985; Midha et al., 1992). In the rat, CsA presents a dose and strain-dependent toxicity which is proportional to its circulatory levels.

The absorption of CsA is slower in the rat than in other animals, but the levels achieved, even at lower doses, are three times higher, implying the existence of species-dependent differences in the absorption of metabolism of the drug (Ryffel, 1982). Thus, the Sprague-Dawley rat presents a lower susceptibility to CsA in comparison with other animals (Whiting et al., 1985). Whiting et al. (1985) suggest that age, size and sex may be important in determining the degree of susceptibility to the drug.

According to these authors, all our animals had the same susceptibility to CsA. Moreover, at the age of three months, and in normal conditions, the BAT is already stabilized in the rat and the number of brown adipocytes hardly varies (Sbarbati et al., 1991). These two factors make the rat the ideal animal in which to assess the pharmacological effects of CsA on the BAT.

Wassef et al. (1985) observed that the subcutaneous route offered a series of advantages with respect to other routes of administration, providing stable plasma CsA levels and increasing the bioavailability of the drug. Using this route, we have obtained plasma CsA concentrations that remained stable throughout the entire treatment period.

In contrast, other authors (Robinson et al., 1983; Wengrovitz et al., 1990; Midha et al., 1992) defend the assessment of CsA in whole blood

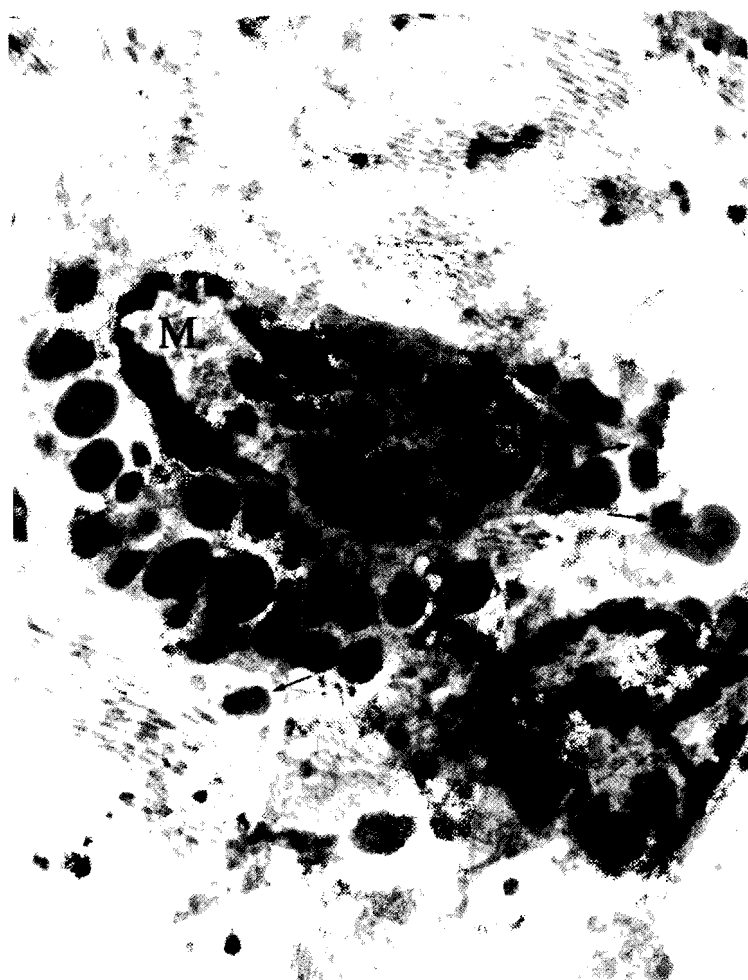


Fig. 6. Mast cell degranulation (arrows) after chronic cyclosporine-A administration. Mast cell (M). x 7,000

since it appears that the drug shows great affinity for binding to the erythrocyte membrane; thus if plasma alone is used, the CsA blood level is only partially determined.

The major negative effect of the dose used by us (5 mg/kg/day) was the increase in blood coagulability, assessed one hour after isolation of the rat plasma. Some of our animals also presented diverse macroscopic alterations, such as renal hypoplasia, hydronephrosis and fatty liver.

We chose BAT as it is highly sensitive to variations in lipid levels, thus constituting a good control of the impact that could be derived from the action of CsA.

The morphological findings reported by Sbarbati et al. (1991) in BAT under normal conditions coincide with those observed in our control group.

The assessment of the morphological response of the BAT during an initial stage that we consider the acute phase, consisting of CsA administration on 4 consecutive days, revealed a significant increase in the number of cells per photographic field with respect to controls. This finding correlated with the reduction in the cell area, from which it can be deduced that there are a greater number of adipocytes of smaller than normal size. This fact led us to assess the most important organelles of each cell (lipid droplets and mitochondria), observing that the lipid area diminished, while the number of lipid droplets per adipocyte grew. Therefore, it could be deduced that there was a lipolytic effect since there was a relationship between the number of lipid vesicles and the cell metabolism, reflected by the fact that when the latter rose, the droplets decreased in size, thus facilitating the release of fatty acids and their uptake by the mitochondria. This would be related to the slight increase in the number of mitochondria that we found in the 4-dose regimen. This lipolytic effect may be due to the mobilization of the triglycerides to produce energy, since other authors have observed diminished blood flow and liver alterations (Montilla et al., 1991) which may oblige the BAT to exert a greater effort in heat production.

Other significant findings are the decreased glycogen deposits and increased endoplasmic reticulum. The former would imply an exhaustion of the carbohydrate metabolism in the adipocyte. Similar effects of the deterioration of the glucose metabolism produced by the use of CsA were observed by Harris (1986), while the vacuolization of the endoplasmic reticulum had been reported earlier by Whiting et al. (1985).

On the other hand, we should point out the greater abundance of white cells (monocytes and polymorphonuclear cells) both in vessels and capillaries and immersed in the tissue. Similar effects were found by Reidy in 1974 (Reidy, 1991). The polymorphonuclear cells and mast cells might act as coparticipants in the activation of the adipocyte lipid metabolism.

In a follow-up of the development of the morphological changes in BAT throughout the entire study period, we find that the effects mentioned above are more marked after the administration of 11 doses of CsA, as demonstrated by morphometric assessment. The

morphology of the adipocytes at this time is totally atypical, with a marked reduction in the cell size and intracellular space.

Within the adipocyte, the most notable findings are the small volume of the lipid droplets, the enclosure and augmented number and size of the mitochondria and the decrease in the remaining cytoplasmic area, as well as the marked pinocytotic activity observed along the entire cell boundary.

Although not all the BAT-related effects have been reported in the literature, Whiting et al. (1985), in a work on CsA toxicity, referred to the existence of large mitochondria in the cells of the renal tubule.

The loss of intracellular space could be the consequence of a cytotoxic effect of the drug aimed at the fibroblasts, resulting in the decrease in this population, whose space is then occupied by brown adipocytes with a high degree of metabolic activity.

Between 25 and 34 days of treatment, a certain balance appears to be reached between lipolysis and lipogenesis, made evident by the recovery of the characteristic morphological pattern of the BAT. At this same number of doses, Montilla et al. (1991) observed a marked increment in the plasma lipid levels which could be the source of lipogenesis for the brown adipocyte. In this period, a new lipid coalescence is initiated since the droplets grow and come into contact with each other (Goglia et al., 1992).

The presence at these stages of fat-containing macrophages, as well as the elevated number of lysosomes observed, are clear signs of functional activity which, together with the increase in the number of vascular buds, could be indicative of tissue recovery or of a certain degree of adaptation to chronic CsA administration.

From the morphological point of view, recovery of zones of the BAT are observed, and there is an increase in the number of white adipocytes.

In conclusion, we can say that CsA, in an acute phase of treatment, induces morphological changes in BAT which are reflected in the variations observed in the brown adipocytes, and which appear to correlate with a lipolytic response to the drug; thus, the BAT may be acting as a system to eliminate the excess of lipids in the blood provoked by CsA administration. However, in cases of continuous treatment, this tissue appears to recover and adapt, finally reaching a balance between the drug and the activity of the brown adipose tissue.

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