EFFECT OF PERIPHERAL BLOOD LYMPHOCYTE ACTIVATION ON CYCLOSPORINE A MEDIATED INHIBITION OF IL-2 PRODUCTION

Thesis submitted for the degree of

Doctor of Philosophy, to the Faculty of Medicine,

University of London.

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1999

ABSTRACT

Cyclosporine A (CsA) is a potent immunosuppressive drug and to make it efficient: effective concentration of CsA is necessary to produce adequate immunosuppression and minimum side effects. In this study, we have tested the hypothesis that "activated T lymphocytes are less sensitive to CsA". We have compared the inhibitory effect of CsA on IL-2 production of activated human peripheral blood lymphocytes (PBLs) and naïve PBLs, as well as before and after haemodialysis. Results: *in vitro* PHA preactivation of T cells shifted the inhibitory dose-response curves to the right and significantly increased the IC_{50} (concentration causing 50% inhibition of IL-2 production) of CsA, *($p < 0.05$).* In 10 normal subjects the IC_{50} increased from 50 to 380 ng/ml, in 10 chronic ambulatory peritoneal dialysis (CAPD) patients from 20.5 to 95.5 ng/ml, and in 12 chronic haemodialysis patients from 56 to 283 ng/ml before haemodialysis and from 99 to 721ng/ml post-haemodialysis. CAPD did not affect the CsA sensitivity of PBLs by comparison with normal volunteers, (CsA IC_{50} was 20.5 ng/ml vs 50 ng/ml, $p > 0.05$). In contrast the IC_{50} was significantly higher after haemodialysis, (99 ng/ml vs 50 ng/ml, $p < 0.05$).

Using CTLL-2 cells as detector of IL-2, the post-haemodialysis IC_{50} was significantly higher than the pre-haemodialysis IC_{50}, (99 ng/ml vs 56 ng/ml, $p < 0.05$). Even after *in vitro* PHA prestimulation of PBLs, the IC_{50} was much higher post- than pre-haemodialysis (721 ng/ml vs 283 ng/ml, $p < 0.05$). Flow cytometry analysis of intracellular IL-2 showed that CsA reduction of the frequency of IL-2 producing human T cells was significantly less post- than pre-haemodialysis at 100 ng/ml CsA, (36.6% and 48.5% respectively, $p < 0.05$). Similarly, CsA inhibition of intracellular IL-2 production in T cells was much less post- than pre-haemodialysis at 100 ng/ml CsA, (37.6% and 54.3% respectively, $p < 0.05$). Measurement of IL-2 concentration in cell culture supernatants by ELISA also showed less CsA inhibition of IL-2 production at 100 ng/ml after haemodialysis by comparison with predialysis, (64.6% and 77.8% respectively, $p < 0.05$).

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Most of chronic haemodialysis patients that we have studied (8 of 12 patients) responded to PHA by producing less IL-2 in T cells and cell culture supernatants post than pre--haemodialysis. This indicates that after haemodialysis the majority of cases do not respond well to mitogen stimulation, a sign of T cell activation during haemodialysis. The expressions of CD25 and CD69 activation markers on circulating T cells of chronic haemodialysis patients were assessed by flow cytometry. The analysis showed abnormally high proportions of circulating T cells spontaneously expressing the activation markers, and no significant difference between pre- and post-haemodialysis in the percentage of T cells expressing CD25 (pre-: 10.42%, post-haemodialysis: 9.17%, $p > 0.05$) and CD69 (pre-: 70.17%, post-haemodialysis: 72.25%, $p > 0.05$). Also when the cells were stimulated with PHA there was no significant difference between the pre- and post-haemodialysis proportions of circulating T cells expressing the activation markers; CD25 (pre-: 13.92%, post-haemodialysis: 12.50%, $p > 0.05$), CD69 (pre-: 71.50%, post-haemodialysis: 71.58%, $p > 0.05$). PHA stimulation of the cells did not enhance expression of CD69, but resulted in slight but significant over expression of CD25.

We found that live-related renal transplant patients who had an additional prophylactic single ATG bolus, have significantly increased *in vitro* CsA sensitivity of circulating T cells at all the tested time points post-transplant as compared to pre-transplant when the patients were maintained on chronic haemodialysis.

These results suggest that preactivation of T cells rendered them less sensitive and resistant to CsA than naïve T cells. Haemodialysis preactivates circulating T cells, and makes them less responsive and resistant to inhibitory effect of CsA as suggested by all parameters. Prophylactic ATG may be effective in removing these preactivated T cells facilitates more effective and potent CsA-based immunosuppression, and effective as prophylaxis against rejection.

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ACKNOWLEDGEMENTS

I would like to thank Professor John Moorhead for his help, guidance and encouragement throughout this project. Similarly, I would like to thank Dr. Zac Varghese for his excellent supervision, help and support. I am grateful to them both for giving me the opportunity to study for this PhD, and for making my time working with them so enjoyable.

I would like to express my appreciation and thanks to all my colleagues at the renal department for their friendly environment, endless contribution, help and technical assistance. My thanks go to all the staff of haemodialysis and radiotherapy departments for their unlimited practical help.

I wish to express my sincere gratitude and thanks to my parents, all members of my family and my wife for their help, encouragement and unending support.

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PUBLICATIONS

Paper

Abudher, M.N., Moorhead, J.F., Fernando, O.N. and Varghese, Z. (1999) Pre-operative ATG bolus treatment increases CsA sensitivity of peripheral blood lymphocytes (PBLs) in renal transplant patients. *Transplant Proc.* **In press.**

Abstract

Varghese, Z., Abudher, M.N. and Moorhead, J.F. (1997) Cyclosporin (CsA) resistance of activated lymphocytes. Presented at the 14th International Congress of Nephrolgy in Sydney Australia, 1997.

ABBREVIATIONS

^{3}H-TdR	Tritiated thymidine
6-MP	6-mercaptopurine
ACE	Angiotensin converting enzyme
ADCC	Antibody-dependent cell-mediated cytolysis
AID	Autoinhibitory domain
AIM	Activation inducer mloecule
ALG	Antilymphocyte globulin
APCs	Antigen presenting cells
ATF	Activating transcription factor
ATG	Antithymocyte globulin
AUC	Area under the curve
Bcl-2	B-cell lymphoma-2
Ca^{2+}	Calcium
CAML	Intracellular calcium-modulating ligand of cyclophilin
CAPD	Chronic ambulatory peritoneal dialysis
CD23	Low affinity Fc receptor of IgE
CD25	Low affinity interleukin-2 receptor
Con.A	Concanavalin A
cpm	Counts per minutes
CREP	Cyclic AMP-response element binding protein
CsA	Cyclosporine A

CTLL-2	IL-2 dependent murine cytotoxic cell line
CyPs	Cyclophilins
DAG	Diacylglycerol
ECD	Phycoerythrin-texas red
ELISA	Enzyme-linked immunosorbent assay
ET-1	Endothelin-1
FcεRII	Low affinity Fc receptor of IgE
FITC	Fluorescence isothiocynate
FKBP	FK binding protein
FPIA	Fluorescence polarisation immunoassay
GFR	Glomerular filtration rate
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HD	Haemodialysis
HEV	High endothelial venules
HIV	Human immunedeficiency virus
HPLC	High pressure liquid chromatography
HUS	Haemolytic uraemic syndrome
IC_{50}	Concentration causing 50% inhibition of IL-2 production
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL-2	Interleukin-2
IL-2R	Interleukin-2 receptor

IP3	Inositol 1,4,5, triphosphate
JNK	Jun N-terminal kinase
KD	Kilo Dalton
LN	Lymph node
MAP	Mitogen-activated protein
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
NF-AT	Nuclear factor of activated T cells
$NF\text{-}AT_c$	Nuclear factor of activated T cells (cytoplasmic component)
$NF\text{-}AT_n$	Nuclear factor of activated T cells (nuclear component)
NK	Natural killer
OKT-3	Muromonab-CD3 (anti-CD3 monoclonal antibody)
PAFs	Platelet activating factors
PBLs	Peripheral blood lymphocytes
PBMCs	Peripheral blood mononuclear cells
PC5, PE-Cy5	Phycoerythrin-cyanin5
PDGF	Platelet-derived growth factor
PHA	Phytohaemagglutinin
PI	phosphoinositide
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PMMA	Polymethylmethacrylate

PTK	Protein tyrosine kinase
RAS	Renin angiotensin system
RBCs	Red blood cells
RIA	Radioimmunoassay
R-PE, RD1	Phycoerythrin
SEM.	Standard error of mean
SH2	src-homology 2 domain
S-S	Disulphide bonds
TCR	T cell antigen receptor
TCR/CD3	T cell antigen receptor complex
TCR-1	TCR alpha/beta
Th	T helper
TNF- α	Tumour necrosis factor-α

1. INTRODUCTION

1.1. HISTORY OF KIDNEY TRANSPLANTATION

The interest in transplantation in the early part of this century was mainly due to the rapidly advancing experimental and clinical surgical skills. The first successful experimental organ transplantation was an autotransplantation of dog kidney from its normal position to neck vessels, which resulted in some urine (Ullmann, 1902). In the same year another Vienna doctor carried out dog-to-dog kidney transplants (Decastello, 1902). The modern method of suturing was established by Carrel, (1902), and lead to further improvements in surgery. Carrel published extensively on organ transplantation, successfully carried out autografts of kidneys in cats and dogs, and showed that allografts failed after functioning briefly. For this work he was awarded a Nobel Prize in 1912.

These early experiments simply demonstrated that such kidney transplants were technically possible. Study of transplant function was impossible, because measurement of renal function was primitive: there was no routine measurement of blood urea and no radiological imaging. This, plus uncertainty of the mechanism of allograft rejection, led to a diminished interest in organ transplantation after about a decade of activity. In future, efforts were directed towards understanding transplant biology in order to prevent the reaction of host against the foreign tissue and allow adaptation of homoplastic grafts to their hosts.

The first recorded human kidney was transplanted in 1906 (Jaboulay, 1906). He carried out two xenograft kidney transplants using pig and goat as donors, transplanting the organ to the arm or thigh of patients with chronic renal failure. Each kidney worked for one hour. Ernst Unger carried experimental work for many years, and by 1909 reported successful transplantation of kidneys en bloc from a fox terrier to boxer dog. The urine output continued for 14 days, and the animal was demonstrated at two medical societies. At the end of 1909, he attempted a transplant using a stillborn child's kidney grafted to a baboon. No urine was produced, and the baboon died shortly after the operation but the postmortem examination showed the operation had been successful. The knowledge that humans and monkeys were serologically similar, and the success of the operation, encouraged Unger to attempt in the same month, monkey-to-human kidney transplant (Unger, 1910). The xenograft kidney from pig ape was sutured to the thigh vessels of a young girl with chronic renal failure.

There was a little new work done, until the revival of interest in transplantation in the 1950s. The first human kidney allograft was carried out in the Ukraine by surgeon Voronory (Voronoy, 1936). In 1933, he transplanted a human kidney of blood group B to a patient of blood group O suffering from acute renal failure caused by mercuric chloride poisoning. The cadaveric donor kidney was transplanted to the thigh vessels under local anaesthetic: the warm time was 6 hours. There was ABO incompatibility and in spite of a modest "exchange transfusion" the kidney never worked, and the patient died two days later. By

1949, Voronory had reported a total of six such transplants, although no substantial function occurred in any.

In 1946, a human allograft kidney transplant to arm vessels under local anaesthetic was attempted by Hufnagel, Hume and Landsteiner. The short period of function of the kidney may have been responsible for the patient's recovery from acute renal failure. It marked the beginning of a major interest in transplantation and dialysis (Francis and Moore, 1972).

In early 1950s, there was increasing interest in experimental and clinical kidney transplantation, accompanied by growing certainty that immunological mechanisms were involved in the destruction and failure of kidney allograft (Simonsen, 1953; Dempster, 1953). Both workers concluded that an immunological mechanism was responsible for failure, and concluded that a humoral mechanism of rejection was likely. Dempster found that radiation, but not cortisone, delayed rejection. In addition, both of them found that the pelvic position of kidney transplant was preferable to a superficial site.

In Paris, several kidney allograft transplants were carried out without immunosuppression in human patients (Kuss *et al.* 1951; Servelle *et al.* 1951; Dubost *et al.* 1951). Several cases were also reported from Boston, and hemodialysis had been used for the first time in preparing the patients. The tentative conclusions drawn from this work, many of which were later confirmed,

were that prior blood transfusion might be beneficial and that host bilateral nephrectomy was necessary for control of post-transplant blood pressure (Hume *et al.* 1955). Unexpected survival of the kidney was obtained in some of these cases. It was thought that the endogenous immunosuppression of uraemia was responsible rather than the small doses of ACTH or cortisone that were used. In Paris, the first live related kidney allograft functioned immediately, but rejected abruptly on the 22nd day (Michon *et al.* 1953). Other cases were reported from Chicago, Toronto, and Cleveland in late 1950s.

With increasing confidence in surgical methods, the fist transplant of a kidney from one twin to another suffering from renal failure was carried out in Boston. From then on, a number of such cases were successfully transplanted in Boston (Murray *et al.* 1958). Many of the recipients are still alive today.

1.2. IMMUNOSUPPRESSION

In Boston, Paris and elsewhere between 1959 and 1962, the first attempts at immunosuppression for organ transplants involved total body irradiation. In Boston, from 12 cases that were treated in this way, long-term survival was achieved only in one man receiving his transplant from his non-identical twin (Murray *et al.* 1960). In Paris, similar success was also obtained with sibling grafts (Kuss *et al.* 1960; Hamburger *et al.* 1959). These results gave hope that success might be obtained in non-twin persons.

Poor results and high mortality were obtained when irradiation was used for bone marrow transplantation. Therefore, some workers looked for alternatives to irradiation and reasoned that an anticancer drugs such as 6-mercaptopurine (6-MP) or methotrexate might immunosuppress their patients. One of the studies showed a poor immune response to foreign protein in rabbits treated with 6-MP (Schwartz and Dameshek, 1959). At the Royal Free Hospital, London, there was a disappointment at failure of irradiation to prolong kidney allograft survival in dogs, but 6-MP was successful (Calne, 1960). In Richmond, the same effect was also noticed (Zukoski *et al.* 1960). In Boston and London, no lasting kidney function was obtained with 6-MP, but one case was reported to have a prolonged survival of a kidney from unrelated donor, when 6-MP was used together with intermittent prednisolone in a recipient who had also irradiation as the main immunosuppressive agent (Kuss *et al.* 1962). This was the first success for chemical immunosuppression.

A new derivative of 6- MP, called BW57-322 (later known as Azathioprine or Imuran) proved to be more successful in dog kidney transplants and less toxic than 6-MP (Calne *et al.* 1962). Subsequently, the first extended successes with human kidney allografts were obtained (Murray *et al.* 1963). Although prednisolone had earlier been added intermittently to Azathioprine therapy, the regular use became a standard regimen after reports by Starzl *et al.* (1963) and Goodwin *et al.* (1962). Until this period, the donor organs had largely been unmatched cadaver kidneys. Remarkably good results were obtained using

combined immunosuppression and live related donors (Starzl *et al.* 1963; Hume *et al.* 1963) and greatly encouraged the practice of transplantation.

In the mid 1960s, the improvements in regular dialysis allowed better preparation for transplantation, and with a return to dialysis being possible, heroic efforts to save a rejecting kidney became unnecessary. In 1962, tissue typing was used routinely (Hamburger *et al.* 1962; Dausset, 1980). Cross matching between donor cells and recipient serum, discovered by Terasaki *et al.* (1965), and introduced into clinical practice by Kissmeyer-Nielsen *et al.* (1966), led to marked decrease in hyper-acute rejection (Kissmeyer-Nielsen *et al.* 1966). All these were responsible for the success of kidney transplantation, and increased interest in cadaveric donor kidney transplantation.

The 1970s ended with two innovations that revived the hopes of reaching the goal of routine, safe and successful kidney transplantation. First, the successful clinical application of HLA-DR matching (Ting and Morris, 1978), and second, the introduction into clinical practice of Cyclosporine, (Cyclosporine A) (Borel, 1976; Calne *et al.* 1978).

1.3. ACUTE REJECTION

Renal graft rejection is defined as renal functional and structural deterioration due to an active immune response expressed by the recipient, and independent of non-immunologic causes of renal dysfuncion (Strom and Suthanthiran, 1996;

Suthanthiran *et al.* 1994). The occurrence of even one reversible rejection is associated with inferior graft survival rates; in the united nations for organ sharing (UNOS) Scientific Renal Transplant Registry, first transplant recipients who were rejection-free at discharge had an 86% one-year graft survival rate compared to 67% for those with one or more rejection episodes ($p < 0.001$) (Cecka and Terasaki, 1993). Acute rejection, which is generally cell-mediated and occurs after days to months, is also considered to be a significant risk factor for the subsequent development of chronic rejection (Almond *et al.* 1993). Chronic rejection is a relentlessly progressive process that occurs over months to years and is a major cause of long-term graft dysfunction and ultimate failure (Bia, 1995). In contrast with chronic rejection, acute rejection is usually reversible with a variety of commonly used immunosuppressive drugs.

1.3.1. The Anti-allograft Response

1.3.1.1. T cell stimulation

T cell activation begins following T cell recognition of intracellularly processed fragments of foreign proteins that are embedded within the groove of the major histocompatibility complex proteins (MHC) expressed on the surface of antigen presenting cells (APCs) (Unanue and Cerottoni, 1989; Germain, 1994). The T cells of the recipient can directly recognise the allograft, that is, (donor) intact allo-MHC molecules on the surface of donor APCs, which are presented with endogenous peptides (direct recognition) (Lechler and Batchelor, 1982; Shoskes and Wood, 1994; Warrens *et al.* 1994; Hornick and Lechler, 1997). At face value

this appears to break the rules of self MHC restriction; however, two hypotheses account for the fact that the high precursor frequency of alloreactive T cells can be accommodated within the context of self MHC restriction. This is easiest to envisage where responder and stimulator MHC molecules are similar, sharing conserved sequences in the exposed TCR-contacting surface of the molecule, differences in the peptide binding groove allow binding and display of different sets of peptides. The alloresponse is thus directed to the multiplicity of different peptides bound by the MHC molecule (multiple binary complex hypothesis). When the exposed surfaces of the responder and stimulator MHC molecules are substantially different, the alternative, high determinant density hypothesis (alloreactive T cell's specificity is for the foreign MHC molecule) may provide a better explanation for the observed strength of the alloresponse. In order to reconcile this with self MHC restriction it only needs to be suggested that a small fraction of T cells whose receptors were selected for self MHC recognition cross-react, by chance with a foreign MHC structure. The T cell response that results in early acute cellular rejection is caused mainly by direct allorecognition. In a rodent model, *in vitro* primed, donor-specific, direct pathway alloreactive T helper cells were shown to effect acute rejection when adaptively transferred into irradiated recipients that had been transplanted with an allogeneic kidney. Rejection occurred only in the presence of donor-derived dendritic cells (Braun *et al.* 1993).

In the indirect pathway, T cells recognise processed alloantigens presented as allopeptides bound to self-MHC by the recipient (self) APCs. Evidence of indirect allorecognition was provided by retransplantation experiments in rat model. MHC incompatible kidney allografts depleted of indigenous dendritic cells (by parking kidneys in intermediate hosts) were permanently accepted without immunosuppression, in certain donor recipient combinations, while suffering others rejection in others (Lechler and Batchelor, 1982). Given that no immunogenic donor-derived cells were present in these retransplanted grafts, they proposed that rejection of these grafts resulted from the sensitisation of T cells with indirect allospecificity. The basic premise for indirect allorecognition as a mechanism for initiation and/or amplification and maintenance of allograft rejection is that donor alloantigens are shed from the graft, taken up by recipient APCs, and presented to $CD4^+$ T cells. The importance of indirect alloresponses is suggested by the downregulation of T cell responses following thymic administration of allogeneic MHC-derived peptides, leading to prolonged survival of subsequent renal allografts. Such peptides could not have affected the direct pathway; this suggests that indirect presentation is critical to the rejection process (Sayegh *et al.* 1994). This indirect pathway can be important especially in chronic rejection.

The T cell antigen receptor complex (TCR/CD3) is composed of clonally variant TCR $\alpha\beta$ peptide chains that recognise the antigenic peptide in the context of MHC proteins and clonally invariant CD3 chains (γ, δ, ε, and ζ) that initiate

intracellular signals originating from antigenic recognition (Wiess and littman, 1994; Clevers *et al.* 1988). The CD4 and CD8 molecules, expressed on reciprocal T cell subsets, bind to non-polymorphic domains of HLA class II (DR, DQ) and class I (A, B, C) molecules, respectively (Miceli and Parnes, 1991). Antigenic recognition stimulates a redistribution of cell-surface proteins and co-clustering of the TCR/CD3 with CD4 or CD8 antigens. This multimeric complex includes additional signalling molecules, CD2 and CD5 proteins, and functions as a unit in initiating T cell activation (Suthanthiran, 1990; Brown *et al.* 1989; Beyers *et al.* 1992).

Following antigenic engagement of the TCR/CD3 complex, signal transduction is initiated and causes an increase in free intracellular Ca^{2+} and sustained protein kinase C (PKC) activation. These events function synergistically in promoting the expression of several nuclear regulatory proteins and transcription of genes important for T cell growth and differentiation, (TCR signal transduction is explained in detail under T cell activation).

If a T lymphocyte encounters the MHC-peptide complex that specifically fits its TCR, it may then be triggered (signal 1: antigen signal). Depending on the circumstances, the T cell may undergo apoptosis, anergy, neglect, partial activation, or full activation with clonal expansion and effector function. Stimulation of T cells via TCR/CD3 complex alone in the absence of co-stimulatory signal (signal 2) induces T cell anergy (Jenkins and Schwartz, 1987;

Schwartz, 1996). Full T cell activation requires both antigenic and co-stimulatory signals engendered by cell-to-cell interactions among antigen-specific T cells and APCs (Jenkins and Schwartz, 1987;Schwartz, 1996; Suthanthiran, 1993). One co-stimulator provided by the APCs is B7-1 or B7-2, which engage the CD28 receptor on the T cell (June *et al.* 1994). Other receptors such as CD43 on T cell may provide similar signal (Sperling *et al.* 1995). Additional co-stimulatory molecules include lymphocyte associated antigen-1 (LFA-1) engaging its ligand intercellular adhesion molecule-1 serves as an adhesion function, the CD2 engages CD58 (Halloran and Miller, 1996). In addition to T cell accessory molecules and their cognate APCs surface proteins, antigen-presenting-cell derived cytokines, such as IL-1 and IL-6, can also provide co-stimulatory signals that result in T cell activation *in vitro* (Williams *et al.* 1985). These events increase the probability that signal 1 will activate cytokine transcription and also help to prepare T cell for cell division. The importance of CD28 co-stimulatory signal transduction is explained in detail under T cell activation.

1.3.1.2. Immune effector mechanisms

T cells not only initiate the immune response, but also mediate antigen-specific effector responses and secrete soluble factors to regulate the activity of other leukocytes. Allograft rejection is contingent upon the co-ordinated activation of alloreactive T cells and APCs. Through the release of cytokines and cell-to-cell interactions, a diverse assembly of leukocytes including $CD4^+$ T cells, $CD8^+$ cytotoxic T cells, natural killer cells, antibody forming B cells, macrophages, and

other inflammatory leukocytes is recruited into the anti-allograft response. The net consequence of cytokine production and acquisition of cell-surface receptors for these transcellular molecules is the emergence of antigen-specific and graft destructive T cells, and activation of macrophages and natural killer cells. The end result of the binding of thrombocytes recruited to the allograft vascular endothelial cells is microvascular thrombosis, leading to reduction in blood flow and necrosis. Cytokines also facilitate humoral arm of the immune response by promoting the production of antibodies that can damage the transplanted organ via complement-dependent and/or antibody-dependent cell-mediated cytotoxicity mechanisms. Moreover, IFN-γ and TNF-α can amplify the ongoing immune response by upregulating the expression of HLA molecules as well as co-stimulatory molecules on graft parenchymal cells and APCs. Donor antigen-specific cytotoxic T lymphocytes and anti-HLA antibodies have been identified during or preceding rejection episodes. (Strom *et al.* 1975; Suthanthiran and Garovoy, 1983).

In vitro study has showed that pro-inflammatory lymphokines like IFN-γ and TNF that implicated in the rejection process (Wu *et al.* 1992; Morgan *et al.* 1993) can activate natural killer cells (Tripp *et al.* 1993), but there is little data to implicate this cell type as direct mediator of graft rejection (Strom *et al.* 1975). Mice bearing two copies of a disrupted perforin gene (perforin gene knockout mice) manifested a gross deficiency in natural killer cell and cytotoxic T cell activity (Kagi *et al.* 1994). Perforin transcripts were shown highly expressed in acute

cellular rejection (Lipman *et al.* 1994). Other study has suggested that natural killer cells are present in organ grafts and that may contribute to acute rejection mediated damage by adhering to exposed Fc portions of antibody molecules during antigen-antibody interactions (Morreta *et al.* 1992).

A large body of data, both clinical and experimental, supports a cardinal role for the IL-2 system in the generation and expression of anti-allograft immunity. Induction of IL-2 gene transcription and expression of interleukin-2 receptor (IL-2R) have been shown in experimental models to precede acute rejection (Bugeon *et al.* 1992). In humans, IL-2 mRNA accumulation can also precede acute rejection episode as evidenced by kinetic studies using fine needle biopsy aspiration technique (Dallman *et al.* 1993). The role of IL-2 has been further suggested by the inhibition of its production during tolerance induction by DST (Josein *et al.* 1995), whereas the injection of recombinant IL-2 could restore rejection (Dallman *et al.* 1991). Other lines of evidence of the central role of IL-2/ IL-2R pathways come from the effect of administration of monoclonal antibodies inhibiting the IL-2R (Kirkman *et al.* 1985; Kupiec-Weglinski *et al.* 1986; Kirkman *et al.* 1991; Soulillou *et al.* 1990). IFN-γ, a secretory product of activated T cell, promotes the expression of HLA antigens that serve as the major stimulus for the initiation of, and subsequently as target for, the anti-allograft response (Whicher and Evans, 1990; Arai *et al.* 1990).

1.4. CHRONIC REJECTION

This phenomenon is characterised by gradual functional deterioration of the grafted organ associated with characteristic histopathologic changes. Its pathophysiology and aetiology are incompletely understood. Chronic rejection affects all solid organ transplants: for instance, renal transplant from cadaver sources have half-life of 6 to 8 years, unchanged over the last two decades despite progressive improvements in immunosuppressive therapy (Cecka and Terasaki, 1994). Thus chronic rejection appears unresponsive to current therapy.

A number of risk factors have been implicated in a chronic rejection. Alloantigen-dependent factors include donor sources, histocompatibility differences between donor and recipients, and the frequency and intensity of acute rejection episodes, which affect long-term graft survival substantially (Matas *et al.* 1993). Alloantigens-independent factors have also been considered, including prolonged cold ischaemia (Van Es *et al.* 1983; Munger *et al.* 1993), infection particularly cytomegalovirus infection (Koskinen *et al.* 1994), and lipid abnormalities (Walli *et al.* 1993).

Acute rejection and chronic rejection operate through significantly different immune response mechanisms. Episodes of acute rejection are characterised by the strong stimulation of T cells by alloantigen presentation and subsequent activation and proliferation of immunocompetent antiallograft T cells. In comparison, chronic rejection appears to be results from ongoing, low-grade

injuries to allograft vascular endothelium. Chronic rejection has as its most prominent feature persistent perivascular inflammation, often with relatively low levels of lymphoid activation, and a general concentric arteriosclerosis (Hayry *et al.* 1992).

The common microscopic features of human allografts arteriosclerosis in kidney, heart, and liver transplants have been catalogued as perivascular inflammation, thinning of vascular media and focal monocye necrosis, focal breaks in the internal elastic lamina, and a generalised concentric intimal thicking characterised by the mingling of T cells and macrophages among the smooth muscle cells of the intima (Demtris *et al.* 1989). Earlier study have postulated that three sets of molecules secreted in response to the inflammation could be responsible for the formation of arteriosclerotic lesions: (1) cytokines secreted by the lymphocyes or macrophages, (2) growth factors secreted by either the immune cells or cells of inflammed tissue, and (3) eicosanoids and platelet-activating factors (PAFs) (Thyberg *et al.* 1990; Foegh, 1988).

Recently, Hayry postulated that a low-level immune response, characterised by perivascular inflammation, induces persistent low-grade damage to the allograft vascular endothelium (Hayry, 1996). The endothelial cells secrete growth factors in response to this damage in an attempt to repair the damage, stimulating the proliferation of smooth muscle cells and the migration of myocytes from the media into the intima, forming the arteriosclerotic lesions.

It is unclear whether the cellular or the humoral arm of the immune response is primarily responsible for ongoing low-grade damage (Hayry *et al.* 1993). As mentioned, lymphocytes and macrophages are commonly found intermingled in vascular endothelia of chronically rejecting allgrafts. Yet, IgG antibodies and complements are found to line the walls of allograft blood vessels, even in well functioning transplants (Baldwin *et al.* 1986). These deposits are not found in normal aorta or syngeneic grafts.

The generality of the low-grade damage also complicates the identification of which cell types secrete the effector cytokines, growth factors, or eicosanoids for allograft arteriosclerosis (Lemstrom *et al.* 1995). The overall picture is a complex series of interrelated response cascades in which generalised damage in the allograft is answered by a generalised stimulation of endothelial immune and repair systems (Orosz, 1994; Jutila, 1994).

1.5. CYCLOSPORINE

1.5.1. Discovery

Cyclosporine A (CsA) is a powerful immunosuppressive drug and has proved to be a potent agent in a wide variety of experimental models in tissue transplantation and in clinical organ transplantation. CsA was first isolated from two strains of fungi imperfecti (Cylinrocarpon lucidum Booth and Trichoderma polysporum) from soil samples by the department of microbiology at Sandoz in

Basle as antifungal agent of limited activity (Dreyfuss *et al.* 1976). The latter, from which CsA is now produced, was shown by Borel to have potent immunosuppressive activity in a variety of *in vitro* and *in vivo* experiments (Borel *et al.* 1976, 1977; Borel, 1982).

1.5.2. Clinical Efficacy

Following initial description of the immunosuppressive properties of CsA, it was shown to suppress rejection of vascularized organ allografts in animals such as rat, dog, rabbit (Kostakis *et al.* 1977; Calne and White, 1977; Green and Allison, 1978). Shortly, afterwards the immunosuppressive effect was seen in various other experimental models of vascularized organ allografts in many species (Morris, 1981). Clinical trials of this drug in kidney transplantation started in Cambridge in 1978 (Calne *et al.* 1979), and CsA is the immunosuppressive drug on which various forms of immunosuppression regimes are based. CsA has molecular weight of 1200 kD and comprise 11 amino acids, one of which is unique and most of that are hydrophobic. Thus, CsA is only soluble in lipids or organic solvents.

The introduction of CsA in 1983 for the treatment of renal allograft rejection had a profound impact on the field of organ transplantation. In the pre-CsA era, one year graft survival was approximately 50% in patients receiving cadaver kidneys and 75% in patients with kidneys from live related donors (reviewed in Kahan, 1989). At present time, use of CsA, along with use of organ preservation, has

increased the one year graft survival to 80-85% in patients receiving cadaver kidneys and 90-95% in patients with kidneys from living related donors (Starzl *et al.* 1982, 1983). After the initial experience, virtually all contraindications to the use of CsA in renal transplantation were disregarded. Even more dramatic improvements have been observed in transplantation situations associated with greater risks of lethal rejection episodes, such as cardiac, liver, heart-lung and multiple organ transplantation. CsA has been shown to be effective in a number of auto-immune diseases such as psoriasis, Behçet's diseases, and insulin- dependent type-I diabetes (Masuada *et al.* 1989; Bougneres *et al.* 1990; Ellis *et al.* 1991). These results document the potential of non-specific immunosuppressive therapy to benefit a wide variety of clinical situations. The early experience with CsA, both in prospective controlled trials and in uncontrolled trials, indicated that CsA was a major advance in immunosuppressive therapy, as was clearly evident in the European collaborative study, which had data from over 200 transplants centres and several thousands renal transplants (Oplez, 1986). However, a number of side effects had become evident, the major one being nephrotoxicity, so subsequent protocols were designed to combine the improved immunosuppression achieved with CsA with a reduction of side effects, by administering lower doses of CsA. There is no convincing evidence that one protocol is superior to another at least for three protocols illustrated by an Australian multicentre trial (Hardie *et al.* 1993): patient and graft survival was identical for CsA and prednisolone; CsA and azathioprine; and CsA, azathioprine and prednisolone. The search for safer and more effective immunosuppressants has more recently led to discovery of FK-506

(Kino *et al.* 1987) in the fermentation broth of streptomyces isukubaensis, an organism obtained from soil samples collected in Tsukuba area in southern Japan. FK-506 is a macrolide, which though structurally unrelated to CsA, is 10-100 more potent than CsA in a variety of experimental models of transplantation and autoimmunity. Except for some experimental differences, both these compounds have an almost identical spectrum of biological activity (Schreiber, 1991; Mckeon, 1991; Schreiber and Crabtree, 1992; Sigal and Dumont, 1992).

1.5.3. Mechanism of Action

It was apparent from a number of *in vivo* experiments that CsA exerts its effect soon after exposure of the recipient to a tissue allograft. This was clearly illustrated in the rat kidney allograft model, showing that CsA relatively ineffective if given either after induction of immune response has taken place or before the recipient animal has been exposed to allogeneic histocompatibility antigens (Homan *et al.* 1980b; Morris *et al.* 1983).

1.5.3.1. Cellular mechanism

In vitro experiments correlated well with these *in vivo* models. In several species, including humans, CsA has been shown to inhibit the lymphocyte proliferative response to concanavalin A (con.A), phytohaemagglutinin (PHA), pokeweed mitogen, monoclonal antibodies to the T- cell receptor (TCR), the CD3 complex or other cell surface receptors, and the combination of the Ca^{2+} ionophore ionomycin in conjunction with the phorbol 12-myristate 13-acetate (PMA) *in*

vitro (Wiesinger and Borel, 1979; Larsson 1980; Borel, 1981; Burckhardt and Guggenheim, 1979; Leapman *et al.* 1981; White *et al.*1979; Sawada *et al.* 1987; Kay *et al.* 1989; Dumont *et al.* 1990). Each of these drug sensitive pathways is associated with initial rise in the intra-cellular Ca^{+2} (Johansson and Moller, 1990), suggesting that a common intermediate may the molecular target of the immunosuppressive drug. Provided CsA is added simultaneously with mitogen, CsA inhibits lymphocyte proliferation and washing the lymphocytes and re-exposing them to the mitogen reverses the CsA effect (Wiesinger and Borel, 1980). But, if CsA is added 48 hours after the addition of mitogen to a culture, then no inhibition of lymphocyte proliferation is observed (Wiesinger and Borel, 1980). Also CsA has little effect on the proliferative responses induced by lymphokines such as IL-2 or IL-4 (Sawada *et al.* 1987; Dumont *et al.* 1990). However, proliferation of T cells stimulated by anti-CD28 antibodies in the presence of PMA is resistant to CsA, indicating not all modes of T-cell activation are susceptible to the suppressive action of CsA (June *et al.* 1987; Lin *et al.* 1991).

Complete inhibition of the mixed lymphocyte reaction (MLR) by CsA has been demonstrated in several species, as well as in humans (Tutschka *et al.* 1982; Leapman *et al.* 1981; Horsburgh *et al.* 1980; Hess and Tutschka, 1980; Keown *et al.* 1981a,b). The generation of cytotoxic T-lymphocyte cells in the MLR is prevented by CsA, but once generated, the CsA has no effect on their cytotoxic activity (Horsburgh *et al.* 1980; Hess and Tutschka, 1980; Keown *et al.* 1981b;

Hess *et al.* 1982b; Bunjes *et al.* 1982). Therefore, theoretically CsA might be expected to be less effective in preventing acute graft rejection in sensitised animals (Homan *et al.* 1980a; Gratwohl *et al.* 1981). Although CsA does not inhibit the secondary MLR response or the generation of cytotoxic T lymphocytes in such a secondary reaction (Leapman *et al.* 1981; Huss and Tutschka, 1980), it does inhibit IL-2 production significantly (Hess *et al.* 1982a; Andrus and Lafferty, 1981), suggesting that CsA could have some efficacy in sensitised recipients in the treatment of ongoing rejection.

The immunosuppressive effect of CsA is considered to result primarily from interference with normal T-cell function. The predominant action of CsA is directed against T-helper lymphocytes (Borel *et al.* 1976; Borel, 1981; Burckhardt and Guggenheim, 1979; Gorden and Singer, 1979; Kunkle and Klaus, 1980; Cammisuli, 1982; Kahan *et al.* 1982). This effect on T-helper lymphocytes inhibits the production of the lymphokines, especially IL-2 previously known as (T cell growth factor) (Larsson, 1980; Palacios and Moller, 1981; Bunjes *et al.* 1982; Lafferty *et al.* 1983). In this way, the generation of cytotoxic T-cells from cytotoxic T-cell precursor is prevented. It is well known one of the principal ways by which CsA exerts its immuosuppressive action is by inhibiting the expression of discrete set of lymphokine genes, including IL-2, IL-3, IL-4, interferon (IFN)-γ, tumour necrosis factor (TNF)-α and others (Kronke *et al.* 1984; Emmel *et al.* 1989). This phenomenon has been demonstrated for CsA, primarily for IL-2 gene, in heterogeneous T-cell populations inhibiting mRNA expression (Kronke *et al.*

1984) and on cloned T-cell lines (Herold *et al.* 1986), and by in situ hybridisation (Granelli, 1988). Furthermore, nuclear run-on transcription studies reveal that both CsA and FK-506 directly interfere with transcription of the IL-2 gene (Tocci *et al.* 1989). That inhibition of IL-2 secretion is crucial for the blockade of T-cell activation is supported by experiments in which the inhibitory effect of CsA was partially counteracted by adding exogenous IL-2 to T cell cultures (Lin *et al.* 1991; Hess, 1985). This finding suggests that with the exception of IL-2 or similar growth factors, other gene products and pathways critical for cell cycle progression remain intact in cells treated with this immunosuppressive agent.

The question whether CsA inhibits expression of cell surface IL-2R has been controversial. Miyawaki *et al.* (1983) demonstrated that CsA did not prevent the expression of Tac antigen on mitogen-stimulated human T cells. Other studies reported that CsA inhibited the expression of IL-2R on stimulated lymphocytes (Bettens *et al.* 1985; Weir *et al.* 1991; Li *et al.* 1992).

1.5.3.2. Molecular mechanism

CsA inhibits T lymphocytes by binding to a family of intra-cellular proteins, termed cyclophilins (CyPs); most of the drug binds to CyPA, 17 kD (Walsh *et al.* 1992; Rosen and Schreiber, 1992). This cytosolic binding protein for CsA was first isolated in 1984 from lymphocytes by Handschumacher *et al.* and dubbed CyP for its high affinity for the drug (Handschumacher *et al.* 1984). It is a highly basic (pI > 9.0) and abundant protein, present in many tissues. When CyP was

sequenced, it was found to be unique among known proteins, giving clues as to its possible physiological function.

In 1989, two groups (Fisher *et al.* 1989; Takahashi *et al.* 1989) discovered that CyP is actually the enzyme peptidyl prolyl cis-trans isomerase (or rotamase). Furthermore, CsA was a potent inhibitor of rotamase activity. First discovered in 1989, rotamase accelerates the interconversion of cis to trans rotamers of proline-containing peptides or proteins, believed to be a rate limiting step in protein folding (Fisher and Schmid 1990). A hypothesis emerged that proline cis-trans isomerization (thereby rotamase activity) may be involved in signal transduction and that the inhibition of this enzymatic activity may be the key to the mechanism of immunosuppression (Fisher *et al.* 1989; Takahashi *et al.* 1989).

The CyPs belong to a larger family of immunophilins (proteins that bind immunosuppressive agents), FK binding protein (FKBP) being another member of that family to which FK-506 and rapamycin bind. The predominant cytoplasmic FKBP is molecular weight of 12 kD and is thus termed FkBP12 (Harding *et al.* 1989; Siekierka, *et al.* 1989). FKBP was identified independently by two groups (Harding *et al.* 1989; Siekierka *et al.* 1989), who showed that although it has no sequence homology with CyP, FKBP also possesses rotamase activity that is potentially inhibited by FK-506 but not by CsA. This finding lent further support for the hypothesis that rotamase activity may be involved in signal transduction and its inhibition might be a convergent step in immunosuppression.

At the cellular level, it was noted that there was a discrepancy between low concentrations of the drugs required to inhibit signal transduction and the concentrations of rotamases present in the cell. Thus, at the optimal level of either drug, only a fraction of corresponding rotamases in the cell was inhibited (Schreiber, 1991; Mckoen, 1991; Schreiber and Crabtree, 1992; Rosen and Schreiber, 1992; Walsh *et al.* 1992; Sigal and Dumont, 1992; Heitman *et al.* 1992). Neither CsA nor FK-506 could have worked if the rotamase activity of either immunophilin was involved in signal transmission. In addition, non-immunosuppressive analogues of CsA, FK-506 and rapamycin have been synthesised that were able to inhibit the rotamase activity of CyP and FKBP respectively (Bierer *et al.* 1990a; Sigal *et al.* 1991; Dumont *et al.* 1992). Like FK-506, rapamycin also bound to FKBP with high affinity and inhibited the rotamase activity of FKBP12 (Bierer *et al.* 1990b). Despite inhibition of rotamase activity, rapamycin was unable to inhibit Cytokine gene transcription (Dumont *et al.* 1990; Bierer *et al.* 1990b). Taken together, these several lines of evidence have led to the conclusion that inhibition of rotamase activity was insufficient to explain drug action and exclude the possibility that inhibition of rotamase activity alone was sufficient for T cell inhibition.

Two models were proposed to explain these conflicting data. The so-called complex model (Bierer *et al.* 1990a; Bierer *et al.* 1990b; Tropschurg *et al.* 1989) assumed a key role for the complexes formed between the immunosuppressants and immunophilins. Thus CyP and FKBP would not be a target of

immunosuppressants, but they would be necessary to mediate the action of these drugs. The second model is identical to the original rotamase hypothesis except that it assumes that the true target are not the abundant isoforms CyPA and FKBP12, but less abundant isoforms with a higher affinity for each of the drugs (Sigal *et al.* 1991).

CALCINEURIN.

The prediction of the complex model is that complexes formed between the drug and its cognate will interact with other molecular target(s) within the cell that may be directly or indirectly responsible for inhibition of signal transduction. The model received support with the findings of (Liu *et al.* 1991; Freidman and Wiessman, 1991), who showed that the complexes of CyPA with CsA or FKBP12 bound to FK-506, when linked to a solid phase, could bind to the same set of proteins in a calcium dependent fashion. These putative target proteins were identified as calmodulin and the catalytic (A) and regulatory (B) subunits of calcineurin (Klee *et al.* 1988). Calcineurin A is 59 kD catalytic subunit, and has an autoinhibitory domain (AID) that ordinarily blocks the phosphatase enzymatic site completely. Calcineurin A has a binding site for calmodulin and for the calcineurin B, 19 kD, regulatory subunit (Cohen, 1989; Stemmer and Klee, 1991). In the presence of calcium, calmodulin and calcineurin B regulatory subunit are activated, then bind to calcineurin A removing the AID from the phosphatase active site and triggering serine phosphatase activity and thus calcineurin is

activated (Griffith *et al.* 1995; Kissinger *et al.* 1995). This perhaps explains the calcium requirements of CsA- and FK506-sensitive pathways.

The complexes of CyP-CsA and FKBP-FK506 inhibited *in vitro* the phosphatase activity of calcineurin (Liu *et al.* 1991). Treatment of Jurkat cells *in vitro* with either CsA or FK506 caused inhibition of calcineurin via complexation with endogenous immunophilins (Fruman *et al.* 1992b). Moreover, concentrations of both CsA and FK506 required to inhibit calcineurin are very similar to those needed to inhibit IL-2 production in activated T-cells, supporting the notion that calcineurin may be the target for both drugs. This was the first evidence supporting the hypothesis that this phosphatase is an important signalling compound in cells activated by the TCR-CD3 complex. These results have now been extended to demonstrate that calcineurin is an essential intermediate in the induction of programmed cell death, or apoptosis (Fruman *et al.* 1992a), and in degranulation, measured by serine esterase release, of cytotoxic T lymphocytes and mast cells (Dutz *et al.* 1993).

Other groups have used different approaches to confirm the role of calcineurin in T-cell activation. In pharmacological studies, various chemical analogs of CsA or FK506 have been tested for their ability to inhibit calcineurin activity *in vitro* in the presence of the appropriate immunophilin (Liu *et al.* 1992; Nelson *et al.* 1993). There was a strict correlation between calcineurin inhibition and immunosuppressive potency of the analogs, as assessed by inhibition of reporter

gene transcription in Jurkat T cells stably transfected with IL-2 promoter-reporter constructs (Liu *et al.* 1992).

Other investigators have employed genetic over-expression studies to analyze the role of calcineurin in T-cell activation pathways (Clipstone and Crabtree, 1992; O'keefe *et al.* 1992). In Jurkat cells, transfection of a cDNA encoding calcineurin resulted in over-expression of this phosphatase, the cells became more sensitive to stimulation from the TCR, and there was concomitant decrease in sensitivity of the transfectants to CsA and FK506 (Clipstone and Crabtree, 1992; O'Keefe *et al.* 1992). Using a truncated, constitutively active form of calcineurin, it was shown that PMA, which activates PKC, could on its own stimulate IL-2 transcription, eliminating the requirement of calcium (O'Keefe *et al.* 1992). These results clearly demonstrated that calcineurin is a rate limiting signal-transducing molecule and has been confirmed in another study (Batiuk *et al.* 1997). Uniquely calcium- and calmodulin- dependent, the protein phosphatase calcineurin is well positioned to receive the signal from the TCR in the form of the second messenger calcium. Recently, an *in vivo* study has shown that the current clinically used concentration of CsA inhibits 50% of calcineurin activity as measured in the peripheral blood leukocytes of renal transplant patients (Batiuk *et al.* 1995a). This explains the safety of calcineurin inhibition: it leaves sufficient activity to mount immune response for host defence. However, it also explains why the degree of immunosuppression may not adequate as monotherapy in some patients.

NF-AT.

As both CsA and FK506 specifically inhibit the TCR-mediated transcription of the IL-2 gene, its promoter region has become the focus of study in a number of laboratories. Of the many enhancer binding elements present in the IL-2 promoter region, the binding sites for two factors: the nuclear factor of activated T cells (NF-AT) (Emmel *et al.* 1989; Brabletz *et al.* 1991; Granelli-Piperno *et al.* 1990), and the Oct-1/ Oct-1-associated protein site (Ullman *et al.* 1991) were found to be specifically affected by CsA and FK506. The transcriptional activity of NF-κB was found to be inhibited about twofold and certain AP-1 sites three to fourfold, but these effects are quite small compared to the several hundred- or thousand-fold inhibition of properly initiated transcription at the NF-AT site (Emmel *et al.* 1989). In particular, NF-AT is a T-cell-specific transcription factor whose activity correlates with the level of IL-2 transcription after the TCR is activated (Shaw *et al.* 1988).

Further characterisation of NF-AT demonstrated that this factor has two compenents, a nuclear subunit ($NF\text{-}AT_n$) and a cytoplasmic subunit ($NF\text{-}AT_c$) (Flanagen *et al.* 1991). NF-AT has been identified as the well-known transcription factor AP-1 (Jain *et al.* 1992). It is newly synthesised through a CsA/FK506 resistance, PKC dependent sub-pathway mediated by CD28 (Flanagen *et al.* 1991; Jain *et al.* 1992). The pre-existing $NF\text{-}AT_c$ translocates into the nucleus in a calcium dependent fashion to form transcriptionally competent NF-AT (Flanagen

et al. 1991). It is the nuclear translocation of NF-AT_c that is inhibited by CsA and FK506 (Fig. 1).

The existence of a subunit of NF-AT in the cytoplasm offered an attractive connection between calcineurin and IL-2 transcription on the two sides of the nuclear membrane. NF-AT_c (which they termed NF-AT_p, where p stands for pre-existing) is indeed a phosphoprotein in resting T-cells and can be dephosphorylated by calcineurin (McCaffrey *et al.* 1993). They further showed that the dephosphorylation of NF-AT_c by calcineurin in cell lysates could be inhibited by EGTA and a specific peptide inhibitor of calcineurin, as well as by prior treatment of cells with CsA or FK506. These data provide the first evidence that NF-AT_c is a candidate for the direct substrate for calcineurin, and may thus serve as the last protein messenger of a signal transduction cascade to relay from the TCR on the cell surface, through calcineurin in the cytoplasm, to the nucleus (Fig. 2).

In summary, the major action of CsA is the inhibition of the transcription of the genes for IL-2 and other cytokines such as IFN-γ, thus acting at an early phase of T-cell activation. This in turn leads to inhibition of lymphokine-induced signals, such as proliferation and generation of cytotoxic T-cell precursors.

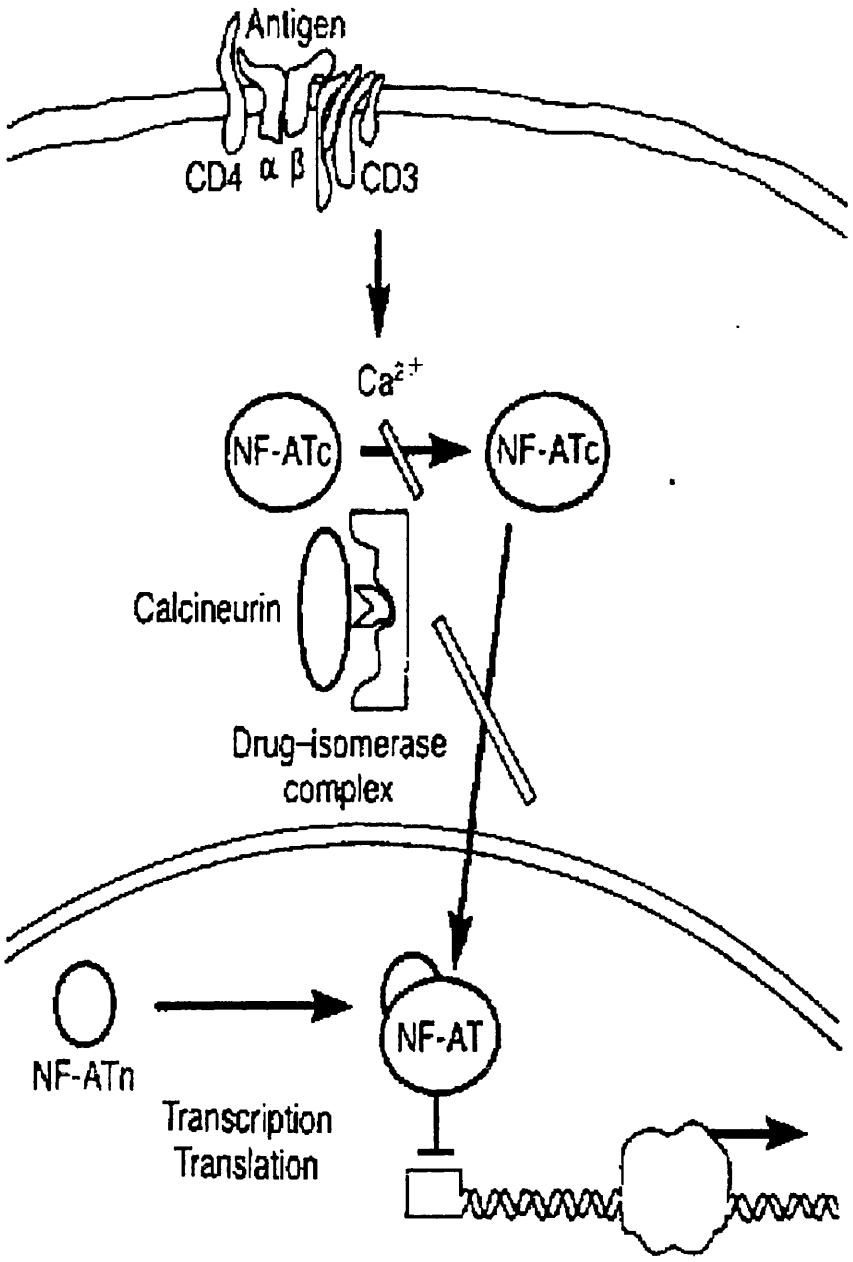

Figure 1. An inhibitory drug-immunophilin complex blocks nuclear translocation of NF-ATc. The nuclear gene shown in the illustration is a representative early gene such as IL-2.

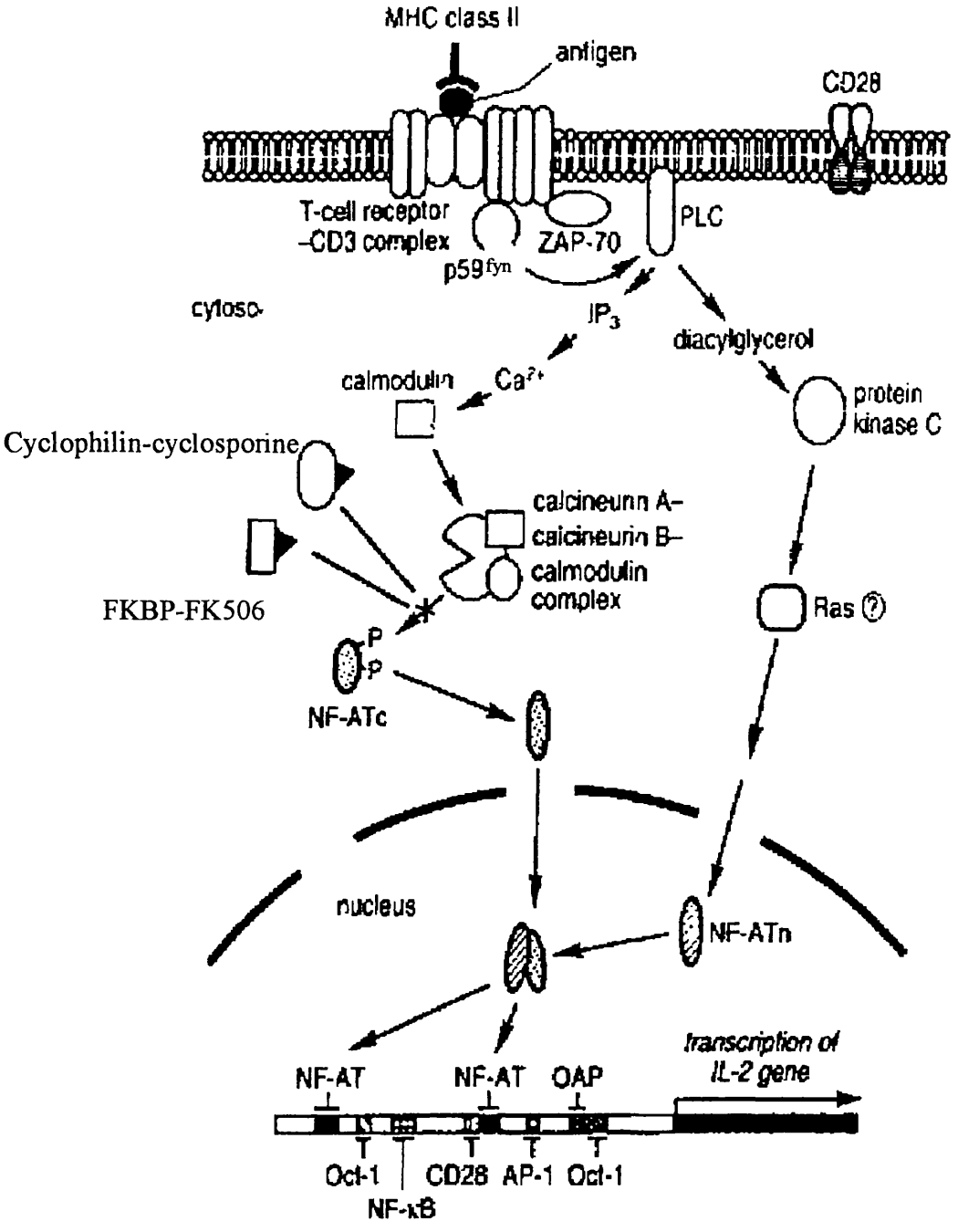

Figure 2. T cell receptor-mediated signal transduction pathway leading to IL-2 transcription. The schematic representation demonstrates the mechanism of action of CsA and FK506.

1.5.4. Pharmacokinetics

Therapeutic drug monitoring of CsA is recommended for three main reasons. Firstly, the bioavailability and metabolism of CsA, and consequently the correlation between dose and concentration varies widely between patients (Bertault-peres *et al.* 1985; Lindberg *et al.* 1986; Ptachcinski *et al.* 1985,1986). Secondly, a relationship between CsA concentrations and clinical events has been demonstrated. Episodes of acute rejection were studied because they indicate insufficient immunosuppression. A decrease in CsA concentration was noticed during a week prior to the diagnosis of acute rejection (Lindholm *et al.* 1990). This indicated that a low CsA concentration might have triggered acute rejection. This assumption was in accordance with other reports of lower area under the curve (AUC) (Kasiske *et al.* 1988a) or lower trough concentrations in rejecting as compared with non-rejecting or toxic patients (Holt *et al.* 1986b; Irschik *et al.* 1984; Klintmalm *et al.* 1985; Rogerson *et al.* 1986). Thirdly, the range between toxic and subtherapeutic concentrations of CsA narrow (Khan and Gravel, 1988; Ptachcinski *et al.* 1986; Shaw *et al.* 1987).

Regarding the large inter-individual variability in the kinetics of CsA, the drug shares this feature with a number of drugs that are metabolised by the cytochrome P-450 iso-enzymes. For such drugs plasma concentrations should predict drug response much better than ingested dose but the formation of active metabolites and inter-individual differences in plasma protein binding may complicate the relationship between plasma/blood concentrations of parent drug and its effects.

Most of the drug is excreted in the bile, only trace amounts being excreted unchanged in the urine. CsA is converted by isoenzymes of the hepatic cytochrome P-450 enzyme superfamily into metabolites with high polarity that retain the cyclic structure (Bertault-Peres *et al.* 1987). Co-administration of drugs that interact with the cytochrome P-450 system may affect CsA metabolism (Kahan and Grevel, 1988). Cytochrome P-450 inhibitors that increase CsA levels include ketoconazole, erythromycin, oral contraceptives, androgens, corticosteroids, and diltiazem. On the other hand, some agents that induce cytochrome P-450 enzymes decrease CsA levels. These include rifampicin, phenobarbitone, phenytoin and isoniazid. Although drug interactions may complicate treatment, no agents are absolutely contraindicated in patients receiving CsA treatment. However, even when clinical judgement is assisted by measurement of circulating levels of the drug, the use of agents affecting CsA metabolism is hazardous, and patients treated with such agents have a higher incidence of allograft rejection or drug-induced toxicity.

Only about 10% of an oral dose is absorbed, and there is considerable variation between individual patients. It is for this reason that Kahan (1985) suggested that CsA pharmacokinetic patterns should be established for individual patients before transplantation. This was supported by other work showing a considerable variation in the pharmacokinetics of CsA as well as circadian variation in the same patient (Ohlman *et al.* 1993). Nevertheless, close monitoring of CsA levels is indicated in the first two weeks after transplantation. Although there is a good

correlation between dosage and levels in individual patients once stability is achieved, there is a poor correlation between dosage and levels between patients. Thus, CsA levels help to determine the appropriate dosage for an individual patient. In general, after a single dose, a peak level is reached within 2-6 hours and trough levels are reached between 12-16 hours.

Since currently used doses of CsA deliver about 50% inhibition of calcineurin (Batiuk *et al.* 1995a), reliable delivery is crucial for predictable immunosuppression. The standard preparation of CsA (Sandimmune), an oily emulsion, was not well absorbed under some circumstances and may have unpredictably left some patients with inadequate levels of calcineurin inhibition. The absorption of Sandimmune is influenced by many factors such as bile flow food intake, or gastrointestinal motility. As a consequence, bioavailability is low and variable. Sandoz has optimised this formulation by the development of a new galenical formulation that incorporates CsA in a microemulsion preconcentrate (Sandimmune Neoral) containing a surfactant, lipophilic and hydrophilic solvents, and Ehanol (Meinzer and Vonderscher, 1993). Pharmacokinetic studies in renal transplant patients demonstrated that microemulsion formulation increases the reliability of CsA absorption (Kahan *et al.* 1994; Kahan *et al.* 1995). It was also confirmed in healthy volunteer studies in which an improvement in the dose-linearity of AUC (Mueller *et al.* 1994) and reduced pharmcokinetic variability (Kovarik *et al.* 1994). Thus, Sandimmune Neoral is associated with an improved

bioavailability and less variability, and represents a clinical advance as compared with the standard formulation.

Most of the CsA in the plasma is associated with lipoproteins of high, low, or very low density (34%, 34% and 10%, respectively) and with chylomicrons (Ryffel *et al.* 1988). Some CsA circulates free unbound in plasma; this fraction does not correlate with the total blood level of CsA or with adverse clinical events (Lindholm *et al.* 1988). Since lipoprotein moieties serve as a reservoir for CsA, probably directly transferring the drug into plasma membranes, lipid binding may buffer its effects.

CsA is almost completely metabolised and less than 1% is eliminated unchanged (Venkataramanan *et al.* 1985). Most of the isolated metabolites are inactive, but some of the major metabolites may be weakly immunosuppressive (Freed *et al.* 1987; Hartman and Jardine, 1987; Hartman *et al.* 1985; Maurer, 1985). In summary, the tremendous intra- and inter-individual variations in absorption, distribution, metabolism and elimination of CsA complicate its safe and effective use.

1.5.5. Monitoring of CsA

Although the measurement of CsA blood or serum levels is not mandatory for the clinical use of CsA, it does provide valuable information in a number of situations, provided that it is remembered that the correlation between high

trough-levels and nephrotoxicity, and low-trough levels and rejection is by no mean an exact one (Holt, 1986; Kahan, 1989). Three methods have been employed to monitor CsA levels in serum or blood: high pressure liquid chromatography (HPLC), radioimmunoassay (RIA) and fluorescence polarisation immunoassay (FPIA). The development of I^{125} labels (Mahoney and Ort, 1985), the availability of monoclonal antibodies for CsA monitoring (Quesniaux *et al.* 1987) and the adaptation of enzyme immunoassay techniques (Oellerich, 1980) allow the parent compound to be measured by all three techniques. Plasma or whole blood may be utilised in the RIA and FPIA, but the binding of CsA to red blood cells (RBCs) is both temperature- and time-dependent (Hows and Smith, 1983; Yatscoff *et al.* 1984). Thus, this influence serum or plasma levels, depending on the time of separation after sampling and the temperature at which it is done. Furthermore, the haematocrit may influence the CsA levels in both plasma and whole blood, although this is of less important with whole blood measurements (Rosano, 1985). However, most centres now use whole blood measurements.

The correlation between CsA levels and either nephrotoxicity or rejection is poor (Holt, 1986). This is apparent from the difficulties in technique just mentioned. However, there is some correlation in that a patient with very high levels in the presence of deteriorating renal function is most likely to be due to CsA nephrotoxicity, whereas conversely, very low levels would suggest that rejection is the cause of deteriorating renal function. Because of all these factors that can

affect CsA levels, it is difficult to obtain appropriate therapeutic CsA levels. However, it is necessary that CsA levels must be considered with other clinical, biochemical and histological information.

1.5.6. Nephropathy

Nephrotoxicity is the most adverse side effect of CsA and is of particular importance in renal transplantation where it has to be distinguished from rejection as cause of renal deterioration. Although nephrotoxicity was not detected in early animal models of transplantation, it became apparent very soon after CsA's initial clinical use (Calne *et al.* 1979). These investigators suggested the use of CsA only in patients whose kidneys were diuresing after transplantation (Calne and White, 1982). Furthermore, nephrotoxicity was demonstrated in animal models using larger doses and more sophisticated evaluation of renal function, and some morphological changes attributed to nephrotoxicity in humans were also observed (Whiting *et al.* 1982). Similarly, renal dysfunction was noted following CsA therapy for polyarthritis (Marbet *et al.* 1980), bone marrow transplantation (Shulman *et al.* 1981), liver transplantation (Iwatsuki *et al.* 1983) and inflammatory eye disease (Palestine *et al.* 1984), suggesting that native kidneys as well as transplanted kidneys are susceptible to its nephrotoxic effects. Shortly thereafter, a chronic irreversible pattern of CsA nephrotoxicity was also recognised in renal transplantation (Klintmalm *et al.* 1984), cardiac transplantation (Myers *et al.* 1984), and therapy for uveitis (Palestine *et al.* 1986).

1.5.6.1. Acute nephrotoxicity

It is evident that CsA causes an intense renal vasoconstriction that is largely reversible with reduction of the dose (Remuzzi and Perico, 1995). In early clinical trials with CsA, a high incidence of oliguric acute renal failure was reported, especially if ischaemia was prolonged (Novick *et al.* 1986). Subsequent clinical trials using lower doses of CsA showed that these problems are dose-related. However, a degree of potentially reversible renal vasoconstriction, with or without an increase in serum creatinine, may occur in all patients who receive effective CsA therapy. Maintenance CsA therapy is associated with transient reductions in renal blood flow and glomerular filtration rate (GFR), both of which correlate with dose and peak CsA levels reached 2 to 4 hours after an oral dose (Ruggenenti *et al.* 1993). The haemodynamic effect of a single dose of CsA is well studied and demonstrated a daily renal hypoperfusion (Perico *et al.* 1992). Discontinuing CsA in stable kidney transplant recipients results in a 30% in improvement in renal hemodynamics and function (Curtis *et al.* 1986). Because acute CsA nephrotoxicity is usually reversible, the important clinical problem in kidney transplantation is to differentiate CsA-induced renal dysfunction from acute rejection. Although there are no specific pathological changes induced acutely by CsA (Mihatsch *et al.* 1995). The absence of cellular or vascular rejection on biopsy suggests acute CsA nephrotoxicity. On the other hand, the presence of acute rejection does not exclude concomitant CsA toxicity.

1.5.6.2. Chronic nephrotoxicity

Although CsA has a major positive impact on renal allograft survival by reducing acute rejection episodes, the half-lives of renal allografts in CsA era are not different from those seen before CsA was available (Cecka and Terasaki, 1994). One possible explanation is that CsA itself causes a chronic lesion similar histologically to chronic rejection, and may lead to chronic allograft dysfunction and ultimate graft loss. Progressive renal failure has been well documented in patients with renal and non-renal transplants and autoimmune diseases, and in experimental animals on CsA (Myers *et al.* 1984). Patients exposed to high blood concentrations of CsA for sustained periods of time may exhibit the changes of long-term CsA nephrotoxicity (Feutren and Mihatsch, 1992). These changes are largely preventable by adjusting CsA dosage early after transplant surgery to the currently accepted target blood concentration ranges (Feutren and Mihatsch, 1992; Morozumi *et al.* 1992). These changes were seen in patients exposed to the high-dose CsA regimens in use when CsA was first introduced into clincal practice, but are less frequently seen in patients receiving the current low-dose CsA-based immunosuppression regimens with dosage guided by therapeutic drug monitoring (Feutren and Mihatsch, 1992). Although excessive initial doses of CsA are known to contribute to chronic nephrotoxicity, the condition may be progressive despite dose reduction. Histologically, long-term CsA nephrotoxicity is characterised by striped tubulo-interstitial fibrosis, tubular atrophy and afferent arteriolopathy (Myers *et al.* 1984). The characteristic vascular lesion of chronic CsA nephrotoxicity is an obliterative arteriolopathy with degenerative changes in

the walls of afferent arterioles (Mihatsch *et al.* 1995). These changes are not necessarily dose-related and can be noticed with doses of CsA as low as 2 to 4 mg/kg, although tends to occur earlier with higher doses (Myers *et al.* 1988a). Most of the reports concerning long-term CsA nephropathy are based on functional data such as serum creatinine or GFR determinations. These data are insufficient, because serum creatinine and GFR can remain stable for relatively long period of time despite progressive tubulo-interstitial disease.

1.5.6.3. Haemolytic uraemic syndrome

Rarely, vascular lesions similar to those encountered in haemolytic uraemic syndrome (HUS) are seen in patients on CsA therapy. Both de novo and recurrent HUS have been described, presumably initiated by CsA injury to the vascular endothelial cells (Van Buren *et al.* 1985). The lesion is usually irreversible (Sommer et el., 1985) and may lead to graft loss, although few reports have described partial recovery if CsA is discontinued (Wolfe *et al.* 1986).

1.5.6.4. Electrolytes and acid-base abnormalities

CsA may reduce potassium excretion both by decreasing the activity of the renin angiotensin system (RAS) and by impairing tubular response to aldosterone (Kamel *et al.* 1992). *In vitro* studies have shown that CsA inhibits the luminal potassium channel (Tulmin and Sands, 1993). As a result, hyperkalaemia is commonly observed in CsA-treated patients. Hyperkalaemia may be sever if angiotensin converting enzyme (ACE) inhibitor, which reduces aldosterone

release, is also used. A hyperchloraemic metabolic acidosis has been observed, as a result of decreased aldosterone activity and suppression of ammonia synthesis by hyperkalaemia (Stahl *et al.* 1986). In addition, CsA has been shown to decrease uric acid renal clearance with resultant hyperuricaemia and occasionally gout, especially if diuretics are used (Lin *et al.* 1989). A correlation between hypomagnesaemia and CsA blood level has been described (Barton *et al.* 1987).

1.5.6.5. Pathophysiology of CsA nephropathology

Previous studies have shown that CsA decreases renal blood flow as a result of afferent arteriolar vasoconstriction (English *et al.* 1987). The analysis revealed that in the rats there was little structural damage despite considerable reduction in GFR, and tubular function remained well preserved. Sympathetic nerve stimulation, imbalance between vasodilating and vasoconstricting prostaglandins, and increased responsiveness to pressors were shown to be involved in the CsA-induced renal vasoconstriction (Rossi *et al.* 1989; Bobadilla *et al.* 1994). Short-term CsA administration has been shown to increase efferent nerve activity significantly (Scherrer *et al.* 1990). In addition, *in vivo* study on rats revealed that α-adrenergic blockade mitigates CsA-induced vasoconstriction (Auch-Schwelk *et al.* 1994). Earlier studies have shown that a concomitant reduction in prostacyclin and increase in thromboxane synthesis may exacerbate the vasoconstrictive effect of CsA (Stahl *et al.* 1989; Erman *et al.* 1989). The role of endothelin (ET)-1 in short-term CsA nephrotoxicity is well established. The *in vitro* exposure of human endothelial cells to CsA was found to induce ET-1 release (Bunchman *et al.*

1991), and the administration of high doses of CsA to rats increased the circulating ET-1 levels (Lanese *et al.* 1993). Additionally, further study, on patients with solid organ transplantation, showed elevated peak ET-1 levels in association with peak CsA levels (Grieff *et al.* 1993). The administration of an ET-1 receptor antagonist was shown to improve renal haemodynamics (Lanese *et al.* 1993; Hunley *et al.* 1995). These *in vivo* studies have also examined the ability of thromboxane receptor blockers, dopamine, prostaglandin analogs, platelet-activating factor antagonists, and ϖ-3 fatty acids to modify or reverse CsA-induced vasoconstriction. Most of these studies showed partial improvements in renal haemodynamics without complete return to normal values.

Most workers in this field assume that the long-term form of CsA renal injury is a result of sustained renal vasoconstriction. According to this notion impairment in renal blood flow leads to tubulo-interstitial fibrosis. Data to support the role of ischaemia as a stimulus for fibrogenesis has been described (Truong *et al.* 1992). CsA-induced arteriolopathy was proposed to produce a pattern of stripped interstitial fibrosis (Mihatsch *et al.* 1995). Furthermore, other investigators suggested that chronic afferent vascular injury caused by CsA leads to irreversible changes (Myers *et al.* 1988b). However, this mechanism of action was recently studied in animal model of long-term CsA nephrotoxicity produced by salt depletion, there was dissociation between functional and histological changes induced by CsA (Elzinga *et al.* 1993). Although, CsA withdrawal led to improved GFR, tubular atrophy and interstitial fibrosis progressed.

Studies have shown that certain matrix components are elevated with *in vitro* administration of CsA. In cell cultures treated with CsA, collagen type I mRNA and IV are shown to be elevated in murine tubular cells. In addition, collagen type III synthesis is increased in human mesangial cells and renal fibroblast cells treated with CsA, and procollagen α1 (I) mRNA is elevated in the renal cortex of CsA-treated rats (Wolf *et al.* 1990; Ghiggeri *et al.* 1994; Nast *et al.* 1991). Recently, *in vivo* study of the components of the extra-cellular matrix in a salt-depleted rat model of long-term CsA nephropathy, by 28 days, showed a histological lesion of fibrosis similar to the lesion seen in patients on long-term CsA therapy (Shihab *et al.* 1996a). The mRNA expression of the proteoglycans biglycan and decorin, and of type I collagen was elevated, indicating active matrix synthesis, and the glycoproteins tenascin and fibronectin EDA+ were elevated mostly in the vessels and interstitium in a manner characteristic of the histology of CsA nephrotoxicity. Additionally, the study demonstrated a strikingly higher mRNA expression in medulla as compared with cortex for all matrix protein examined. This is in accordance with previous data showing that the early changes of long-term CsA nephropathy are first observed in the medulla (Young *et al.* 1995).

A mononuclear cellular infiltrate occurs early in CsA nephtrotoxicity and is associated with upregulation of the macrophage chemoattractant osteopontin (Young *et al.* 1995). These infiltrate cells are a source of cytokines and other inflammatory mediators. Platelet-derived growth factor (PDGF) was shown to be

present in the arteriolar walls of CsA-treated rats (Shehata *et al.* 1994). Another cytokine, transforming growth factor (TGF)-β, is elevated in human T cells exposed to CsA (Khanna *et al.* 1994) and in the juxtaglomerular cells of CsA-treated rats (Shehata *et al.* 1995). CsA was also shown to enhance TGF-β1 secretion and to upregulate its receptor in activated T lymphocytes (Ahuja *et al.* 1995). TGF-β is implicated in the fibrosis of a number of chronic diseases by directly stimulating the synthesis of individual matrix components and by blocking matrix degradation through its action on proteases and their inhibitors (Border and Noble, 1994). Similarly, *in vivo* studies on CsA treated rats suggested that the fibrosis of long-term CsA nephrotoxicity involves increased expression of TGF-β which results in increasing matrix deposition and reducing matrix degradation (Shihab *et al.* 1996a).

TGF-β is also known to induce ET-1 mRNA expression by vascular endothelial cells (Kurihara *et al.* 1989). Because the structural changes in this chronic model of CsA nephrotoxicity are induced by salt depletion, the RAS is expected to be upregulated and was studied. In CsA-treated renal transplantation patients, there is evidence of juxtaglomerular cell hyperplasia (Bennett and Porter, 1988). In this model, the renal RAS is induced by CsA and plays an important role, not only in glomerular haemodynamics, but also in glomerular growth and sclerosis (Mason *et al.* 1991). The medullary location of Angiotensin II type 1 receptor corresponds to the lesion of CsA nephrotoxicity (Meister *et al.* 1993). In addition, Angiotensin II has been shown, separately from CsA, to induce considerable interstitial

fibrosis (Johnson *et al.* 1992). Further study has suggested that the effect of TGF-β1 on fibrosis in long-term CsA nephrotoxicity is at least partly modulated by Angiotensin II (Shihab *et al.* 1996b). Other mechanisms may also be operating to produce long-term CsA nephropathy.

The discovery CyPA, a 17 kD protein that binds to CsA, led to a better understanding of CsA mediated-events (Liu *et al.* 1991). Since then, additional CsA-binding proteins were identified, namely CyP-B, C and D. CyP-C has been shown to have a restrictive tissue distribution and occurs only in the immune system and the kidney (Friedman and Weissman, 1991). These findings may explain the specificity of CsA organ toxicity, i.e., nephrotoxicity.

1.6. ANTITHYMOCYTE GLOBULIN

The polyclonal antithymocyte globulin (ATG) and antilymphocyte globulin (ALG) have been available for use as immunosuppressive agent in organ transplantation since the late 1960s. They have proved to be effective either as rescue treatment of the first rejection episodes and graft versus host reaction or as prophylactic treatment of rejection (Cosmi, 1988; Najarian *et al.* 1976). These preparations were the first anti-T cell antibody preparations. Although the sera were purified, these polyclonal products resulted in development of antibodies against several cell types, and often depleted patients of granulocytes and platelets as well as peripheral blood lymphocytes. ATG is an equine- or a rabbit-derived, immunoglobulin-containing, lymphocyte-selective immunosuppressive agent. It

exerts its immunosuppressive effects by inhibiting cell-mediated immune responses. This involves the opsonization of antigen-reactive T cells in peripheral blood, followed by lysis and sequestration in the reticuloendothelial system (Krensky and Clayberger, 1994).

Several studies have shown the presence in ATG of specific antibodies directed against functional molecules on T cells (e.g., CD2, CD3, CD4, and CD8), B cells (CD19, CD20, and CD21), and non-lineage-specific markers such as HLA-class I and class II antigens, adhesion molecules (CD11a/CD18), and activation markers (CD25) (Smith *et al.* 1985; Raefsky *et al.* 1986; Bonnefoy-Berard *et al.* 1991; Bonnefoy-Berard *et al.* 1992c).

1.6.1. Lymphocyte Depletion

ATG treatment induces a strong lymphocyte depletion, which persists during the entire treatment period. The number of circulating T cells will gradually increase after the cessation of treatment and reach pre-treatment values within 1 to 6 weeks, with major variability among patients (Bonnefoy-Berard and Revillard, 1996). After the first muromonab-CD3 (OKT3) injection most T cells disappear from peripheral blood rapidly (30-60 minutes). Subsequently, by days 2 to 5 of treatment, small but significant numbers of T cells become detectable (Chatenoud *et al.* 1983). A study comparing OKT3 with ATG-Fresenius in lymphocyte subpopulations showed that during the first week after transplantation, no major differences in CD2, CD4, CD8 counts, although CD3 counts and CD4/CD8 ratios

were significantly lower in OKT3-treated than in ATG-treated patients (Bock *et al.* 1995). It has been shown in cadaveric renal transplant patients, that ATG induced intense depletion of lymphocyte subsets including memory cells, and that T cell populations remained depleted and did not recover at one month post-transplantation (Mestre *et al.* 1999).

1.6.2. Antigenic Modulation

Although antigenic modulation of the TCR complex has not been directly shown as yet during ATG treatment, modulation of specific lymphocyte membrane molecules is likely to occur. In addition to a partial T cell depletion, OKT3 injection induces antigenic modulation of TCR complex. These molecules are reexpressed with in a few hours both *in vitro* when cells are incubated in the absence of OKT3 and *in vivo* when OKT3 serum level decreases because of immunisation or cessation of treatment (Chatenoud *et al.* 1983; Chatenoud *et al.* 1982). Functional evaluation of these antigenically modulated cells from patients receiving OKT3 with no other immunosuppressive drugs has confirmed their total unresponsiveness to mitogens and alloantigens *in vitro*. This correlated with the profound immunosuppression exhibited by these patients.

1.6.3. T cell Activation Mediated In vitro and In vivo

Several studies have shown that *in vitro* ATG obtained from animal species with different cell sources as immunogen, are mitogenic for peripheral blood T lymphocytes in a dose dependent manner with a maximum of proliferation

observed at 72 hours (Grasbeck *et al.* 1963; Bonnefoy-Berard *et al.* 1992b). Furthermore, this activation was shown to be monocyte independent (Bonnefoy-Berard *et al.* 1992b). The studies suggested that ATG stimulation may result from combined effects of different antibodies, including CD3, CD2 and CD28 antibodies, and thus bypass the need for a second signal delivered by accessory cells. This activation was shown to be associated with the production of lymphokines, for instance TNF-α, IL-2 and IFN-γ as well as upregulation of the α chain of the IL-2 receptor (CD25) (Bonnefoy-Berard *et al.* 1992b). However, some ATG may lack CD3 and CD2 specificities and be devoid of mitogenic activity despite their documented immunosuppressive effectiveness in transplantation (Bonnefoy-Berard *et al.* 1991; Bock *et al.* 1995).

Despite the ATG mitogenic activity documented *in vitro*, ATG induces only minor first infusion reactions in the patients. This is in contrast to the activation syndrome follows the first injection in most of the OKT3-treated patients (Chatenoud and Bach, 1993), confirmed with the finding of peaked IL-2 and IFN-γ in the circulation within 4 hours (Chatenoud *et al.* 1990b). Reasons for the difference between ATG and OKT3 in this respect are not clear. They may be related to dosage but also to relative proportions of activating (anti-CD2, -CD3) and blocking (anti-CD4, -CD11a, -CD18, anti-HLA DR) antibodies in ATG.

1.6.4. Interference with Activation Signals

ATG display multiple functional effects on T cells as shown by *in vitro* assays. Precise relevance of such functional activities to the immunosuppessive of antibody treatment remains uncertain. As mentioned early, ATG can activate a large proportion of T cells and trigger their proliferation. *In vivo*, however, the net result is lymphocytopenia rather than lymphocytosis. This paradox may be explained by inhibition of T cell activation at high ATG concentrations and by occurrence of activation-induced cell death after stimulation by ATG.

At supramitogenic concentrations, ATG was shown to inhibit T cell responses in monocyte-dependent (OKT3, lectins) and monocyte-independent (D66 + X11 CD2 pair; phorbol esters plus ionomycin) suggesting that ATG did not only supress cognate interaction or co-stimulatory signals delivered by monocytes but also generated off signals to T cells (Bonnefoy-Berard *et al.* 1992a). Precise analysis of this inhibitory effect showed a post-transcriptional inhibition of CD25 expression by ATG with a dissociation between decreased IFN-γ and unimpaired IL-2 secretion (Bonnefoy-Berard *et al.* 1992a), which is different from the mechanisms of action of other immunosuppressive drugs (e. g., CsA and FK506) (Schreiber and Crabtree, 1992).

An important issue regarding the functional effects of ATG is whether some degree of selectivity toward transplant antigen-specific alloactivated T cells can

be reached. Three mechanisms were considered (1) interference with activation signals, (2) anergy, and (3) clonal deletion.

ATG was shown to inhibit mixed lymphocyte reaction when added at low concentrations *in vitro* (Revillard *et al.* 1970). This inhibition could be attributed to the presence of specific antibodies to CD11a/CD18, CD4, CD45, MHC class II molecules, and other cell surface molecules involved in the generation of co-stimulatory signals.

Clonal anergy can be defined as state of specific unresponsiveness initiated by a first exposure to antigen under conditions which prevent complete activation (Schwartz, 1990), as for instance blockade of co-stimulatory signals such as the CTLA4/CD28 pathway (Tan *et al.* 1993). Mitogenic antilymphocyte antibodies could induce a non-specific state of anergy towards any subsequent activation by specific antigen, and this may contribute to their immunosuppressive activity. Alternatively, they may interfere with activation of transplant specific alloreactive T cell clone and thus contribute to the induction of anergy. Alloactivation of unprimed human T cells in the presence of soluble anti-CD3 antibody was shown to induce a sustained state of anergy (Anasetti *et al.* 1990). This effect was not observed with other antibodies directed against CD2, CD4, CD5, CD8, CD18, and CD28.

Activation-induced cell death appears as a major mechanism of lymphocyte homeostasis, which prevents excessive clonal expansion after antigenic stimulation (Lynch *et al.* 1995). It involves apoptosis of activated T and B cells after interaction between the Fas molecules (Apo-1/CD95) expressed on activated lymphocytes ans its ligand Fas-L expressed by subset of activated T cells. A direct evidence for this mechanism to occur in ATG therapy is still lacking but is possibly involved. Clonal deletion is characterised by apoptosis of antigen-specific T cell clones. This deletion could be achieved by cytotoxic antibodies or immunotoxins targeted to surface molecules exclusively expressed by alloactivated T cells after transplantation *in vivo*.

1.6.5. Other Cellular effects

The effect of ATG may not be restricted to T cells, since ATG reacts strongly with T and B cells, monocytes/macrophages, polymorphonuclears, platelets and endothelial cells (Bonnefoy-Berard *et al.* 1991; Bonnefoy-Berard *et al.* 1992c). This explains that some ATG may induce a dose-related drop in neutrophil and platelet counts during the first days of treatment, and, may cause thrombosis and phlebitis at high concentration of ATG. Unlike other mitogens, ATG was shown not to activate B cells but prevented B cell proliferation and differentiation induced by a variety of stimuli (Bonnefoy-Berard *et al.* 1992c). The absence of B cell response was suggested due to a direct effect of ATG on B cells because fixation of ATG prevented the proliferation of highly purified B cells stimulated by anti-IgM or anti-CD40 plus IL-4 (Bonnefoy-Berard *et al.* 1992c).

ATG is among the most powerful immunosuppressive agents. Unlike corticosteroids, azathioprine, CsA, FK506, and rapamycin, ATG and OKT3 can destroy resting memory T cells, a property which may be advantageous in prophylactic treatments of presensitised organ graft recipients. ATG exerts its immunosuppressive activity mainly through its capacity to induce peripheral lymphocytopenia via various mechanisms including activation-induced cell death. The long-lasting immunosuppressive effect after cessation of treatment is accompanied by a high risk of overimmunosuppression, and increased susceptibility to viral infection and virus-associated lymphoproliferative syndromes. ATG contains a mixture of multiple antibody specificities, which account for complex functional effects.

1.7. T CELL ACTIVATION

1.7.1. Introduction

Triggering the antigen receptor of either T or B cells initiates a sequence of events that activates these cells for immunlogic function (Crabtree, 1989). Over 200 genes were known to be activated or inactivated in the T cells and studies in transgenic mice indicated a considerable portion of the genome might be made transcriptionally active as T lymphocytes pass from a resting dormant state to an immunologically active state in preparation for cell division (Verweij *et al.* 1990). A large number of genes are probably activated to handle the increased demand

for protein synthesis, more rapid rates of metabolism, and the initiation of cell cycle which required that nearly all proteins be doubled prior to cell division.

The net effect of the process of T cell activation is to co-ordinate the actions of many immune cells that do not express specific-antigen receptor yet must respond to specific antigen. This immune regulatory function is exerted through the secretion of cytokines and expression of cell surface molecules that mediate direct intercellular contact with other cell types. The spectrum of lymphokines secreted and thus the type of immune response initiated depends largely on the particular subset of T cell activated.

Signal transduction events leading to function are not the result of a single wave of gene activation events, but rather of a cascade of sequential gene activation. The timing of events during activation very likely has a critical function in co-ordinating the action of other cell type. For example, certain molecules like the chemokine RANTES were known to be produced after entry into S phase and completion of cell division (Schall *et al.* 1990). The study indicated that RANTES might have a stringent requirement for completion of S phase. Furthermore, the late action of RANTES might reflect a local requirement for chemotaxis late in the activation process after cells have lost their homing receptors and migrated to tissues where an immune response is underway.

An aspect of T cell activation that mimics many developmental events is a change of homing receptors during the process. Naïve post-thymic T cells express a lymph node (LN) homing receptor, Mel 14 [leukocyte cell adhesion molecule (LCAM)-1] that directs attachment to the high endothelial venules (HEV) of LNs, and enable them to pass between endothelial cells and gain entry to the paracortical regions of LNs. Some investigators showed that about 3 days after T cell activation in LN, T cells lose Mel 14 by a process that has not been elucidated and accompanied by the appearance of very late activation antigens (Jung *et al.* 1988). These allow the attachment of lymphocytes to capillaries and blood vessels at the site of inflammation. This sequential exchange of homing receptor might be critical to the final outcome of immune response.

The final act of T cells activation appears to involve suicide of mature activated T cells to terminate an immune reaction. Mutations in either the fas ligand, as in *gld* mice, or tumour necrosis (TNF) receptor family molecule fas (CD95), as in *lpr* mice, have shown to cause a syndrome characterised by the accumulation of large numbers of $CD4^{-}CD8^{-}$, TCR^{+}, and $B220^{+}$ T lymphocytes (Watanabe-Fukunaga *et al.* 1992). These mutations were deficient in activation-induced T cell death. The authors concluded that the T cells accumulation reflected a deficiency in activation-induced T cell death, thereby implicating fas in the elimination of these cells and terminating an immune response. Further studies demonstrated that both fas and B-cell lymphoma (Bcl)-2 induced within 24 hours of TCR stimulation, but the cells become sensitive to deletion via fas only several days later (Itoh *et al.*

1993; Ogasawara *et al.* 1993). These reports suggested that Bcl-2 might act to prevent cell death early in the process of T cell activation before the cell has performed its normal function. The information further confirmed that the timing of events during activation very likely has a critical function in co-ordination of immune response.

1.7.2. TCR Signal Transduction

The time that T lymphocytes must interact with antigens before committing to the final response was initially defined from studies in which T cells were exposed to APCs and later the interaction terminated with a monoclonal antibody to the tumour antigen (Lowenthal *et al.* 1985). These studied defined a period of 2 hours for commitment to the process of T cell activation. This period corresponds roughly to the time required for the activation of many of the early genes, about 40 minutes for IL-2 and 30 to 90 minutes for most others. Most of the known immunosuppressants work within this time period. TCR signalling leads to the generation of second messengers that elevate intracellular Ca^{2+} and activate *ras* through PKC activation. These third messengers then give rise to distinct, but connected signalling pathways that culminate in the activation of a group of transcription factors, such as NF-AT, Oct/Oap, AP-1and NF-κB, cyclic AMP response-element binding protein (CREB), and perhaps members of the activating transcription factor (ATF) family (Durand *et al.* 1987; Shaw *et al.* 1988) (Fig. 3). The net effect of this signalling pathway is to activate the cytokines, growth factors and cell-surface molecules that co-ordinate the immune response.

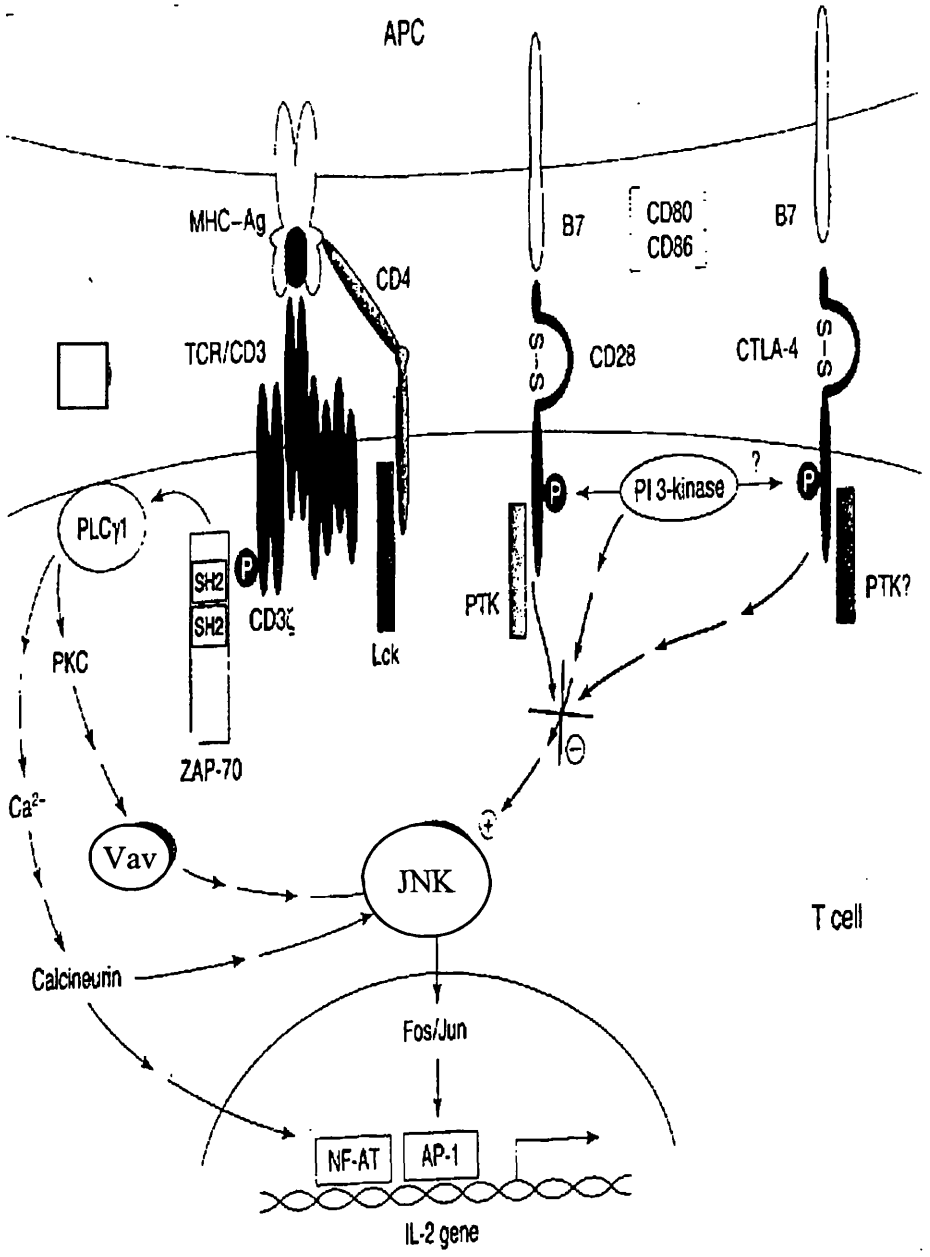

Figure 3. Schematic diagram of the signalling pathways mediated by the T cell receptor complex, CD28 and CTLA-4 after ligation with their respective ligands. TCR and CD28 signals appear to be integrated at the level of JNK. Although the level at which CTLA-4 signalling interferes with T cell activation is unknown, it seems that the effect occurs at some stage of the CD28 pathway.

1.7.2.1. The *ras*-AP-1 pathway

TCR signalling activates *ras* by mechanisms that are not completely understood. The present evidence supports a series of signalling events that are initiated by oligomerization of antigen receptors due to binding of MHC-bound antigen, with activation of the *src*-like tyrosine kinases *lck* and perhaps *fyn* as well as the *syk* family tyrosine kinase *ZAP-70,* reviewed by Weiss and Littmann (1994). TCR ligation is followed by phosphorylation of CD3ζ, perhaps by the *src*-family kinase *lck*. CD3ζ then binds and activates the *syk* family tyrosine kinase *ZAP-70.* Subsequently, phospholipase C (PLC)-γ-1 becomes phosphorylated and activated via tyrosine phosphorylation to convert membrane lipids to second messengers inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). DAG regulates members of the PKC of Ca^{2+}/phospholipid-dependent serine/threonine kinases. PKC has been shown to contribute to the activation of the *ras* pathway by phosphorylation of *ras*GAP (Downward *et al.* 1990). The conclusion was that the latter initiates a complex feedback mechanism that makes dissection of biochemical relationships in the pathway extremely difficult. The way that early events of T cell signal transduction activate *ras* via the PKC-independent pathway is debated, but three mechanism have been proposed and are supported by the following evidence. Phosphorylation of the *vav* rac/rho-like exchange factor indicates that it might have a role as a *ras* exchange factor (Gulbins *et al.* 1993). Second, the linker protein *Shc* has been suggested to directly couple TCR-ζ to the *Sos ras* exchange protein via membrane recruitment of the *Grb-2/Sos* complex (Ravichandran *et al.* 1993). Finally, a 36 kD tyrosine-phoshorylated protein has

been found to coprecipitate with *Sos*, *Grb-2*, and perhaps PLC-γ–1 in T cells (Sieh *et al.* 1994; Buday *et al.* 1994). The latter findings suggested a cell type-specific coupling mechanism to *ras*.

The activation of *ras* probably occurs at a site where the signalling pathways including *ras* pathway, Ca^{2+} dependent pathway and CD28 mediated transduction pathway converge to a single necessary and sufficient step, which, although subject to feedback controls through PKC, is sufficient for activating transcription factors such as AP-1. McCormick (1993) has reviewed the mechanisms controlling ras. The process of ras activation leads to several events in T cells including recruitment of *c-raf* to the cell membrane and activation of IP3 kinase, p70S6 kinase, *jun* N-terminal kinase (JNK), and the mitogen-activated protein (MAP) cascade. The sequence of signalling events that lead to the activation of the transcription factor AP-1 involves either one or both kinase cascades activated by the MAP kinase family members. The well studied MAP kinase cascade initiated by *raf-ras* interactions leads from *ras* to *raf* to MAP kinase-kinase to MAP kinase to the serum response factor, which activates the *c-fos* gene. Alternatively, but not exclusively, *ras* activation can lead to the phosphorylation and activation of JNK. JNK phosphorylates and induces the transcriptional activity of the *jun* transcription protein. These two *ras*-induced events, i.e., induction of *fos* protein and activation of the pre-existing *jun* protein, result in assembly of the AP-1 transcription factor.

1.7.2.2. Ca^{2+} signalling pathway

Understanding the site of action of CsA requires an explanation of Ca^{2+} signalling pathway in T cells. TCR ligation causes activation of PLC-γ-1 via tyrosine kinases, which results in the production of IP3 and DAG by breakdown of the membrane phospholipid phosphatidylinositol 4,5-biphosphate. The IP3 generated interacts with the IP3 receptor in endoplasmic reticulum, resulting in the leakage of Ca^{2+} out of this intracellular compartment (Clapham, 1995). At this point, the mechanisms that generate a sufficient rise in Ca^{2+} from basal levels of about 100 nM to a level of about 1μM to propagate the signalling pathway unclear. Two hypothesises were proposed. Either Ca^{2+} leakage leads to sensing of the loss and induction of a rapid influx of Ca^{2+} from the external media by unknown mechanisms, referred to as capacitive calcium entry (reviewed in Clapham, 1995), likely involving the intracellular calcium-modulating ligand of cyclophylin (CAML) protein, or Ca^{2+} released from internal stores is sufficient for activation (Bram and Crabtree, 1994). Most workers in this field favour the former hypothesis. Regardless of the mechanism by which Ca^{2+} is elevated, the next step in signalling pathway seems clear. The sustained rise in intracellular Ca^{2+} activates the calcium/calmodulin-dependent serine/threonine phosphatase calcineurin (Hubbard and Klee, 1989). The study demonstrated that calcineurin is composed of an A chain, which is a catalytic subunit, and a B chain, which is a Ca^{2+} binding regulatory subunit. CsA and FK506 exert their immunosuppressive action by inhibiting calcineurin phosphatase activity via binding to immunophilins to form inhibitory complexes. The complexes of CyP-CsA and

FKBP-FK506 inhibited, *in vitro*, the phosphatase activity of calcineurin (Liu *et al.* 1991). Treatment of Jurkat cells *in vivo* with either CsA or FK506 caused inhibition of calcineurin via complexation with endogenous immunophilins (Fruman *et al.* 1992b).

The consequence of calcineurin activation is a nuclear translocation of the NF-AT_c (Clipstone and Crabtree, 1992; Flanagan et al 1991), most likely occur by dephosphorylation of NF-AT_c by calcineurin (McCaffrey *et al.* 1993). Once NF-AT_c has translocated to the nucleus it combines with NF-AT_n, bind to consensus sites in several cytokine promoters and initiates gene transcription, including IL-2. The net effect of inhibition of calcineurin by CsA or FK506 is inhibition of the translocation of NF-ATc from the cytoplasm to the nucleus (Flanagen *et al.* 1991) and lack of cytokine gene transcription. The nuclear subunit could be isolated from any tissue if the cells have been stimulated with PMA (Flanagan *et al.* 1991). Furthermore, the study showed that the production of nuclear subunit required protein synthesis and RNA synthesis and hence must require the activation of a new gene, and AP-1 replaced this component both in transfection assays and *in vitro* transcription.

1.7.3. CD28 Signal Transduction

In the last seven years, a massive amount of *in vitro* and *in vivo* data had implicated CD28 as a major costimulatory receptor on T cells, and B7 as its costimulatory ligand on professional antigen presenting cells. The importance of

the CD28-B7 interaction in T cell response has been recently confirmed by analysis of CD28-deficient mice. T cell dependent antibody responses were essentially absent in these mice, as were T cell responses to some, but not all, viruses (Shahinian *et al.* 1993). A subsequent analysis revealed that T cell proliferation in the presence of antigen presenting cells was markedly reduced (Green *et al.* 1994). Together, these data suggest that CD28 is the major, if not the only, receptor for antigen presenting cells-derived signals that costimulate TCR signals and augment proliferation.

Unlike the TCR-induced signalling pathways, the CD28 signal transduction route does not involve the formation of IP3 or translocation of PKC and is resistant to the immunosuppressive effects of CsA (June *et al.* 1990; June *et al.* 1987). Recent evidence suggests that the CD28-induced signal is mediated via phosphoinositide (PI) 3-kinase (Ward *et al.* 1993; Pages *et al.* 1994; Rudd *et al.* 1994). In addition, further study revealed that simultaneous activation of TCR and CD28 results in a synergistic activation of JNK (Su *et al.* 1994). The latter observation indicates that TCR and costimulatory signals become integrated at the level of JNK (Fig. 3).

Human T cells respond to CD28 costimulation by a dramatic enhancement in the induction of IL-2 mRNA. Two mechanisms account for the CD28 co-induced expression of the IL-2 gene. CD28 costimulation has been shown to activate IL-2 gene transcription (Fraser *et al.* 1991; Verweij *et al.* 1991) and to prolong IL-2 mRNA half-life (Lindsten *et al.* 1989; Umlauf *et al.* 1995).

Earlier studies have shown that the immunosuppressive drug CsA drastically inhibits, but does not completely block, IL-2 production following stimulation by anti-CD3 and anti-CD28 antibodies (Thompson *et al.* 1989). The drug-resistant activity can most likely be traced to the CD28 signalling pathway, since stimulation of cells with PMA (to mimic the Ca^{2+} independent aspects of TCR signalling) and anti-CD28 antibody is entirely resistant to CsA (Thompson *et al.* 1989). In this case, IL-2 is produced at significant levels in the continued presence of CsA or FK506. A recent study has shown an alternative pathway for NF-AT activation that is calcineurin independent (Ghosh *et al.* 1996), in addition to the well known Ca^{2+} dependent pathway. Since dephosphorylation of NF-AT appears to be necessary for its activity, a different phosphatase must be involved in this CD28 signalling pathway. They suggested a hypothesis that this different phosphatase, like ceramide-induced phosphatase can lead to NF-AT activation in CsA resistant manner. A candidate second messenger (instead of Ca^{2+}) is ceramide, which has been reported to be involved in CD28 signalling pathway (Chan and Ochi, 1995; Boucher *et al.* 1995).

1.7.4. T Cell Memory

Immunological memory can be defined as the altered response of immune system following re-exposure to the same antigen. In general, the immune response is faster and stronger. The reduced responses seen when anergy or tolerance ensue are not usually considered in the same context.

CD45 has widely used, with the higher molecular weight isoforms defining the pool containing naïve cells and the lower molecular weight isoforms, the pool in which memory cells reside. In humans, naïve cells are held to be CD45RA positive, CD45RO negative, and memory T cells to be CD45RA negative and CD45RO positive (Trowbridge and Thomas, 1994; Bunce and Bell, 1997). The CD45 profile can also change with activation or with cytokine treatment (Bunce and Bell, 1997), so other adhesion molecules such as CD44 and CD62L are used. Naïve cells are CD44 low, and memory T cells are CD44 high. Here again problems arise because the clear pattern seen in C57BL/6 mice is not seen in BALB/c and other strains (Budd *et al.* 1987). Naïve cells are also CD62L high and lose expression of antigen on encountering antigen (Bradley and Duncan, 1991). Naïve cells are thus readily distinguished from effector T cells. Subsequently, some memory cells appear to regain CD62L expression and again the marker does not provide a definitive identification of the memory cell. The best that can be done is to apply multiple criteria to identify memory cells. CD44 high memory cells should also be low in the expression of activation markers such as CD25 and CD69 (Dutton *et al.* 1998).

From polyclonal models, it has been suggested that memory cells are more easily triggered than naïve cells, responding at lower antigen dose (Pihlgren *et al.* 1996), without the stringent requirements for co-stimulation (Mullbacher and Flynn, 1996) that tightly delimit the response of naïve T cells to antigen. Memory cells secrete a wider variety of cytokines than do naïve cells. Naïve CD4 cells produce

only IL-2 and IL-3, and naïve CD8 cells produce only IFN-γ, whereas memory cells can be induced to secrete the whole range of T cell cytokines (Swain, 1994). Moreover, populations of polarised effector cells will, upon adoptive transfer, give rise to resting memory cells that retain the polarised pattern (Swain, 1994). Thus, not only do memory cells secrete a potentially broader range of cytokines upon re-stimulation, they can also be specialised to perform unique functions specified by the patterns of cytokines they secrete. The cytokine production capabilities of memory cells endow them with a broad spectrum of functions that naïve cells cannot emulate. Naïve CD4 T cells are very poor at helping B cells, whereas CD4 memory T cells mediate vigorous B cell response (Swain, 1994; Croft and Swain, 1992). Memory cells also give rise to a second generation of effectors (memory effectors) after re-stimulation with antigen, and this response seems to peak earlier than the response of naïve cells, supporting a further functionally significant difference between naïve and memory cells (Bradley *et al.* 1993). Activated cells and effectors can be induced to undergo rapid apoptosis upon re-stimulation (Zhang *et al.* 1997; Van Parijs *et al.* 1996: Critchfield *et al.* 1994), while naïve cells respond under same conditions by secreting cytokines and proliferating (Croft *et al.* 1992). The susceptibility to rapid activation-induced cell death develops during the generation of effectors (Zhang *et al.* 1997). The susceptibility to rapid activation-induced cell death seems to disappear when CD4 T cells become memory cells (Swain *et al.* 1996).

1.7.5. CD25 and CD69 Activation Markers

The effects of IL-2 on the activation of its target cells are mediated through specific cell surface receptors (IL-2R). IL-2 comprises at least three sub-units encoded by different genes. The first component to be identified, IL-2Rα (CD25) is a 55 kD. The other two sub-units identified are IL-2Rβ and IL-2Rγ. IL-2Rβ is a 75 kD protein encoding a large intra-cytoplasmic domain, whereas IL-2Rγ is a 64 kD protein (reviewed in Theze, 1994). Precise measurements of the binding affinities between IL-2 and human IL-2R sub-units have been performed using plasmon resonance (Balasubramanian *et al.* 1995). The study confirms that CD25 or IL-2Rβ can directly bind IL-2, whereas IL-2Rγ has no measurable affinity for this cytokine. In addition, they demonstrated that CD25, but not IL-2Rγ, interacts with IL-2Rβ to form a stable heterodimer in the absence of ligand. The results suggest a sequential mechanism for the formation of high affinity IL-2R (αβγ) in activated T cells. CD25 is expressed on activated human T cells, B cells, and macrophages (Greene and Leonard, 1986). In IL-2R deficient mouse models, all the chains of IL-2R are required to form a functional receptor. This characteristic is due to the inability of mouse IL-2Rβ chain to directly bind IL-2 in the absence of CD25 (Chastagner et al. 1996). Therefore, in this species, binding of IL-2 is strictly dependent on the induction of CD25; this explains why expression of this chain is highly regulated (Froussard *et al.* 1991).

CD69 is a surface phosphorylated homodimer formed by the association of 28 kD and 32 kD chains, which are held by disulfide bridges. Cloning and expression of

human CD69 gene has revealed that the two chains composed of differentially glycosylated sub-units (Ziegler *et al.* 1993; Hamann *et al.* 1993; Lopez-Cabrera *et al.* 1993). CD69 was initially described as an antigen expressed early in the activation of lymphoid cells (Lanier *et al.* 1988; Hara *et al.* 1986; Cebrian *et al.* 1988). Although resting peripheral blood lymphocytes do not express CD69, it is quickly induced following stimulation of TCR/CD3 complex in T cells; cross-linking of the surface immunoglobulin in B cells; and by IL-2, IFN-α or CD16 stimulation in natural killer cells (Lanier *et al.* 1988; Testi *et al.* 1989b; Risso *et al.* 1989). CD69 surface expression can be detected by 30 minutes (Cebrian *et al.* 1988), reaches peak levels by 18-24 hours (Testi *et al.* 1989b; Mardiney *et al.* 1996), and remained stable throughout the 72-hour culture period (Mardiney *et al.* 1996). Other investigators have reported that after peaking, the CD69 expression declines with a $T_{1/2}$ of 24 hours if the stimulus is withdrawn (Testi *et al.* 1989b).

Initial evidence for the ability of CD69 to generate intracellular signals derived from the observation that anti-CD69 monoclonal antibodies were able to induce lymphocyte proliferation in the presence of PMA (Cebrian *et al.* 1988). Human T cells, B cells and thymocytes, as well as murine T cells, can be induced to proliferate by stimulation of CD69 in the presence of PMA (Yokoyama, 1993, Sanchez-Mateos *et al.* 1989; Testi *et al.* 1989a). Cross-linking of CD69 on activated T lymphocytes was shown to generate extracellular Ca^{+2} influx and, upon simultaneous PKC stimulation, induced the expression of the genes encoding IL-2, IFN-γ (Testi *et al.* 1989a; Nakamura *et al.* 1989) and TNF-α

(Santis *et al.* 1992). It has been shown that CD69 stimulation in the presence of PMA induces transient *c-fos* gene expression and the formation of specific AP-1 DNA-binding complexes in T cells (Tugores *et al.* 1992). Furthermore, it has also been shown that the induction of AP-1 complexes, together with the induction of NF-AT complexes, following CD69 cross-linking in the presence of PMA, participates in the transcriptional regulation of IL-2 gene expression in T lymphocytes (D' Ambrosio *et al.* 1993). Signal generation from CD69 in mature T cells require the expression of the TCR/CD3 complex at the cell surface (Testi *et al.* 1988), suggesting that CD69 signalling may act in conjunction with TCR/CD3 signalling. The wide spectrum of cellular end responses that follow CD69 cross-linking suggests that CD69 molecules may act as common triggers for a variety of haematopoietic cells at different stages of their development. Signals generated through CD69 may be sufficient to act as a co-stimulus that is only required during activation, as observed in lymphoid cells. However, the biological significance of CD69-induced cell activation is still unresolved, and will probably remain elusive until CD69 ligands have been identified.

1.8. T CELL AND B CELL IMMUNE DYSFUNCTION IN CHRONIC RENAL FAILURE

Independently of the cause, end-stage renal disease is associated with a major impairment of both non-specific and specific immunity. This was reported in 1950s, when it was observed that patients with end-stage renal disease exhibited prolonged survival of skin homografts (Dammin *et al.* 1957). Since then, several

reports have confirmed the presence of a clinically relevant immunodeficiency syndrome in patients with end-stage renal disease (reviewed in Descamps-Latscha *et al.* 1994). Such immunodeficiency was well documented by reports indicating that bacterial infections still represent a major cause of morbidity and mortality in end-stage renal disease, (reviewed in Keane and Maddy, 1989;Tolkoff-Rubin and Rubin, 1991; Haag-Weber and Horl, 1993). Likewise, the high susceptibility of uraemic patients to viral infection suggests an underlying immunodeficiency state. The haemodialysis centre has long been recognised as a high risk environment for contamination by hepatitis B virus (HBV) (reviewed in Mioli and La Greca, 1992) and, more recently, hepatitis C virus (HCV) (Jadoul *et al.* 1993) and human immunodeficiency virus (HIV) (Glassock *et al.* 1990; Marcus *et al.* 1991; Tebben *et al.* 1993).

Another clinical observation was cutaneous anergy indicating an abnormal, T cell-mediated, delayed-type hypersensitivity response (reviewed in Chatenoud *et al.* 1990a). Progressive disappearance of established autoimmunity, was well documented in patients having systemic lupus erythematosus with uraemia (Coplon *et al.* 1983). An increased incidence of autoantibodies has also been reported in haemodialysis patients and in patients treated by peritoneal dialysis (Nolph *et al.* 1976; Gagnon *et al.* 1983). Furthermore, the presence of hypersensitivity reactions (Hakim *et al.* 1984) and IgE antibodies to the ethylene oxide used for sterilising dialysis equipment (Rumpf *et al.* 1985; Bommer and Ritz, 1987) is also suggestive of abnormal B cell behaviour in these patients.

1.8.1. T Cells

A paradoxical observation first shown in chronic renal failure was the coexistence of a functional deficiency state with phenotypic signs of T cell activation (Chatenoud *et al.* 1986). This paradoxical observation has been extended to most immunocompetent cells of uraemic patients (Descamps-Latscha, 1994).

1.8.1.1. T cell deficiency

Most reports showed that the number of circulating lymphocytes in uraemic patients is generally reduced, but this abnormality affects both $CD4^+$ and $CD8^+$ T cells so the $CD4^+/CD8^+$ ratio fell within normal range (Raska *et al.* 1983; Castiglione *et al.* 1991; Rabb, *et al.* 1994; Deenitchina *et al.* 1995).

Earlier studies yielded controversial results for T cell function; some reports showed normal proliferative responses to lectins or alloantigens, but others revealed reduced or even enhanced *in vitro* proliferation to the same stimuli (reviewed in Chatenoud *et al.* 1990a). Later, T cells functions have been studied in a greater detail in chronic renal failure and dialysis, and clearly demonstrated that T cells exhibit impaired proliferative responses to most mitogens or allogeneic lymphocytes (Shasha *et al.* 1988; Swirski *et al.* 1996). The defective proliferation of T cells in uraemia is associated with abnormally low IL-2 and IFN-γ production, and enhanced expression of IL-2 receptor (Dumann *et al.* 1990; Meuer *et al.* 1987; Descamps-Latscha, 1993). Study of mRNA expression of IL-2

and IFN-γ revealed low mRNA expression, more pronounced in haemodialysis patients (Gerez *et al.* 1991).

Regarding the underlying mechanisms of T-cell deficiency, some studies showed that the deficiency is more marked with autologous serum, and that uraemic serum highly reduced the proliferative response of normal T cells (Newberry *et al.* 1971; Girndt *et al.* 1993). These observations suggested the involvement of circulating inhibitory substances. The responsible toxins included the guanidine derivatives, notably methylguanidine, low-density lipoproteins, other middle-sized molecules and prostaglandin E_2 (Modai *et al.* 1990; Kamata *et al.* 1983; Barsotti *et al.* 1975; Chouaib *et al.* 1988). They concluded there was a high possibility that these toxins could play a significant role in the T cell defect (Vanholder *et al.* 1994). There is also evidence that parathyroid hormone might suppress T cell function in early phases of hyperparathyroidism (Massry *et al.* 1994).

Several studies showed the role of monocyte in impaired uraemic T cell function. Earlier studies on the function of different cell populations, demonstrated that the *in vitro* abnormalities in peripheral blood mononuclear cells (PBMCs) of haemodialysis patients were normalised by combination of the patient's T cells with monocytes from healthy control persons. On the other hand, normal T cells showed less proliferation and IL-2 secretion in the presence of monocytes from renal failure patients on haemodialysis (Meuer *et al.* 1987). T cell dysfunction

could be mediated directly via impaired antigen processing in monocytes, and altered antigen presentation to T cell (Ruiz *et al.* 1990) and/or, indirectly, because of defective delivery of costimulatory signals, to the T cell such as monocyte derived cytokines (Dinarello, 1992) and monocyte surface molecule (B7) (Matthias *et al.* 1993). The demonstration that blockade of T cell activation was observed in monocyte-dependent stimulation (which is the case for all antigens, most mitogens and anti-CD3 antibody), but not in monocyte-independent stimulation (by anti-CD2 antibody), strongly supports the possibility that monocytes contribute to T cell deficiency (Meuer *et al.* 1987).

A downregulation of the TCR/CD3 complex by uraemic milieu has been proposed (Stachowski *et al.* 1991). The study showed that the density of TCR alpha/beta (TCR-1) and CD3 molecule density on uraemic $CD4^+$ T cells was related to the vigour of the T cell proliferative response induced by anti-CD3 monoclonal antibodies. Furthermore, the percentage of TCR-1 and CD3 positive cells of $CD4^+$ T cells was the same in controls and patients with end stage renal failure, but the TCR/CD3 receptor density was lower in uraemic $CD4^+$ T cells. Also, the study demonstrated that incubation of T cells for 24 hours with uraemic serum lowered TCR/CD3 receptor density on normal and uraemic $CD4^+$ T cells, and showed a positive correlation between TCR/CD3 receptor density and anti-CD3 induced lymphocyte proliferation. These results support the hypothesis that the impaired T cell response to antigen in uraemia is due to downregulation of TCR/CD3 complex by the uraemic environment.

Alteration in the expression of TCR variable (V) beta chain, which can affect the development of tolerance, autoimmunity and the response to external agents, has been shown in haemodialysis patients (Sunder-Plassmann *et al.* 1992). A great increase in the TCR V beta 6.7 chain positive T cells and a massive deletion of TCR V beta 8 chain were observed. The conclusion was responses to infectious agents or uraemic toxins may contribute to these T cell dysfunction mechanisms in uraemia.

There is a hypothesis that blood transfusion and/or subsequent iron overload may exert a suppressive effect on T cell functions (Klatzmann *et al.* 1983; Gafter *et al.* 1992; Keown and Descamps, 1979; Keown *et al.* 1984; De sousa, 1989). The wide spread use of erythropoietin therapy with the reduction of the frequency of blood transfusion has shown to improve T cell functions (Collart *et al.* 1990; Grimm *et al.* 1990). A possible role of malnutrition in the mechanisms of T cell deficiency was reported (Mattern *et al.* 1982).

1.8.1.2. T cell activation

Despite the deficient responses to most pathogens and mitogens, T cells from end stage renal disease and chronic haemodialysis patients showed clear-cut signs of activation. Study of T cell activation process revealed increased numbers of cells expressing low affinity IL-2R (CD25 activation marker) (Beaurain *et al.* 1989). Furthermore, other reports demonstrated a higher density of IL-2R at cell surface, and increased levels of soluble IL-2R and IL-2 in the circulation (Di Stefano *et al.*

1990; Walz *et al.* 1990; Nassberger *et al.* 1992; Caruana *et al.* 1992), as well as increased proportion of a subset of activated T cells (TCR αβ) expressing HLA-DR antigens (Deenitchina *et al.* 1995). In a study of large cohort of chronic renal failure patients, found that the plasma level of CD25 molecule increases with the progression of renal insufficiency in undialyzed patients, being negatively correlated with creatinine clearance levels. In addition, CD25 level was significantly higher in haemodialysis patients especially after dialysis session than in chronic ambulatory peritoneal dialysis (CAPD) and undialyzed patients (Decamps-Latscha *et al.* 1995). However, there are discrepancies between studies in the expression of T cell activation markers. In 1994, a report studying the responses to a panel of activation markers of T cells including CD25, CD38, VLA, CD71 (transferrin receptor) and HLA-DR as well as surface antigens CD3, CD4, CD7 and CD8 by two colour flow cytometry in haemodialysis patients did not confirm that there is an upregulation of activated T cell marker in haemodialysis patients (Rabb *et al.* 1994).

Regarding the role of activated T cell in immunodeficiency state, there was a report suggesting increased IL-2 consumption by its own receptor leading to decreased bioavailability of IL-2 (Chatenoud *et al.* 1990a). A more precise evaluation of T cell function taking into account the T cell subsets on the basis of cytokine profiles, may be required to sort out the importance of cytokine secretion.

The prominent role of dialysis-membrane bioincompatibility in the upregulation of T cell activation using CD25 expression has been shown in a crossover study that compared complement-activating and nonactivating dialysis membranes (Zaoui *et al.* 1991). The study design was a crossover study using cuprophane and polymethylmethacrylate (PMMA) membranes. Chronic dialysis with new cuprophane membrane leads to an increase in baseline expression of IL-2Rα and β in PBMCs. This finding is reversed when patients were dialysed with a PMMA membrane, which is also associated with minimal complement activation. The increased expression of IL-2R sub-units is reproduced *in vitro* by direct contact of PBMCs with cuprophane membrane and by the addition of the anaphylatoxin C5a. Furthermore, it has been shown that a single session of *in vitro* dialysis induced the transcription of mRNA for IL-2R, but not the surface expression of IL-2R or the IL-2 production by normal lymphocytes (Donati *et al.* 1991). These observations were made with both cuprophane and polysulphone membranes that differed in permeability, biocompatibility and their long-term effects on lymphocytes of haemodialysis patients (Degiannis *et al.* 1990), but not sham dialysis (connecting directly the arterial and venous lines to each other without the interposition of the dialyser). The authors suggested that the limited activation events observed with *in vitro* dialysis model might be amplified by repeated exposure. These studies confirm the participation of lymphocytes in the complex blood-membrane interactions that occur during dialysis. It has been reported that haemodialysis induces the production of IL-1 (Haeffner-Cavaillon *et al.* 1989; Dinarello *et al.* 1988). Furthermore, direct *in vitro* exposure of monocytes or

lymphocytes of healthy volunteers to cellulose or cuprophane membrane particles stimulated monocytes to produce more IL-1 and T lymphocytes to produce more IL-2, suggesting a direct effect of membrane materials on monocytes and T cells (Luger *et al.* 1987). Moreover, IL-1 that is well known to increase IL-2 production by activated T cells (Luger *et al.* 1982), may account for additional T cell activation in haemodialysis.

1.8.2. B Cells

During the last years, evidence of a dual state (deficiency and activation) for B cells has also been obtained.

1.8.2.1. B cell deficiency

Although plasma levels of IgG, IgM and IgA are usually in normal range in end stage renal disease patients, specific antibody responses are significantly depressed (Borradori *et al.* 1990; Beaman *et al.* 1989; Paczek *et al.* 1990a). This is most evident when antibody responses to vaccines were tested, especially against T cell dependent antigens such as Haemophilus influenzae (Rautenberg *et al.* 1988) and HBV (Stevens *et al.* 1984; Benhamou *et al.* 1986; Weissman *et al.* 1988). There is also evidence that parathormone has inhibitory effect on B cells (Alexiewicz *et al.* 1990). However, it is not certain whether the defect is intrinsic to B lymphocytes or linked to a defect in T cell-B cell co-operation. Importantly, some reports demonstrated that administration of erythropoietin improved B cell

function (Paczek *et al.* 1990b; Pfaffi *et al.* 1988) and antibody response after hepatitis B vaccination (Sennesael *et al.* 1991).

1.8.2.2. B cell activation

B cell activation in uraemic patients is not as well documented as T cell activation in haemodialysis patients. The observation of elevated plasma levels of the low affinity Fc receptor of IgE (FcεRII or CD23), which is predominantly expressed on activated B cells, was suggestive of an intrinsic B cell activation state (Descamps-latscha *et al.* 1993). Furthermore, in haemodialysis patients, another study showed that the soluble CD23 level was markedly higher than in CAPD and undialyzed patients, and at the end than at the beginning of dialysis session. (Desamps-latscha *et al.* 1995) There was a demonstration that soluble CD23 can be a cofactor in IL-1-induced early human thymocyte maturation and T cell activation (Mossalayi *et al.* 1990).

1.9. AIM OF THE STUDY

Since CsA was introduced into clinical practice in late 1983 to prevent rejection in transplant patients, there has been a rapid growth in the number and types of transplants, the number of transplant centres, an increase in the life expectancy of the transplanted organ, and substantial decreases in the rates of acute rejection and life-threatening infections. Despite these successes, major improvements in immunosuppressive therapy are needed, especially reduction in acute rejection

episodes and toxic side effects, and rigorous definition of the relationship between drug concentration and clinical effects. We still do not know what is a "safe" dose of CsA that is immunologically effective and at same time does not cause progressive long-term nephrotoxicity.

Presently, it is not clear what role long-term CsA treatment plays in the development of chronic graft dysfunction. Clearly, the drug produces deleterious pathological changes in the kidney. Inadequate doses of CsA may actually contribute to progressive graft failure and, alternatively, the renal toxic effects of CsA could coexist with the changes of chronic rejection. Whether long-term immunosuppression with CsA inevitably leads to renal fibrosis is unknown and has not been excluded by any properly controlled study.

1.9.1. Hypothesis

Because of the above-mentioned problems associated with CsA-based immunosupression, we have hypothesised that "activated T lymphocytes are less sensitive to CsA". This may be at least partially, responsible for inadequate immunosuppression and on other hand, the high CsA-dosage required may lead to increase incidence of CsA nephrotoxicity. In this project, we have tested this hypothesis, in normal volunteers and chronic renal failure patients on dialysis mainly by determining *in vitro* CsA sensitivity of peripheral blood lymphocytes (PBLs) of these volunteers. We have also tested the effect of ATG therapy to renal transplant patients on *in vitro* CsA sensitivity of PBLs. This has a very

important clinical implication on the regulation of CsA immunosuppressive therapy. Concomitant use of another immunosuppressive drug targeting towards the activated T cells may give more effective and potent immunosuppression, allows further reduction of CsA dosage, better drug monitoring with good CsA concentration-clinical effects correlation and fewer overt side effects such as nephrotoxicity.

2. MATERIALS AND METHODS

2.1. Volunteers

Four groups of volunteers were included in our study as follows: (1) a control group of ten normal healthy subjects, (2) ten chronic renal failure patients with end stage renal disease on CAPD, (3) twelve chronic renal failure patients with end stage renal disease on maintenance haemodialysis (Appendix 4), and (4) six perioperative live-related renal transplant patients who had one haplotype match with their respective donors, were maintained before transplantation on chronic haemodialysis, and had received one single bolus injection of ATG (Fresenius, Germany) therapy (9 mg/Kg body weight) 1 hour before the operation as part of the immunosuppressive regimen (CsA, azathioprine and steroids).

2.2. Blood Samples

30ml of blood was taken from each HD patients before and immediately after their dialysis session. Similarly, 30 ml of blood was taken from each healthy donors, and CAPD patients. In renal transplant patients at induction stage, 30 ml of blood was taken 2 days pre-, and 3 days and one month post-renal transplantation. The post-transplant blood samples taken from the patients before the morning CsA dose. Samples from all these volunteers were collected in heparinized universal tubes (Sterilin, UK), kindly supplied by renal department of Royal Free Hospital, London.

2.3. CsA

CsA (Sandoz, Basle, Switzerland) was dissolved in 100% ethanol at 500 μg/ ml and further diluted with human AB serum (Quest Biomedical, UK) for the treatment of human peripheral blood mononuclear cells (PBMCs) *in vitro*. A range of CsA concentrations was used in the cultures. CsA was added 1hour before stimulation of PBLs and remained in the cultures for the duration of CsA sensitivity assays.

2.4. Culture Medium

The tissue culture medium used for culture of human PBMCs was RPMI 1640 (Life Technologies, Paisley, Scotland) supplemented with L-glutamine (2mM), 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma, Poole, Dorset, UK). This culture medium is referred to in the text as incubation medium. Laterally, 10% human AB serum (Quest Biomedical, UK) was added to the cells containing incubation medium, since the human AB serum contained the appropriate concentrations of CsA.

2.5. CTLL-2

The IL-2 dependent murine cytotoxic cell line (CTLL-2, kindly supplied by tissue typing department of Hammersmith hospital, European Collection of Animal Cell Cultures, Salisbury, UK) was cultured in RPMI 1640 supplemented with sodium bicarbonate (0.24% final concentration), L-glutamine (2mM), penicillin (100

units/ml), streptomycin (100 μg/ml), sodium pyruvate (1mM), (all from Sigma, Poole, Dorset, UK), human recombinant IL-2 (rIL-2, 10 U/ml, Fluka, England) and heat inactivated 10% Foetal Calf's Serum (FCS, Labtech., Sussex, UK). The cells were cultured in 25 cm^2 flasks (Costar, High Wycombe, UK) and subcultured every three days. Prior to use in a CsA sensitivity assay, the CTLL-2 cells were washed four times with RPMI 1640 medium and cultured overnight with normal culture medium, but without added rIL-2.

2.6. Isolation of PBMCs

Mononuclear cells were separated from samples of peripheral blood by ficoll-gradient centrifugation (Lymphoprep, Nycomed Pharma As, Oslo, Norway). Blood from volunteers was double diluted with sterile Dulbecco's Phosphate Buffered Saline (PBS, Pharmacy of Royal Free Hospital), 2 volumes of the diluted blood laid slowly down on 1 volume of lymphoprep and centrifuged at 400 *g* without break for 20 minutes. The interface layer, which contains the PBMCs, was carefully collected and washed once with sterile PBS at 250 *g* for 15 minutes. Two further washes were carried with incubation medium at 150 *g* for 10 minutes. The PBMCs were resuspended in incubation medium and the differential viable lymphocyte counts were quantified after staining of this preparation with 0.2% trypan blue. The cells were used immediately in the intended assay, no cryopreserved cells were used in the analysis of the groups studied.

2.7. CsA Sensitivity Assay Using CTLL-2 as Detector of IL-2

The read out of this assay was IL-2 production by PBLs in response to PHA (Sigma, Poole, Dorset, UK) in the presence of appropriate concentrations of CsA. The IL-2 production was measured by the addition of CTLL-2. This indicator cell line has been shown to proliferate only in the presence of human or murine IL-2 and murine IL-4 (Gillis and Smith, 1977).

1 X 10^5 viable PBMCs per 90 µl incubation medium per well were plated in 96-well U-bottom tissue culture microtiter plates (Greiner Labortechnik, UK). For preactivation of T cells before adding CsA, PHA (0.5 µg/ml) was added and the T cell preactivation assay plates were incubated for 2 hours at 37°C in 5% CO_2 humidified incubator. The normal assay without T cell preactivation was also performed at the same time for each patient, in this assay the T cell preactivation step was omitted in order not to activate T cells before adding CsA. The only difference in the CsA sensitivity assay between normal and preactivation was the T cell preactivation step, otherwise, all other steps were identical. 10 µl/well of each CsA concentration dissolved in human AB serum was added in 5 replicates to the plated cells and incubated for 1 hour at 37°C in 5% CO_2 humidified incubator. The final CsA concentration used in the cultures was 0, 10, 50, 125, 250, 500, 1000, and 10000 ng/ml. PHA (0.5 µg/ml) was added and the culture plates were incubated overnight at 37°C in 5% CO_2 humidified incubator.

After approximately 18 hours of incubation, the culture plates were gamma-irradiated at 3360 rads (kindly done by radiotherapy department of Royal Free hospital, London) to prevent proliferation of responder PBLs, and 3 X 10^3 indicator CTLL-2 cells were added to each well to detect IL-2 production. After 8 hours of incubation the plates were labelled with 1 μCi/well of tritiated thymidine (^{3}H-TdR) (Amersham International plc, Amersham, England), and proliferation of CTLL-2 was assessed by ^{3}H-TdR incorporation after a further 18 hours incubation. The cellular ^{3}H-TdR incorporation was assessed by harvesting cells onto glass fibre filters (Titertek, Flow Laboratories, Irvine, Scotland) using micro cell harvester (Flow Laboratories, Irvine, Scotland), and measured in a β-spectrometer (1217 Rack Beta, LKB Wallac, Milton Keynes, UK) after liquid scintillation fluid (OptiScint Hi safe, Fisions-Chemicals, England) was added to the filter discs.

In each culture plate background control wells were included in addition to the positive control (PHA in the absence of CsA). In order to determine background CTLL-2 proliferation 5 replicate background control wells contained PBMCs and indicator CTLL-2 cells without CsA or PHA. The other 5 replicate background control wells contained PBMCs and PHA, but no CsA, nor indicator CTLL-2 cells in order to determine background radioresistant lymphocyte proliferation. The adducted means of background counts per minutes (cpm) were deducted from the mean counts at each CsA concentration, in order to determine the absolute CTLL-2 proliferation (Appendix 1).

In all experiments CTLL-2 proliferation to a range of rIL-2 concentrations was measured, to ensure that the CTLL-2 cells gave a dose-dependent response to IL-2. The CTLL-2 cells were plated out at 3 X 10^3 cells per well. Various concentrations of rIL-2 were added to the culture wells, such that the final concentration of rIL-2 varied between 0 and 2 U/ml. The plate was incubated for 8 hours and labelled with 1 μCi/well of ^{3}H-TdR. Proliferation of CTLL-2 cells was assessed by ^{3}H-TdR incorporation after a further 18 hours incubation. The assay was carried out in triplicate and the mean of CTLL-2 proliferation was determined for each rIL-2 concentration used.

In all experiments the lymphoproliferative response to PHA was measured after 72 hours of culture, to ensure that the PBLs were responsive to PHA stimulation, and only patients who had lymphocyte proliferation exceeded 20 X 10^3 cpm were included. The PBMCs were plated out at 1 X 10^5 cells per well and PHA (0.5μg/ml) was added to positive control wells, negative control wells without PHA stimulation and pre-activated PBMCs wells were included. The assay was incubated for 72 hours and labelled with 1 μCi/well of ^{3}H-TdR. Proliferation of lymphocytes was assessed by ^{3}H-TdR incorporation after a further 18 hours incubation. The assay was carried out in 5 replicates and the mean of lymphoproliferative response was determined.

2.8. Antibodies

All antibodies used were fluorescence labelled. The antibodies were labelled with either fluorescence isothiocynate (FITC), phycoerythrin (R-PE or RD1), phycoerythrin-texas red (ECD) or phycoerythrin-cyanin5 (PE-Cy5 or PC5). Monoclonal antibodies used in our study to detect surface antigens of human PBMCs were:- anti-human CD25-FITC (mouse IgG_1; clone M-A251, Pharminogen, Cambridge Biosciences, Cambridge, UK), anti-CD3-ECD (mouse IgG_{2a}; clone HIT3A, Coulter Corporation, Miami, Florida, USA) used as a marker of human T cells, and anti-human CD69-PE-Cy5 (mouse IgG_{2b}; clone TP1.55.3, Immunotech, A Coulter Company, France). CYTO-STAT tetraCHROME [CD45-FITC/CD4-RD1/CD8-EDC/CD3-PC5] monoclonal antibody (Coulter Corporation, Miami, Florida, USA) was used as a cell surface staining control for set up flow cytometric gating, PMT voltage and compensation. CD25 molecule is a 55 kD glycoprotein that is low affinity IL-2 receptor expressed on activated human T cells, B cells, and macrophages (Greene and Leonard, 1986). CD69 molecule also designated as activation inducer mloecule (AIM) is a phosphorylated disulfide linked 27/33 kD homodimer composed of differentially glycosylated subunits. It is the earliest inducible surface glycoprotein to appear upon activation of T cells, natural killer (NK) cells and B cells. CD69 is undetectable on most circulating PBLs (Cebrian *et al.* 1988). Resting T cells do not express CD69 but its expression may be rapidly induced by triggering of their TCR/CD3 complex. We used the CD69 molecule as an activation marker of T cells. The monoclonal antibody used to stain intracellular IL-2 and its isotype

matched control antibody were both obtained from Pharminogen (Cambridge Biosciences, Cambridge, UK). The anti-cytokine antibody was R-PE-rat anti-human IL-2 (rat IgG_{2a}; clone MQ1-17H12). The isotype matched control antibody used was clone R35-95 (rat IgG_{2a}, PE conjugated).

2.9. Multicolour Staining for Cell Surface Antigens and Intracellular Cytokine (IL-2)

This technique was used to study the effect of the haemodialysis process on T cell activation, and was directed towards the expression of activation markers (CD25, CD69 molecules), intracellular IL-2 positive T cells, and intracellular IL-2 intensity per T cell. The CsA sensitivity before and after the dialysis session was studied by investigating mainly the percentage of reduction of the frequency of IL-2 positive T cells and intracellular IL-2 intensity per T cell. The staining procedure was carried out using CytoStain Kit (Cytofix/Cytoperm Plus [with GolgiPlug] Kit, Pharminogen, San Diego, CA, USA). The components of the kit were; Cytofix/Cytoperm solution, 10X concentrate Perm/Wash solution (were diluted 1:10 with in distilled water prior to use), and GolgiPlug. The GolgiPlug contains Brefeldin A, a protein transport inhibitor that results in accumulation of most intracellular cytokine proteins and thereby enhances cytokine staining signals. The protocol of the technique was followed precisely.

2.9.1. T cell stimulation

PBMCs were isolated from blood of chronic haemodialysis patients both pre- and post-haemodialysis and 1 X 10^5 viable PBMCs per 90 µl incubation medium per well were plated in 96-well U-bottom tissue culture microtiter plates. 10 µl/well of each CsA concentration dissolved in human AB serum were added to the plated cells and incubated for 1 hour at 37°C in 5% CO_2 humidified incubator. The final CsA concentration used in the cultures was 0, 10, 100, and 1000 ng/ml. PHA (0.5 µg/ml) and GolgiPlug (1 µl/ml) were added and the culture plates were incubated overnight at 37°C in 5% CO_2 humidified incubator. Negative control (without PHA stimulation) was included in the assay.

2.9.2. Staining of cell surface antigens

The cells were protected from light throughout staining and storage stages. After 18 hours of initiation of culture, the plated cells in 96-well U-bottom tissue culture microtiter plates were washed twice with (250 µl/well) staining buffer (Dulbecco's PBS from Gibco BRL Paisley Scotland, 1% heat-inactivated FCS, 0.1% (w/v) sodium azide from BDH Laboratory supplies, Poole, UK; pH 7.4-7.6) and pellet by centrifugation at 250 *g* for 5 minutes. 50 µl of staining buffer plus 20µl anti-human CD25-FITC, 10 µl anti-CD3-ECD and 20 µl anti-human CD69-PE-Cy5, were added to the resuspended 10^5 cells/well. For cell surface staining control, an additional cell sample of healthy donor was stained with CYTO-STAT tetraCHrome monoclonal antibody (7 µl). The concentrations of all these monoclonal antibodies were ≤ 0.5µg/well. The cells were incubated for 30

minutes at 4°C, washed twice with staining buffer (250 µl/well) and pellet by centrifugation at 250 *g* for 5 minutes.

2.9.3. Fixation and Permeabilization of cells

The cells were thoroughly resuspended by vortexing and 100 µl per well of Cytofix/Cytoperm solution were added. The cells were incubated for 20 minutes at 4°C, washed twice with 1X Perm/Wash solution (250 µl/well) and pellet by centrifugation at 250 *g* for 5 minutes.

2.9.4. Staining for intracellular IL-2

The fixed permeabilized cells were thoroughly resuspended in 50 µl of Perm/Wash solution containing R-PE-rat anti-human IL-2 (2.5 µl/well) or the isotype matched control antibody (rat IgG_{2a}, PE conjugated). The cells were incubated for 30 minutes in dark at 4°C, washed twice with 1X Perm/Wash solution (250 µl/well) and resuspended in staining buffer prior to flow cytometric analysis.

2.9.5. Four-colour flow cytometry

Cells were analysed within 24 hours by FACScan flow cytometer after acquiring a minimum of 5000 cells (Coulter, EPICS XL-MCL, Luton, UK). The PMT voltage and compensation were set up using the cell surface staining control, and quadrant markers were set up based on unstained cells. Data were analysed by

LANtastic software (Coulter System II, USA). The absolute counts and the percentage of interested T cell populations were calculated and recorded. The intracellular IL-2 in T cell was calculated from median fluorescence intensity. In order to overcome the flow cytometric day to day variation in median fluorescence intensity readings, daily monitoring of the flow cytometry was performed using multi beads (Dako FluoroSpheres, Dako A/S, Denmark). A computer-generated calibration median fluorescence intensity-IL-2 molecules/single $CD3^+$ cell curve was performed each time the flow cytometric analysis was carried out, and the intracellular IL-2 in T cell of each sample was determined (Appendix 3, TallyCAL software, Dako A/S, Denmark).

2.10. IL-2 Measurement by ELISA

The estimation of *in vitro* IL-2 production by an enzyme-linked immunosorbent assay (ELISA) technique was carried out in the haemodialysis patients group, mainly for comparison between pre- and post-haemodialysis levels of IL-2 production and sensitivity to CsA. PBMCs were isolated from both pre- and post-haemodialysis blood and 1 X 10^6 viable PBMCs per 180 µl incubation medium per well were plated in 96-well U-bottom tissue culture microtiter plates. 20 µl/well of each CsA concentration dissolved in human AB serum were added in duplicate to the plated cells and incubated for 1 hour at 37°C in 5% CO_2 humidified incubator. The final CsA concentration used in the cultures was 0, 10, 100, 500, and 1000, ng/ml. PHA (0.5 µg/ml) was added and the culture plates

were incubated overnight at 37°C in 5% CO_2 humidified incubator. A negative control (without PHA stimulation) was included in the assay.

After 24 hours of initiation of culture, the culture supernatants were harvested and stored at –70°C until IL-2 was measured. The supernatants were assayed for IL-2 by ELISA test kits (Genzyme Corporation, Cambridge, MA, USA). The protocol of the kit was followed precisely. 100 µl of each requested standard concentration and culture supernatant samples were plated in the appropriate test wells in duplicate in the kit's IL-2 microtiter plate, and incubated at 37°C for 60 minutes. The test wells were washed 5 times with the wash reagent (50 ml wash concentrate diluted with 950 ml distilled water) using an automated washer (Novapath Washer, BIO-RAD, Brussels, Belgium). 100 µl of IL-2 biotinylated antibody were added into each test well and incubated at 37°C for 60 minutes. After washing 5 times the test wells, 100 µl of streptavidin reagent were added into each test well and incubated at 37°C for 15 minutes. A further washing step (5 times) was carried out, and 100 µl of the freshly prepared working substrate (combined equal volumes of substrate A with substrate B) were added into each test well and incubated at room temperature for 10 minutes. The enzymatic reaction was terminated by the added 100 µl of stop solution into each test well. The absorbance was read at 450 nm using (Model 3550 Microplate Reader, BIO-RAD, Brussels, Belgium).

2.11. Statistical Analysis

2.11.1. CsA sensitivity assay using CTLL-2 cells

Dose-response curves were generated using a one-site competition model for non-linear regression (Scientific Graphic Software, Jandel Sigma Plot, CA, USA) for every individual involved in the study (Appendix 1). The 50% inhibitory concentration (IC_{50}) of CsA points were interpolated from the computer generated curves. CsA IC_{50} is the concentration of CsA causing 50% inhibition of IL-2 production. The IC_{50} of the patient groups were compared with the control group using the unpaired student t test. The IC_{50} of preactivation assays were compared with those of the normal assay of the same group, those of the post- were compared with those of the pre-haemodialysis and those of post- transplant after prophylactic single ATG bolus were compared with those of the pre-transplant of the same patients. Paired student t tests were used for these intra-group comparisons (Appendix 2). The difference was considered significant at $p < 0.05$.

2.11.2. Four-colour flow cytometric analysis

The numbers of intracellular IL-2 molecules per $CD3^+$ cells were compared post- and pre-haemodialysis using the paired student t test. The data analysis of percentage of CsA reduction of frequency of $CD3^+$ cells positive for IL-2 and CsA inhibition of intracellular IL-2 production in T cells for comparison between post- and pre-haemodialysis were also done using the paired student t test.

2.11.3. IL-2 measurement by ELISA

In order to determine the IL-2 concentrations by ELISA, the computer-generated standard curve was determined each time the assay was performed (BIO-RAD Microplate Manager, Brussels, Belgium). Then, the corrected absorbency and from which IL-2 concentration were calculated for each sample also by the computer analysis. Comparisons between the post- and the pre-haemodialysis IL-2 concentrations in cell culture supernatants and between post- and pre-haemodialysis in CsA inhibition of IL-2 secretion in cell culture supernatants were performed using the paired student t test.

For all the chapters, Sigma Plot© for Windows was used to present the data graphically. The values in the figures and tables are expressed as mean ±. standard error of mean (SEM). The differences were considered significant when $p < 0.05$.

3. VALIDATION OF THE ASSAYS

3.1. Introduction

It was essential for the reliability of the assays used in this study to be validated and standardised. For this reason, the critical steps in the assays were evaluated. We therefore determined the best gamma radiation dose and incubation period of PBMCs to give the most effective inhibition of lymphocyte proliferation, aiming at minimising the background ^{3}H-TdR incorporation due to lymphocyte proliferation. Since we used CTLL-2 as a detector of IL-2, it was essential to make sure dose-dependent CTLL-2 proliferation to IL-2 and that CsA did not interfere with CTLL-2 dose-response to IL-2. We determined the effect of centrifuge speed on CsA sensitivity of lymphocytes and selected the lowest centrifuge speed (low speed) compatible with a minimum disturbance in CsA sensitivity. To avoid any alteration in CsA sensitivity, fresh rather than cryopreserved PBMC cells were used. Regarding flow cytometry analysis, we investigated the incubation periods of cell cultures to give maximum expression of activation marker. The validation of these critical steps will be explained in detail in this chapter. We assumed this standardisation is very essential for the data to be reliable.

3.2. Standardization of CsA Sensitivity Assay

3.2.1. Proliferative response of CTLL-2 cells to IL2

In all experiments the CTLL-2 proliferative response to a range of rIL-2 concentrations was measured, to ensure that the CTLL-2 cells gave a dose-dependent response to IL-2. The procedure was carried out as mentioned (Materials and Methods chapter). The CTLL-2 cells proliferated to rIL-2 in a dose-dependent manner, with significant proliferation above background control levels occurring at a rIL-2 concentration of 0.0125 U/ml (Fig. 4). In order to demonstrate the response of CTLL-2 cells to IL-2, the cells were exposed to a range of rIL-2 concentrations in each experiment.

3.2.2. CTLL-2 cells response to IL-2 in the presence of CsA

Since CsA is an immunosuppressive agent that inhibits T cell activation, it was necessary to find out whether CsA interfered with the dose-dependent response of CTLL-2 cells to IL-2. The CTLL-2 cells were plated out at 3 X 10^3 cells per well. Various concentrations of CsA were added to the culture wells in triplicate, such that the final concentrations of CsA were in the range of 0, 125, 250, 500, 1000, and 10000 ng/ml. The assay was incubated for 1 hour and then rIL-2 (2 U/ml) was added to culture wells. The assay was incubated for further 8 hours and then labelled with 1 μCi/well of ^{3}H-TdR. Proliferation of CTLL-2 cells was assessed by ^{3}H-TdR incorporation after 18 hours of incubation. The assay was done six

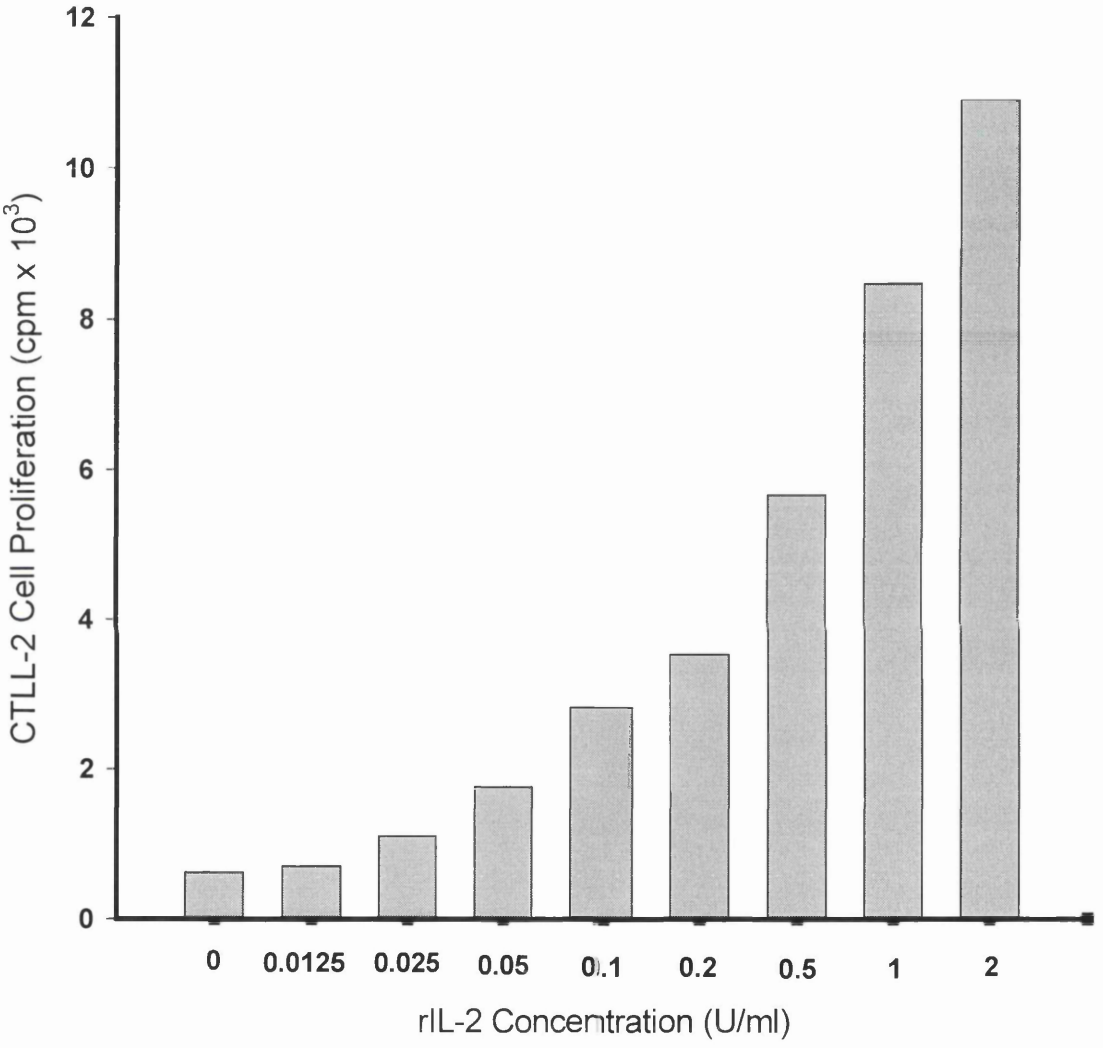

Figure 4. Proliferative response of CTLL-2 cells to IL-2. The CTLL-2 cells proliferate to rIL-2 in a dose-dependent manner. Values are expressed as mean cpm of ^{3}H-TdR incorporation of triplicate cultures.

times and the paired student t test was used to analysis the data. The samples without CsA were used as a control.

The CTLL-2 proliferation to the rIL-2 concentration with or without various concentrations of CsA were statistically similar *($p > 0.05$)* (Table 1, Fig. 5). The result showed that CTLL-2 response to IL-2 was not altered at any of the CsA concentrations tested.

CsA concentration	CTLL-2 Proliferation (cpm x 10^3)	
0 (control)	10.161 ± 0.011	
125 ng/ml	10.178 ± 0.010	*$p = 0.44$*
250 ng/ml	10.163 ± 0.016	*$p = 0.92$*
500 ng/ml	10.141 ± 0.016	*$p = 0.14$*
1000 ng/ml	10.149 ± 0.012	*$p = 0.40$*
10000 ng/ml	10.157 ± 0.016	*$p = 0.89$*

Table 1. Response of CTLL-2 cells to IL-2 in the presence of various concentrations of CsA. The CTLL-2 cells respond normally to rIL-2 (2 U/ml) in the presence CsA. Values are expressed as mean ± (SEM) cpm of ^{3}H-TdR incorporation, p values: particular CsA concentration compared with the control (without CsA).

3.2.3. Inhibition of lymphocyte proliferation

Inhibition of lymphocyte proliferation was an essential step in the CsA sensitivity assay. After 18 hours of cell culture, the IL-2 is being secreted and lymphocytes are in S phase to start proliferation. Since CTLL-2 cells are the detectors of IL-2

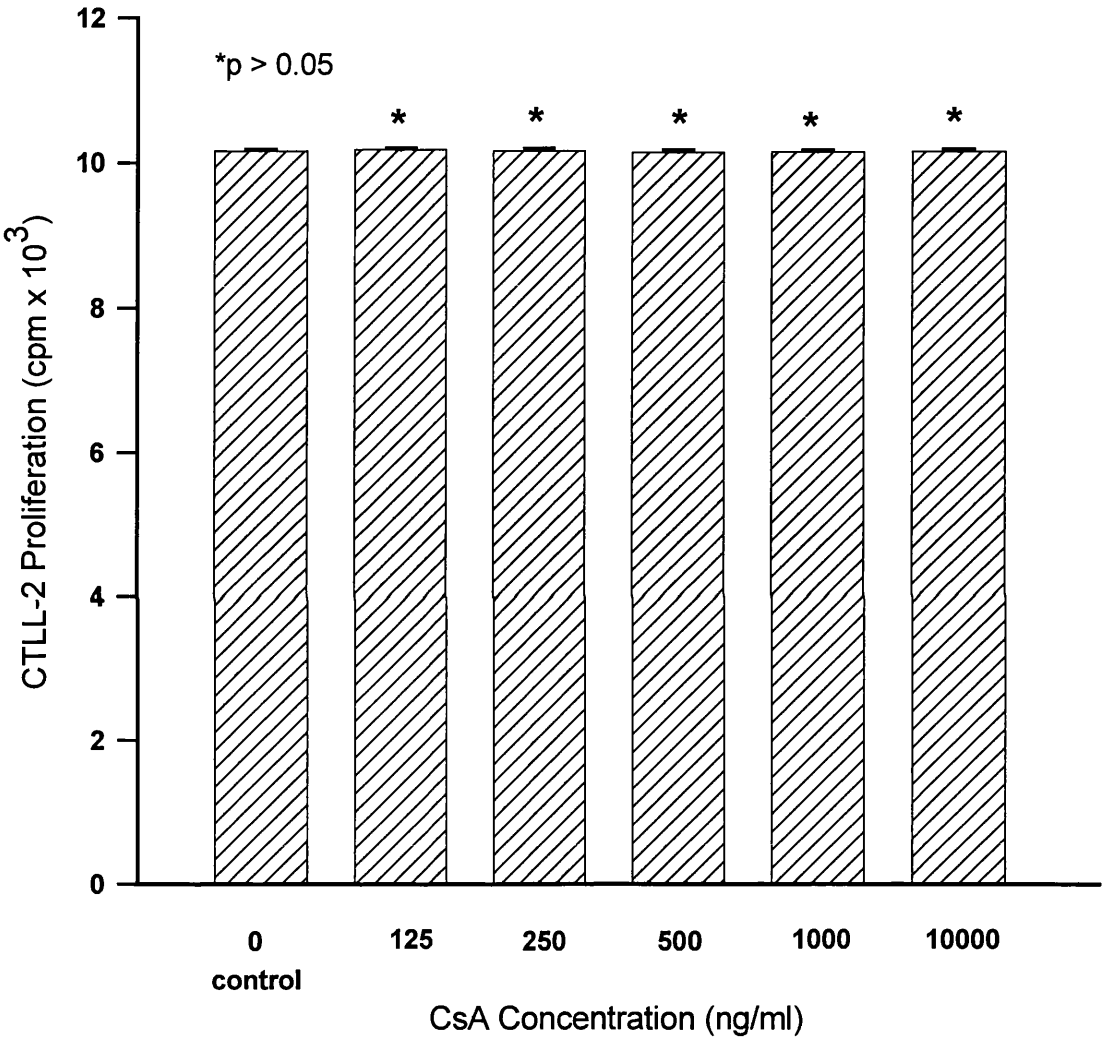

Figure 5. CTLL-2 Proliferative response to IL-2 in the presence of CsA. The CTLL-2 cells respond normally to rIL-2 (2 U/ml) in the presence of various concentrations of CsA. Values are expressed as mean ± SEM cpm of ^{3}H-TdR incorporation, p value: particular CsA concentration compared with the control (without CsA), n=6 experiments.

molecules in this assay, and they respond to secreted IL-2 by proliferation, it is necessary to stop any further lymphocyte proliferation when adding CTLL-2 cells to the cultured plates, so that the ^{3}H-TdR incorporation reflects CTLL-2 proliferation. In order to achieve this, it was essential to determine the dose of gamma irradiation that should be given to cultured PBMCs immediately before adding CTLL-2 cells, and to determine the incubation period of cell culture (1 or 3 days) associated with minimal proliferation of radio-resistant lymphocytes.

3.2.3.1. Dose of gamma irradiation. PBMCs were isolated from blood of 10 normal volunteers as mentioned in the chapter Materials and Methods. 1 X 10^{5} viable PBMCs per 100 µl incubation medium with 10% human AB serum per well were plated in 96-well U-bottom tissue culture microtiter plates in 10 replicates and PHA (0.5 µg/ml) was added. The 3 sets of culture plates were incubated for 72 hours and then irradiated differentially by gamma irradiation doses (2000, 3360, 4000 rads), and labelled with 1 µCi/well of ^{3}H-TdR. Proliferation of lymphocytes was assessed by ^{3}H-TdR incorporation after a further 18 hours incubation. Lymphocyte proliferation (cpm) was determined at each radiation dose and the effect of these different doses of gamma irradiation was analysed by paired student t test. A set of culture plates without irradiation was included, and regarded as a control.

Analysis of data showed that lymphocyte proliferation inhibited significantly by gamma irradiation at these doses ($p < 0.0001$: vs control) (Table 2, Fig. 6), and

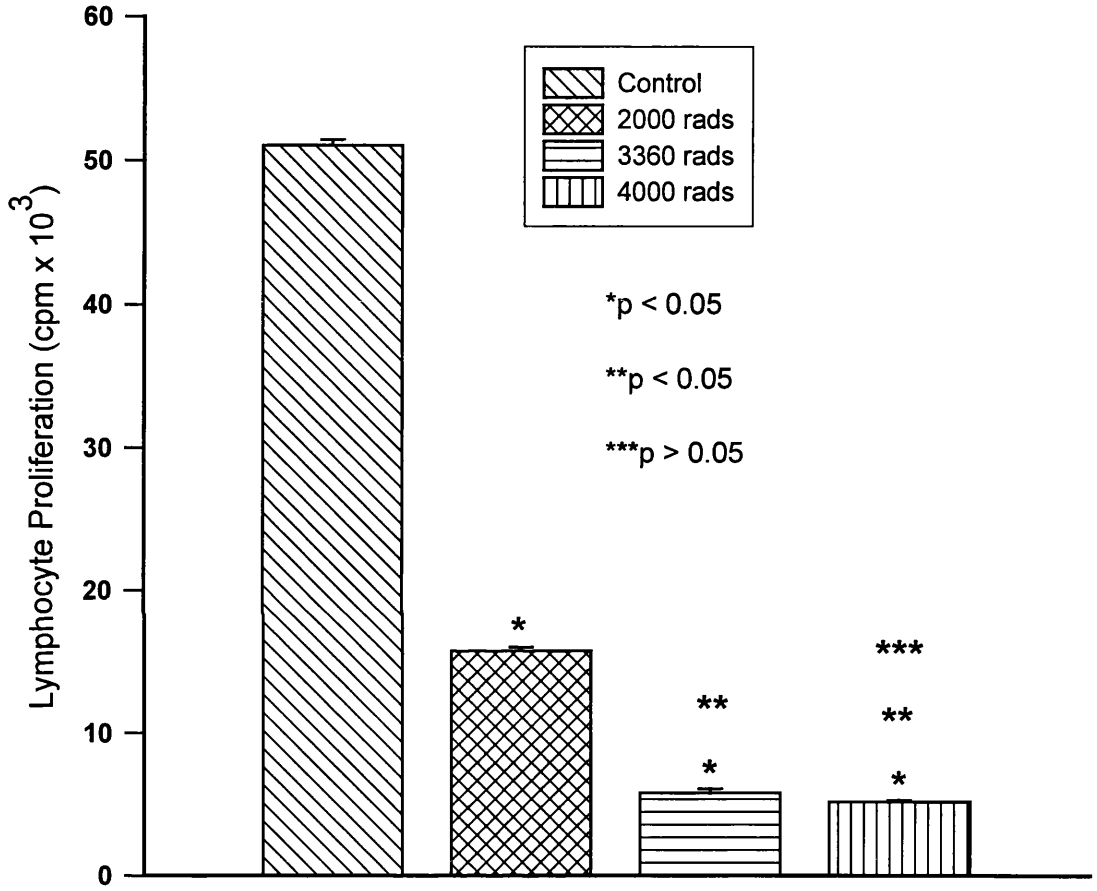

Figure 6. Comparison between gamma radiation doses in inhibiting lymphocyte proliferation. After 72 hours of culture, PHA-stimulated PBMCs of 10 normal volunteers were gamma irradiated with the indicated doses. The graph shows 4000 rads gives maximum inhibition of lymphocyte proliferation. In comparison with 3360 rads, radiation at 4000 rads does not achieve statistically better reduction in lymphocyte proliferation. Values are expressed as mean ± SEM cpm of. ^{3}H-TdR incorporation, *p: radiation doses vs control (without irradiation), **p: other radiation doses vs 2000 rads, ***p: 4000 rads vs 3360 rads.

maximum inhibition of lymphocyte proliferation was achieved with 3360 and 4000 rads, with lymphocyte proliferation 5779 and 5166 cpm respectively, ($p < 0.0001$: vs 2000 rads) (Table 2, Fig. 6). There was statistically no significant difference in radio-resistant lymphocyte proliferation after irradiation at 3360 or 4000 rads, ($p > 0.05$). The results indicated that lymphocyte proliferation could be inhibited with gamma irradiation and maximum inhibition of lymphocyte proliferation could achieved with gamma irradiation at 3360 rads, since there was no significant difference in lymphocyte proliferation between 3360 and 4000 rads.

Dose of gamma radiation (rads)	Lymphocyte proliferation (cpm)	Lymphocyte proliferation (% of control)
0 (Control)	51050 ± 380	
2000	15772 ± 248 *(*p < 0.0001)*	30.9 ± 0.49% *(*p < 0.0001)*
3360	5779 ± 303 *(*p < 0.0001)* *(**p < 0.0001)*	11.32 ± 0.60% *(*p < 0.0001)* *(**p < 0.0001)*
4000	5166 ± 122 *(*p < 0.0001)* *(**p < 0.0001)* *(***p > 0.05)*	10.12 ± 0.24% *(*p < 0.0001)* *(**p < 0.0001)* *(***p > 0.05)*

Table 2. Effectiveness of different gamma radiation doses in inhibiting lymphocyte proliferation. After 72 hours of culture, PHA-stimulated PBMCs of 10 normal volunteers were irradiated with particular gamma radiation dose. Radiation at 3360 and 4000 rads gave maximum inhibition of lymphocyte proliferation. In comparison with 3360 rads, radiation at 4000 rads did not achieve statistically better reduction in lymphocyte proliferation. Values are presented as mean ± SEM cpm of. ^{3}H-TdR incorporation, *p: other gamma radiation doses vs control (without irradiation), **p: other gamma radiation doses vs 2000 rads, ***p: 4000 vs 3360 rads.

3.2.3.2. Effect of gamma radiation on IL-2 integrity. It was essential to find out whether the gamma radiation doses we intended to use had any adverse effects on

the structure and function of IL-2 molecules or interfered with detection of IL-2 molecules by CTLL-2 cells. Specifically did the gamma radiation doses denature IL-2 molecules? So that CTLL-2 cells could not detect them so that CTLL-2 proliferation did not reflect the quantity of secreted IL-2.

We tested CTLL-2 proliferation after irradiating rIL-2 at radiation doses of 3360 and 4000 rads, and expressed CTLL-2 proliferation as percentage of the control (without radiation). Various concentrations of rIL-2 were added in triplicates to the culture wells in 3 sets of 96-well U-bottom tissue culture microtiter plates, such that the final concentration of rIL-2 varied between 0 and 2 U/ml. One set of the culture plates was irradiated at 3360 rads, the other at 4000 rads while the other one was not irradiated and kept as a control for CTLL-2 proliferation. The CTLL-2 cells were plated out at 3 X 10^3 cells per well. The assay was incubated for 8 hours and labelled with 1 μCi/well of ^{3}H-TdR. Proliferation of CTLL-2 cells was assessed by ^{3}H-TdR incorporation after a further 18 hours incubation. The assay was carried out in triplicate and the mean of CTLL-2 proliferation was determined for each rIL-2 concentration used, and expressed as a percentage of the control CTLL-2 proliferation. The data were analysed with paired student t test comparing the percentage of control CTLL-2 proliferation at 4000 rads with that of 3360 rads; results were expressed as mean ± SEM % of the control.

Radiation at 3360 rads was associated with almost normal detection of IL-2 molecules by CTLL-2 cells, while radiation at 4000 rads was associated with

reduction in detection of IL-2 molecules by CTLL-2 cells. After radiation of rIL-2 molecules at 3360 rads, the CTLL-2 proliferation was 99.6 ± 3.0% of the control, whereas at 4000 rads, the CTLL-2 proliferation was 90.1 ± 1.9% of the control, (*p = 0.014:* 4000 rads vs 3360 rads*)* (Fig. 7). Statistically, there was significant difference in CTLL-2 proliferation to irradiated rIL-2 between 3360 rads and 4000 rads, hence radiation at 4000 rads might have caused sufficient damage to IL-2 molecules to interfere with the normal proliferative response of CTLL-2 cells to IL-2.

According to the above-mentioned results, radiation of PBMCs at 3360 rads was preferable to 4000 rads in the CsA sensitivity assay. Statistically there was no significant difference in the degree of inhibition of lymphocyte proliferation at 3360 rads and 4000 rads, so that radiation at 3360 rads is sufficient for inhibition of lymphocyte proliferation. However, radiation of IL-2 molecules at 4000 rads interfered with the normal proliferative response of CTLL-2 cells to IL-2, while the proliferative response of CTLL-2 cells to irradiated IL-2 molecules at 3360 rads was normal.

3.2.3.3. Incubation period of cell culture. After determining the radiation dose needed to give maximum inhibition of lymphocyte proliferation, it was necessary to define the incubation period of cell culture associated with minimum lymphocyte proliferation. IL-2 secretion of mitogenic-stimulated T cells was

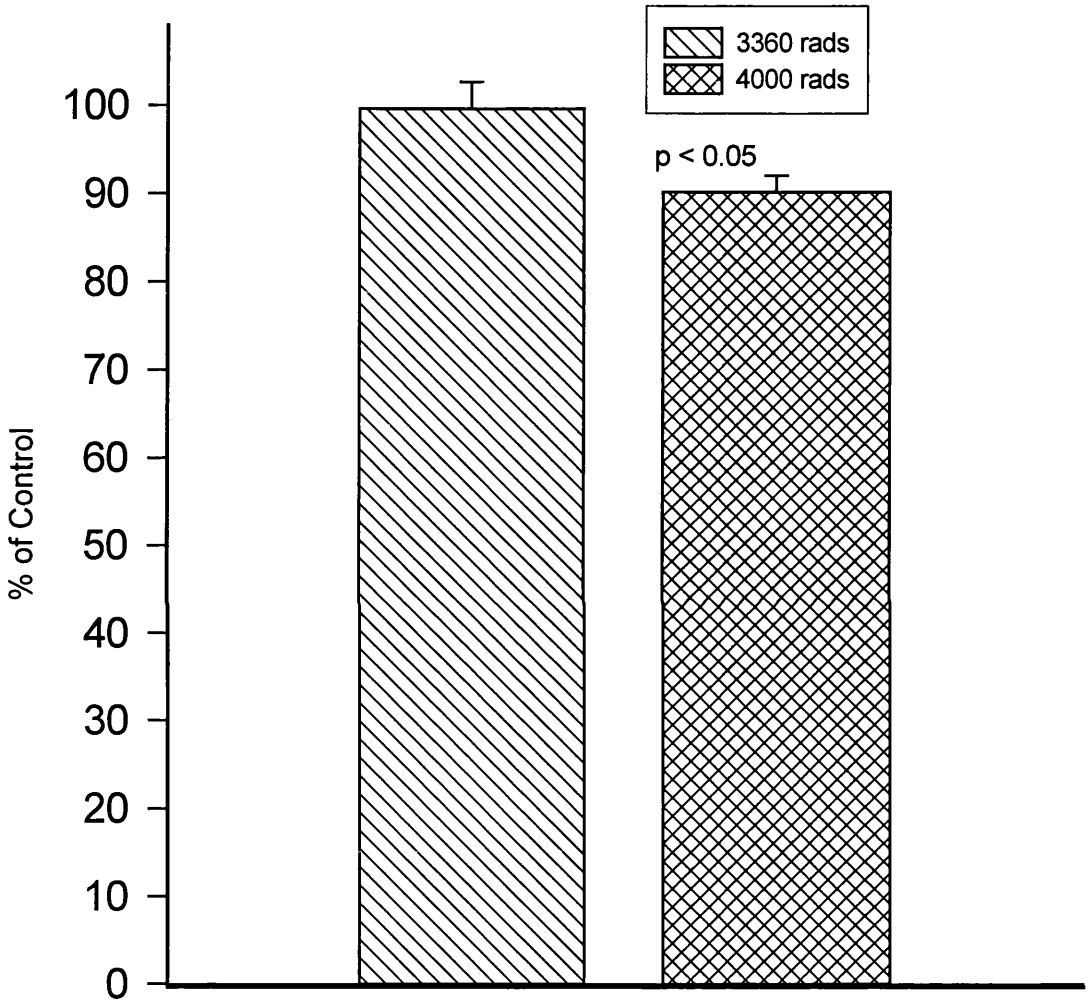

Figure 7. Effect of gamma radiation doses on IL-2 integrity. Various concentrations of rIL-2 were irradiated at 3360 or 4000 rads. CTLL-2 cells responded normally to rIL-2 irradiated at 3360 rad, but a irradiation at 4000 rads led to a 10% reduction in CTLL-2 proliferation. Values are expressed as mean ± SEM % of the control (CTLL-2 proliferation without irradiation of IL-2).

abruptly terminated between 18 and 24 hours after the onset as demonstrated by Gullberg *et al.* (1981), and Deacock *et al.* (1992) had established an assay in which cell cultures were incubated for only 18 hours to detect IL-2 production. We therefore designed experiments with different incubation periods (18 and 72 hours) aiming to determine the effect of varying incubation periods on lymphocyte proliferation after gamma irradiation.

PBMCs were isolated from blood of six normal volunteers as mentioned in the Materials and Methods chapter. 1 X 10^5 viable PBMCs per 100 µl incubation medium with 10% human AB serum per well were plated in 96-well U-bottom tissue culture microtiter plates in 10 replicates and PHA (0.5 µg/ml) was added. The 2 sets of culture plates were then incubated one set for 18 hours and the other for 72 hours. After the varying periods of cell culture, the culture plate sets were gamma irradiated (3360 rads), and labelled with 1 µCi/well of ^{3}H-TdR. Proliferation of lymphocytes was assessed by ^{3}H-TdR incorporation after a further 18 hours incubation. Lymphocyte proliferation (cpm) was determined for each set of incubation periods. Data were analysed with paired student t test comparing the effect of these varying periods of cell culture, and the values are mean ± SEM cpm.

Radio-resistant lymphocyte proliferation was much higher after 72 hours than after 18 hours of culture with 11,650 ± 820 and 349 ± 20 cpm respectively, ($p < 0.0001$) (Fig. 8). Our result showed that an 18-hour culture period was associated

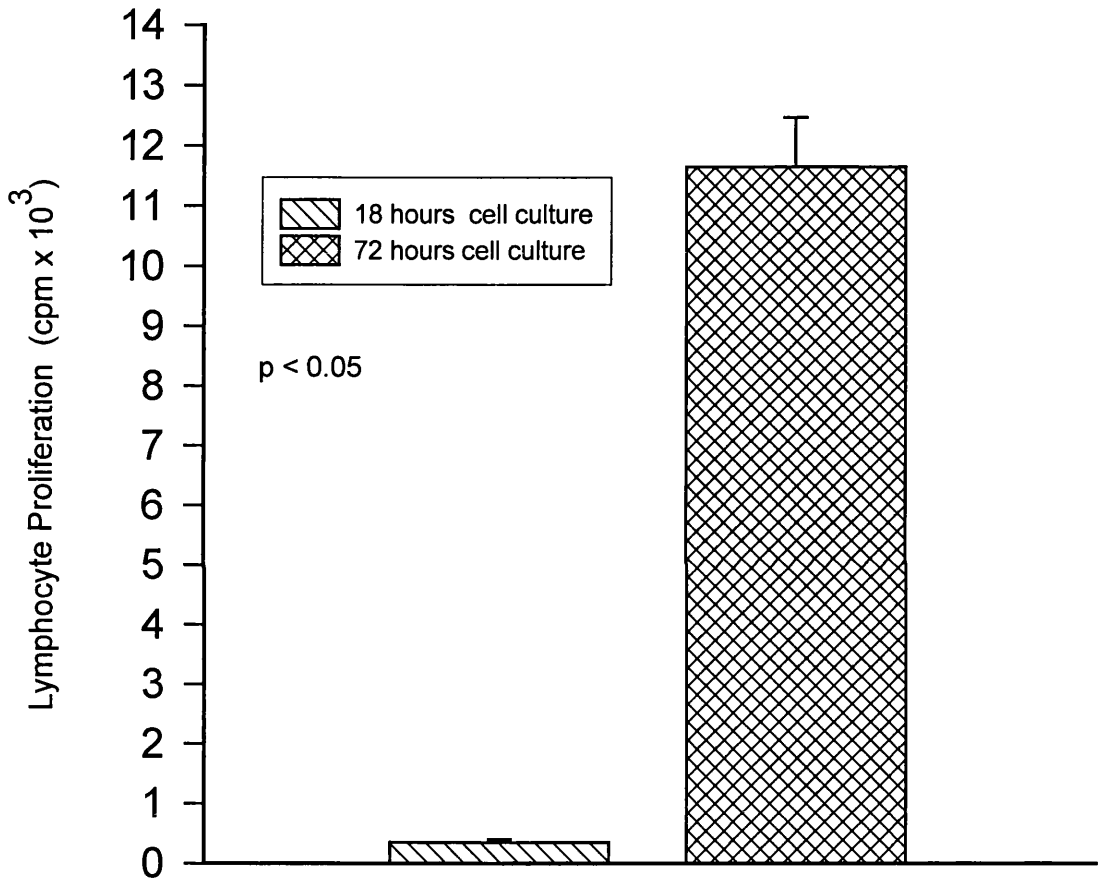

Figure 8. Radio-resistant lymphocyte proliferation after various incubation periods of cell culture. After 18 hours or 72 hours of cell culture, PHA-stimulated PBMCs of 6 normal volunteers were irradiated with gamma radiation at 3360 rad. 18-hour culture period is associated with less radio-resistant lymphocyte proliferation in comparison with that of 72-hour culture period. Values are expressed as mean ± SEM cpm of ^{3}H-TdR incorporation.

with less lymphocyte proliferation than with a 72-hour culture period. This indicated that in order to achieve minimum backgrounds of ^{3}H-TdR incorporation due to lymphocyte proliferation, it was necessary to incubate the culture plates for the CsA sensitivity assay for only 18 hours before gamma irradiation. The CTLL-2 cells begin to show significant proliferation above background control levels at a rIL-2 concentration of 0.0125 U/ml. From earlier work using the indicator CTLL-2 cells to detect IL-2 production (Vie and Miller, 1986), this concentration of IL-2 could be achieved from IL-2 production from a single cell only after 3 days of culture of the cell under optimal conditions including mitogenic stimulation. Nonetheless, this does not reduce the value of the CsA sensitivity assay, providing that CsA dose-response curves are generated using the same experimental protocol.

3.2.4. Effect of centrifuge speeds on CsA sensitivity

Since the state of lymphocytes is critical and has a major influence on CsA sensitivity, it was necessary during the process of PBMCs isolation and washing to avoid any vigorous manoeuvres that might cause lymphocyte activation leading to an alteration in CsA sensitivity. To set up a precise protocol for the CsA sensitivity assay, we simultaneously isolated and washed PBMCs from four healthy subjects under two different conditions of the isolation process and determined their effects on CsA sensitivity. High centrifuge speed isolation of PBMCs consisted of isolation of PBMCs from blood at 2060 *g* for 20 minutes without break, then washing twice at 1430 *g* for 10 minutes and twice at 514 *g* for

3 minutes. This isolation procedure has been validated by the tissue typing lab of our department. The low centrifuge speed isolation of PBMCs consisted of isolation of PBMCs at 400 *g* for 20 minutes without break, then washing once at 250 *g* for 15 minutes and twice at 150 *g* for 10 minutes, the procedure is recommended by Hudson and Hay (1989). We carried out CsA sensitivity assay, using CTLL-2 as a detector of IL-2, on PBMCs of four normal volunteers as mentioned in the chapter of materials and methods, with isolation of PBMCs under high- and low-centrifuge speeds. The dose-response curves were computer-generated for these volunteers under both conditions of the isolation.

The dose-response curves showed a clear difference in CsA sensitivity between high- and low-centrifuge speed isolation procedures (Fig. 9). After high centrifuge speed isolation, the curves of all volunteers showed that PBLs were resistant to CsA at concentrations expected to cause immunosuppression of IL-2 production and as compared with low-centrifuge speed isolation. Furthermore, under the high-centrifuge speed isolation procedure, enhanced production of IL-2 occurred at low concentrations of CsA, while considerable inhibition of IL-2 production existed with low concentrations of CsA in the low-centrifuge speed isolation procedure. Other investigators have observed CsA resistance or even enhancement. Earlier studies have shown that following injection of alloantigen into the footpads of mice, the number of cells in the draining popliteal lymph nodes, the percentage of IL-2Rα-positive cells, and their spontaneous proliferative response were either totally resistant or enhanced by the

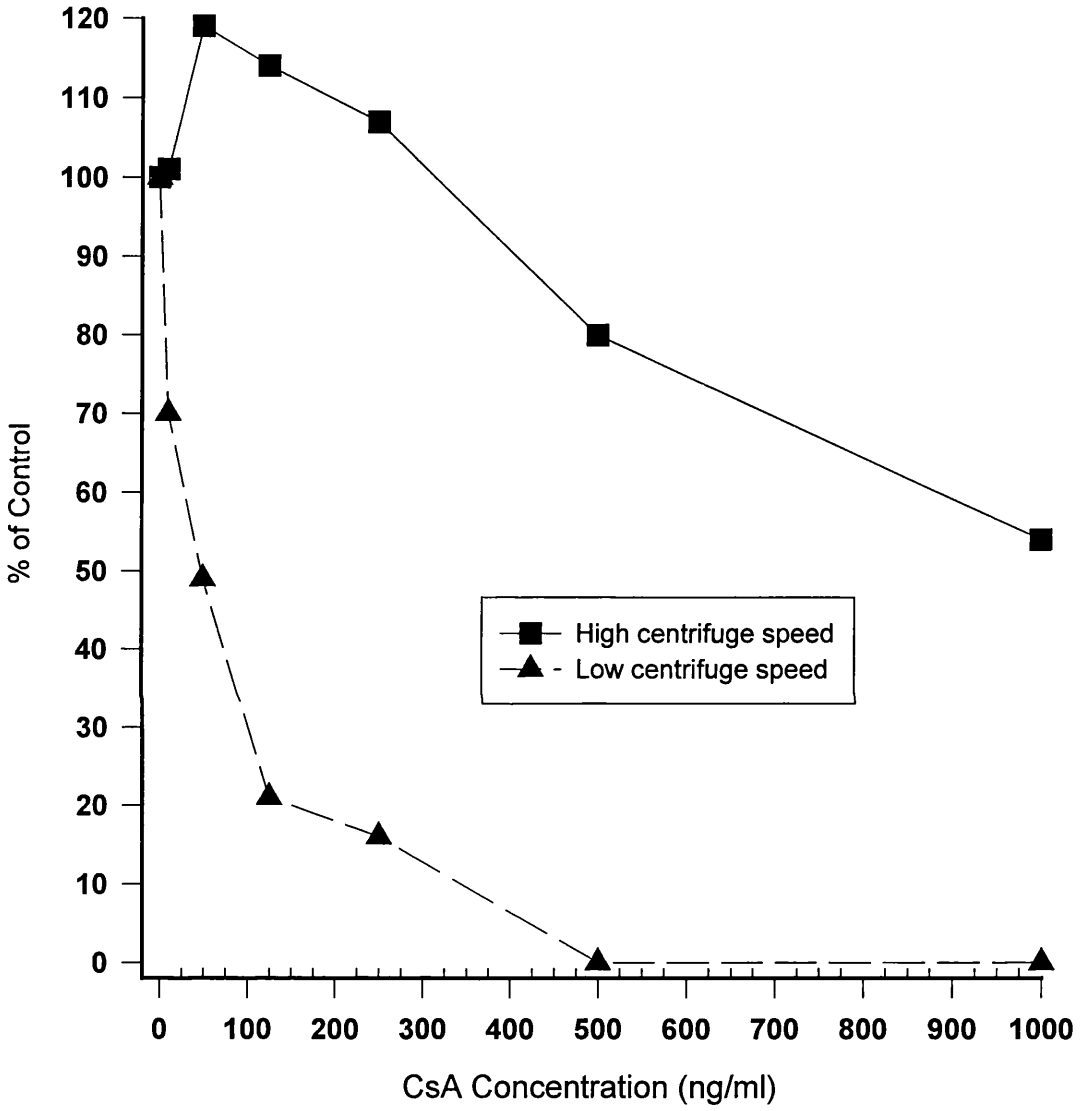

Figure 9. Representative dose-response curves demonstrating the alteration of CsA sensitivity of T cells with different centrifuge speeds of PBMCs isolation. The graph shows resistance to CsA and even a paradoxical enhancement of IL-2 production at low concentrations of CsA with high centrifuge speed. Control is defined as CTLL-2 proliferation reflecting the IL-2 production with no added CsA, similar graphs were obtained with 3 other normal volunteers.

administration of CsA (Kroczek *et al.* 1987; Pereira *et al.* 1990). The authors suggested that in certain experimental situations, alternative pathways for T cell activation, as exemplified by the CD28 pathway, that are resistant to CsA may contribute significantly to *in vivo* immune responses. Furthermore, *in vitro* study on PBMCs of healthy subjects reported that 35 of 99 tests have shown paradoxical proliferation of T cells with lower concentrations ($\leq$ 50 ng/ml) of CsA (Masy *et al.* 1994).

From these data, the importance of avoiding vigorous manoeuvres of PBMCs during the assay work was clear, and the low-centrifuge speed isolation procedure was therefore adopted in all subsequent experiments as recommended by Hudson and Hay (1989).

3.2.5. Monitoring for reproducibility of the assay

To confirm that the CsA sensitivity assay gave reproducible results, CsA sensitivity was determined repeatedly over a period of time using PBMCs from the same volunteers. CsA sensitivities as determined by CsA IC_{50} were found to remain approximately constant over time (Fig. 10).

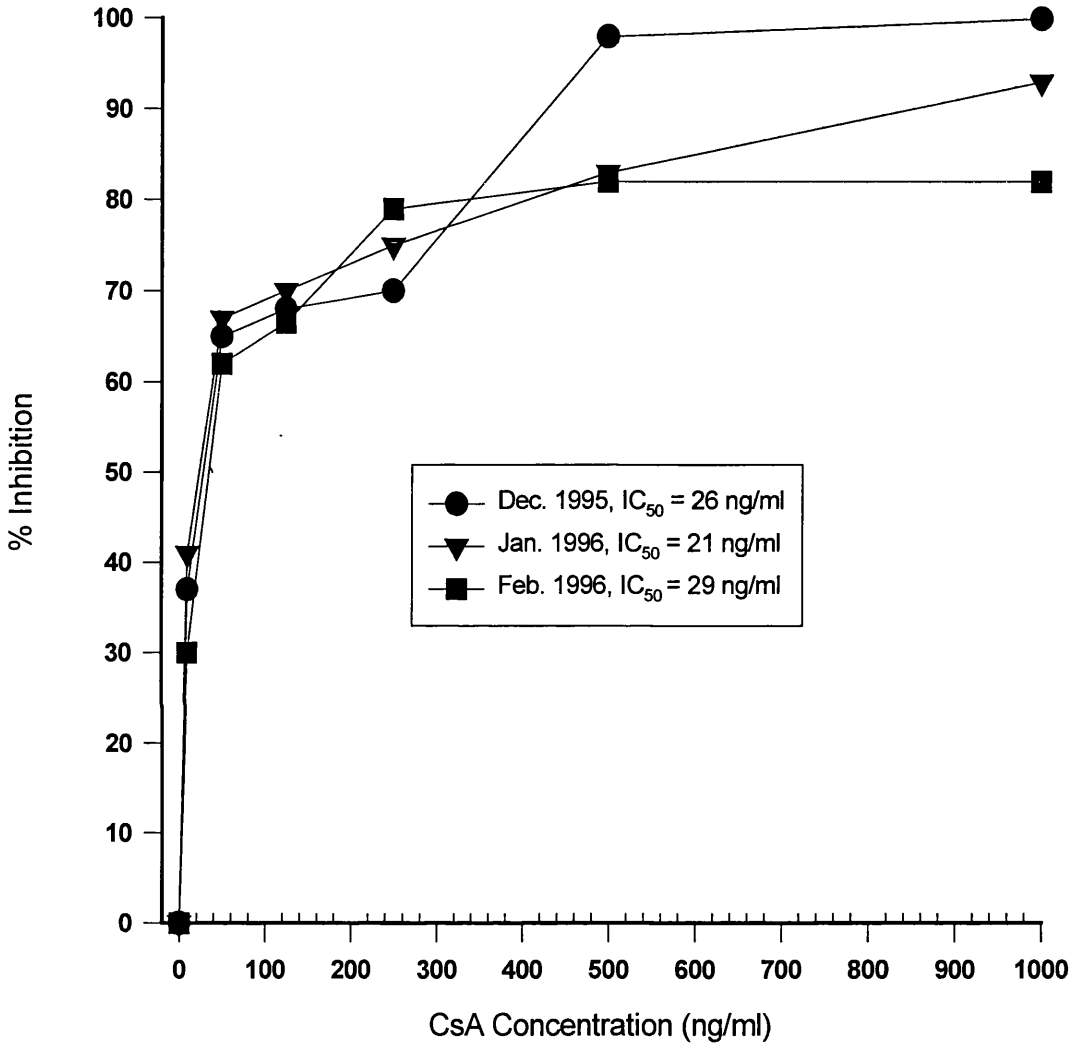

Figure 10. CsA sensitivity of T cells over a period of time tested on PBMCs of the same volunteer. Repeatedly determined IC_{50} of CsA remained approximately constant over period of several months using the CsA sensitivity assay. Control is defined as CTLL-2 proliferation reflecting the IL-2 production with no added CsA, and was taken as 0% inhibition. The figure is presented as % inhibition of IL-2 production as detected by CTLL-2 proliferation, IC_{50}: Concentration of CsA that achieved 50% inhibition of IL-2 production, similar results were obtained with 5 other normal volunteers.

3.3. Flow Cytometry Analysis

3.3.1. Kinetics of CD69 expression

The CD69 molecule is one of the activation markers of T cells that we have intended to use in our study as an indication of T cell activation. For this reason, we decided to determine the kinetics of expression of CD69 molecules on T cells.

PBMCs were isolated from blood of four normal volunteers as mentioned in Materials and Methods chapter. 1 X 10^5 viable PBMCs per 100 µl incubation medium with 10% human AB serum per well were plated in 96-well U-bottom tissue culture microtiter plates, and PHA (0.5 µg/ml) or PMA (5 ng/ml) were added. Unstimulated (resting) cells were included and referred as negative control. The 3 sets of culture plates were incubated, the first for 2 hours only, the second for 18 hours and the third for 48 hours. After the varying periods of cell culture, the plated cells were washed twice with staining buffer (250 µl/well) and pelleted by centrifugation at 250 *g* for 5 minutes. The staining of cell surface molecules was carried out using conjugated monoclonal antibodies. 50 µl of staining buffer plus 10 µl anti-CD3-FITC (Coulter Corporation, Miami, Florida, USA) used as a marker of human T cells, and 20 µl anti-human CD69-PE (Immunotech, A Coulter Company, France), were added to the resuspended 10^5 cells/well. An extra cell sample was stained with 7 µl CYTO-STAT tetraCHrome monoclonal antibody which was used as a cell surface staining control to set up flow cytometric gating, PMT voltage and compensation. The cells were incubated

for 30 minutes at 4°C in the dark, washed twice with staining buffer (250 µl/well) and resuspended in 500 µl staining buffer.

Cells were analysed immediately by FACScan flow cytometer after acquiring 10000 cells. The PMT voltage and compensation were set up using the cell surface staining control, and quadrant markers were set up based on unstained cells. Data were analysed by LANtastic software. The percentage of T cells expressing CD69 at each time point was calculated from the absolute counts.

The analysis showed that resting circulating T cells did not express CD69 molecules but that a proportion of T cells expressed CD69 after 2 hours of stimulation. These are in agreement with previous reports that showed CD69 is not detectable on resting T cells but appears very early after T cell activation (Cebrian *et al.* 1988). The percentage of T cells expressing CD69 molecules was higher with PMA than with PHA at all the three periods tested, the difference being accounted for by the fact that PMA is known to provide a very strong stimulus to T cells via PKC. The percentage of T cells expressing CD69 molecules increased with incubation period and peaked after 18 hours with both PMA and PHA stimulation (Fig. 11, 12). Subsequently, the CD69 expression remained stable throughout the 48-hour culture period. These observations were similar with all the four volunteers tested. The findings are consistent with a report that showed that maximum expression of CD69 occurred 24 hours after mitogen stimulation and remained stable throughout the 72-hour culture period

(Mardiney *et al.* 1996). In practice the cells were incubated for 18 hours in the experiments that used this activation marker.

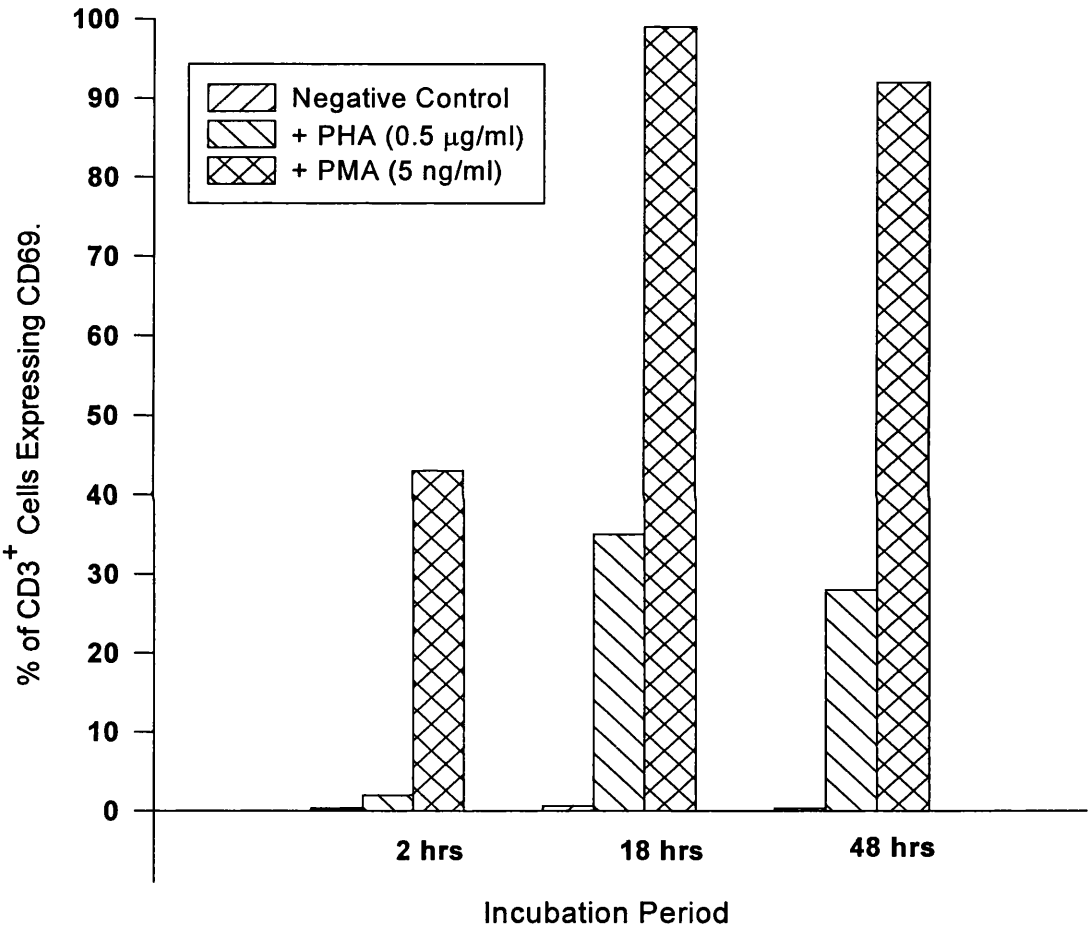

Figure 11. Kinetics of CD69 expression on T cells. Representative graph of one of the volunteers tested. PBMCs were stimulated with PHA or PMA and incubated for the indicated periods of time. Unstimulated cells were also included and regarded as negative control. Cells were dual labelled with FITC-antibody against CD3 and PE-antibody against CD69. Similar results were obtained with 3 other normal volunteers.

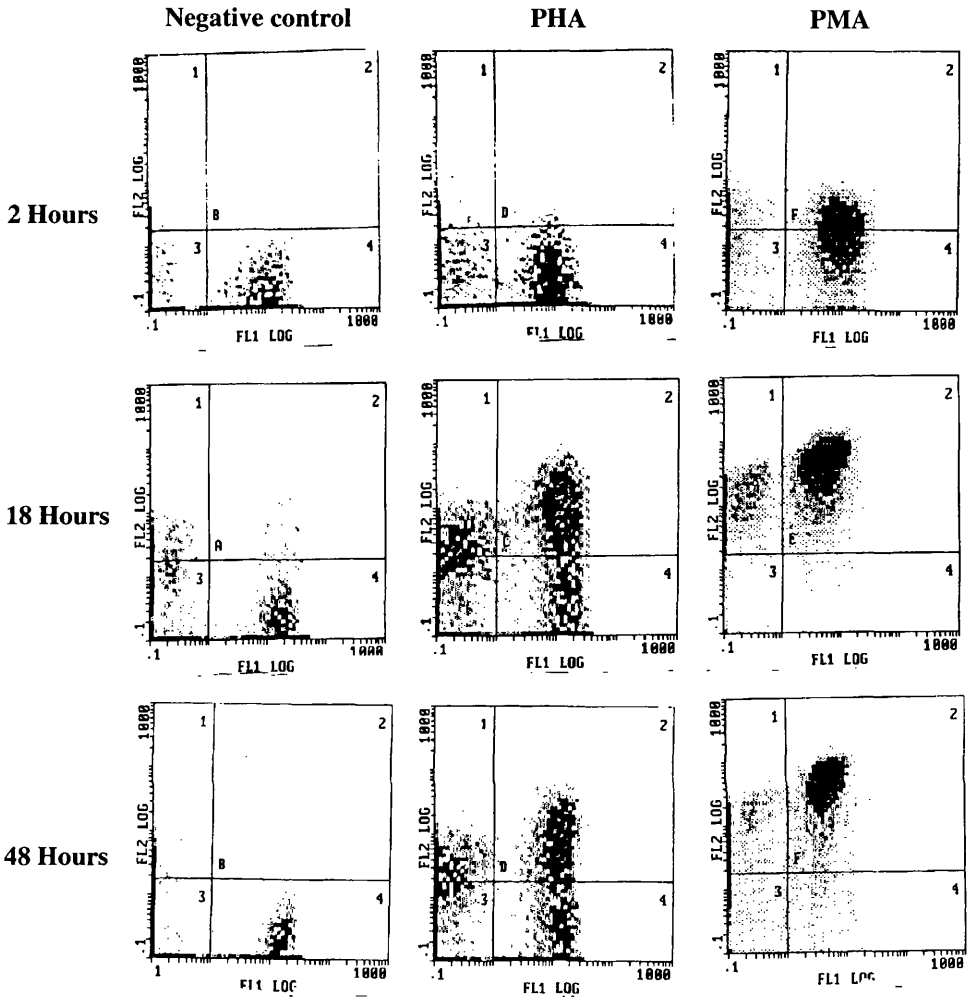

Figure 12. Flow cytometric determination of the kinetics of CD69 expression on T cells. T cells were stimulated with PHA or PMA and incubated for the indicated periods. Unstimulated cells were also included and regarded as negative control. Cells were dual labelled with FITC-antibody against CD3 (FL1) and PE-antibody against CD69 (FL2).

4. REDUCED CsA SENSITIVITY AFTER T CELL PREACTIVATION

4.1. Rationale for Study

In this chapter, the effects of *in vitro* prestimulation of T cells with PHA for 2 hours on CsA sensitivity of T cells are reported. The purpose of PHA prestimulation of T cells was to activate T cells before adding CsA. CsA sensitivity of T cells after *in vitro* preactivation was studied in different groups of volunteers in comparison with that of normal resting T and was determined using CTLL-2 as a detector of IL-2 production. The percent inhibition of IL-2 production was then calculated from which we deduced CsA sensitivity. In clinical transplantation, CsA based-immunosuppression is associated with problems such as inadequate immunossupression, CsA nephrotoxicity and a poor correlation between CsA concentration and clinical effect. We hypothesised that this may be at least partially related to the presence of activated T cells. In this chapter the hypothesis that "Activated T cells are less sensitive to CsA" is tested by demonstration of the effect of *in vitro* preactivation of T cells on CsA sensitivity, and any alteration of CsA sensitivity of T cells in chronic renal failure patients on dialysis in comparison with that of normal healthy volunteers.

4.2. Results

4.2.1. Preactivation of T cells of normal volunteers

In vitro prestimulation of T cells with PHA was carried out in normal healthy volunteers and the inhibitory effects of various CsA concentrations on IL-2 production, as detected by CTLL-2 cells, were assessed in comparison with the normal unpreactivated T cells. In all the experiments of the normal volunteers tested, there was a clear shift in dose-responsive curves to the right after *in vitro* preactivation of T cells (Fig. 13). This implied that T cells are less sensitive to CsA after the preactivation and more CsA concentration required to achieve the same inhibition of IL-2 production of normal unpreactivated T cells. The IC_{50} of CsA was higher after T cell preactivation compared with that of normal unpreactivated T cells. The IC_{50} of CsA was 380 ng/ml and 50 ng/ml with and without preactivation of T cells respectively, ($p = 0.013$) (Fig. 14, Table 3).

4.2.2. Preactivation of T cells of chronic renal failure patients on dialysis

The effect of *in vitro* preactivation of T cell on CsA sensitivity was then determined in CAPD and haemodialysis patients. Although the immune system is impaired in patients with end stage renal failure, it was necessary to investigate whether *in vitro* preactivation of T cells of these patients reduced CsA sensitivity.

In all the ten CAPD patients on whom CsA sensitivity assays performed, the inhibitory dose-response curves were clearly shifted to the right after *in vitro* T

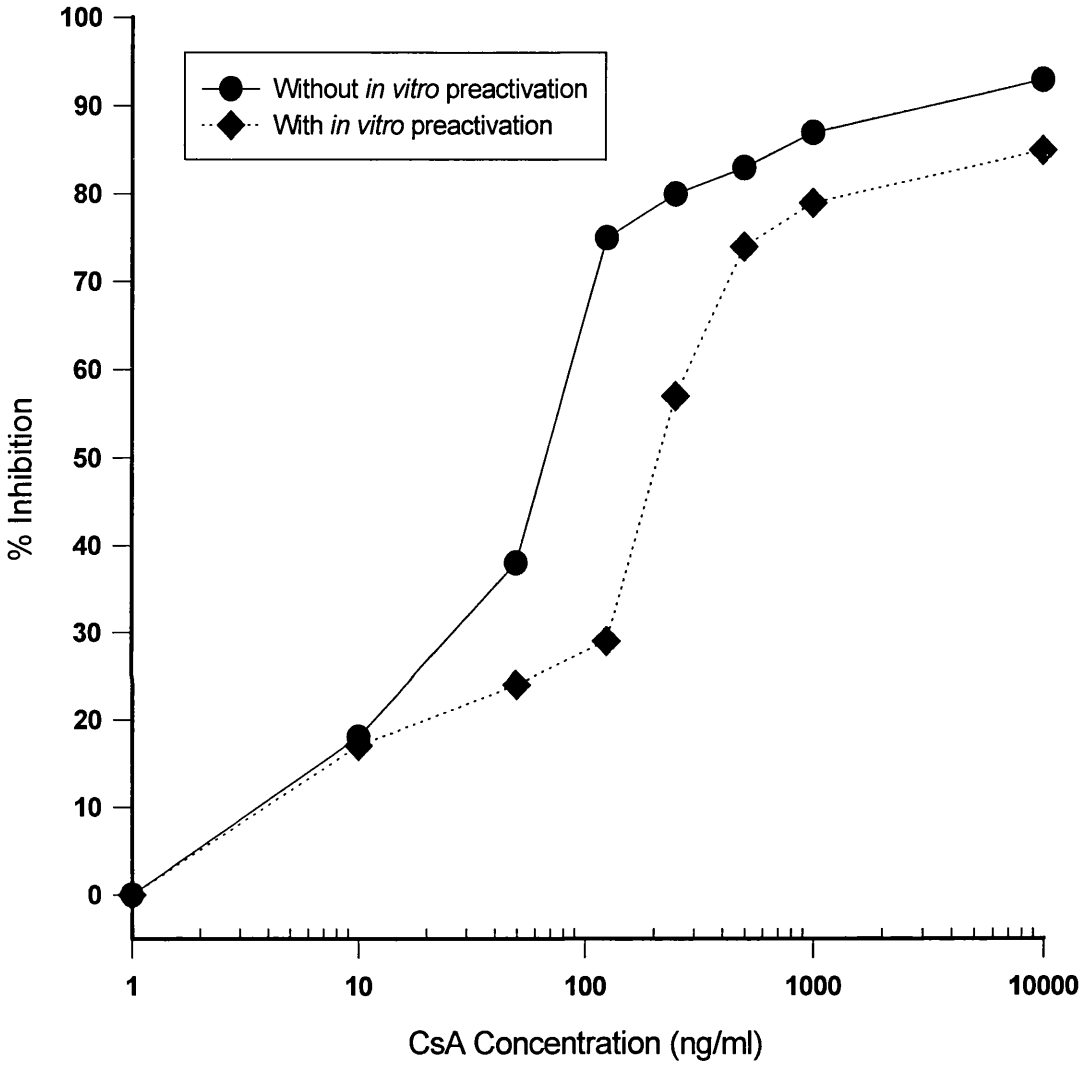

Figure 13. CsA sensitivity of T cells with and without *in vitro* preactivation in a normal volunteer. CsA sensitivity assay using CTLL-2 as a detector of IL-2 production was carried out with and without PHA preactivation on PBMCs. The representative inhibitory dose-response graph demonstrates a clear shift to the right in the dose-response curve after T cell preactivation. IL-2 production with no added CsA was taken as 0% inhibition (control). The figure is expressed as % inhibition of IL-2 production as reflected by CTLL-2 proliferation, similar graphs were obtained with 9 other normal volunteers.

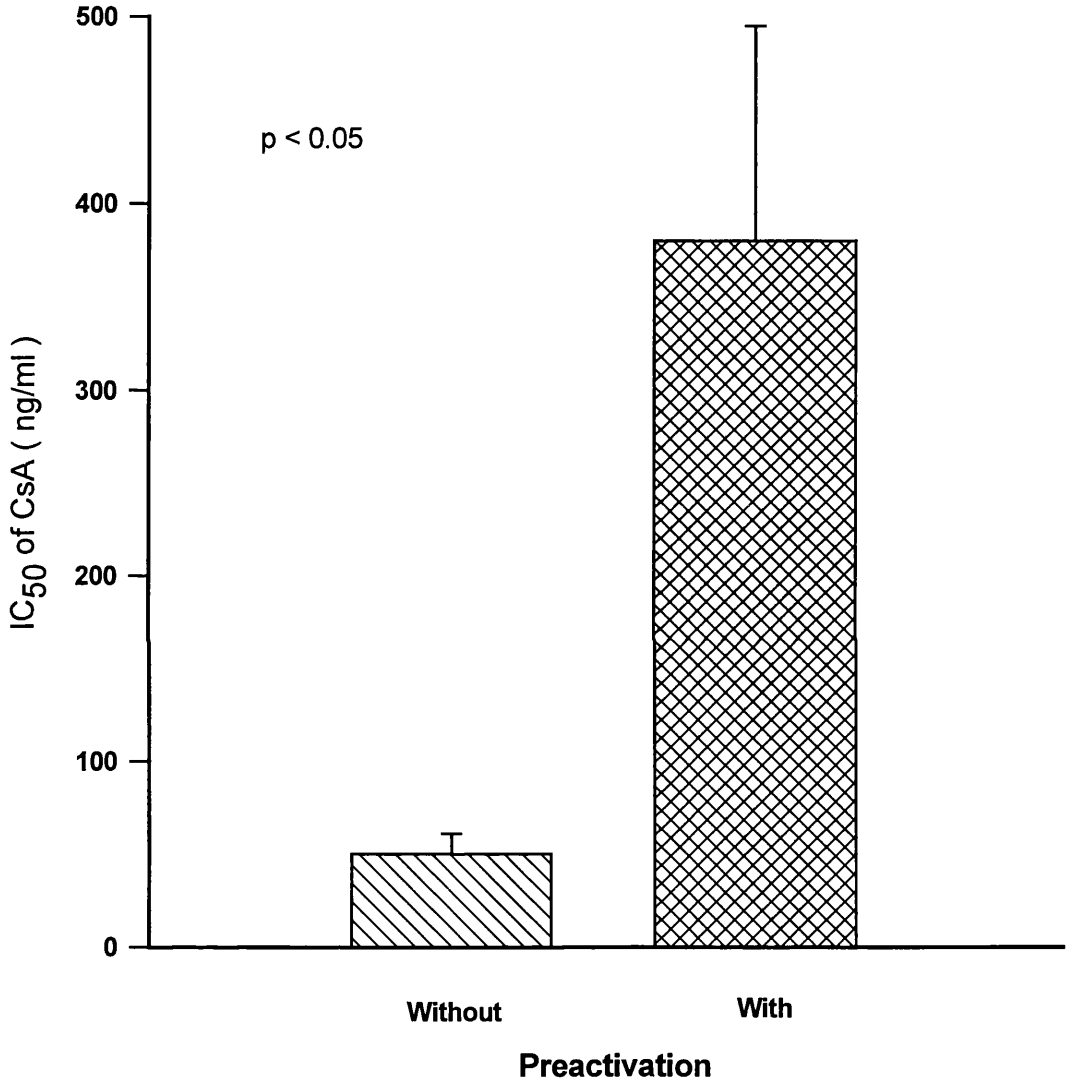

Figure 14. Effect of *in vitro* T cell preactivation on CsA sensitivity in normal volunteers. CsA sensitivity assay using CTLL-2 as a detector of IL-2 was carried out with and without PHA preactivation on PBMCs of normal volunteers. The IC_{50} of CsA was plotted from the inhibitory dose-response curves. The IC_{50} of CsA is markedly increased after T cell preactivation. Values are presented as mean ± SEM, p value: with preactivation vs without preactivation, n=10.

cell preactivation with PHA (Fig. 15). The IC_{50} of CsA after the *in vitro* preactivation was 95.5 ng/ml compared with the IC_{50} of CsA without the preactivation of T cells which was 20.5 ng/ml, (*p = 0.023*) (Fig. 16, Table 3). The shift to the right of dose-response curves and the statistically significant increase in the IC_{50} of CsA after preactivation, demonstrated that the *in vitro* preactivation of T cells rendered the T lymphocytes more resistant to CsA and reduced their CsA sensitivity, despite immune dysfunction caused by uraemia.

Volunteers group	(CsA IC_{50}) Without preactivation	(CsA IC_{50}) With preactivation	*p value*
Normal volunteers (n=10)	50 ± 11	380 ± 115	*0.013*
CAPD patients (n=10)	20.5 ± 3	95.5 ± 26	*0.023*
HD patients (predialysis) (n=12)	56 ± 16	283 ± 41	*0.0007*
HD patients (postdialysis) (n=12)	99 ± 18	721 ± 158	*0.0033*

Table 3. Effect of *in vitro* T cell preactivation on CsA sensitivity of T cells. CsA sensitivity assay, using CTLL-2 cells as detector of IL-2 production, was carried out with and without *in vitro* preactivation of T cells with PHA and the IC_{50} of CsA was plotted from the inhibitory dose-response curves. The data indicate that preactivation of T cells increased the IC_{50} of CsA significantly in all the groups. IC_{50} values: mean ± (SEM).

We also examined the effect of *in vitro* PHA preactivation of T cells in haemodialysis patients before and after dialysis on CsA sensitivity. *In vitro* preactivation with PHA caused apparent shift to the right in the inhibitory dose-

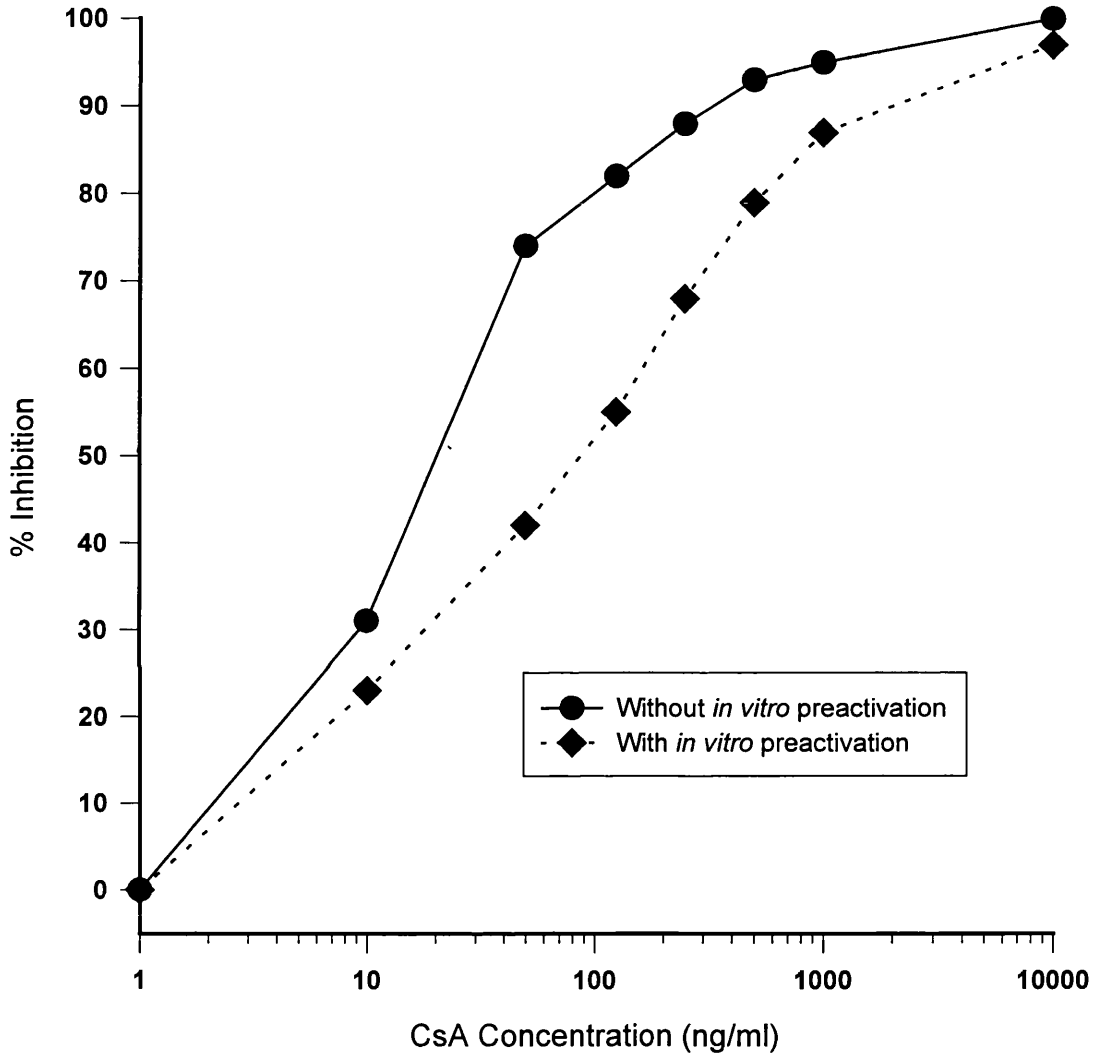

Figure 15. Representative inhibitory dose-response graph demonstrating reduction of CsA sensitivity of T cells after *in vitro* preactivation in a CAPD patient. CsA sensitivity assay using CTLL-2 as a detector of IL-2 production was carried out with and without PHA preactivation on PBMCs. A clear shift to the right occurs in dose-response curve after T cell preactivation. IL-2 production with no added CsA was considered as 0% inhibition. The response is expressed as % inhibition of IL-2 production as reflected by CTLL-2 proliferation, similar graphs were obtained with 9 other CAPD patients.

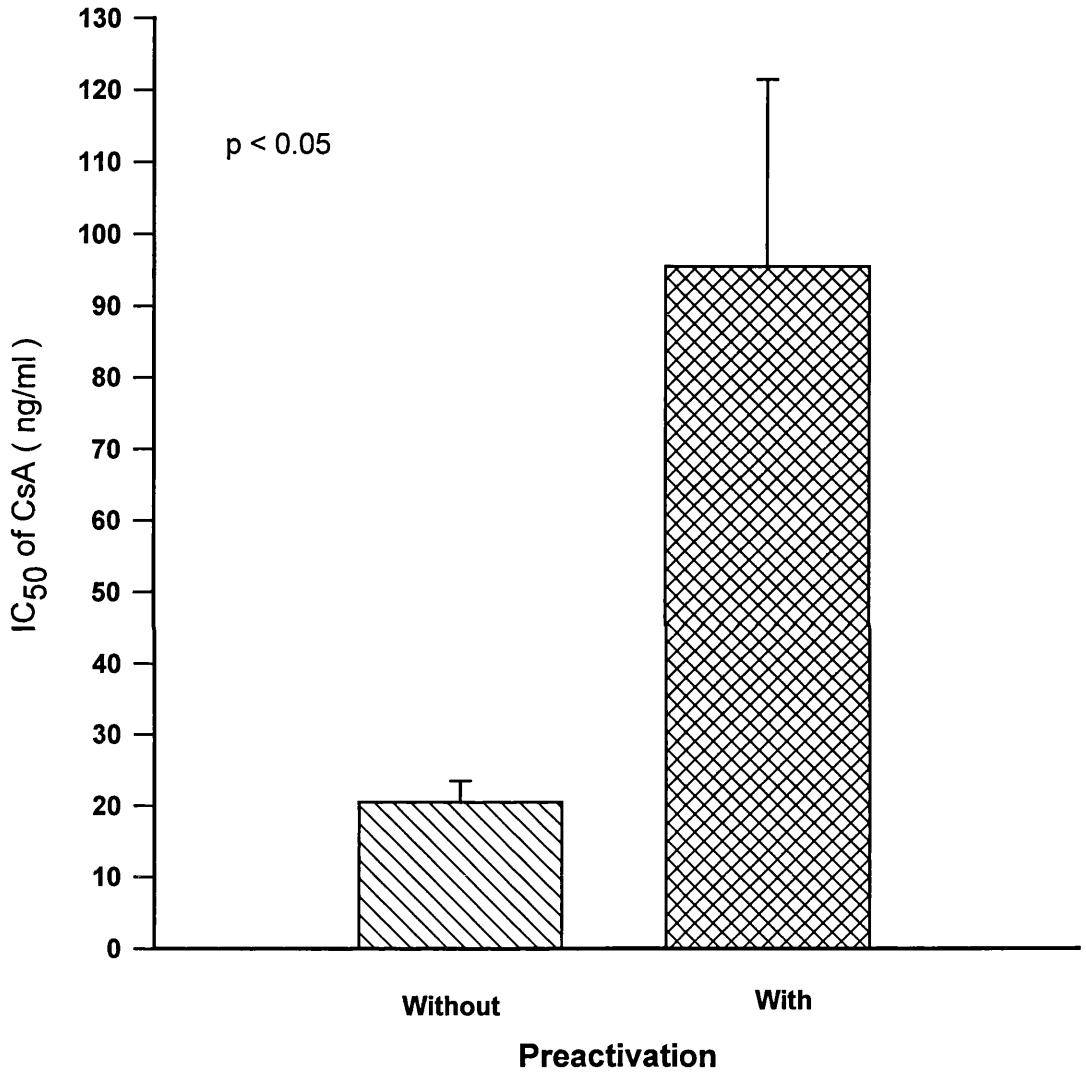

Figure 16. Effect of *in vitro* T cell preactivation on CsA sensitivity of T cells in CAPD patients. CsA sensitivity assay using CTLL-2 as a detector of IL-2 production was carried out with and without PHA preactivation on PBMCs of CAPD patients. The IC_{50} of CsA was determined from the inhibitory dose-response curves. The IC_{50} of CsA is markedly increased after T cell preactivation. Values are presented as mean ± SEM, p value: with preactivation vs without preactivation, n=10.

response curves pre- and post-haemodialysis (Fig. 17). *In vitro* preactivation of T cells with PHA yielded a statistically significant increase in the IC_{50} of CsA, whether pre- or post-haemodialysis. Predialysis, the IC_{50} of CsA without *in vitro* preactivation was 56 ng/ml; after *in vitro* preactivation with PHA the IC_{50} of CsA increased significantly to 283 ng/ml, ($p = 0.0007$) (Fig. 18, Table 3). Postdialysis, the IC_{50} of CsA without *in vitro* preactivation was 99 ng/ml, and increased significantly after *in vitro* PHA preactivation to 721 ng/ml, ($p = 0.0033$) (Fig. 18, Table 3). These results demonstrated that even in chronic renal failure patients on dialysis who have immune dysfunction, the *in vitro* preactivation of T cells made the T lymphocytes resistant to CsA and reduced the CsA sensitivity of T cells.

4.2.3. CsA sensitivity of T cells in CAPD patients

After demonstrating the effect of *in vitro* preactivation of T cells with PHA on CsA sensitivity in different volunteer groups, we investigated any alteration in CsA sensitivity of T cells in chronic renal failure patients on dialysis in comparison with that of normal volunteers. We found that the IC_{50} of CsA in CAPD patients was 20.5 ng/ml, while the IC_{50} of CsA in normal volunteers was 50 ng/ml, ($p = 0.21$) (Fig. 19, Table 4). The result showed that there was statistically no significant change in IC_{50} of CsA in CAPD patients, although the IC_{50} of CsA was lower in CAPD patients than in normal volunteers. This indicated that the CsA sensitivity of T cells is not altered in CAPD patients as compared with normal volunteers.

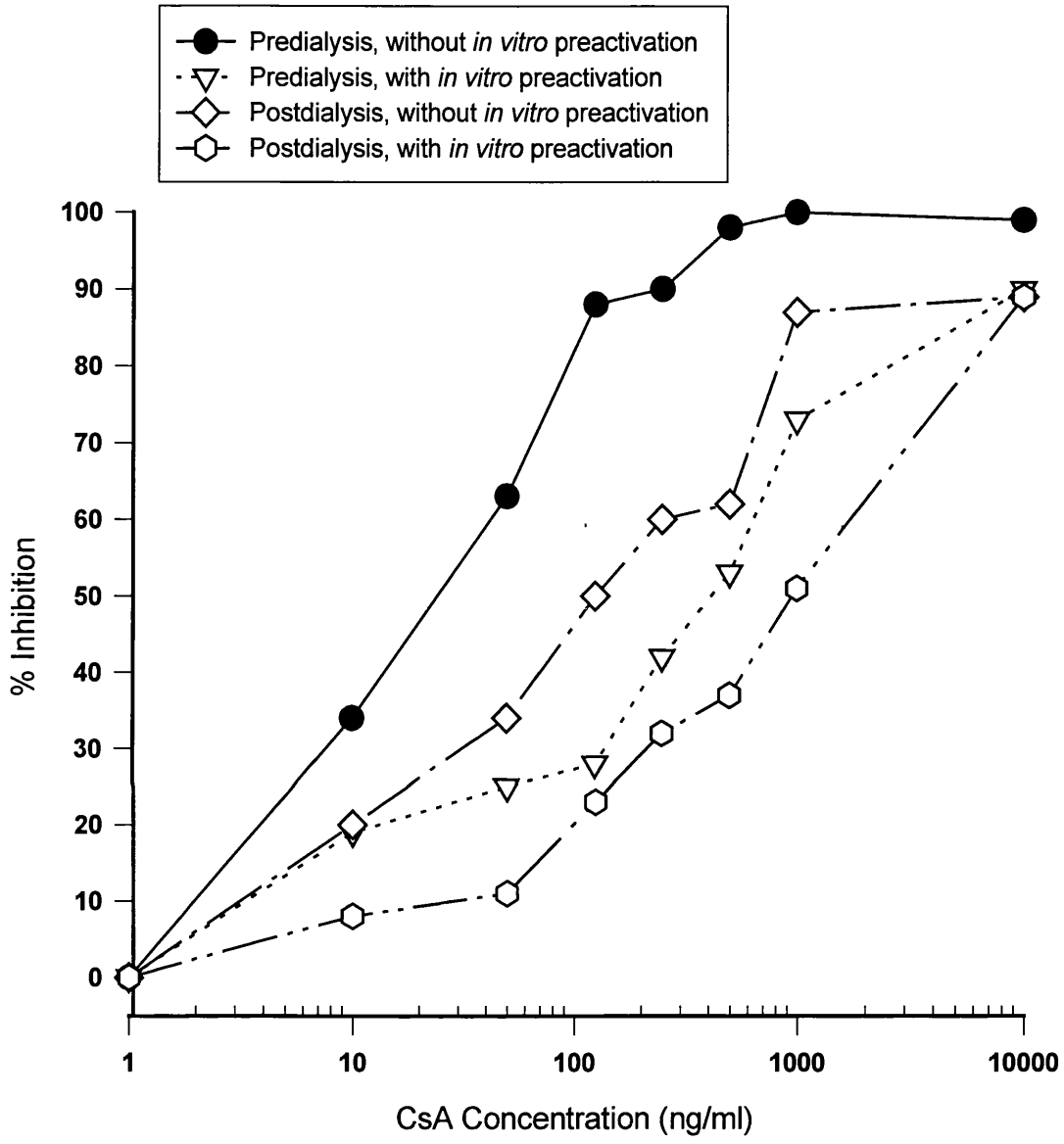

Figure 17. Representative inhibitory dose-response graph of a chronic haemodialysis patient showing reduction of CsA sensitivity of T cells after *in vitro* preactivation. CsA sensitivity assay using CTLL-2 as a detector of IL-2 production was carried out with and without PHA preactivation on PBMCs isolated before and after dialysis A clear shift to the right occurs in the dose-response curves after *in vitro* T cell preactivation. IL-2 production with no added CsA was regarded as 0% inhibition. The response is expressed as % inhibition of IL-2 production as reflected by CTLL-2 proliferation, similar graphs were obtained with 11 other chronic haemodialysis patients.

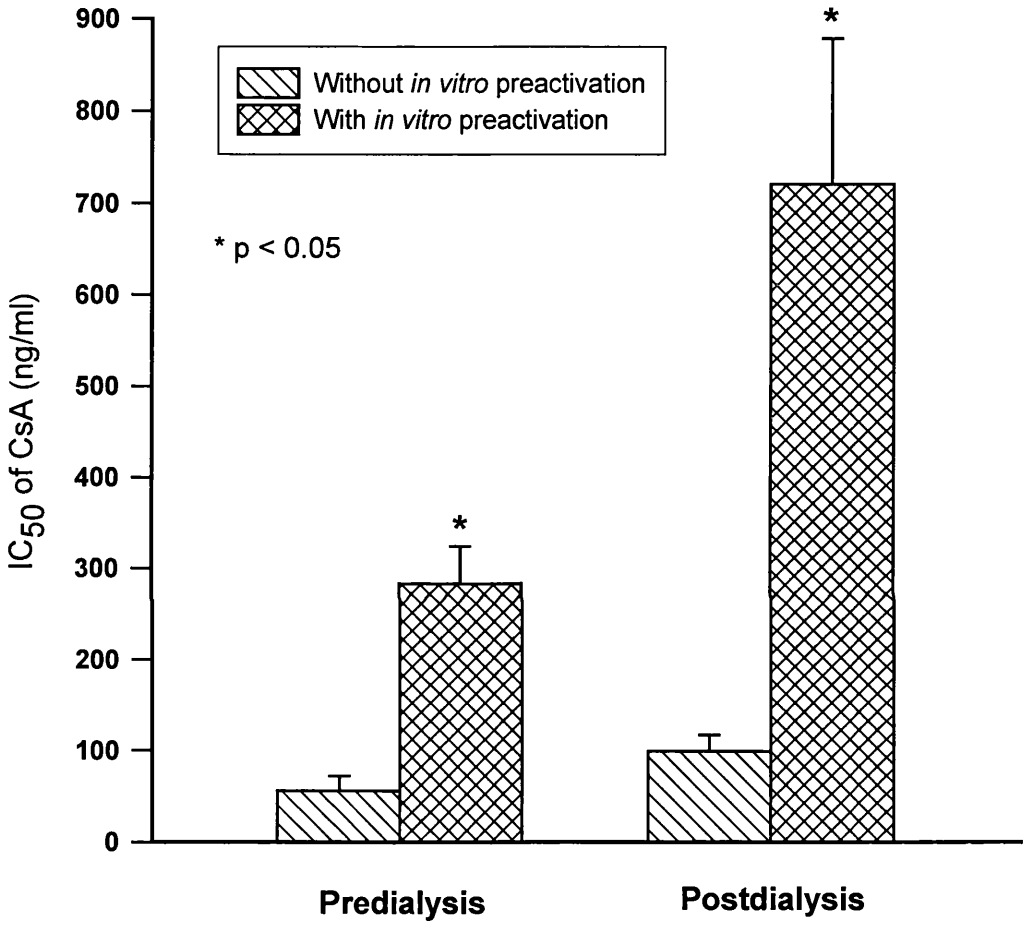

Figure 18. Effect of *in vitro* T cell preactivation on CsA sensitivity of T cells in chronic haemodialysis patients. CsA sensitivity assay using CTLL-2 as a detector of IL-2 production was carried out with and without PHA preactivation on PBMCs of chronic haemodialysis patients before and after dialysis. The IC_{50} of CsA was determined from the inhibitory dose-response curves. Both the pre- and post-haemodialysis IC_{50} of CsA markedly increased after *in vitro* T cell preactivation. IC_{50} values of CsA are presented as mean ± SEM, *p value: with *in vitro* preactivation vs the corresponding without preactivation, n=12.

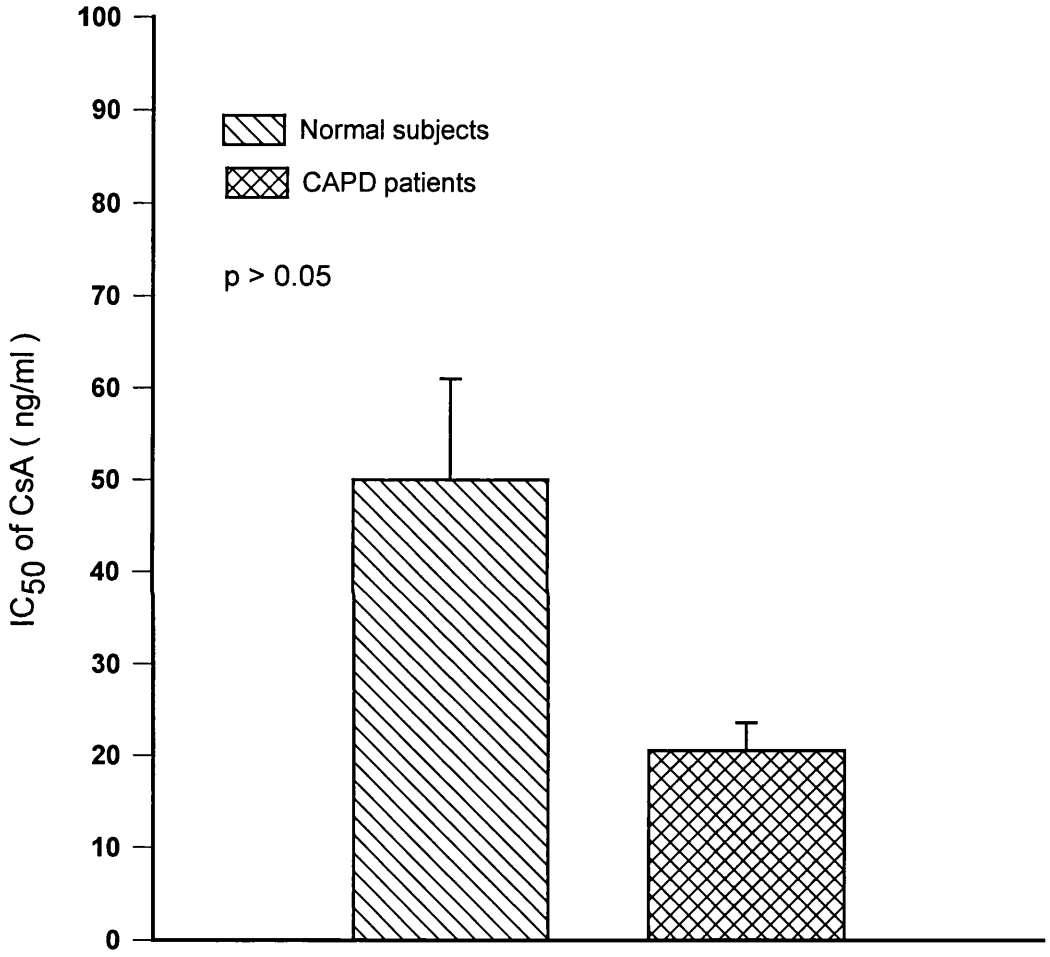

Figure 19. Comparison in CsA sensitivity of T cells between normal subjects and CAPD patients. CsA sensitivity assay using CTLL-2 as a detector of IL-2 production was carried out without *in vitro* preactivation on PBMCs. The IC_{50} of CsA was determined from the inhibitory dose-response curves. Statistically, there is no significant difference in IC_{50} of CsA between the two groups. IC_{50} values of CsA are presented as mean ± SEM.

Volunteers group	(CsA IC_{50})	*p value
Normal volunteers (control) (n=10)	50 ± 11	
CAPD patients (n=10)	20.5 ± 3	*0.21*
HD patients (predialysis) (n=12)	56 ± 16 **p = 0.13	*0.75*
HD patients (postdialysis) (n=12)	99 ± 18 **p = 0.006	*0.030*

Table 4. Effect of dialysis on CsA sensitivity of T cells in chronic renal failure patients. CsA sensitivity assay without *in vitro* PHA preactivation, using CTLL-2 cells as detector of IL-2 production was carried out and the IC_{50} of CsA was plotted from the inhibitory dose-response curves. The data indicate that the IC_{50} of CsA increases significantly in post-haemodialysis patients. Normal volunteers were regarded as a control, (*p vs control, **p vs CAPD patients). IC_{50} values: mean ± (SEM).

4.2.4. CsA sensitivity of T cells in chronic haemodialysis patients

In order to investigate any alteration in CsA sensitivity of T cells in chronic renal failure patients on haemodialysis, we determined the CsA sensitivity of T cells in twelve chronic renal failure patients on chronic haemodialysis before and after the dialysis session and compared with CsA sensitivity of ten normal volunteers. In order to determine the IC_{50} of CsA in the haemodialysis patients, blood was drawn immediately before dialysis and again after dialysis session of 4 hours. The IC_{50} of CsA of normal controls was 50 ng/ml. The predialysis IC_{50} of CsA was 56 ng/ml *($p = 0.75$)*, and the postdialysis IC_{50} of CsA was 99 ng/ml *($p = 0.030$)*, (Fig. 20, Table 4). In comparison to CsA IC_{50} of CAPD patients (20.5 ng/ml), the

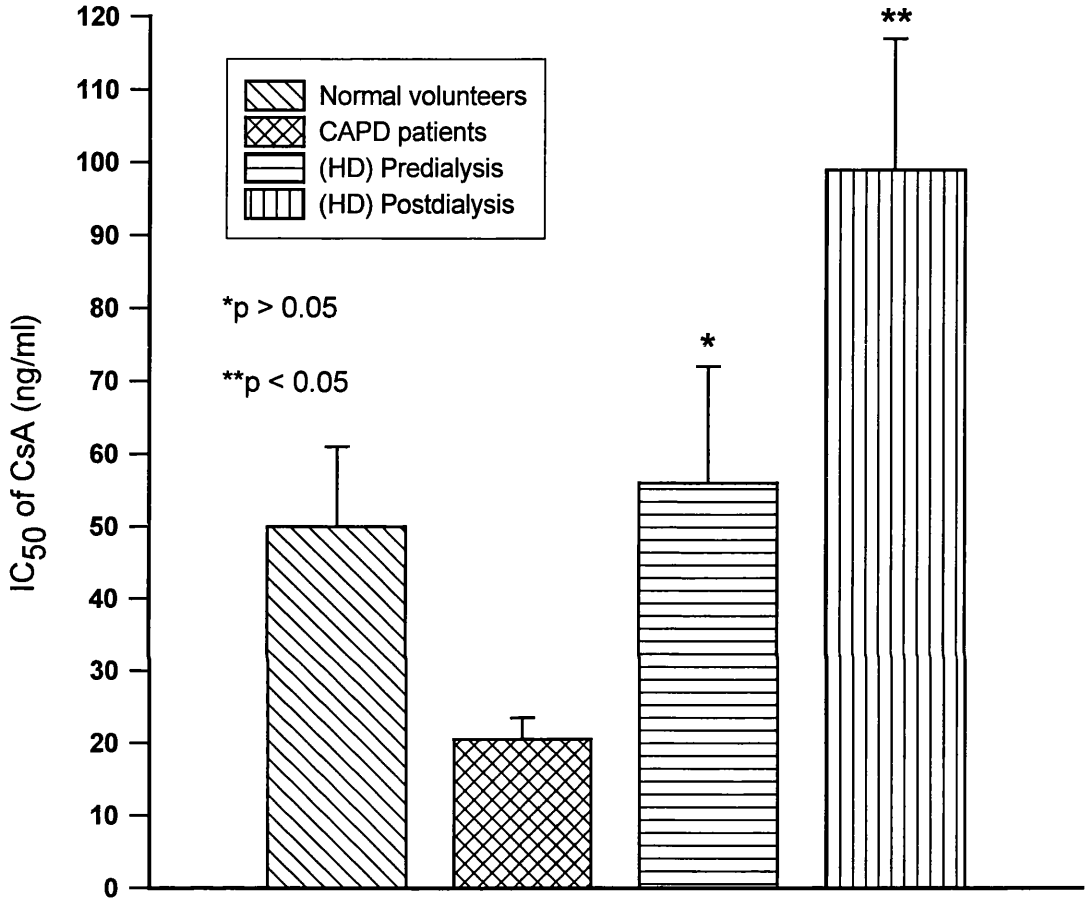

Figure 20. CsA sensitivity of T cells of chronic haemodialysis patients compared to normal volunteers and CAPD patients. CsA sensitivity assay using CTLL-2 as a detector of IL-2 production was carried out without *in vitro* preactivation on PBMCs. The IC_{50} of CsA was determined from the inhibitory dose-response curves. Statistically, there is significant rise in the IC_{50} of CsA after haemodialysis as compared to normal volunteers and CAPD patients (control groups). IC_{50} values of CsA are presented as mean ± SEM, *p value: predialysis vs control groups, **p value: postdialysis vs control groups.

predialysis IC_{50} of CsA was not significantly different *($p = 0.13$),* but IC_{50} of CsA was significantly higher after haemodialysis *($p = 0.006$),* (Fig. 20, Table 4). The results showed that there was statistically significant rise in the IC_{50} of CsA after haemodialysis as compared to normal volunteers and CAPD patients. On the other hand, there was statistically no significant change in the predialysis IC_{50} of CsA as compared to that of normal volunteers and CAPD patients. The results indicated that the CsA sensitivity of T cells was reduced in haemodialysis patients after dialysis but had returned to normal before the next dialysis (48-72 hours).

For all the volunteers included in the study, control wells of 72 hours incubation showed lymphocyte proliferation to PHA and there was no proliferation in the wells without PHA.

4.3. Discussion

In addition to normal volunteers group, we included CAPD patients and haemodialysis patients in our study to investigate the inhibitory effect of CsA on IL-2 production after *in vitro* preactivation. These two groups are waiting for renal transplantation and will receive CsA immunosuppression, so it was interesting to know their CsA sensitivity. We investigated IL-2 production in response to PHA because it is a well established *in vitro* model of T cell activation and is inhibited by moderate doses of CsA (Hess *et al.* 1988).

The initial experiments showed that *in vitro* preactivation with PHA in normal volunteers rendered the IL-2 production of PBLs less sensitive to CsA-mediated inhibition, and reduced the CsA sensitivity of T lymphocytes. It is well known that one of the principal ways by which CsA exerts its immunosuppressive action is by inhibiting the expression of a discrete set of lymphokine genes, notably IL-2 (Kronke *et al.* 1984; Emmel *et al.* 1989). Inhibition of IL-2 secretion is crucial for the blockade of T-cell activation as suggested by experiments in which the inhibitory effect of CsA was partially counteracted by adding exogenous IL-2 to T-cell cultures (Lin *et al.* 1991; Hess, 1985). A sustained rise in intracellular Ca^{2+} as a result of TCR ligation activates the calcium/calmodulin-dependent serine/threonine phosphatase calcineurin pathway (Hubbard and Klee, 1989). CsA blocks the Ca^{2+}-dependent signal transduction pathway initiated by TCR-mediated signal transduction by PHA, apparently through its ability to bind to and inactivate calcineurin. The CsA binds to CyP to form an inhibitory complex. The inhibitory complex of CyP-CsA binds to and inhibits the phosphatase activity of calcineurin (Liu *et al.* 1991; Fruman *et al.* 1992b). The consequence of calcineurin activation is a nuclear translocation of NF-AT_c (Clipstone and Crabtree, 1992; Flanagan *et al.* 1991), most likely occurring by dephosphorylation of NF-AT_c by calcineurin (McCaffrey *et al.* 1993). Once NF-AT_c has translocated to the nucleus it combines with NF-AT_n, binds to consensus sites in several cytokine promoters and initiates gene transcription, including IL-2. Earlier studies have shown that NF-AT is T-cell-specific transcription factor whose activity correlates with the level of IL-2 transcription after TCR is activated (Shaw *et al.*

1988). The net effect of CsA-mediated calcineurin inhibition is inhibition of the translocation of NF-ATc from the cytoplasm to the nucleus (Flanagen *et al.* 1991), hence lack of cytokine gene transcription. CsA and FK-506 titration experiments demonstrated that the inhibition of cellular calcineurin closely paralleled the inhibition of T cell activation as assessed by IL-2 production (Fruman *et al.* 1992b). All these reports indicated that calcineurin is essential, plays a critical role in Ca^{2+}-dependent T cell activation, and is the target for the drug. A period of 2 hours has been defined for commitment to the process of T cell activation; the time required for the activation of many of the early genes is about 40 minutes for IL-2 and 30 to 90 minutes for most of the others (Lowenthal *et al.* 1985). Thus in our preactivation experiments, the calcineurin in T cells was already activated and the IL-2 gene was transcriptionally active by the time CsA was added. The reduction in CsA sensitivity of T cells after preactivation indicates that the susceptibility of activated and inactivated calcineurin to inhibition by CsA is different, and that activated calcineurin is less sensitive to inhibition by CsA. This implies that CsA may be unable to suppress activated T cells, even if the Ca^{2+}-dependent (CsA-sensitive) pathway was responsible for activating the T cells. In agreement with our findings, other studies have demonstrated CsA resistance in mice following injection of alloantigen into the footpads (Kroczek *et al.* 1987; Pereira *et al.* 1990), that might be related to the presence of activated T cells. Furthermore, it has been reported *in vitro* that at least one third of healthy subjects have shown paradoxical proliferation of T cells with lower concentrations ($\leq$ 50 ng/ml) of CsA (Masy *et al.* 1994). In view of our

findings, this failure of inhibition of T cells by CsA might be also related to the presence of Ca^{2+}-dependent activated T cells in these experimental situations. The presence of these activated T cells in these animal studies could be related either to inadequate immunosuppression, to the process of animal experiments or to the preparation of the cells.

Studies have shown that the calcineurin of PBLs is only partially inhibited in CsA-treated patients despite therapeutic CsA blood levels that would be completely inhibitory *in vitro* (Pai *et al.* 1994; Batiuk *et al.* 1995a). Furthermore, they were able to induce mRNA for IFN-γ and IL-2 in the PBLs of these patients in response to calcium ionophore and the cells showed no inhibitory evidence that they were exposed to high CsA concentrations *in vivo* (Batiuk *et al.* 1995a). These observations were confirmed in CsA-treated mice with blood levels in the human therapeutic range which did not manifest complete inhibition of any of the CsA-sensitive systems: calcineurin activity, IL-2 and IFN-γ mRNA etc (Batiuk *et al.* 1996b). These findings are all in agreement with the clinical experience that transplant patients on CsA have a 50% incidence of acute rejection, produce cytokines in response to T cell receptor stimulation by anti-CD3 (Chatenoud *et al.* 1991), and have cytokines detectable in their inflammatory infiltrates (Noronha *et al.* 1992). With all these reports, in addition to our data, we suggest that the *in vivo* immunosuppressive effect of CsA might not adequately inhibit activated T cells. The presence of activated T cells could be the source of acute rejection or chronic rejection, depending on the extent of inhibition of these cells.

The distribution of CsA among the different blood components has been studied extensively. Variations in haematocrit, lipoprotein, and serum protein levels can each alter the distribution of CsA (Lensmeyer *et al.* 1989; Hughes *et al.* 1991; Kasiske *et al.* 1988b). The presence of plentiful elements such as red blood cells in whole blood have been shown to provide irrelevant non-leukocyte binding sites that can prevent CsA from reaching the leukocytes (Batiuk *et al.* 1996a), the presumed targets of CsA immunosuppression. Recently, it has been shown that untreated red blood cells can pull CsA out of previously treated PBLs, enhancing recovery of both calcineurin activity and inducible IFN-γ synthesis, demonstrating that CsA inhibition of calcineurin activity is rapidly reversible (Batiuk, *et al.* 1995b). Finally, CsA also accumulates in many organs (Lensmeyer *et al.* 1991). Thus, since CsA binding sites are not saturated during maintenance immunosuppression, both drug non-compliance, variation in blood composition, and perhaps, organ composition could potentially impact on CsA availability to PBLs, and thus inhibition of calcineurin activity and cytokine synthesis, especially in the presence of inadequate inhibition of activated T cells. Together these factors predict risk of graft rejection.

We also noticed this observation of reduced CsA sensitivity of circulating T cells after PHA preactivation of T cells in patients with end stage renal disease on both CAPD and haemodialysis. It is well known that the immune system is impaired in chronic renal failure and dialysis, and several studies have shown defective proliferation of T cells to most mitogens (Shasha *et al.* 1988), and abnormally low

IL-2 and IFN-γ production, (Meuer *et al.* 1987; Descamps-Latscha, 1993). A study on mRNA expression of IL-2 and IFN-γ revealed low mRNA expression, more pronounced in haemodialysis patients (Gerez *et al.* 1991). The reduced CsA sensitivity of T cells that we have demonstrated after PHA prestimulation in dialysis patients, suggests that the T lymphocytes of dialysis patients are activated by the PHA stimulus, and the extent of CsA inhibition of calcineurin in the PBLs with and without preactivation are dissimilar. Thus, T lymphocyte function at the level of the Ca^{2+}-dependent (CsA-sensitive) pathway is physiologic in dialysis patients, in agreement with a previous report that T cell function was found to be physiologic in chronic haemodialysis patients (Meuer *et al.* 1987). Extra T cell mechanisms have been proposed underlying uraemic T cell deficiency, namely: uraemic toxins (Newberry *et al.* 1971; Girndt *et al.* 1993; Vanholder *et al.* 1994), impaired monocyte function (Meuer *et al.* 1987; Ruiz et al 1990; Dinarello, 1992; Matthias *et al.* 1993), hyperparathyrodism of chronic renal failure (Massry *et al.* 1994), and a downregulation of the TCR/CD3 complex by the uraemic environment (Stachowski *et al.* 1991).

Our study showed that the CsA sensitivity of PBLs in CAPD patients and in haemodialysis patients predialysis was not different from that of normal volunteers as defined by IC_{50} of CsA. In contrast, the CsA sensitivity of PBLs was reduced in haemodialysis patients after a 4 hours haemodialysis session as the IC_{50} for CsA was significantly increased over that of normal volunteers and CAPD patients. This suggests that chronic haemodialysis patients are less

sensitive to CsA after dialysis, and the reduced CsA sensitivity that we have seen after haemodialysis could be contributed to by the presence of a preactivated population of T cells in these patients (Chatenoud *et al.* 1986).

5. T CELLS PREACTIVATION IN CHRONIC HAEMODIALYSIS PATIENTS

5.1. Rationale for Study

The effect of chronic haemodialysis in end stage renal disease patients on T cell activation and its sensitivity to CsA is reported in this chapter. The findings of reduced CsA sensitivity of T cells after *in vitro* PHA preactivation, and the reduced CsA sensitivity of T cells of chronic haemodialysis patients as compared to that of normal volunteers and CAPD patients suggested the need for a study of the effect of haemodialysis on T cell activation comparing before and after a dialysis session (predialysis, postdialysis). In addition to the expression of activation markers, intracellular IL-2 molecules and the ability to secrete IL-2, the CsA sensitivity of T cells of peripheral blood was studied using different approaches. This study was considered important because fits potential to explain inadequate CsA-based immunosuppression and rejection in relation to the presence of a specific population of T cells due to chronic haemodialysis.

5.2. Results

5.2.1. CsA sensitivity of T cells using CTLL-2 cells

We determined the CsA sensitivity of circulating T cells using CTLL-2 cells as detector of IL-2, pre- and post-haemodialysis. In individual chronic haemodialysis

patients, there was a clear shift to the right in the inhibitory dose-response curve after haemodialysis as compared to predialysis (Fig. 17). When PBLs of these patients were preactivated with PHA before adding CsA, there was a more shift to the right in the inhibitory dose-response curves after dialysis as compared to predialysis (Fig. 17). The shift to the right in the curves after dialysis indicated that CsA sensitivity of T cells was reduced by haemodialysis. The predialysis IC_{50} of CsA, was 56 ng/ml, and increased to 98 ng/ml after haemodialysis, *($p = 0.018$)* (Fig. 21, Table 5). When the IC_{50} of CsA was determined pre- and post-dialysis after *in vitro* prestimulation with PHA, the predialysis IC_{50} of CsA was 283 ng/ml, while the postdialysis IC_{50} of CsA was 721 ng/ml, *($p = 0.042$)* (Fig. 21, Table 5). The statistically significant increase in IC_{50s} for CsA post-haemodialysis clearly demonstrated that haemodialysis leads to resistance to CsA and reduction of CsA sensitivity of T cells.

Stimulus	Predialysis (IC_{50})	Postdialysis (IC_{50})	*p value*
Normal	56 ± 16	98 ± 18	*0.018*
PHA Prestimulation	283 ± 41	721 ± 158	*0.042*

Table 5. CsA sensitivity of T cells in relation to haemodialysis in end stage renal disease patients on maintenance haemodialysis. CsA sensitivity assay using CTLL-2 as a detector of IL-2 production was carried out with and without PHA prestimulation on PBMCs isolated before and after dialysis. The IC_{50} of CsA was plotted from the inhibitory dose-response curves. The data show that the IC_{50} of CsA increases significantly after haemodialysis even with prestimulation of T cells. IC_{50}: concentration of CsA that achieved 50% inhibition of IL-2 production, IC_{50} values (ng/ml) are mean ± SEM of n=12.

For all patients, control wells of 72 hours incubation showed lymphocyte proliferation in response to PHA and no proliferation in the wells without PHA.

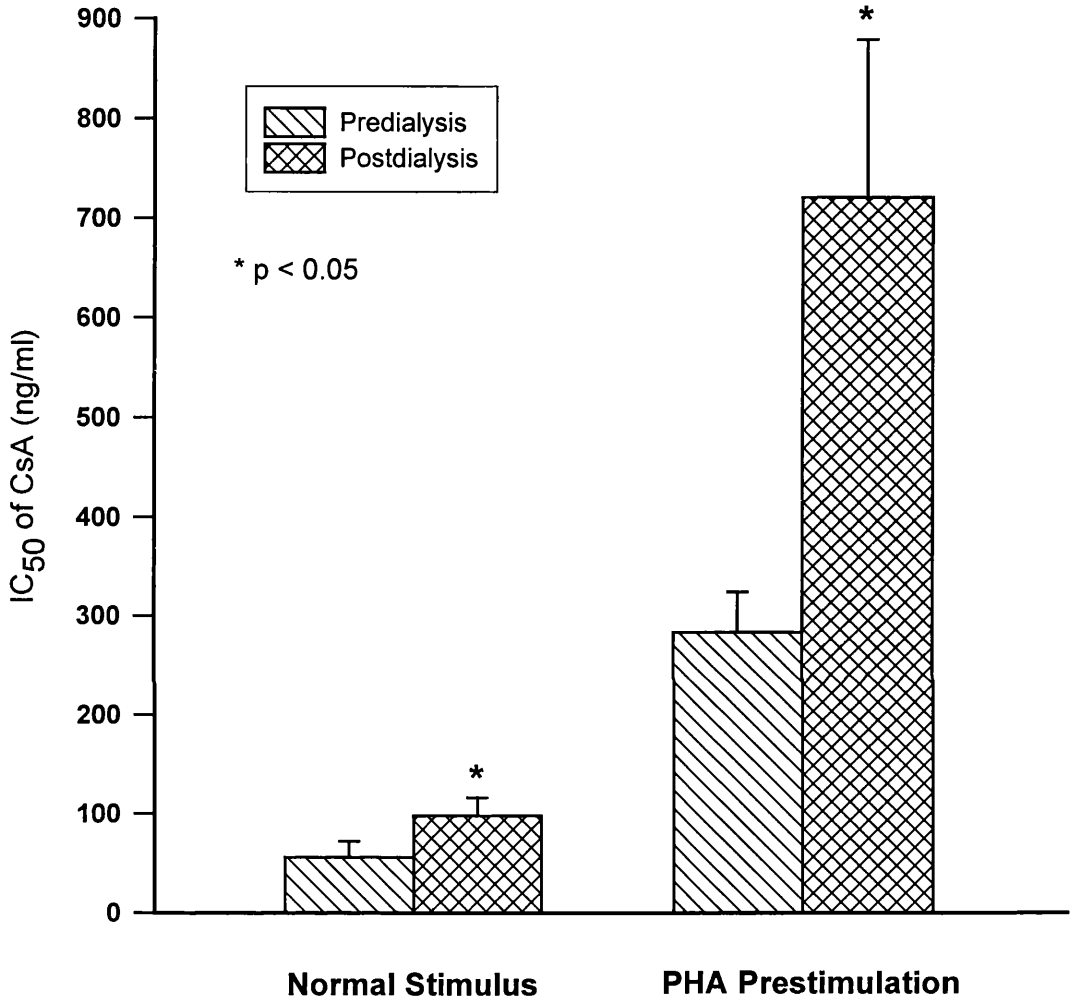

Figure 21. Effect of haemodialysis on CsA sensitivity of T cells. CsA sensitivity assay using CTLL-2 as a detector of IL-2 production was carried out with and without *in vitro* PHA prestimulation on PBMCs of 12 chronic haemodialysis patients pre- and post-dialysis. The IC_{50} of CsA was determined from the inhibitory dose-response curves. The IC_{50} of CsA is markedly increased after haemodialysis. IC_{50} values of CsA are presented as mean ± SEM; *p value: postdialysis compared with predialysis for the corresponding cell stimulation state.

5.2.2. Effect of CsA on frequency of IL-2 Producing T cells

The *in vitro* inhibitory effect of CsA on the frequency of T cells positive for IL-2 was studied in chronic renal failure patients on chronic haemodialysis by flow cytometric analysis before and after dialysis. The purpose of this analysis was to determine the effect of haemodialysis on the CsA reduction of the frequency of IL-2 producing T cells. After PBMCs were exposed to various concentrations of CsA, stimulated by PHA, and the stained cells analysed by flow cytometry, the percentage of CsA reduction of the frequency of T cells positive for IL-2 was determined pre- and post-haemodialysis. The percentage of CsA reduction of the frequency of $CD3^+$ cells positive for IL-2 was significantly different between predialysis and postdialysis at 100 ng/ml of CsA, 48.5% and 36.6% reduction respectively, *($p = 0.0057$)* (Fig. 22, Table 6). The data showed that the 100 ng/ml of CsA did not achieve the same percent reduction, and that the degree of CsA reduction of the frequency of IL-2 producing T cells was less post- than pre-haemodialysis.

The degree of CsA reduction of the frequency was nearly the same for pre-and post-haemodialysis at the extremes of CsA concentration (10 and 1000 ng/ml) (Fig. 22, Table 6). At 10 ng/ml of CsA, the percentage of reduction of frequency of $CD3^+$ cells positive for IL-2 was 24.4% pre- and 22.7% post-haemodialysis, *($p= 0.67$)*. The percentage of reduction of frequency of $CD3^+$ cells positive for IL-2 at 1000 ng/ml of CsA was 54.9% pre- and 53.1% post-haemodialysis, *($p = 0.66$)*. At the very high *in vitro* CsA concentration, i. e. 1000 ng/ml, which is at

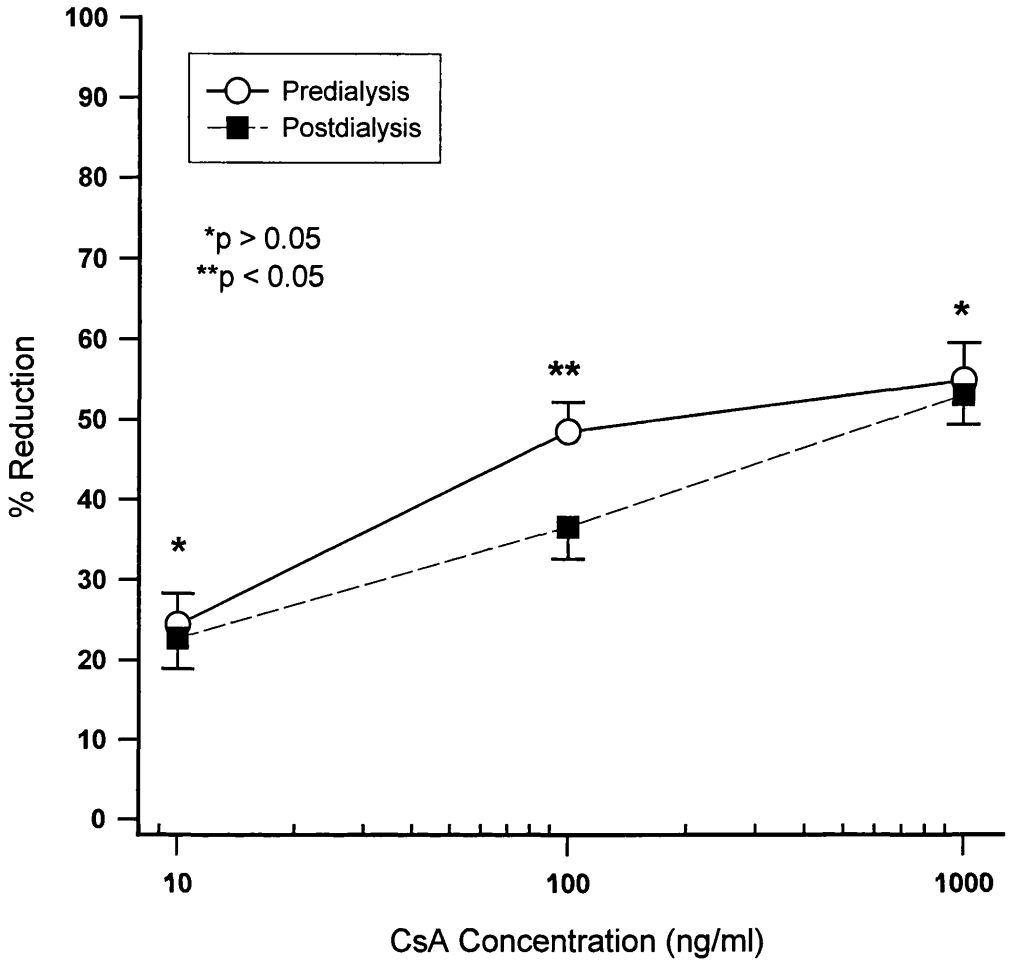

Figure 22. Effect of haemodialysis on CsA reduction of the frequency of IL-2 producing T cells. CsA sensitivity assay was carried on PBMCs of 12 chronic haemodialysis patients pre- and post-dialysis. After 18 hours of incubation, the cells were stained with monoclonal antibodies and analysed by flow cytometry. The cell labelling was with ECD-antibody against CD3 and PE-antibody against intracellular IL-2. The graph shows that the degree of reduction of the frequency is less post- than pre-haemodialysis at 100 ng/ml of CsA. Frequency of $CD3^+$ cells positive for IL-2 with no added CsA was taken as 0% reduction (control). Response is expressed as % reduction of the frequency of $CD3^+$ cells positive for IL-2. Values are mean percent ± SEM, p value: postdialysis vs predialysis at each CsA concentration.

least one order of magnitude more potent than that seen *in vivo*, maximum inhibition of IL-2 is achieved. This may not have any clinical relevance since there is no significant difference in the degree of CsA reduction of the frequency pre-and post-haemodialysis. The reduced reduction by CsA of the frequency of IL-2 producing T cells at 100 ng/ml of CsA after dialysis indicates that the CsA sensitivity of T cells was reduced and that T cells were made resistant to CsA during haemodialysis.

CsA concentrations (ng/ml)	Predialysis	Postdialysis	*P value*
10	24.4 ± 3.9	22.7 ± 3.8	*0.67*
100	48.5 ± 3.7	36.6 ± 4	*0.0057*
1000	54.9 ± 4.7	53.1 ± 3.7	*0.66*

Table 6. CsA reduction of the frequency of IL-2 producing T cells in relation to haemodialysis. CsA sensitivity assay was carried on PBMCs of 12 chronic haemodialysis patients before and after-dialysis. After 18 hours of incubation, the cells were stained with monoclonal antibodies and analysed by flow cytometry. The cells Labelling was with ECD-antibody against CD3 and PE-antibody against intracellular IL-2. The data show at 100 ng/ml of CsA, there is less degree of CsA reduction of the frequency after haemodialysis. Frequency of $CD3^+$ cells positive for IL-2 with no added CsA was taken as 0% reduction (control). Results are expressed as % reduction of the frequency of $CD3^+$ cells positive for IL-2. Values are mean percent ± SEM.

For all volunteers, control wells of 72 hours incubation showed lymphocyte proliferation with PHA and no proliferation in the wells without PHA.

5.2.3. Intracellular IL-2 in T cells in relation to haemodailysis and its sensitivity to CsA

We studied the effect of haemodialysis on intracellular IL-2 response to PHA stimulation and on *in vitro* CsA inhibition of intracellular IL-2 production in T

cells. The study was carried out on PBMCs of chronic haemodialysis patients and analysed by flow cytometry. We found statistically no significant difference in the number of intracellular IL-2 molecules in $CD3^+$ cells pre- and post-haemodialysis, for either PHA-stimulated or unstimulated cells (Fig. 23, Table. 7). The data were widely scattered since the intracellular IL-2 of T cells showed very wide inter–individual variation in both pre- and post-haemodialysis (Fig. 24). However, the percentages of the cases that had higher number of intracellular IL-2 molecules in predialysis than in postdialysis was 66.6% for unstimulated cells and 58.3% for PHA stimulated. Although there was statistically no significant difference in the number of intracellular IL-2 molecules due to haemodialysis, in the majority of volunteers studied the number of intracellular IL-2 molecules in $CD3^+$ cells was less post- than pre-haemodialysis, whether the cells were PHA-stimulated or not.

Cells	Predialysis	Postdialysis	*p value*
Unstimulated	205199 ± 30299	158441 ± 18301	*0.38*
PHA-stimulated	257910 ± 39248	221064 ± 24480	*0.19*

Table 7. Effect of haemodialysis on intracellular IL-2 production by T cells. After 18 hours of incubation, the unstimulated and PHA-stimulated PBMCs isolated pre- and post-haemodialysis were stained with monoclonal antibodies and analysed by flow cytometry. The cell Labelling was with ECD-antibody against CD3 and PE-antibody against intracellular IL-2. Results are expressed as intracellular IL-2 molecules per 3000 $CD3^+$ cells. Values are mean ± SEM, n=12 chronic haemodialysis patients.

We also determined the effect of dialysis on the *in vitro* CsA inhibition of intracellular IL-2 production in T cells, analysed by flow cytometry. There was a

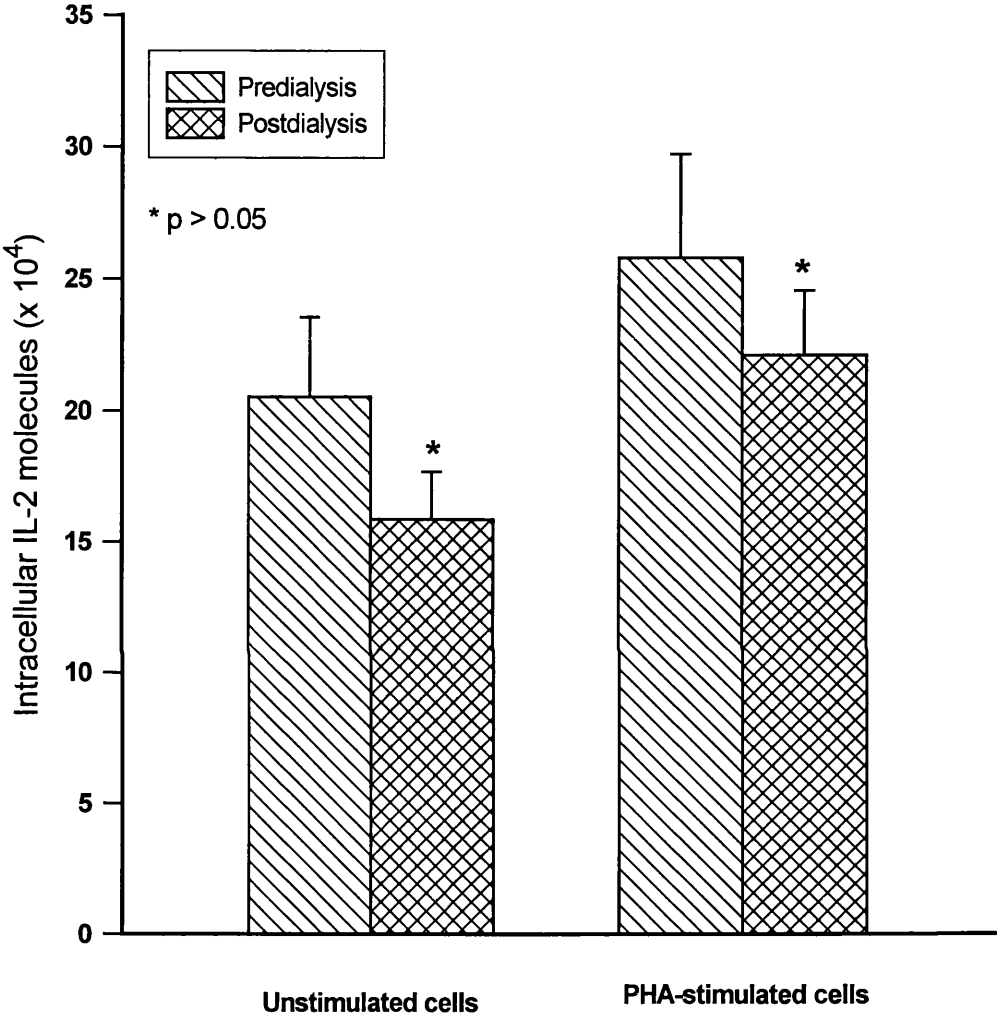

Figure 23. Intracellular IL-2 production in T cells in relation to haemodialysis. After 18 hours of incubation, the unstimulated and PHA-stimulated PBMCs isolated before and after haemodialysis were stained with monoclonal antibodies and analysed by flow cytometry. The cell labelling was with ECD-antibody against CD3 and PE-antibody against intracellular IL-2. Results are expressed as intracellular IL-2 molecules per 3000 $CD3^+$ cells. Values are mean ± SEM, *p value: postdialysis vs predialysis for each cell stimulation state, n=12 chronic haemodialysis patients.

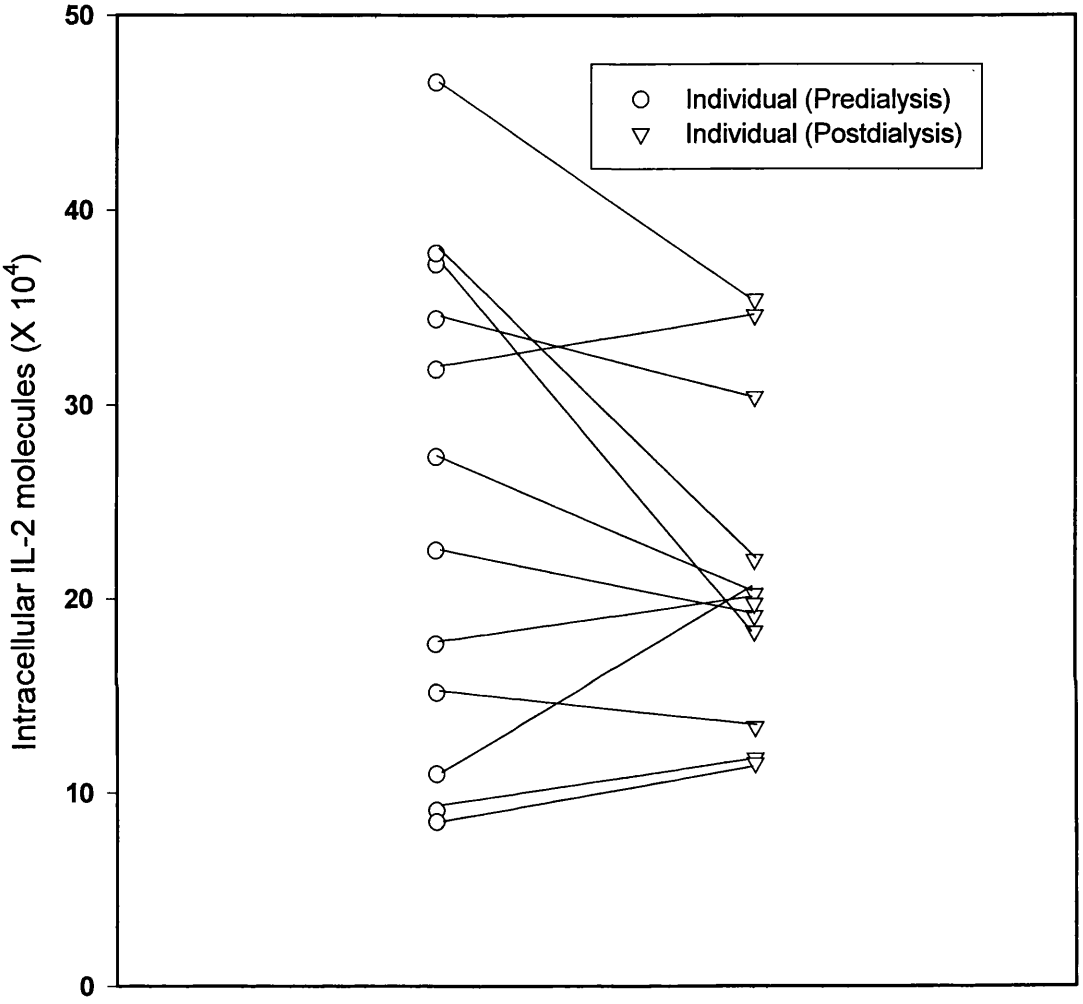

Figure 24. A wide variety in the number of intracellular IL-2 molecules within T cells in chronic haemodialysis patients. After 18 hours of incubation, the PHA-stimulated PBMCs were labelled with ECD-antibody against CD3 and PE-antibody against intracellular IL-2. The graph shows a wide individual scatter of the number of intracellular IL-2 molecules both pre- and post-haemodialysis. Results are expressed as intracellular IL-2 molecules per 3000 $CD3^{+}$cells, n=12.

statistically significant difference between pre- and post-haemodialysis samples at 100 ng/ml of CsA in the percentage of CsA inhibition of intracellular IL-2 production in $CD3^+$ cells, 54.3% and 37.6% inhibition respectively, *(p = 0.017)* (Fig. 25, Table 8). The data showed that at 100 ng/ml of CsA the percent inhibition achieved was not the same, and that the degree of CsA inhibition of intracellular IL-2 production in T cells was less post- than pre-haemodialysis. The percent CsA inhibition of intracellular IL-2 production was statistically similar pre- and post-haemodialysis at the extremes of CsA concentration (10 and 1000 ng/ml) (Fig. 25, Table 8). At 10 ng/ml of CsA, the percent inhibition of intracellular IL-2 production in $CD3^+$ cells was 28.4% pre- and 22.9% post-haemodialysis, *(p = 0.33)*. The percent inhibition of intracellular IL-2 production in $CD3^+$ cells at 1000 ng/ml of CsA was 63.8% pre- and 64.2% post-haemodialysis, *(p = 0.55)*. The achievement of minor or huge inhibition of intracellular IL-2 production at the extremes of CsA concentration did not elicit the CsA resistance of T cells after haemodialysis. The inhibition of intracellular IL-2 production in T cells at these CsA concentrations was either insufficient or so high that the difference in the inhibition of intracellular IL-2 production between pre- and post-haemodialysis cannot be determined at these CsA concentrations. The lower CsA inhibition of intracellular IL-2 production in T cells at 100 ng/ml of CsA after dialysis session indicates that the CsA sensitivity of T cells was reduced and that T cells became resistant to CsA during haemodialysis.

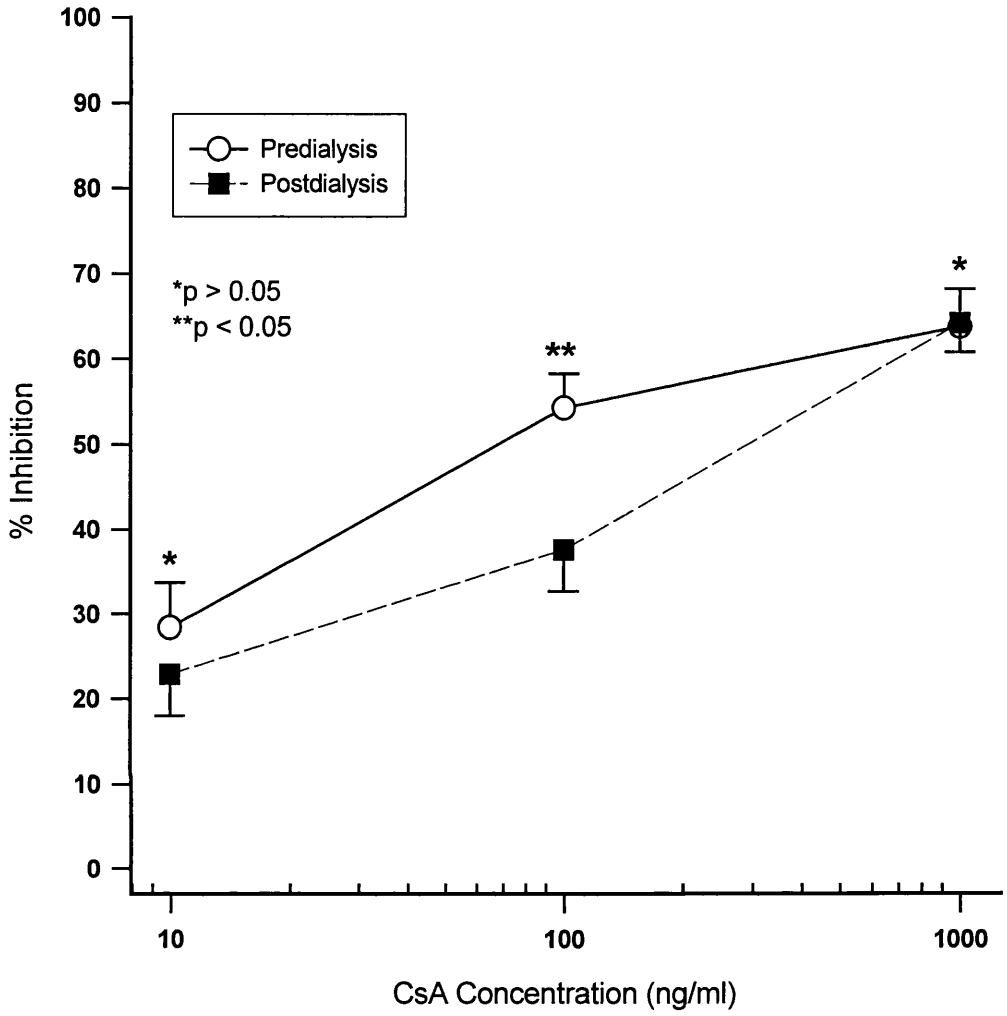

Figure 25. Percentage of CsA inhibition of intracellular IL-2 production in T cells in relation to haemodialysis. CsA sensitivity assay was carried on PBMCs of 12 chronic haemodialysis patients pre- and post-dialysis. After 18 hours of incubation, the cells were stained with monoclonal antibodies and analysed by flow cytometry. The cell labelling was with ECD-antibody against CD3 and PE-antibody against intracellular IL-2. The graph shows at 100 ng/ml of CsA, there is less degree of inhibition of intracellular IL-2 production post- than pre-haemodialysis. Intracellular IL-2 production within $CD3^+$ cells with no added CsA was taken as 0% inhibition (control). Results are expressed as % inhibition of intracellular IL-2 production in $CD3^+$ cells. Values are mean percent ± SEM, p value: postdialysis vs predialysis at each CsA concentration.

CsA concentrations (ng/ml)	Predialysis	Postdialysis	*p value*
10	28.4 ± 5.3	22.9 ± 4.9	*0.33*
100	54.3 ± 4	37.6 ± 4.9	*0.017*
1000	63.8 ± 4.4	64.2 ± 3.4	*0.55*

Table 8. Effect of haemodialysis on CsA inhibition of intracellular IL-2 production in T cells. CsA sensitivity assay was carried on PBMCs of 12 chronic haemodialysis patients before and after dialysis. After 18 hours of incubation, the cells were stained with monoclonal antibodies and analysed by flow cytometry. The cell Labelling was with ECD-antibody against CD3 and PE-antibody against intracellular IL-2. The data show that the degree of CsA inhibition of intracellular IL-2 production was less post- than pre-haemodialysis at 100 ng/ml of CsA. Intracellular IL-2 production within $CD3^+$ cells with no added CsA was taken as 0% inhibition (control). Results are expressed as % inhibition of intracellular IL-2 production in $CD3^+$ cells. Values are mean percent ± SEM.

When we looked at the number of the intracellular IL-2 molecules per 3000 $CD3^+$ cells in response to various CsA concentrations in relation to dialysis, we found that there was statistically no significant difference in the number of intracellular IL-2 molecules in $CD3^+$ cells between pre- and post-haemodialysis at each CsA concentration tested (10, 100, 1000 ng/ml) (Table 9, Fig. 26). Careful examination of the later (Fig. 26) showed that the number of intracellular IL-2 molecules in $CD3^+$ cells were reduced in parallel between pre- and post-haemodialysis at 10 and 1000 ng/ml of CsA, but at 100 ng/ml of CsA the proportion of reduction of intracellular IL-2 molecules in $CD3^+$ cells was more pre- than post-haemodialysis.

This may indicates the presence of CsA resistance after haemodialysis that was masked by inter-individual variation.

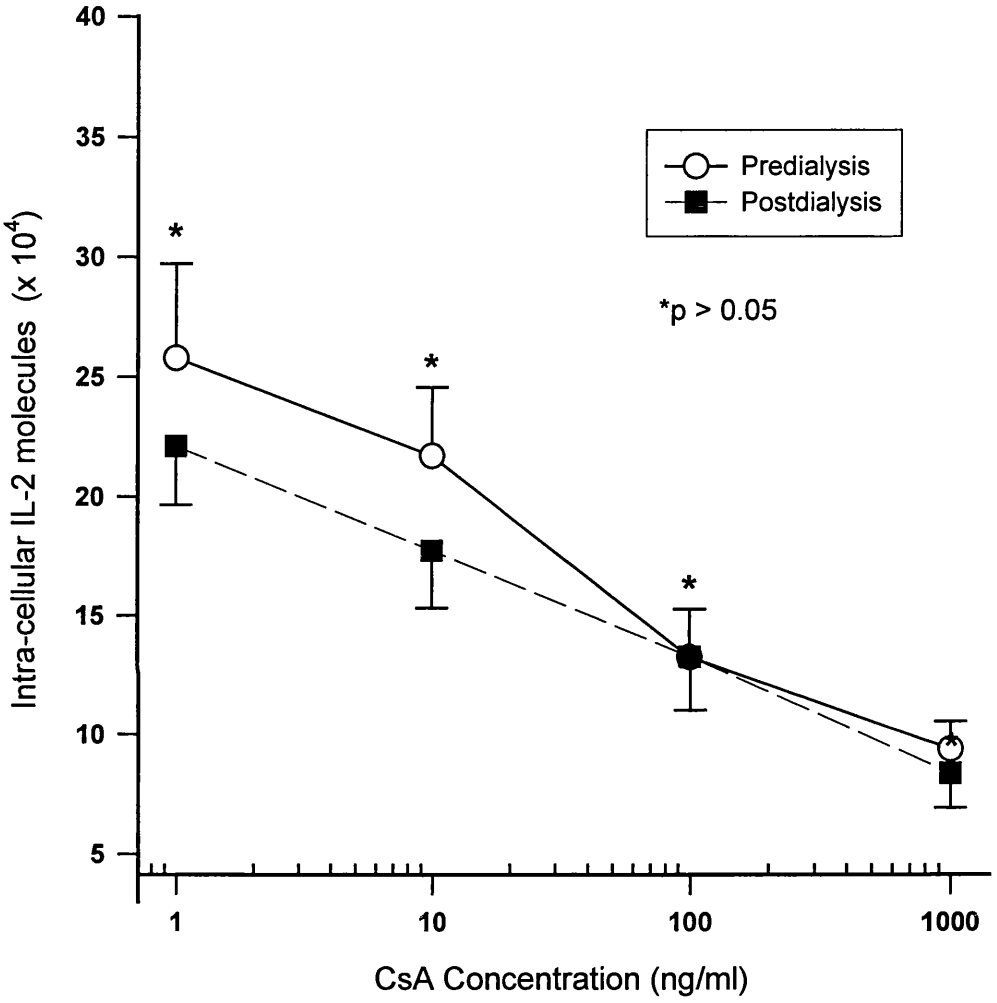

Figure 26. Effect of haemodialysis on CsA reduction of intracellular IL-2 within T cells. CsA sensitivity assay was carried on PBMCs of 12 chronic haemodialysis patients pre- and post-dialysis. After 18 hours of incubation, the cells were stained with monoclonal antibodies and analysed by flow cytometry. The cell labelling was with ECD-antibody against CD3 and PE-antibody against intracellular IL-2. Results are expressed as intracellular IL-2 molecules per 3000 $CD3^+$ cells. Values are mean ± SEM, *p value: postdialysis vs predialysis at each CsA concentration.

CsA concentrations (ng/ml)	Predialysis	Postdialysis	*p value*
0	257910 ± 39248	221064 ± 24480	*0.19*
10	217163 ± 28559	177329 ± 24261	*0.28*
100	132454 ± 20164	132543 ± 22457	*0.70*
1000	93704 ± 11591	83662 ± 14486	*0.60*

Table 9. Intracellular IL-2 production in T cells after CsA inhibition in relation to haemodialysis. CsA sensitivity assay was carried on PBMCs of 12 chronic haemodialysis patients before and after dialysis. After 18 hours of incubation, the cells were stained with monoclonal antibodies and analysed by flow cytometry. The cell Labelling was with ECD-antibody against CD3 and PE-antibody against intracellular IL-2. Results are expressed as intracellular IL-2 molecules per 3000 $CD3^+$ cells. Values are mean ± SEM.

For all volunteers, control wells of 72 hours incubation showed lymphocyte proliferation in response to PHA and no proliferation in the wells without PHA.

5.2.4. *In vitro* IL-2 secretion and sensitivity to CsA in relation to haemodialysis

We tested T cells ability to secrete IL-2 and its sensitivity to CsA before and after dialysis session, by measuring IL-2 concentration in cell culture supernatants. The purpose of the analysis was to determine the effect of haemodialysis on T cell capacity of IL-2 production and on the degree of CsA inhibition. IL-2 production and its sensitivity to CsA were tested on PBMCs of chronic renal failure patients on maintenance haemodialysis in response to PHA and in the presence of various CsA concentrations; ELISA was used to measure secreted IL-2. Without cell stimulation, IL-2 concentrations were undetectable in cell culture supernatants both pre- and post- haemodialysis. Both the pre- and post-haemodialysis T cells

responded to PHA stimulation, and there was statistically no significant difference in IL-2 concentration when postdialysis was compared with predialysis (Fig. 27, Table 10). Although there was no significance difference between pre- and post-haemodialysis in IL-2 concentration in response to PHA as there was a wide inter-individual variation in IL-2 concentrations both pre- and post-haemodialysis; 66.6% of cases had higher IL-2 concentrations pre- than post-haemodialysis (Fig. 28).

Cells	Predialysis	Postdialysis	*p value*
Unstimulated	1.14 ± 0.80	0.48 ± 0.42	*0.16*
PHA-stimulated	35.6 ± 11	58 ± 31	*0.39*

Table 10. Effect of haemodialysis on *in vitro* IL-2 secretion by PBMCs. After 24 hours incubation of the unstimulated and PHA-stimulated PBMCs of 12 chronic haemodialysis patients, ELISA was used to measure IL-2 concentration in cell culture supernatants. Results are expressed as IL-2 concentration in cell culture supernatant (pg/ml). Values are mean ± SEM.

Regarding the *in vitro* CsA inhibition of IL-2 secretion, we found a statistically significant difference in the percentage of inhibition of IL-2 secretion between pre- and post-haemodialysis at 100 ng/ml of CsA; inhibition was 77.8% and 64.6% respectively, *(p = 0.037)* (Fig. 29, Table 11). This data showed that postdialysis at 100 ng/ml of CsA the degree of CsA inhibition was significantly less when compared to predialysis. Thus, in agreement with previous data, this indicated the existence of CsA resistance of T cells after haemodialysis. At each of the extremes of CsA concentration (10, 500, 1000 ng/ml) tested, no significant

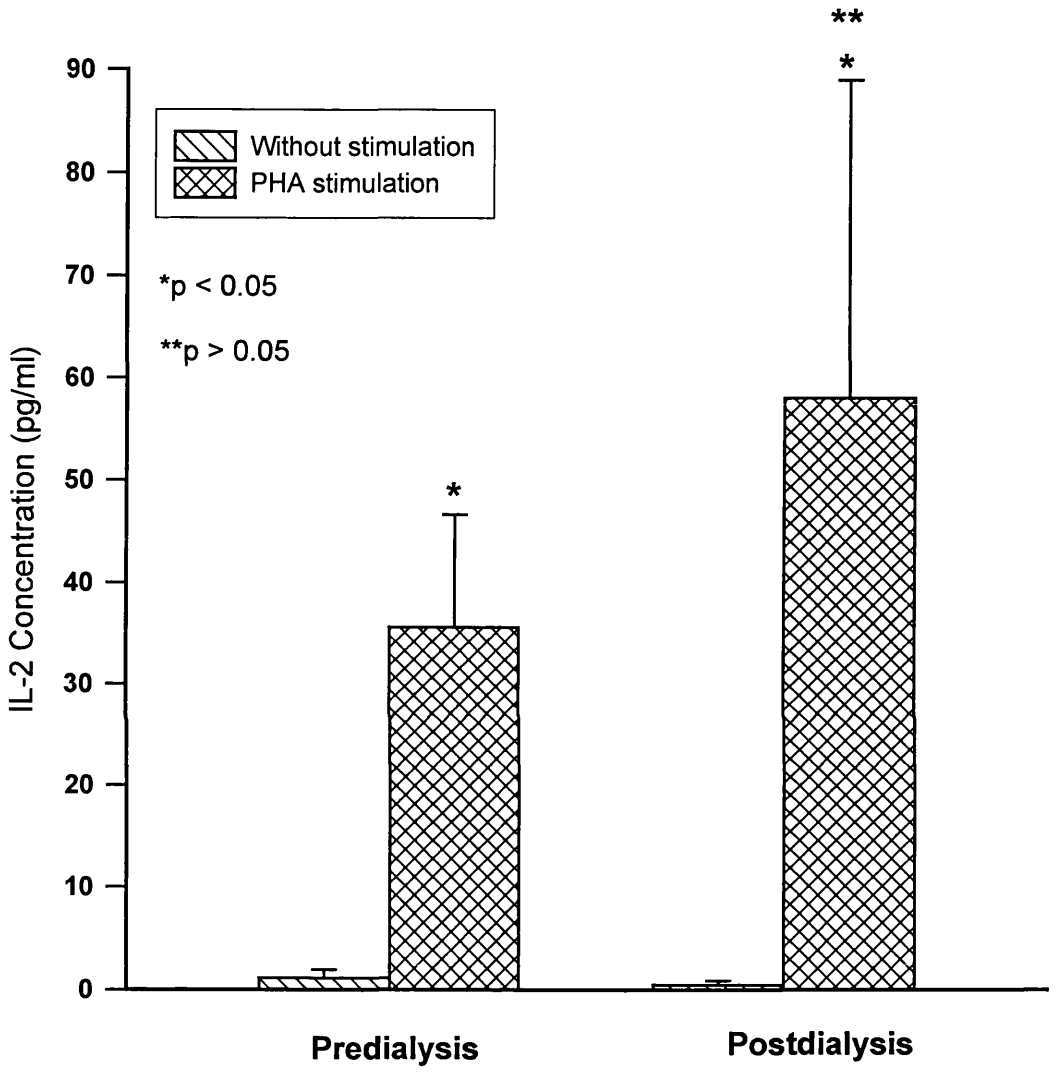

Figure 27. IL-2 secretion by PBMCs isolated pre- and post-haemodialysis. After 24 hours incubation of unstimulated and PHA-stimulated PBMCs of 12 chronic haemodialysis patients, ELISA was used to measure the IL-2 concentration in cell culture supernatants. IL-2 production increased significantly in response to PHA before and after haemodialysis. Results are expressed as IL-2 concentration in cell culture supernatant (pg/ml). Values are mean ± SEM, *p value: PHA-stimulated compared with the unstimulated cell state, **p value: postdialysis vs predialysis for PHA-stimulated cells.

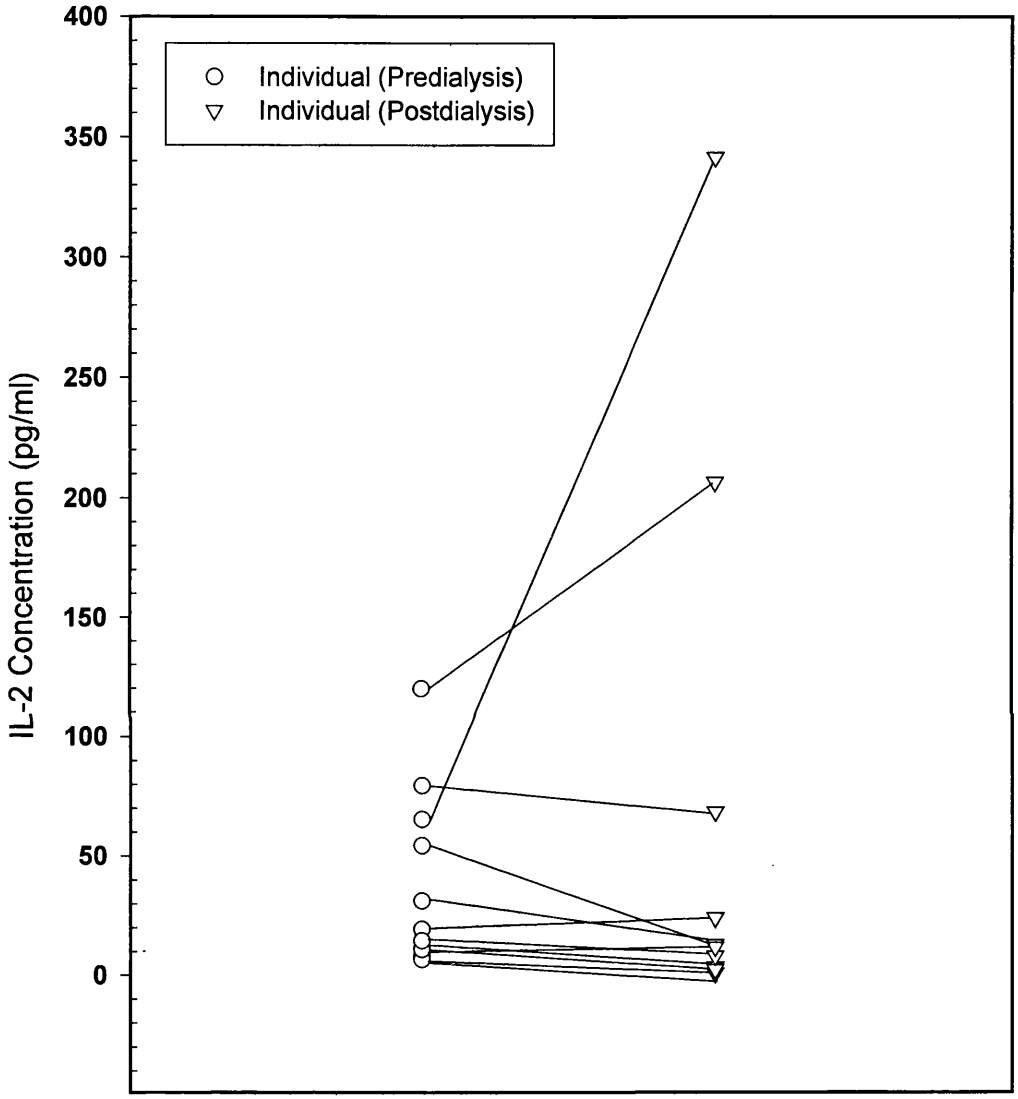

Figure 28. A wide variation of *in vitro* IL-2 production by PBMCs isolated from chronic haemodialysis patients. After 24 hours incubation of PHA-stimulated PBMCs of pre- and post-haemodialysis, ELISA was used to measure the IL-2 concentration in cell culture supernatants. The graph shows a wide individual scatter of IL-2 concentration both pre- and post-haemodialysis, and also indicates that PBLs of majority of the patients after haemodialysis produced less IL-2 in response to PHA stimulation, n=12.

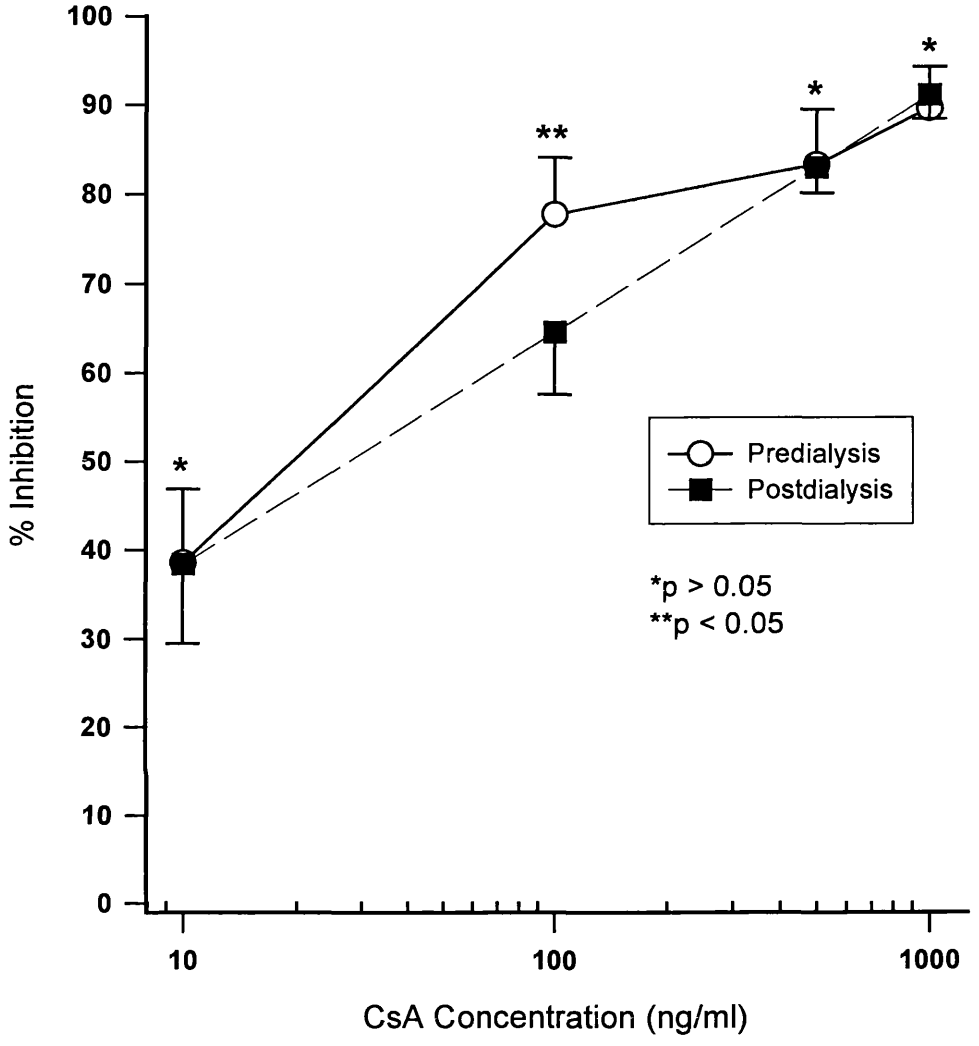

Figure 29. CsA inhibition of IL-2 secretion in relation to haemodialysis. CsA sensitivity assay was carried on PBMCs of 12 chronic haemodialysis patients before and after dialysis. After 24 hours of incubation, ELISA was used to measure the IL-2 concentration in cell culture supernatants The graph shows that at 100 ng/ml of CsA, there is significantly less CsA inhibition of IL-2 secretion after haemodialysis as compared to predialysis. IL-2 secretion with no added CsA was taken as 0% inhibition (control). Results are expressed as % inhibition of IL-2 secretion as determined by measurement of the IL-2 concentration. Values are mean ± SEM, p value: postdialysis compared with predialysis at each CsA concentration.

difference was observed in the percentage of *in vitro* CsA inhibition of IL-2 secretion when postdialysis was compared with predialysis for the corresponding CsA concentration (Fig. 29, Table 11). The achievement of minor or huge inhibition of IL-2 production at the extremes of CsA concentration did not elicit the CsA resistance of T cells postdialysis. The inhibition of IL-2 production at these CsA concentrations was either insufficient or so high that the difference in CsA sensitivity of T cell in the inhibition of IL-2 production between pre- and post-haemodialysis cannot be determined at these CsA concentrations.

CsA concentrations (ng/ml)	Predialysis	Postdialysis	*p value*
10	38.6 ± 8.3	38.4 ± 8.9	*0.99*
100	77.8 ± 6.4	64.6 ± 7	*0.037*
500	83.4 ± 6.2	83.1 ± 2.9	*0.96*
1000	89.7± 4.7	91.2± 2.7	*0.79*

Table 11 Effect of haemodialysis on CsA inhibition of IL-2 secretion. CsA sensitivity assay was carried on PBMCs of 12 chronic haemodialysis patients pre- and post-haemodialysis. After 24 hours incubation of the cells, ELISA was used to measure IL-2 concentration in cell culture supernatants. The data show at 100 ng/ml of CsA, there is significantly less degree of CsA inhibition of IL-2 production after haemodialysis. IL-2 production into cell culture supernatant with no added CsA was taken as 0% inhibition (control). Results are expressed as % inhibition of IL-2 secretion. Values are mean ± SEM.

For all volunteers, control wells after 72 hours incubation showed lymphocyte proliferation in response to PHA and no proliferation in the wells without PHA.

5.2.5. CD25 and CD69 as markers of T cell activation

The expression of CD25 and CD69 on peripheral blood T lymphocytes was investigated by flow cytometry analysis in chronic renal failure patients on maintenance haemodialysis before and at the end of dialysis. The expression of these molecules was used to indicate T cell activation. CD25 was clearly expressed on T cells both pre- and post-haemodialysis, and there was no significant difference in the percentage of T cells expressing CD25 pre- and post-dialysis (Fig. 30, Table 12). The percentage of $CD3^+$ cells expressing CD25 was 10.42% for predialysis, and 9.17% for postdialysis, *($p = 0.063$)*. However, when PBMCs were stimulated with PHA, the percentage of T cells expressing CD25 was slightly elevated both pre- and post-haemodialysis, being statistically significant (Fig. 30, Table 12). PHA stimulation did not result in any significant difference in percentage of T cells expressing CD25 compared the post- with the pre-haemodialysis (Fig. 30, Table 12). In normal controls, the percentage of unstimulated $CD3^+$ cells expressing CD25 was almost undetectable (0.96 ± 0.15%, *$p \leq 0.0001$*, n=6).

	Predialysis	Postdialysis	**p value (Post- vs Pre-dialysis*
Unstimulated cells	10.42 ± 1.3	9.17 ± 1.1	*0.063*
PHA-stimulated cells	13.92 ± 1.1	12.50 ± 1.5	*0.12*
***p value (stimulated vs unstimulated)*	*0.0049*	*0.00019*	

Table 12. CD25 expression on T cells in chronic haemodialysis patients. After 18 hours of incubation, the PBMCs isolated before and after dialysis were stained with monoclonal antibodies and analysed by flow cytometry. The cells Labelling was with ECD-antibody against CD3 and FITC-antibody against CD25. No significance difference in the expression of CD25 between pre- and post-haemodialysis. Results are expressed as percentage of $CD3^+$ cells expressing CD25. Values are mean ± SEM, n=12.

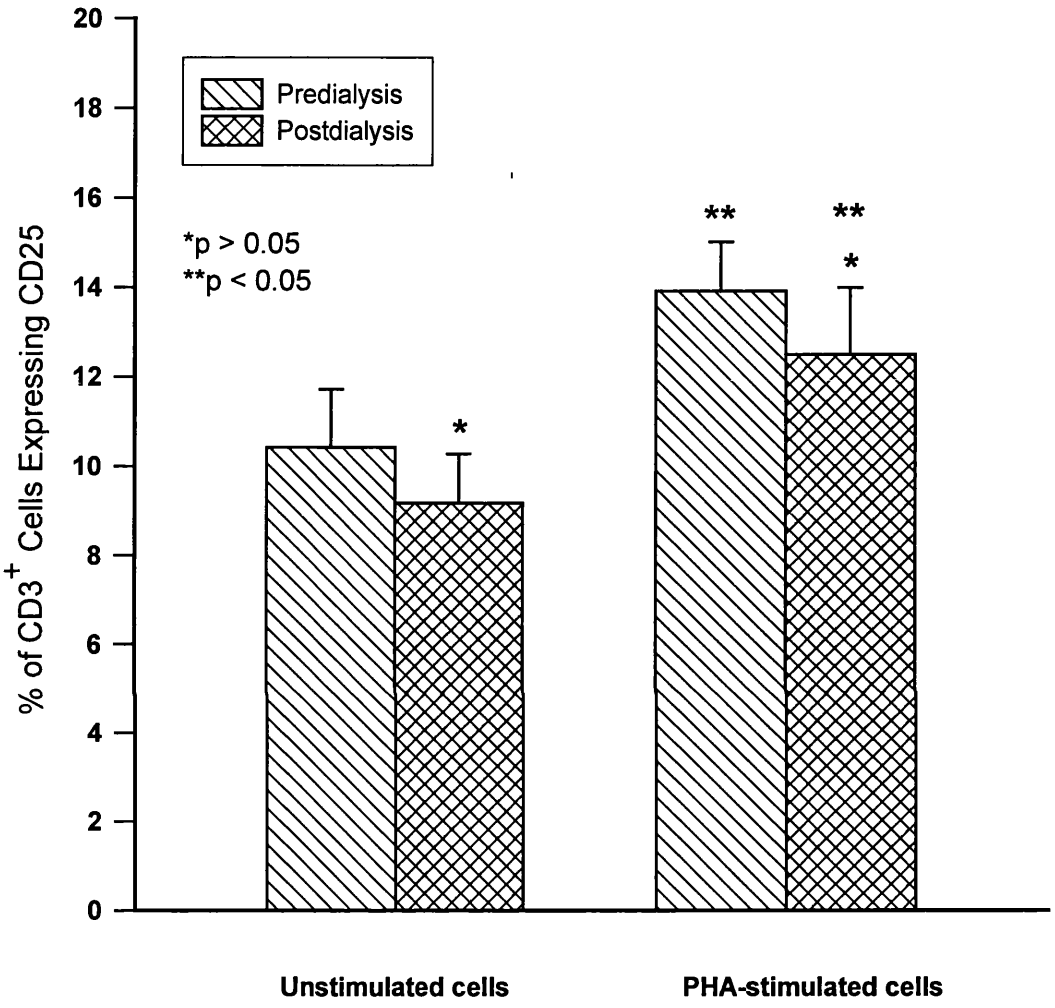

Figure 30. Pre- and post-haemodialysis CD25 expression on T cells. After 18 hours of incubation, the PBMCs isolated pre- and post-haemodialysis were stained with monoclonal antibodies and analysed by flow cytometry. The cell labelling was with ECD-antibody against CD3 and FITC-antibody against CD25. The graph shows expression of CD25 on T cells and that PHA stimulation enhances the expression of CD25 both pre- and post-haemodialysis. Values are mean ± SEM, *p value: postdialysis compared with predialysis for each cell stimulation state, **p value: PHA-stimulated compared with the unstimulated state, n=12 chronic haemodialysis patients.

When we tested the expression of CD69 on T cells in relation to dialysis, CD69 was expressed on the majority of T cells before and after dialysis, and the percentage of T cells expressing CD69 was almost identical when postdialysis was compared with predialysis (Fig. 31, Table 13). The percentage of $CD3^+$ cells expressing CD69 was 70.17% pre- and 72.25% post-haemodialysis, *($p = 0.10$).* PHA simulation of PBMCs did not enhance the expression of CD69 on T cells, as the percentage of T cells expressing CD69 remained almost similar after PHA stimulation for both the pre-and post-haemodialysis. Furthermore, PHA stimulation did not result in any significant difference in percentage of T cells expressing CD69 between pre- and post-haemodialysis (Fig. 31, Table 13). In normal control subjects, the percentage of unstimulated $CD3^+$ cells expressing CD69 was almost undetectable ($0.61 \pm 0.13\%$, *$p < 0.0001$,* n=6).

	Predialysis	Postdialysis	**p value (Post- vs Pre-dialysis*
Unstimulated cells	70.17 ± 2.2	72.25 ± 1.6	*0.10*
PHA-stimulated cells	71.50 ± 2.5	71.58 ± 2.1	*0.96*
***p value (stimulated vs unstimulated)*	*0.43*	*0.63*	

Table 13. CD69 expression on T cells in chronic haemodialysis patients. After 18 hours of incubation, the PBMCs of pre- and post-haemodialysis were stained with monoclonal antibodies and analysed by flow cytometry. The cell Labelling was with ECD-antibody against CD3 and PE-Cy5-antibody against CD69. Pre- and post-haemodialysis CD69 expression is statistically similar. Results are expressed as percentage of $CD3^+$ cells expressing CD69. Values are mean ± SEM, n=12.

For all volunteers, control wells of 72 hours incubation showed lymphocyte proliferation with PHA and no proliferation in the wells without PHA.

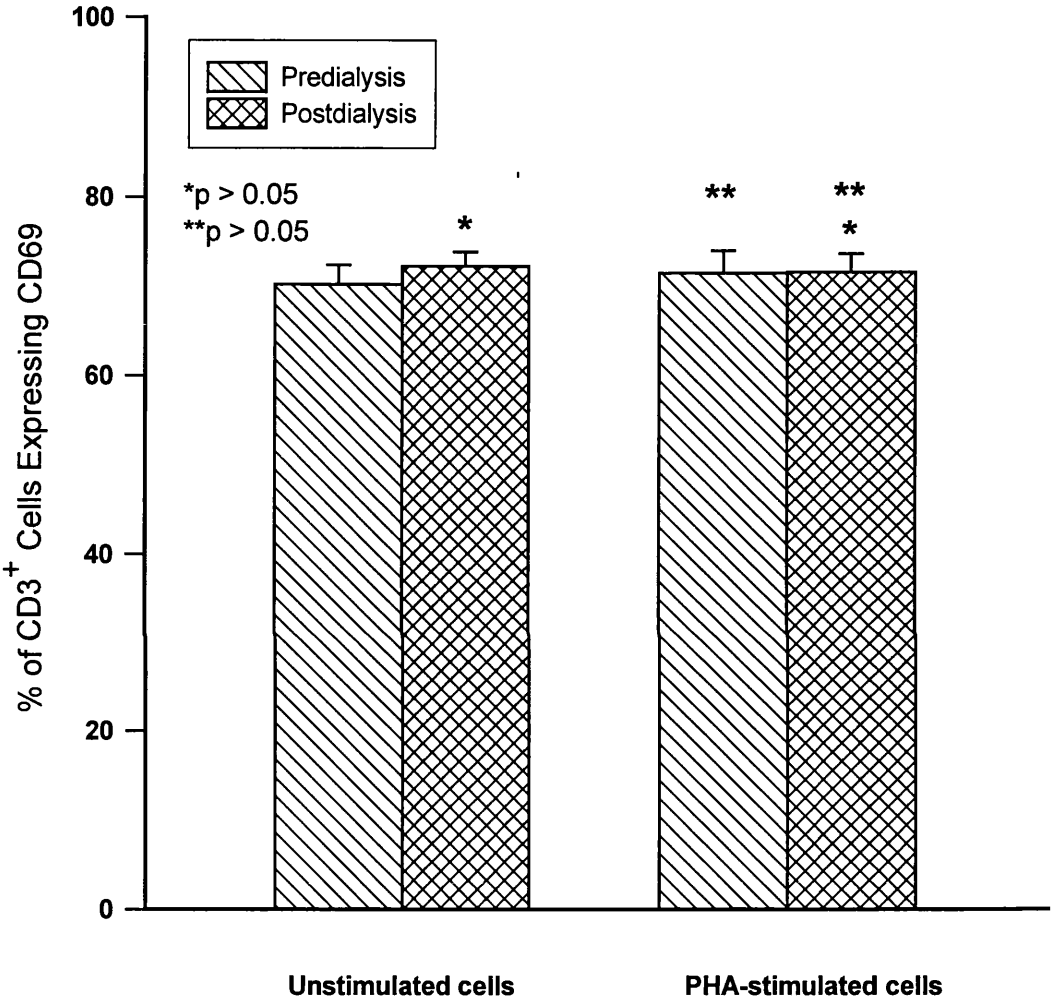

Figure 31. Pre- and post-haemodialysis CD69 expression on T cells. After 18 hours of incubation, the PBMCs isolated pre- and post-haemodialysis were stained with monoclonal antibodies and analysed by flow cytometry. The cell labelling was with ECD-antibody against CD3 and PE-Cy5-antibody against CD69. The graph shows expression of CD69 and it is similar in pre- and post-haemodialysis. Values are mean ± SEM, *p value: postdialysis compared with predialysis for each cell stimulation state, **p value: PHA-stimulated compared with the unstimulated state.

5.3. Discussion

The process of Haemodialysis is well recognised as an immunologic challenge due to passage of blood over a foreign membrane, which may well induce lymphocyte activation. T lymphocyte activation is a normal response to immunologic stimuli, which enables the body to regulate its response to foreign and unwanted native antigens. However, lack of T lymphocyte activation or inappropriate T lymphocyte activation are associated with a dysfunctional immune response that may enhance susceptibility to infection and tumours, and may increase the risk of allograft rejection after transplantation. It is well known that the immune system is impaired in patients with chronic renal failure and in patients on regular haemodialysis, and these patients have frequent infectious complications (reviewed in: Descamps-Latscha *et al.* 1994), and reduced efficacy of clinical vaccinations (Kohler *et al.* 1984; Cappel *et al.* 1983). Many *in vitro* studies correlated these clinical findings with reduced proliferation of PBMCs to various stimuli (Donati *et al.* 1991; Kurz *et al.* 1986; Ghio *et al.* 1985). Paradoxically, despite deficient responses to most pathogens and mitogens, several reports have shown that T cells from end stage renal disease and chronic haemodialysis patients showed functional and phenotypic signs of activation. Thus it is important to establish the sensitivity of T cells to CsA in chronic haemodialysis patients who are known to have the paradoxical coexistence of a functional deficiency state with phenotypic signs of T cell activation (Chatenoud *et al.* 1986), since these patients will be candidates for renal allografts.

In the previous chapter we showed that there was significant reduction of CsA sensitivity of T cells in chronic haemodialysis patients postdialysis as compared with healthy volunteers and CAPD patients. In the current chapter we have reported the immediate effect of haemodialysis on CsA sensitivity of circulating T cells. This was done by testing several parameters before and at the end of a dialysis session of 4 hours. The data on IL-2 secretion in cell culture supernatant measured by either CTLL-2 proliferation or ELISA, frequency of IL-2 producing T cells, and intracellular IL-2 production in T cells demonstrated that the susceptibility of T cells to CsA pre- and post-haemodialysis was different with less susceptibility and less sensitivity to CsA after haemodialysis. We found no correlation between the value of individual postdialysis CsA IC_{50s} and haemodialysis duration of each patient (appendix 4).

Thus, we are able to show that the phenomenon of haemodialysis rendered circulating T cells resistant to and less sensitive to CsA. Recently, it has been reported that haemodialysis patients have a higher incidence of acute cellular rejection following renal transplantation as compared to CAPD patients (Zamauskaite *et al.* 1999). The authors speculated that the mechanism of this observation could be because of preserved T helper (Th) 1 cytokines (IL-2, IFN-γ and TNF-α) response, responsible for graft rejection, in haemodialysis patients, while the Th 1 response is impaired in CAPD patients (Zamauskaite *et al.* 1999). It has been reported that if the transplanted kidney functioned adequately through the first post-transplant week such that the patient did not require dialysis during

that period, 1-year graft survival was 83% for first and 80% for repeat transplant recipients (Cecka *et al.* 1992). When dialysis was required, survival fell to 64% for first and 52% for repeat transplant recipients (Cecka *et al.* 1992). Although delayed graft function can reduce both graft and patient survival (Sanfilippo *et al.* 1984); dialysis could contribute significantly to poor outcome through reducing CsA sensitivity of PBLs. Lindholm *et al.* (1993) were the first to study the impact of acute rejection on the long term renal allograft survival. They treated a group of 951 cadaveric renal allograft recipients with three different doses of CsA together with steroids and azathioprine. In the low dose CsA group, they found an estimated graft half-life of 6.6 years for the 562 patients (59.1%) who had a history of acute rejection and of 12.5 years for those without rejection ($p < 0.0001$). An even more impressive impact of acute rejection on graft half-life was found by Matas *et al.* (1994). In a group of 278 cadaveric kidney graft recipients treated with quadruple induction immunosuppression, the half life was 33 years in the 150 patients without rejection and 22 years in the 64 patients with one rejection within the first year of transplantation. The half-life decreased sharply in the group of 50 patients with multiple rejection episodes (5 years) and in the 14 patients with a first rejection episode after the first year (2 years). The same effect was also seen in the living-donor graft recipients. Other investigators on a group of 861 first cadaveric kidney graft recipients transplanted under dual immunosuppressive therapy with CsA and steroid have shown that 761 (88.3%) patients still had a functioning graft at the end of the first post-transplant year (Vanrenterghem, 1995; Vanrenterghem and Peeters, 1997). Of these 761 patients,

54.8% had no rejection during the first six months after transplantation, 31.8% had one rejection episode and 13.4% had multiple rejection episodes. Actuarial graft survival at five years was 86.8%, 88.4% and 83.1%, respectively, and at nine years was 75.4%, 75.2% and 62.8%, respectively. Estimated graft half-life was 20 years for the patients without rejection and 17.1 years for the patients with one or more rejection episodes. For the group with only one rejection episode, however, the half-life was identical to that of the group without rejection (20.1 years). The half-life was only 12.7 years for those with more than one rejection episode. Furthermore, it has been reported that multiple rejection episodes increase significantly the occurrence of chronic rejection (Vanrenterghem and Peeters, 1994). Clearly, these reports indicate that acute rejection is indeed a risk factor for chronic rejection and late graft loss. Thus, haemodialysis could has an impact on late graft outcome, since haemodialysis reduces the *in vitro* CsA sensitivity of PBLs, preserves Th 1 response, and increases the incidence of acute rejection episodes following kidney transplantation.

A majority of the studied haemodialysed patients had higher number of intracellular IL-2 molecules in T cells and extracellular IL-2 concentration pre- than post-haemodialysis. Thus, the findings suggest that after haemodialysis session the majority of cases do not respond well to mitogen stimulation which agrees with the observation that Con A-activated T cells responded poorly to a new mitogenic challenge (Gullberg *et al.* 1981; Gullberg and Larsson, 1982). This suggests that haemodialysis preactivates the circulating T cells of chronic

haemodialysis patients. Several reports have shown that in normal subjects and mice, recombinant IL-2 exclusively induced proliferation of T cells that had been previously activated by a mitogen (Chatenoud *et al.* 1986; Bemer and Truffa-Bachi, 1996). This was also noticed in chronic haemodialysis patients: resting T cells from haemodialysis patients responded vigorously to exogenous IL-2 alone, but normal T lymphocytes required initial activation by antigen or mitogen (Chatenoud *et al.* 1986). Furthermore, in agreement with our findings a significant increase in IL-2 production occurred within the first hour of the haemodialysis session and persisted throughout dialysis (Luger *et al.* 1987). Our data suggests that preactivated T cells in haemodialysed patients could result from haemodialysis itself. Activated T cells and effectors can undergo a rapid apoptosis upon re-stimulation (Zhang *et al.* 1997; Van Parijs *et al.* 1996: Critchfield *et al.* 1994), whereas naïve cells respond to stimulation by secreting cytokines and proliferating (Croft *et al.* 1992). The susceptibility to rapid activation-induced cell death seems to disappear when $CD4^+$ T cells become memory cells (Swain *et al.* 1996). The ability of resting PBLs of chronic haemodialysis patients to respond vigorously to exogenous IL-2 in the absence antigen or mitogen (Chatenoud *et al.* 1986) could be related to memory cells. The requirements for the activation of memory cells for proliferation and cytokine production are not so quite strict as those of naïve cells (Duncan and Swain, 1994; Dubey *et al.* 1996). Although most memory cells are low for CD25 and CD69, it has been reported that memory cells can be $CD25^+$ and *ex vivo* cytolytic (Selin and Welsh, 1997). Tough *et al.* (1996) reported that unrelated viral infections stimulate IFN production, which causes

proliferation of unrelated memory cells. Indeed, renal transplantation inflammatory reactions may stimulate the assumed haemodialysis memory cells, which may increase the risk of acute rejection.

In addition to abnormal IL-2 production pre- and post-haemodialysis, the activation markers CD25 and CD69 were strongly expressed on the circulating T cells of chronic haemodialysis patients. This strong expression of CD25 is in agreement with previous reports that revealed an increased number of T cells expressing the CD25 activation marker in chronic haemodialysis patients (Chatenoud *et al.* 1986; Beaurain *et al.* 1989). The percentage of T cells expressing CD25 in chronic haemodialysis patients that we found was closer to that determined by Beaurain *et al.* (1989). Furthermore, we noticed that there was no significant difference between pre- and post-haemodialysis expression of CD25 and CD69. This is in agreement with a study that showed that CD25 expression on T cells did not change significantly after haemodialysis (Beaurain *et al.* 1989). It was shown earlier that CD25 appears within 6 hours after PHA stimulation on normal adult T cells, and that the receptor number peaks after 48-72 hours (Depper *et al.* 1984) or even later up to 4-5 days (Yokoi *et al.* 1982; McDouall *et al.* 1994). Subsequently, it was reported that the number of CD25 molecules progressively decreased unless there was further stimulation by re-exposure to PHA or other stimulus (Depper *et al.* 1984). We also found that the percentage of T cells expressing CD25 was slightly elevated pre- as compared to post-haemodialysis, although not significantly *($p = 0.063$)*. This may be because

haemodialysis activated T cells, and the expression of CD25 peaked several days later before the next haemodialysis. Haemodialysis may then have caused immediate shedding of CD25 from the activated T cells resulting in acute postdialysis reduction of the percentage of circulating T cells expressing CD25. Furthermore, we found that PHA treatment of the cells resulted in significant increase in the percentage of T cells expressing CD25 for both pre- and post-haemodialysis as compared with untreated cells. It has been reported that CAPD and haemodialysis patients have higher plasma level of soluble CD25 than undialysed patients (Walz *et al.* 1990). It has recently been reported that pre-haemodialysis patients have higher soluble CD25 than CAPD patients and that the level of soluble CD25 becomes significantly higher after haemodialysis, suggesting that hemodialysis caused shedding of these molecules from activated T cells (Descamps-Latscha *et al.* 1995). Thus, the over-expression of CD25 on T cells upon PHA stimulation that we have noticed can be explained by the fact that constant shedding of CD25 molecules from activated T cells decreased the number of CD25 molecules on T cells unless further stimulation occurred, as with PHA stimulation.

The acute effects of haemodialysis on the activation state of T cells have been evaluated by *in vitro* dialysis of the blood of healthy donors, and showed induction of transcription of IL-2R mRNA, but no expression of IL-2R on the lymphocyte surface after a single dialysis session (Donati *et al.* 1992). These authors suggested that repeated stimuli, such as that seen in regular maintenance

haemodialysis, however, might result in T cell activation as shown by increased CD25 expression on lymphocytes of haemodialysed patients. Although clinical haemodialysis is complicated and a variety of factors may be involved in determining the CD25 expression, this *in vitro* study is in agreement with our findings and suggests that the phenomenon of T cell activation is likely to be the result of direct physical interaction between the dialysis membranes and lymphocytes circulating through the hollow fiber dialyzers.

Our analysis showed that CD69 was expressed on a majority of the circulating T cells of chronic haemodialysis patients and did not change significantly after dialysis. These observations are consistent with the expression of CD25, and that CD69 expression further indicates that the T cells are preactivated in chronic haemodialysis patients. CD69 is not detectable on resting normal T cells and is the earliest activation marker to be expressed (within 30 minutes) upon mitogen stimulation (Cebrian *et al.* 1988). It is maximally expressed at 24 hours, and expression remained stable throughout 72-hour culture period (Mardiney *et al.* 1996). These reports and our initial experiments on the kinetics of CD69 expression may explain the insignificant change in CD69 expression on T cells before and after haemodialysis. Haemodialysis activation of T cells and maximum expression of CD69 on T cells was achieved by the time the experiment was completed. Subsequently, the expression of CD69 on T cells remained stable until the next haemodialysis.

In conclusion, it has been demonstrated that CsA sensitivity of circulating T cells postdialysis is reduced as compared to predialysis in chronic haemodialysis patients, using several parameters focused on IL-2. We also demonstrated in these patients the presence of preactivated T cells as indicated by expression of T cell activation markers, and the abnormal IL-2 response in relation to haemodialysis that suggest that haemodialysis preactivates T cells in chronic haemodialysis patients. Thus, our data clearly show the existence in chronic haemodialysed patients of abnormally high proportions of T cells presenting phenotypic and functional signs of preactivation as a result of haemodialysis. These preactivated T cells may offer an explanation of reduced *in vitro* CsA sensitivity of T cells after haemodialysis. The presence of these preactivated T cells may have potential relevance to CsA resistance, and to the risk of acute rejection episodes and chronic rejection after renal transplantation.

6. *IN VITRO* CsA SENSITIVITY OF T CELLS IN RENAL TRANSPLANT PATIENTS WHO RECEIVED ATG

6.1. Rationale for study

We have shown in previous chapters that *in vitro* CsA resistance of circulating T cells is attributed to T cell preactivation as shown by *in vitro* PHA prestimulation, and the functional and phenotypic evidence of peripheral blood T lymphocytes of patients on chronic haemodialysis. According to this data we decided to determine the *in vitro* perioperative CsA sensitivity of circulating T cells of renal transplant patients who received single ATG bolus induction therapy as part of the immunosuppressive regimen. ATG is lymphocyte-selective immunosuppressive agent inhibiting cell-mediated immune response through various mechanisms. This involves the opsonization of antigen-reactive T cells in peripheral blood, followed by lysis and sequestration in the reticuloendothelial system (Krensky and Clayberger, 1994). Measuring the *in vitro* CsA sensitivity of circulating T cells in these patients may help indirectly to show the effect of elimination of preactivated T cells by ATG-based immunosuppressive regimen since this T cell subpopulation is responsible for T-cell CsA resistance.

6.2. Results

We have determined the *in vitro* CsA-mediated inhibition of IL-2 production before and at intervals after transplantation in live-related kidney transplant patients who had one haplotype match with their respective donors, had received single prophylactic ATG bolus and were maintained before transplantation on chronic haemodialysis. The assay was carried on PBMCs obtained from renal transplant patients before the morning CsA dose. Increasing amounts of CsA were added to these cell cultures and PHA-induced IL-2 production was detected by CTLL-2 proliferation. The CsA sensitivity was determined 2 days pre-transplant, and 3 days and 1 month post-transplant. The six transplant patients studied routinely received CsA, azathioprine, steroids and single intravenous bolus injection of ATG-Fresenius (9 mg/Kg body weight) 1 hour before operation. The regression inhibition curves were constructed and the IC_{50} was plotted for each patient from these inhibitory dose-response curves. The inhibitory dose-response curves of individual patients showed a shift to the left at 3 days post-transplantation, which remained shifted to the left at 1 month post-transplantation in comparison to the pre-transplantation curve. This was similar for all the renal transplant patients studied who received ATG induction therapy (Fig. 32).

We have monitored the CsA IC_{50} of each live-related renal transplant patient at the above mentioned intervals in relation to the transplant and we found that all the individual patients who received single ATG bolus induction as part of the immunosuppressive regimen had a similar IC_{50} pattern; this decreased acutely at 3

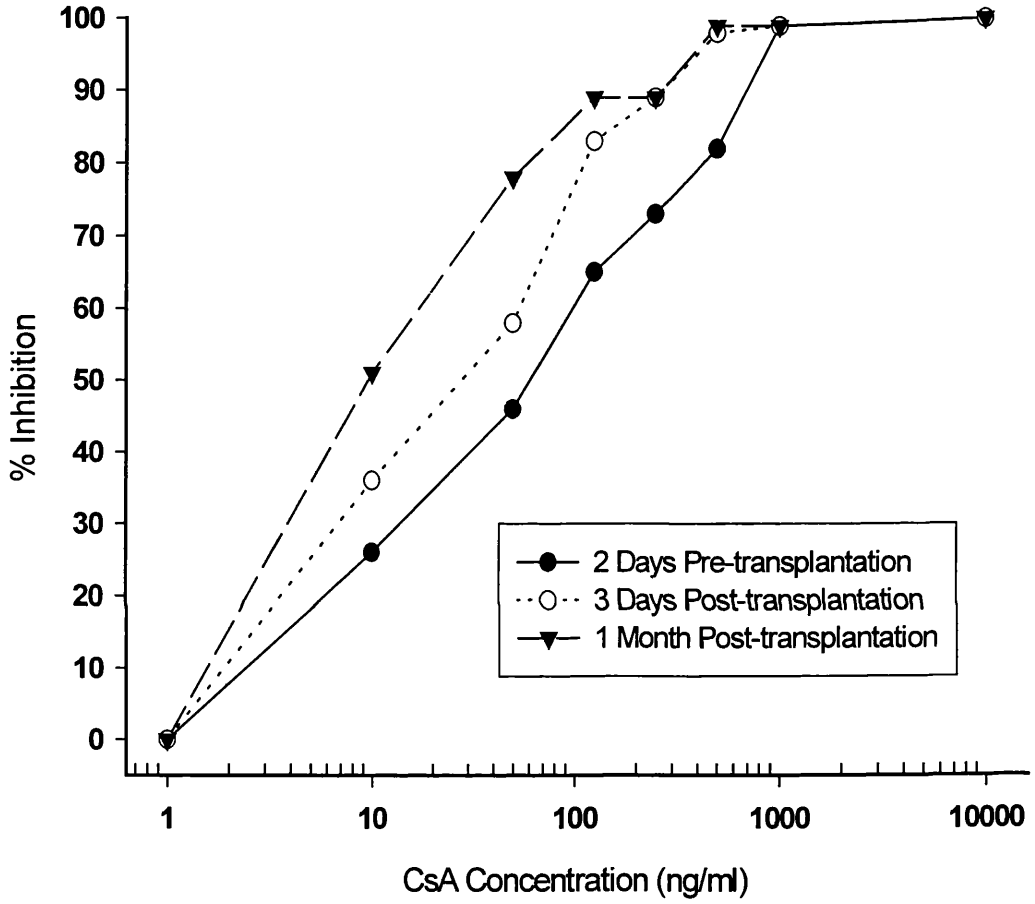

Figure 32. Representative inhibitory dose-response graph of an individual renal transplant patient who received a prophylactic preoperative single ATG bolus as part of the immunosuppressive regimen. CsA sensitivity assay using CTLL-2 cells as detector of IL-2 production was carried out on PBMCs at the time points indicated on the graph. A shift to the left occurs in the dose-response curves after transplantation. IL-2 production with no added CsA was taken as 0% inhibition (control). The response is expressed as % inhibition of IL-2 production as reflected by CTLL-2 proliferation, similar graphs were obtained with the other 5 live-related renal transplant patients.

days post- and maintained low at 1 month post-transplant as compared to the pre-transplant IC_{50} (Fig. 33).

Statistical analysis of IC_{50} of these renal transplant patients who received ATG induction therapy as part of immunosuppressive regimen showed a significant decrease of IC_{50} at 3 days and 1 month post-transplantation to 21.5 ng/ml and 12.1 ng/ml respectively as compared to the pre-transplant IC_{50}, which was 61.17 ng/ml, ($p < 0.05$) (Table 14, Fig. 34).

Timing	CsA IC_{50}	*p value*
Pre-transplant	61.17 ± 17.04	*control*
3 days post-transplant	21.50 ± 4.41	*0.028*
One month post-transplant	12.1 ± 2.34	*0.021*

Table 14. *In vitro* CsA inhibition of IL-2 production in live-related renal transplant patients who received a prophylactic preoperative single ATG bolus as part of the immunosuppressive regimen. CsA sensitivity assay using CTLL-2 cells as detector of IL-2 production was performed on PBMCs, and the IC_{50} of CsA was plotted from the inhibitory dose-response curves. The data indicate that the IC_{50} of CsA decrease significantly post-transplant. Pre-transplant IC_{50} was regarded as a control, (p vs control). IC_{50} values: mean ± (SEM), n=6 live-related renal transplant patients.

These *in vitro* data indicate that renal transplant patients who received ATG induction therapy as part of the immunosuppressive regimen are more sensitive to CsA post-transplant as compared to the pre-transplant.

For all volunteers, control wells of 72 hours incubation showed lymphocyte proliferation in response to PHA and no proliferation in the wells without PHA.

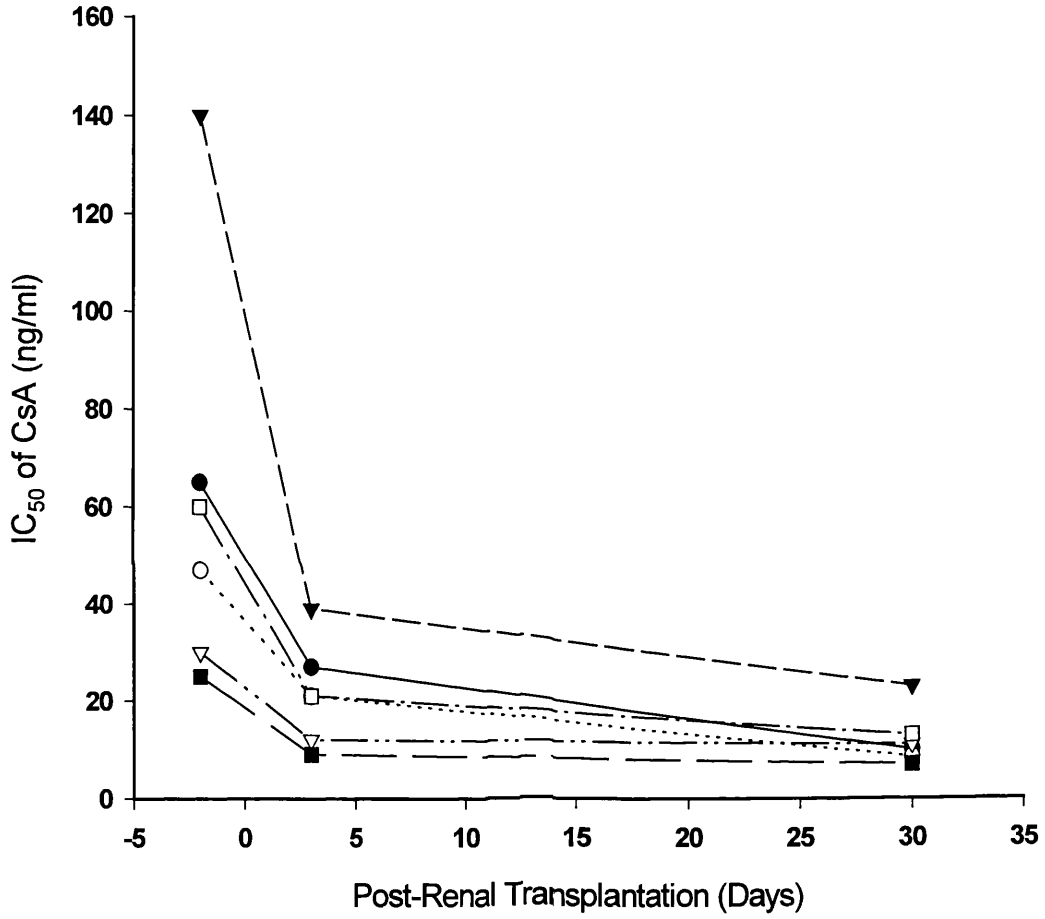

Figure 33. Longitudinal quantitative perioperative CsA IC_{50} monitoring of individual renal transplant patients who received a prophylactic preoperative single ATG bolus as part of the immunosuppressive regimen. CsA sensitivity assay using CTLL-2 cells as detector of IL-2 was carried out on PBMCs. The *in vitro* IC_{50} of CsA was plotted from the inhibitory dose-response curves. All the 6 live-related renal transplant patients who received an additional single ATG bolus induction therapy are shown to have a similar pattern of *in vitro* CsA IC_{50}, which acutely decreased after transplantation.

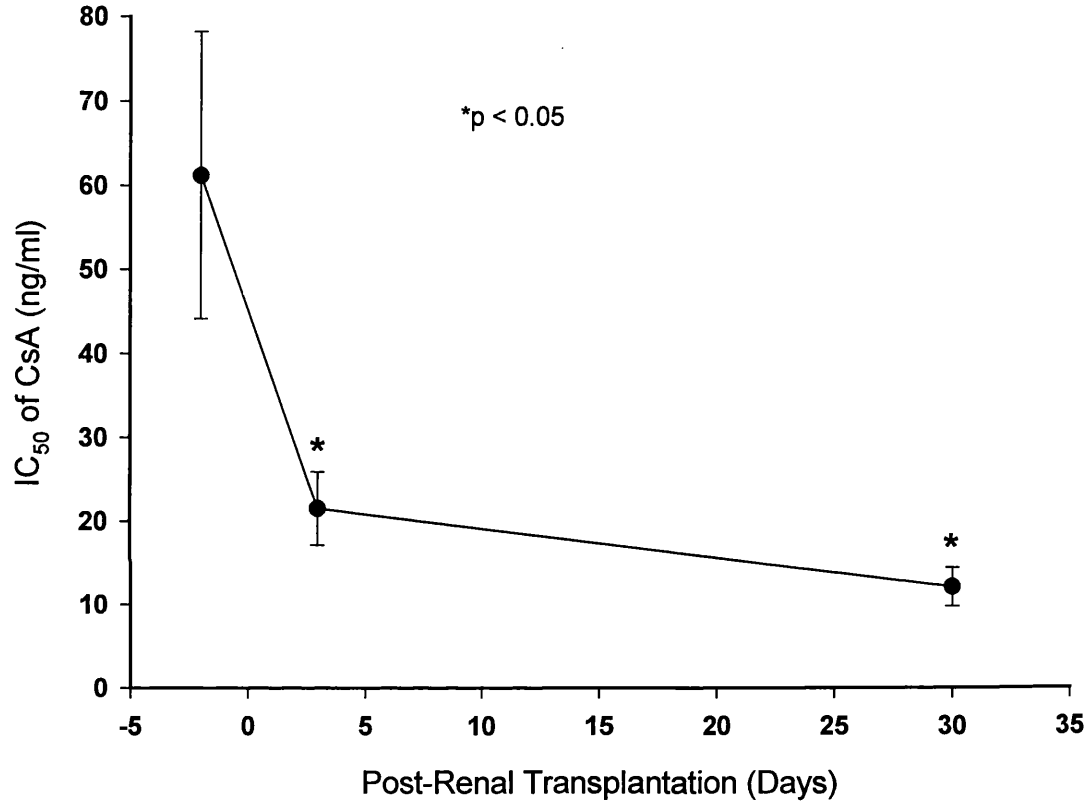

Figure 34. Perioperative CsA IC_{50} of renal transplant patients who received a prophylactic preoperative single ATG bolus as part of the immunosuppressive regimen. CsA sensitivity assay using CTLL-2 as a detector of IL-2 was carried out on PBMCs. The *in vitro* IC_{50} of CsA was plotted from the inhibitory dose-response curves of each individual patient. The graph shows significantly acute decrease of *in vitro* CsA IC_{50} after transplantation. IC_{50} values of CsA are presented as mean ± SEM; *p: post-transplant IC_{50} values compared with pre-transplant IC_{50}, n=6 live-related renal transplant patients.

6.3. Discussion

The rationale for the prophylactic use of ATG is the avoidance of early exposure of the transplanted organ to rejection. Acute rejection episodes develop on CsA maintenance therapy in 59-63% of patients although most of the episodes are reversed by a short course of high dose steroid pulse therapy (Deierhoi *et al.* 1988). Our previous data have demonstrated CsA resistance of T cells of patients on chronic haemodialysis, due to the preactivation of peripheral blood T lymphocytes by haemodialysis. We attempted to determine the *in vitro* perioperative CsA sensitivity of peripheral blood T cells of renal transplant patients maintained on chronic haemodialysis before transplantation, and a prophylactic single ATG bolus induction was included in the immunosuppressive regimen, since lymphocyte depletion is a major immunosuppressive effect of ATG. The live-related donor kidney transplant patients received one bolus intravenous injection of ATG-Fresenius (9 mg/kg body weight) one hour before transplant operation in addition to the standard immunosuppressive regimen including prednisolone, azathioprine and CsA. The rationale of injecting ATG 1 hour before the operation was to produce maximal immunosuppression when the recipient was most likely to respond to the foreign organ. A marked T cell depletion was evident before completion of anastomoses and lasted for at least 5 days (Kaden *et al.* 1992,1995).

ATG treatment induces strong lymphocyte depletion, and several investigators have monitored the lymphocyte counts during and after ATG treatment. Reports

have shown that lymphocytopenia persists during the entire treatment period and the number of circulating T cells gradually increases after the cessation of treatment (Bonnefoy-Berard and Revillard, 1996). A study has shown that 1-hour following the first OKT3 injection most T cells disappear from peripheral blood. Subsequently, by the second to the fifth day post-transplant, small but significant levels of T cells were observed in all patients (Chatenoud *et al.* 1983). The observations shown with OKT3 may be applicable to ATG, since ATG contains CD3-specific antibodies. A comparative study of lymphocyte subpopulations showed that beyond day 10 of treatment after renal transplantation, the CD3 counts were lower in the ATG-Fresenius-treated than in OKT3-treated patients, as were CD2 counts, CD4 counts and the CD4/CD8 ratio, suggesting a more prolonged immunosuppressive effect of ATG-Fresenius (Bock *et al.* 1995). Several mechanisms could be relevant to this depletion. Opsonization, and subsequent phagocytosis of lymphocytes and sequestration in the reticuloendothelial system are generally considered as a major mechanism for lymphocyte depletion (Krensky and Clayberger, 1994). Other proposed mechanisms for T cell depletion are complement-dependent lysis, and antibody-dependent cell-mediated cytolysis, all of which depend on the structure and the level of aggregation of antibody Fc regions (Bonnefoy-Berard and Revillard, 1996). However, it is difficult to evaluate the relative contribution each of these three mechanisms *in vivo*. More recently new mechanisms were proposed. A group of investigators have suggested that T cells may lyse other T cells (homocytolysis) in the presence of OKT3 (Wong *et al.* 1990). Bivalent CD3

monoclonal antibody would cross link CD3 molecules on two different T cells, mimicking the encounter of a T cell with its native target and inducing TCR-dependent antibody-bridged cell-mediated cytolysis. This mechanism requires bivalency of anti-CD3 monoclonal antibody, but it is independent of the Fc region or complement. Lymphocytopenia may also result from a rapid margination caused by adhesion of lymphocytes to endothelium, as a result of a rapid upregulation and increased avidity of adhesion molecules (CD11a and CD11b) on TCR/CD3-mediated activation (Ten Berge *et al.* 1995). This margination of T cells does not involve physical destruction but may account for the rapid disappearance of T cells from peripheral blood. Although these two mechanisms that were shown *in vitro* with OKT3, they could be applicable to ATG, in as much as ATG contains CD3-specific antibodies.

In the view of the mechanisms of lymphocyte depletion induced by ATG, it is important to know the relative contribution of cell destruction (cytolysis) and reversible margination of lymphocytes to the lymphocytopenia observed during treatment. In the latter case one would expect that memory alloreactive T cells would be rapidly re-expressed after cessation of treatment, whereas if major destruction of the T cell peripheral pool occurred, repopulation may involve naïve T cells readily susceptible to anergy or tolerance induction. Although we are not aware of any study that defined the relative contribution of cytolysis and reversible margination of lymphocytes to the ATG-induced lymphocytopenia, we think that cytolysis contributes considerably to lymphocyte depletion. Our results

have shown that these live-related renal transplant patients who had an additional prophylactic single ATG-bolus, have significantly increased *in vitro* CsA sensitivity of peripheral blood T cells post-transplant as compared to pre-transplant when the patients were on chronic haemodialysis. This increased *in vitro* CsA sensitivity is observed in all the parameters that we have tested. Due to shortage of live-related renal transplant patients, it was not possible to find live-related renal transplant patients who did not receive prophylactic ATG induction therapy as part of immunosuppressive therapy as controls for statistical comparison with the study group; a limited number of study group patients were investigated. The cadaveric renal transplant patients were unsuitable because it was practically impossible to determine pre-transplant *in vitro* CsA sensitivity of their T cells. However, our results indicate that prophylactic use of ATG as in our study facilitates more effective and potent CsA-based immunosuppression. This may have a potential role in preventing acute rejection episodes especially during the early period of a transplant and in improving the late outcome of graft function by preventing chronic rejection.

The use of ATG as induction therapy was evaluated by several studies to determine its impact on the incidence of acute rejection, and patient and graft survival. The benefit of prophylactic ATG induction therapy is shown in several large studies of cadaveric renal transplant patients who received (9 mg/kg body weight) intravenously intraoperative single ATG bolus just before completion of anastomoses in addition to the standard triple immunosuppressive therapy:

azathioprine, prednisolone and CsA (Kaden *et al.* 1992, 1995, 1997). These studies showed that the additional prophylactic single ATG bolus induced a drastic T cell reduction lasting for at least 5 days and resulted in a slightly reduced rejection rate in comparison with kidney graft recipients on standard triple immunosuppressive therapy, and a significantly delayed onset of rejection crises as well as a significant improvement of 6, 12, 24 and 36 months graft survival. Interestingly, the 3-year patient survival was also significantly increased and there was no increase in major infections. Since these patients received an intravenous single ATG bolus dose similar to the patients in our study before completion of anastomoses, our findings of post-transplant drastically increased *in vitro* CsA sensitivity of circulating T cells correlates well with the T cell depletion shown by Kaden *et al.* (1992, 1995). In addition, the post-transplant increased *in vitro* CsA sensitivity of circulating T cells that we found gives the underlying explanation of delayed onset and a decreased incidence of rejection, and improved graft and patient survival as was demonstrated by Kaden *et al.* (1992, 1995,1997). Also, it has been shown that a prophylactic single ATG (8 mg/kg body weight) intravenous injection administered 6 hours after kidney transplantation to 58 renal allograft patients induced T cell depletion with a low incidence of delayed graft function and acute rejection episodes (Zietse *et al.* 1993). In this study, the concomitant medication for all patients including the control patients who did not receive ATG consisted of steroids and azathioprine, and CsA. Furthermore, a study of 65 transplanted cadaveric kidneys showed that induction therapy with ATG reduced the incidence of acute rejection episodes and improved the graft

survival at 1 and 2 years (Abouna *et al.* 1993). In this study the post-transplant immunosuppression included azathioprine, prednisone and ATG for 7-14 days, after which CsA therapy was initiated, and ATG was discontinued after 72 hours. In agreement with our result of increased *in vitro* CsA sensitivity of T cells of renal transplant patients who received ATG induction therapy, this data can be explained by the fact that ATG induction therapy causes lymphocyte depletion by removing the activated T cells, hence achieving adequate and potent immunosuppression. However, in this study of 65 cadaveric renal transplants leukopenia and/or thrombocytopenia requiring dose reduction of ATG developed in 23% of patients. Also, a significant number of patients developed cytomegalovirus infection, gram negative septicaemia and oral/oesophageal infection (Abouna *et al.* 1993). Attempts have been made to reduce the total amount of ATG given to patients in order to reduce the side effects of ATG. Abouna *et al.* (1995) conducted a randomised trial of ATG induction therapy in 45 recipients of cadaveric renal allografts and compared a fixed daily dose with an adjusted dose based on T cell monitoring. Both groups were controlled for similar baseline characteristics. Patients in group 1 (n = 23) received ATG at variable daily dose to maintain the concentration of $CD3^+$ at 50-100 cells/μl. Group 2 (control) received a fixed daily dose (15 mg/kg body weight/day) of ATG. All the patients received immunosuppression therapy with azathioprine, prednisone and CsA (started when serum creatinine reached 3 mg/dl with a 3-day overlap with ATG). The main daily dose (9 ± 1.9 mg/kg body weight/day) of ATG used for group 1 was considerably less than that for the control group. This

low dose of ATG resulted in a significant reduction in leukopenia and thrombocytopenia in addition to a reduction in the incidence of infection that was not statistically significant. It also resulted in substantial cost savings. No significant differences were observed for patient and graft survival at 6 months, 1 year and 2 years. The incidences of acute rejection, steroid resistant rejection or recurrent rejection were not found to be significantly different. This study suggested that low-dose prophylactic ATG induction therapy is associated with significant reduction of the side effects, and is as effective and potent immunosuppression as high-dose prophylactic ATG induction therapy. All these studies are in agreement with our findings and indicate that even a single prophylactic ATG injection is effective in improving short- and long-term results after kidney transplantation. From an immunological point of view, this suggests that prevention of immune response is better and easier to achieve than interrupting the immune response.

Other investigators compared regimens of CsA and prednisone (n = 75); and CsA, prednisone, and ATG (n = 60), (Lloveras *et al.* 1992). ATG was administered on alternated days during the first 15 days after the transplantation. Actuarial patient and graft survival at 6 months, 1 year and 3 years was not statistically different between the two regimens. Acute rejection episodes within the first 6 months were 33.3% and 31.6% respectively, but no statistical analysis was performed. The authors concluded that the prophylactic ATG induction therapy employed did not offer additional long-term benefits for patient and graft survival. The shortage

of data on improved graft function and patient survival at various periods raised questions about the long-term impact of prophylactic ATG induction therapy on the success of renal transplantation. This is supported by the United Network for Organ Sharing Registry (Cecka *et al.* 1992), which showed that graft function and survival rates are minimally, if at all, affected by prophylactic ATG therapy. However, variations in the immunosuppressive regimens of study centres, different doses of concomitant therapies and the use of prophylactic antibiotics distort the accuracy of meta-analysis of treatment outcomes. Furthermore, it is difficult to establish long-term graft and patient survival because of the variation of follow up periods.

From the benefits of prophylactic ATG induction therapy in our study and in the studies of Abouna *et al.* (1993, 1995); Kaden *et al.* (1992, 1995, 1997); Zietse *et al.* (1993) it appears that prophylactic ATG is effective in removing activated T cells and effective as prophylaxis against rejection. Our limited pilot study demonstrates the importance of establishing a large prospective, controlled, randomised study aiming to determine the effect of prophylactic ATG induction therapy on patient and graft survival, graft function, and allograft immune response in renal transplant patients maintained on chronic haemodialysis before transplantation.

7. CONCLUSIONS

Since late 1983 when CsA was introduced into clinical practice to prevent rejection in transplant patients, there has been a rapid growth in the number and types of transplants, and substantial decrease in the rates of acute rejection and life-threatening infections. Despite these successes, major improvements in immunosuppressive therapy are needed, especially reduction in acute rejection episodes and toxic side effects. The occurrence of even one reversible rejection is associated with inferior graft survival rates; in the UNOS Scientific Renal Transplant Registry, first transplant recipients who were rejection-free at discharge had an 86% one-year graft survival rate compared to 67% for those with one or more rejection episodes ($p < 0.001$) (Cecka and Terasaki, 1993). A long-term adverse consequence also is reported following acute rejection. The estimated half-life (time taken for 50% of the grafts functioning at one year to fail) for patients without acute rejection was 8.6 years, and 7.4 years for the recipients with one or more rejections (Cecka and Terasaki, 1993). Acute rejection is also considered to be a significant risk factor for the subsequent development of chronic rejection (Almond *et al.* 1993). These reports demonstrate the importance of improving immunosuppressive therapy and preventing the occurrence of acute rejection in order to improve graft function and survival. Because of the above mentioned problems associated with CsA-based immunosupression, we have proposed a hypothesis "activated T lymphocytes are less sensitive to CsA" that may be at least partially, responsible for inadequate

immunosuppression and on other hand, the high CsA-dosage required may lead to increase in the incidence of CsA nephrotoxicity. We have tested this hypothesis, in normal volunteers and chronic renal failure patients on dialysis mainly by determining *in vitro* CsA sensitivity of PBLs of these volunteers.

Our initial experiments showed that the *in vitro* preactivation with PHA in normal volunteers reduced the CsA sensitivity of T lymphocytes, as demonstrated by shift to the right in representative inhibitory dose-responsive curves and a statistically significant increase in IC_{50} of CsA. We also noticed this observation of reduced CsA sensitivity of circulating T cells after PHA preactivation of T cells in patients with end stage renal disease on dialysis: CAPD and haemodialysis. It has been reported that the concentration of CsA required to inhibit calcineurin is very similar to that needed to inhibit IL-2 production in activated T-cells (Fruman *et al.* 1992b), and 2 hours has been defined for commitment to the process of T cell activation, (Lowenthal *et al.* 1985). Thus, we can conclude from our preactivation experiments that the susceptibility of activated and inactivated calcineurin to CsA-mediated inhibition is not the same, and the activated calcineurin is resistant and less sensitive to the inhibition by CsA. This indicates that CsA, even if the Ca^{2+}-dependent (CsA-sensitive) pathway is activated, may not suppress activated T cells. Other studies have also demonstrated CsA resistance (Kroczek *et al.* 1987; Pereira *et al.* 1990; Masy *et al.* 1994). Since *in vivo* CsA inhibition of calcineurin activity is partial (Pai *et al.*1994; Batiuk *et al.*1995a) and rapidly reversible (Batiuk, *et al.*1995b), it follows that drug non-compliance and variation

in blood composition (Lensmeyer *et al.*1989; Hughes *et al.*1991; Kasiske *et al.*1988b) could potentially affect CsA availability to PBLs, and thus inhibition of calcineurin activity and cytokine synthesis. Thus, the *in vivo* immunosupressive effect of CsA might not be able to adequately inhibit activated T cells, and would carry high risk of acute graft rejection or chronic rejection depending on the extent of inhibition of these cells. We could also conclude that reduction in CsA availability to PBLs might contribute to the presence of alloantigen-activated T cells, which may be inhibited with difficulty by maintenance CsA-based immunosuppression and can be a source of rejection.

The reduction in CsA sensitivity of T cells after preactivation in dialysis patients indicates that even in chronic uraemic patients who have T cell deficiency, T lymphocytes are activated by PHA, and that activated calcineurin is resistant and less sensitive to inhibition by CsA. Thus, at the level of the Ca^{2+}-dependent (CsA-sensitive) pathway, the function of the T cell is physiologic in dialysis patients. Studies on the mechanisms underlying the uraemic T cell deficiency have revealed several extra T cell factors (Girndt *et al.* 1993; Vanholder *et al.* 1994; Meuer *et al.* 1987; Ruiz *et al* 1990; Matthias *et al.* 1993; Massry *et al.* 1994; Stachowski *et al.* 1991).

Haemodialysis is a life-sustaining procedure; it has become clear that haemodialysis can no longer be considered a simple process whereby blood and dialysate are separated by an inert semi-permeable membrane. Significant

changes may occur because of the interactions of blood with various components of haemodialysis equipment (Basile and Drueke, 1989; Cheung, 1990). We have studied the immediate effect of haemodialysis on CsA sensitivity of circulating T cells through testing several parameters before and at the end of a dialysis session of 4 hours. Initially, using CTLL-2 cells as detector of IL-2 production, we tested the CsA sensitivity of circulating T cells. The shift to the right in representative inhibitory dose-response curves and the significant increase in IC_{50} values of CsA at the end of dialysis session, which became more pronounced with *in vitro* PHA prestimulation, all showed that haemodialysis reduced CsA sensitivity of T cells. Several other parameters, both intra- and extra-cellular, which focused on CsA inhibition of IL-2 production, have also been examined to determine the immediate effect of haemodialysis on the CsA sensitivity of T cells. We found that the degree of reduction of the frequency of IL-2 producing T cells at 100 ng/ml of CsA was significantly less post- than pre-haemodialysis. The significant difference in the percentage of CsA reduction of the frequency of IL-2 producing T cells between pre- and post-haemodialysis indicates indirectly that there is less inhibition of intracellular IL-2 production post- than pre-haemodialysis. Similarly, our data regarding intracellular IL-2 production in T cells showed a significantly smaller degree of CsA inhibition of intracellular IL-2 production after haemodialysis at 100 ng/ml CsA. Furthermore, measurement of IL-2 concentration in cell culture supernatants indicated that the degree of CsA inhibition of IL-2 secretion at 100 ng/ml of CsA was less after haemodialysis in comparison with predialysis. Thus, all the *in vitro* experiments that focused at

different levels of IL-2 including biological and quantitative assays showed that haemodialysis renders T cells less sensitive to CsA. This correlates with the reduced CsA sensitivity of T cells after haemodialysis as compared to normal volunteers and CAPD patients obtained from our previous experiments when CTLL-2 proliferation was used as an indicator of IL-2 production.

The analysis of CsA inhibition of quantitative intracellular IL-2 production in T cells in relation to haemodialysis did not show any statistical difference between pre- and post-haemodialysis. The wide inter-individual variations in the ability to produce IL-2 may mask the statistical significant difference in the CsA inhibition of intracellular IL-2 production before and after haemodialysis, when analysed by the number of intracellular IL-2 molecules in T cells. However, taking in consideration our previous data, the CsA resistance of IL-2 production after haemodialysis could also be demonstrated by analysis at level of intracellular IL-2. The explanation of no significant difference pre- and post-haemodialysis in CsA reduction of the frequency of IL-2 producing T cells, and CsA inhibition of intracellular IL-2 production in T cells and IL-2 secretion at the extremes of CsA concentration is that at these concentrations the CsA effect is not a significantly difference pre- and post-haemodialysis. At 10 ng/ml of CsA the degree of inhibition of IL-2 production did not reach significance pre- and post-haemodialysis. However, at a CsA concentration of 500 or 1000 ng/ml, which at least one order of magnitude more effective than seen *in vivo*, IL-2 production was maximally inhibited and abolished the difference in CsA sensitivity of T cells

pre- and post-haemodialysis. However, this does not have clinical relevance. The *in vitro* experiments showed that the degree of CsA reduction of the frequency of IL-2 producing T cells and CsA inhibition of intracellular IL-2 production in T cells were much lower than the degree of CsA inhibition of IL-2 production into cell culture supernatant as detected by CTLL-2 proliferation or measured by ELISA. This may be attributed to the variation of the sensitivity of the assays, and also to the fact that *in vitro* CsA quantitatively reduces IL-2 synthesis per T cell in a large number of T cells.

Our study showed that there is no significant difference in either the number of intracellular IL-2 molecules in $CD3^+$ cells or in the IL-2 concentration of the cell culture supernatant before and after dialysis, but there was wide inter-individual scatter of the data. This inter-individual variation is in agreement with previous observations in healthy volunteers and in pre-transplant patients (Koutouby *et al.*1993; Zucker *et al.*1996). However, most of the patients studied had lower numbers of intracellular IL-2 molecules and extracellular IL-2 concentration post- than pre-haemodialysis, which is a sign of T cell activation (Gullberg *et al.* 1981; Gullberg and Larsson, 1982). Thus, haemodialysis may preactivate the circulating T cells of chronic haemodialysis patients. The activation markers, CD25 and CD69 were strongly expressed on the circulating T cells of chronic haemodialysis patients, and showed no significant difference between the pre- and post-haemodialysis expression, in agreement with previous reports (Beaurain *et al.*1989). The constant shedding of CD25 molecules from haemodialysis activated

T cells (Walz *et al.*1990) could explain the PHA-induced over expression of CD25 on T cells seen pre- and post-haemodialysis. It seems that unlike CD25, CD69 is stable on T cells and is not susceptible to shedding, since we found that PHA treatment did not increase the percentage of T cells expressing CD69 before or after haemodialysis, and the percentage of T cells expressing CD69 was much higher than the percentage of T cells expressing CD25. In contrast to chronic haemodialysis patients, it has been shown in healthy individuals that the T cells expressing CD25 are always more numerous than the T cells expressing CD69 (Caruso *et al.* 1997).

Although less IL-2 was produced after haemodialysis, the presence of CsA resistance of IL-2 inhibition after haemodialysis and the observation that haemodialysis T cells responded vigorously to IL-2 alone (Chatenoud *et al.*1986) suggest the existence in haemodialysed patients of an abnormal T cell sub-population with signs of preactivation, which might not be adequately inhibited by CsA-based immunosuppression and carries high risk of graft rejection after transplantation. The haemodialysis-related T cell activation is likely to be a result of direct physical interaction between the dialysis membranes and lymphocytes circulating through the hollow fiber dialyzers (Donati *et al* 1992; Luger *et al.* 1987) and most likely is a non-specific activation. Although CsA sensitivity of predialysis PBLs returns to normal, haemodialysis T cells are still capable of responding strongly to IL-2 (Chatenoud *et al.*1986) and could be a source of rejection. The difference in the T cell activation state before and after

haemodialysis that was demonstrated by reduced CsA sensitivity of T cells after haemodialysis was not paralleled by expression of the T cell activation markers, due to the kinetic pattern of expression of these activation markers on T cells. This indicates that expression of T cell activation markers does not necessarily reflect the activation state of T cells at that time, but is historically valid.

Because we found that CsA resistance of T cells of patients on chronic haemodialysis is due to T cell preactivation, we decided to test whether a prophylactic single ATG bolus aimed at removing these preactivated T cells could achieve adequate immunosuppression. We determined the *in vitro* perioperative CsA sensitivity of peripheral blood Lymphocytes of renal transplant patients maintained on chronic haemodialysis before transplantation, and included a single ATG bolus induction in the immunosuppressive regimen. Our results showed that live-related renal transplant patients given a prophylactic single preoperative intravenous ATG-Fresenius bolus as part of the immunosuppressive regimen had significantly increased *in vitro* CsA sensitivity of peripheral blood T cells at the tested time points post-transplant as compared to pre-transplant. In association with the other reports (Kaden *et al.* 1992,1995, 1997; Zietse *et al.* 1993; Abouna *et al.* 1993, 1995) we concluded that prophylactic single ATG bolus delayed the onset and reduced the incidence of acute rejection, and improved graft and patient survival through effective removing CsA resistant preactivated T cells that enhanced CsA sensitivity. The effective prophylactic action of ATG may have very important clinical implications on the regulation of CsA-based

immunosuppressive therapy. Prophylactic use of ATG induction therapy targeted towards removing activated T cells may allow further reduction of CsA dosage, better drug monitoring with good CsA concentration-clinical effects correlation, and fewer overt side effects such as nephrotoxicity. Thus, in addition to the fact that ATG may be effective as prophylaxis against acute rejection, prophylactic ATG induction therapy may improve the late graft function by minimising chronic rejection and CsA nephrotoxicity.

In summary, we have confirmed our proposed hypothesis that "activated T lymphocytes are less sensitive to CsA":

- Initially we found that PHA preactivation of T cells reduced the CsA sensitivity of circulating T cells in healthy volunteers, CAPD patients and chronic haemodialysis patients.

- Then, in comparison to normal volunteers, and CAPD and pre-haemodialysis patients, we demonstrated a reduction of CsA sensitivity of circulating T cells after haemodialysis in chronic haemodialysis patients using several parameters focused on IL-2.

- We also demonstrated in these chronic haemodialysis patients the presence of preactivated T cells as indicated by the expression of T cells activation markers and abnormal IL-2 production. This suggested that haemodialysis activated T cells in chronic haemodialysis patients. Thus, our findings clearly showed the existence in chronic haemodialysis patients of abnormally high

proportions of T cells presenting haemodialysis-related phenotypic and functional signs of preactivation. These preactivated T cells offer an explanation of reduced *in vitro* CsA sensitivity of T cells after haemodialysis. The presence of preactivated T cells may have potential relevance to CsA resistance, and to the risk of acute rejection episodes and chronic rejection after renal allograft transplantation.

- Our findings of increased post-transplant *in vitro* CsA sensitivity of circulating T cells in renal transplant patients who received a single prophylactic bolus of ATG indicates that prophylactic use of ATG induction therapy aimed at removing activated T cells facilitates more effective and potent CsA-based immunosuppression. This may have a potential role in preventing acute rejection episodes during the early period of a transplant, improve graft and patient survival, and improve long-term graft function.

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9. APPENDIX

CsA IC_{50} Theory

CsA IC_{50} is the concentration of CsA causing 50% inhibition of IL-2 production. An issue of interest is the vastly reduced concentration of CsA that is needed *in vitro* to achieve the comparable *in vivo* effect (Batiuk *et al.* 1996b). They found that the IC_{50} *in vivo* for inhibition of calcineurin and cytokine production by CsA, which are correlated (Batiuk *et al.* 1994), was at least 310 ng/ml, whereas *in vitro* concentrations never exceeded 25 ng/ml. Similarly, it has been shown that calcineurin is partially inhibited in leukocytes of CsA treated-patients (Batiuk *et al.* 1995a). This discrepancy is likely due to the fact that the usual culture medium conditions fail to allow for CsA's extreme hydrophobicity. *In vivo*, CsA distribution is affected by many factors, including haematocrit, lipoprotein and serum protein levels (Kasiske *et al.* 1988b; Hughes *et al.* 1991), which can reduce the availability of CsA to the relevant sites in leukocytes. In contrast, isolated leukocytes in aqueous culture medium are the only available hydrophobic compartment, creating an unrealistic degree of partitioning of CsA into the lymphocytes. When CsA is added to whole blood *in vitro*, the IC_{50}s for inhibition of leukocyte calcineurin activity and cytokine production approach those seen *in vivo* (Batiuk *et al.* 1996a). This *in vitro/in vivo* discrepancy is now widely acknowledged and must be taken into consideration when attempting to simulate the clinical effects of CsA. In other words, the CsA concentration *in vitro* which models the CsA effect *in vivo*, i.e., around 50% inhibition. Furthermore, since complete inhibition of calcineurin and cytokine production is rarely seen in patients, even at peak CsA levels, *in vitro* complete inhibitory effects of CsA which are seen only at very high concentrations may not be relevant to clinical conditions.

Appendix 1: Calculation of CsA inhibition of IL-2 production from CsA sensitivity assay using CTLL-2 as detector of IL-2 (e.g. Volunteer).

1.1. Without *in vitro* preactivation

	BaCTLL-2	*Rres lymph*	*CsA 0*	*CsA 10*	*CsA 50*	*CsA 125*	*CsA 250*	*CsA 500*	*CsA 1000*	*CsA 10000*
cpm1	318	286	11199	6795	3151	2413	1820	1383	1097	1319
cpm2	274	218	10458	7207	2932	2650	1895	1324	1057	1205
cpm3	570	310	9075	7455	2414	3743	2174	1549	862	1262
cpm4	404	311	10471	8411	3862	2531	1463	1441	1446	843
cpm5	472	258	10471	6685	2830	2651	1867	1178	1444	815
mean cpm	407.6	276.6	10334.8	7310.6	3037.8	2797.6	1843.8	1375	1181.2	1088.8
SD	118.8	39.3	772.4	689.1	532.8	537.6	253.9	137.9	256.7	240.8
cor. mean			9650	6626	2353	2113	1159	690	496	404
% control			control	69 ± 6.5	24 ± 4.21	22 ± 4.23	12 ± 1.65	7 ± 0.70	5 ± 1.08	4 ± 0.88
log (cor.mean + control)			9.1747132	9.6974469	9.3929119	9.3727143	9.2881344	9.2437751	9.2248348	9.2157258
% log control			control	52.2734	21.8199	19.8001	11.3421	6.9062	5.0122	4.1013

KEYS.

- CsA concentration (ng/ml)
- Background CTLL-2 proliferation: BaCTLL-2.
- Radio-resistant lymphocyte proliferation: Rres lymph
- Count per minute: cpm
- Standard deviation: SD
- Corrected mean: cor. mean

1.2. With *in vitro* preactivation

	BaCTLL-2	*Rres lymph*	*CsA 0*	*CsA 10*	*CsA 50*	*CsA 125*	*CsA 250*	*CsA 500*	*CsA 1000*	*CsA 10000*
cpm1	318	365	9559	7694	6536	5134	2828	3689	2607	958
cpm2	274	268	10840	7472	5539	5682	4362	1842	2188	1020
cpm3	570	214	9472	7543	5442	4643	3743	2050	1284	1338
cpm4	404	312	10843	8170	6995	3798	3015	2329	2502	779
cpm5	472	254	8614	7390	5671	3976	4294	3030	2105	871
mean cpm	407.6	282.6	9865.6	7653.8	6036.6	4646.6	3648.4	2588	2137.2	993.2
SD	118.8	57.9	964.4	309.4	689.7	787.3	708.7	761.7	520.9	213.1
cor. mean			9175	6963	5346	3956	2957	1897	1446	302
% control			control	76 ± 3.07	58 ± 6.62	43 ± 7.28	32 ± 6.21	21 ± 6.18	16 ± 3.78	3 ± 0.71
log (cor.mean + control)			9.1242377	9.688932	9.5833512	9.4827311	9.4036019	9.3121747	9.2705885	9.1566231
% log control			control	56.4694	45.9113	35.8493	27.9364	18.7937	14.6351	3.2385

Appendix 2: Individual CsA IC_{50} (ng/ml) of normal subjects.

	CsA IC_{50} from normal values		**CsA IC_{50} from log-transformed values**	
V. code	*without preact.*	*with preact.*	*without preact.*	*with preact.*
MHB	65	220	41	160
TON	10	130	6	40
KAS	98	585	28	180
RYF	70	420	15	115
RUN	85	1000	32	610
VIN	84	1000	29	500
MNA	35	179	12	62
ALS	27	108	11	60
GIT	10	63	7	38
DAR	13	98	8	42
Mean ± (SEM) IC_{50}	**50 ± 11**	**380 ± 115** ***p = 0.013***	**18.9 ± 3.9**	**180.7 ± 64.8** ***p = 0.029***

KEYS. Volunteer: V. Preactivation: preact.

Although the CsA IC_{50} values are reduced after log-transformation, the trend remained the same and the statistical significance with and without pre-activation is maintained.

Appendix 3: Calculation of number of intracellular IL-2 molecules per CD3+ cell using multi beads and TallyCAL software (e.g. volunteer).

	PREDIALYSIS		**POSTDIALYSIS**	
Sample	**MFI**	**IL-2 molecules**	**MFI**	**IL-2 molecules**
CsA 0	6.3	2928.1	7.0	3275.1
CsA 10 ng/ml	5.3	2492.8	5.7	2678.1
CsA 100 ng/ml	4.6	2172.8	5.3	2502.1
CsA 1000 ng/ml	4.2	1973.2	4.9	2302.7

Median fluorescence intensity: MFI

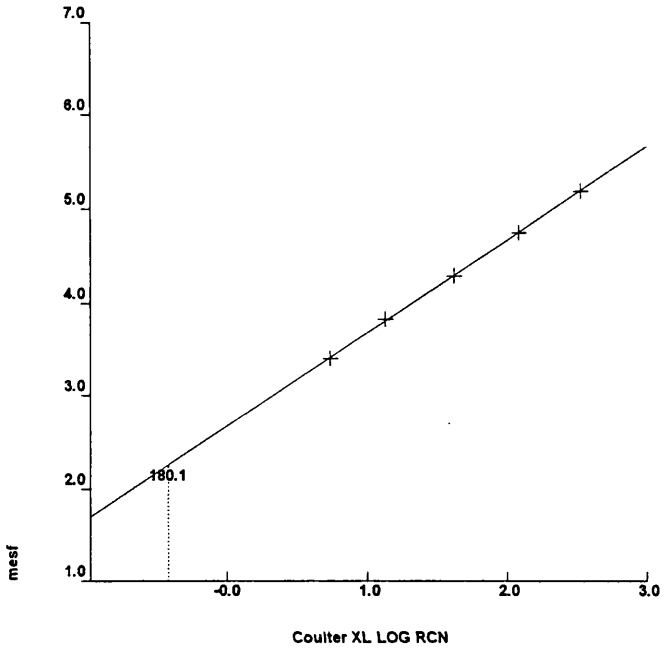
TallyCAL Calibration Report

Institute: ROYAL FREE

Department: TISSUE TYPING

Operator: jb

Date: 13.1.98



Units: mesf

Bead Batch: 036(301)

Parameter: FL1

Calibration Particle: DAKO FLUOROSPHERES

Cytometer ID: FRED

Expiry Date: 0398

LOG Decades: 3.96

Buffer Temperature:

Zero Channel Value: 49.62

Buffer pH:

Regression Coefficient: 0.99963

Analysis Software:

AvgRes%: 2.33151

Region File:

Slope: 0.99101

List Mode File:

Offset: 2.67649

Appendix 4: Chronic haemodialysis patients

Patient code	Date of born	Sex	Duration of haemodialysis
MIS	4/5/1941	M	26 months
ESH	26/4/1973	F	62 months
ACA	1/10/1939	M	50 months
PPR	23/5/1969	M	11 months
JNA	15/7/1949	M	10 months
GBR	12/81947	M	48 months
MEP	28/11/1965	M	163 months
RGO	16/2/1969	M	7 months
ICH	5/4/1940	M	9 months
WDE	25/10/1935	M	27 months
RAR	10/9/1929	M	36 months
NWH	22/8/1961	M	11 months