R O T R F

Roche Organ Transplantation Research Foundation

BIANNUAL REPORT

October 2000

The Roche Organ Transplantation Research Foundation

The mission of the Roche Organ Transplantation Research Foundation (ROTRF) is to advance the science of solid organ transplantation in order to improve the care of the thousands of patients undergoing transplantation every year. The results of the funded research projects will contribute to an understanding of many aspects of the clinical and scientific adventure of transplantation, e.g. the mechanisms of long-term organ deterioration, the consequences of tissue injury, and the opportunities to intervene in these processes.

The Foundation is an independent medical research charity that provides operating funds to established academic staff in universities, transplant centres and research institutes. The Foundation supports research in solid organ transplantation, particularly where there is an unmet medical need.

The funding, a donation from F. Hoffmann-La Roche Ltd., provides the Foundation with 25 million Swiss francs over the first five years. The funds are disbursed as grants of up to 300,000 Swiss francs distributed over three years. The Foundation is legally independent from F. Hoffmann-La Roche Ltd. and is guided solely by the Board of Trustees according to its charter.

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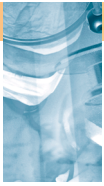


1. Preface

The Roche Organ Transplantation Research Foundation (ROTRF) is very pleased to announce that thirteen research grants have been awarded to scientists around the world following the fourth cycle of grant review. A total of 2 million Swiss Francs has been awarded in this funding cycle.

After the first four ROTRF funding cycles, a number of lessons have already been learnt about international research funding in transplantation. First, it is clearly possible to have an international adjudication process in transplantation that receives widespread interest. The Trustees and the Scientific Advisory Committee have in general been very pleased with the high level of scientific quality and originality shown by the applicants.

Secondly, we have seen that researchers from some countries have much less experience in writing the letter of intent and in particular the full paper submission than others. Thus, the ROTRF will strive to give continuing consideration to developing an understanding of the international granting process, such that "grantsmanship" is equivalent in all countries and that applicants from certain parts of the world are not discriminated against.

Many grant-awarding bodies find that clinical research is the most difficult type of research to fund through a formal process. This does not usually represent a bias on the part of the Scientific Advisory Committee, since the clinical research grants tend to get less favorable ratings overall, even from clinicians. This is particularly puzzling since these projects often eventually lead to very high profile publications. The ROTRF intends to take steps to overcome this analogy, and encourages researchers to submit clinical research proposals in subsequent cycles.

In summary, the Foundation is proving to be a great and unique achievement and all involved can be proud of it. All those who have been involved in supporting the ROTRF to accomplish its mission deserve the Foundation's gratitude.

On behalf of the Board of Trustees

Phil Halloran

2. Facts and Figures

Funding Cycle IV – Letter of Intent Submission in April 2000

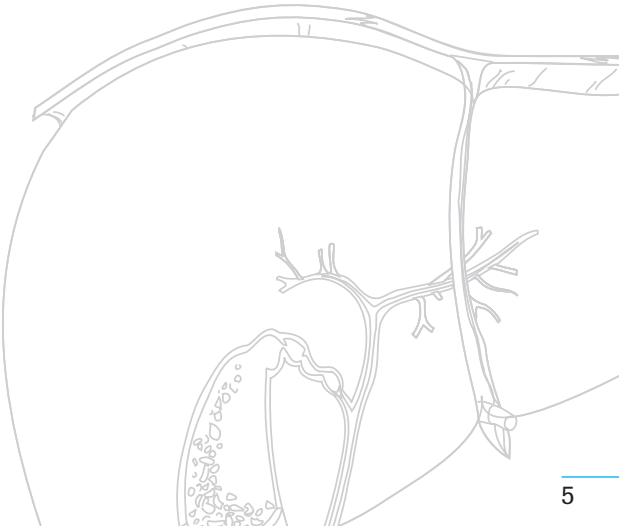
In the fourth ROTRF funding cycle, 134 letters of intent were received from scientists around the world. About a third of the applications came from the United States (36%), while 13% came from the UK. In total, North America accounted for 44% of the applicants and Europe for 46%. The remaining 10% of the applicants came from Australia, New Zealand and Asia.

The Scientific Advisory Committee of the ROTRF evaluated all the applications, which were submitted electronically via the ROTRF's homepage (www.ROTRF.org), for originality and scientific excellence. The top 21 applications were invited to submit a full paper application and subsequently underwent a second thorough review by the Scientific Advisory Committee and the Board of Trustees.

ROTRF Grants were finally awarded to 13 applicants: five from the USA; three from the UK; two from Canada; and one each from France, Israel and Poland (see the orange dots on the world map on the following page). Almost 40% of the Grantees in ROTRF funding cycle IV are female.

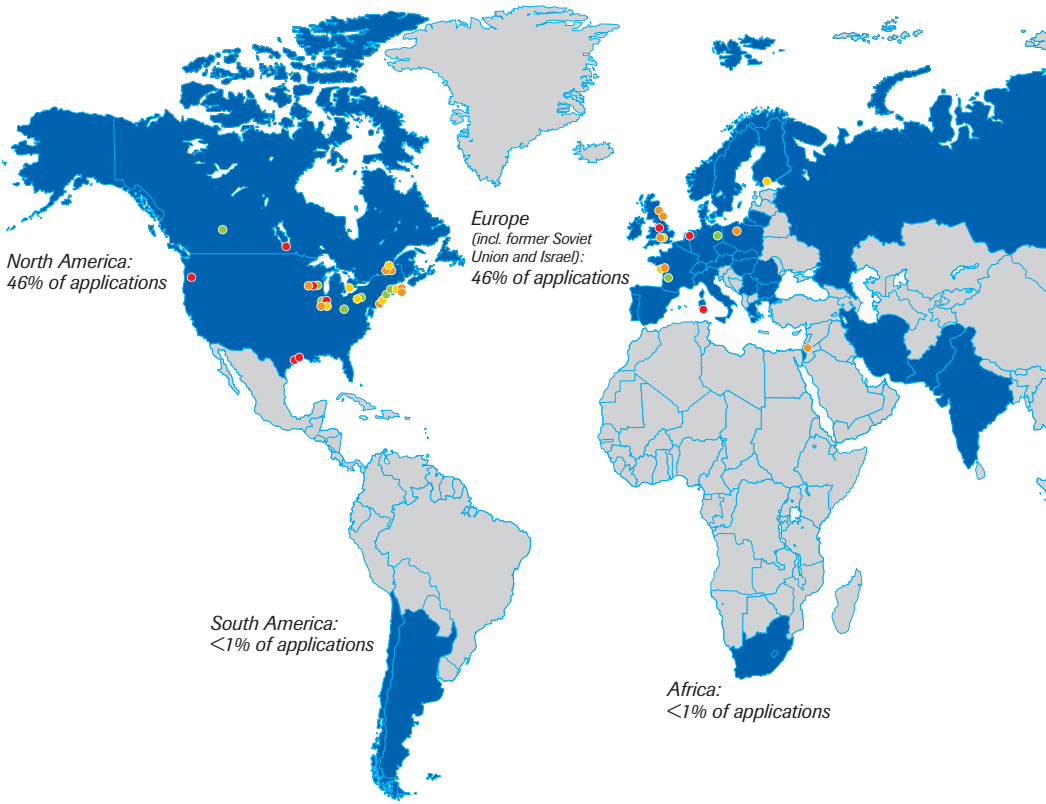
The abstracts of the novel and promising research projects are presented on the following pages. Their research interests focus mainly on the improvement of long-term graft survival and prevention of chronic organ dysfunction, induction of tolerance, the development of new immunosuppressive agents, and the improvement of donor organ preservation.

In this fourth cycle of ROTRF Grant Awards, a total of 2 million Swiss Francs was allocated.



The Global View of Applications to the ROTRF

Distribution of ROTRF applications worldwide



Cycle I Grantees

Berlin, Germany
Bordeaux, France
Boston, USA
Chicago, USA
Cincinnati, USA
Edmonton, Canada
Madison, USA
Melbourne, Australia
New Haven, USA
Pittsburgh, USA

Cycle II Grantees

Boston, USA
Chicago, USA
Helsinki, Finland
London, Canada
Montreal, Canada
Nantes, France
New York, USA
Oxford, UK
Pittsburgh, USA

Cycle III Grantees

Birmingham, UK
Cagliari, Italy
Chicago, USA
Houston, USA
Houston, USA
Madison, USA
Nijmegen, The Netherlands
Portland, USA
Winnipeg, Canada

Cycle IV Grantees

Boston, USA
Boston, USA
Chicago, USA
Dundee, UK
Laval, Canada
Madison, USA
Montreal, Canada
Nantes, France
Newcastle-upon-Tyne, UK
Oxford, UK
Philadelphia, USA
Rehovot, Israel
Warsaw, Poland

3. Grant Awards in Cycle IV

Dr. Maria-Luisa Alegre, Principal Investigator
Dr. J. Richard Thistlethwaite, Research Associate
Dr. Ping Zhou, Research Associate

University of Chicago, Chicago, USA

Role of Costimulatory Molecules in Cardiac Allograft Rejection

T lymphocytes are essential for acute rejection of transplanted organs, and rejection does not occur in the absence of T cells. T cells become stimulated following encounter with antigen (including transplant antigens) by signals delivered by the T cell receptor during antigen recognition together with signals delivered by costimulatory molecules expressed on T cells. Transplant antigens are recognized by T cells either on the graft itself, or on the surface of antigen-presenting cells that also express the ligands for the T cell costimulatory molecules. However, the actual mechanism of rejection is not well understood. For example, neither the type of antigen-presenting cell nor its origin (the transplant recipient or the graft) is clearly defined. In addition, it is not known whether T cells participate in the destruction of the graft, or if they activate other effector cells that reject the transplant.
The availability of mice made genetically deficient in specific costimulatory molecules, costimulatory ligands or signaling pathways has provided tools to dissect the role of these individual molecules in the promotion or prevention of graft rejection. We have recently shown that T cells from recipient mice preferentially receive specific costimulatory signals from host than from graft antigen-presenting cells.
The aims of this study are to characterize the cell types utilized for costimulation and antigen presentation to T cells and the mechanism of rejection operating in mice with defective costimulation capacity. Immunosuppressive regimens currently in use in clinical transplantation induce a global immunosuppression of all T cells, which results in increased susceptibility to infections and cancers. This study may reveal the importance of alternative costimulatory molecules. This has the potential to help design better immunosuppressive regimens applicable to the clinical setting with the ultimate goal of achieving donor-specific tolerance.

Dr. Simi Ali, Principal Investigator
Dr. John A. Kirby, Co-Applicant

University of Newcastle-upon-Tyne,
Newcastle-upon-Tyne, UK

Anti-Rejection Therapy: Modifying Intragraft Immunity by Specific Blockade of Th1 Cell Recruitment

Solid organ transplantation is often complicated by rejection, which is manifested by vigorous inflammation. The Th1 cytokines stimulate the acute rejection process whilst Th2 cytokines tend to downregulate this process. It is also known that Th1 and Th2 lymphocytes produce different chemokine receptors. For example, Th1 lymphocytes express chemokine receptor CCR5. One of the major ligands for CCR5 is the chemokine RANTES. Significantly, lymphocytes expressing CCR5 are known to predominate during acute renal rejection.

The early events in rejection are controlled by small proteins, termed chemokines, which recruit immune cells from the blood and direct them into the transplanted organ. Recent evidence suggests that chemokines anchor themselves to molecules called proteoglycans, which exist on the surface of cells lining the blood vessels. In addition, they also bind to their specific receptors.

We will address the possibility that this anchorage is essential for functional activation of chemokines and that disruption of this process might offer a possibility for anti-inflammatory therapy. Data from our group suggests that the chemokine RANTES contributes to the process of graft rejection. In this study we will use molecular biology methods to produce several versions of RANTES with variable abilities to bind proteoglycans. We will then use these constructs in a range of assays designed to assess the biological activity. The potential anti-inflammatory activity of these molecules will be evaluated by selectively blocking the movement of CCR5-expressing Th1 lymphocytes in laboratory experiments. These molecules should contribute to the rational design of therapeutic agents to selectively block graft-damaging Th1 lymphocytes following transplantation.

Dr. Michael Autieri, Principal Investigator
Christopher Carbone, Research Associate

Temple University, Philadelphia, USA

Characterization of Expression and Growth-Enhancing Properties of AIF-1 in VSMC of Injured Arteries

The initiation of allograft vasculopathy (AV) is believed to involve a chronic immune response of the recipient to the donor vasculature in which activated recipient immune cells produce cytokines that activate medial vascular smooth muscle cells (VSMC). The activation of VSMC is responsible for most of the obliterative arterial intimal thickening and is the major complication that limits long-term survival of transplanted solid organs. Interventions that are successful in patients with conventional coronary artery disease are not applicable to the majority of patients with AV because of its extensive nature. The risk of infection and malignancy associated with aggressive immunosuppressive therapy also advocates the need to identify a molecular target that directly impacts the VSMC response to injury.

We feel that allograft inflammatory factor-1 (AIF-1) is one of several calcium-binding proteins that play a critical role in the regulation of cellular growth. AIF-1 is not expressed in normal arteries, or in arteries from failing human hearts, but is rapidly expressed in VSMC in response to balloon angioplasty or allograft injury. AIF-1 expression correlates with proliferation of VSMC during *in vivo* injury and in cultured VSMC. Correspondingly, stable transfection and constitutive expression of AIF-1 in human VSMC leads to enhanced proliferation and increased expression of proliferative genes in those cells. Conversely, inhibition of AIF-1 expression or modification of AIF-1 calcium binding greatly reduces the proliferative capacity of VSMC.

The specific aims of this project are to define the mechanism of AIF-1 growth-enhancing activity in human VSMC, characterize the expression pattern of AIF-1 and its sensitivity to various therapeutic compounds, and explore the use of AIF-1 as a surrogate marker of VSMC pathophysiology. We anticipate that inhibition of AIF-1 expression and/or activity will reduce or prevent VSMC proliferation, thereby limiting the severity of transplant restenosis and other vascular proliferative diseases.

Dr. Francois Denis, Principal Investigator
Dr. Claude Daniel, Co-Applicant
Dr. Denis Girard, Co-Applicant

INRS-Institut Armand-Frappier, Laval, Canada

Creating Artificial Immunoprivilege for Allograft Acceptance

The eye, testis and placenta are termed immunoprivileged sites because they are protected from the immune system through the expression of the FasL molecule, which causes the death of infiltrating auto-reactive T cells. This permanent removal of auto-reactive T cells prevents any further attacks from the immune system and creates tolerance. While the use of immunosuppressive drugs has afforded success in organ transplantation, permanent graft acceptance is still elusive because of the phenomenon of chronic rejection. Given that inducing immuno-privilege within allografts was expected to induce tolerance, researchers have expressed FasL in grafts, but surprisingly this caused accelerated organ rejection. The cause of this acute rejection was massive neutrophilic infiltration, revealing that FasL had chemotactic properties towards neutrophils. This novel inflammatory property of FasL remains ill defined and requires molecular characterisation before the promise of immunoprivilege can be applied to organ transplantation.

This research project aims at defining the molecular determinants of FasL involved in its chemotactic and apoptotic properties. Through molecular techniques, these two properties will be dissociated, and non-chemotactic pro-apoptotic forms of FasL will be designed.

Subsequent work will attempt to target the engineered FasL molecules to grafts, through the development of high-affinity single-chain antibodies recognising donor-specific molecules. Dual-specificity molecules, consisting of FasL and single-chain antibodies, will then be constructed and tested for their potency *in vitro*. The efficacy of the novel reagents will be tested *in vivo* using a unique murine model of graft rejection that allows evaluation of two important pathways involved in graft rejection. It is anticipated that these studies will allow induction of artificial immunoprivilege and afford permanent allograft tolerance.

Prof. Peter Friend, Principal Investigator
Dr. Susan Fuggle, Co-Applicant
Charles Imber, Co-Applicant

Nuffield Department of Surgery, Oxford, UK

Prolonged Preservation of Donor Livers Using Normothermic Perfusion

At present the preservation of organs for transplantation is based on cooling to ice temperature in order to reduce metabolic activity and the infusion of a solution designed to maintain ionic and fluid balance across cell membranes. Organs preserved in this way suffer injury caused by the period of preservation, due to persisting low-level metabolic activity and the process of reperfusion with warm blood at the time of transplantation. In the case of a liver, additional problems occur because of the deposition of fat within the cells of the liver; this is a response to injury that occurs in many livers before removal from the donor. Whereas this has no measurable effect upon biochemical liver function in the donor at normal body temperature, it is recognised as a serious adverse prognostic factor following transplantation. Cooling a fatty liver increases the susceptibility to preservation-reperfusion injury through a poorly understood mechanism, leading to poor function of the liver within the patient immediately after transplantation. For this reason, many potential livers are discarded before transplantation because of the risk of "primary non-function".

Because much of the injury that occurs is related to cooling and rewarming, a preservation system that enables a liver to be maintained in a viable, non-deteriorating state without recooling would be of enormous benefit. Liver function immediately following transplantation would be no worse than immediately prior to removal from the donor, enabling rapid recovery following surgery. It should enable livers with significant fatty deposition to be used for transplantation, thereby increasing the donor pool by 10–15%. Because the liver would be stored in a functioning state, it would also enable viability to be assessed after removal from the donor and prior to transplantation, thereby enabling the safe use of more marginal donors. It might also allow time for damage to the liver to recover before transplantation.

The ability to maintain a donor organ for several days might also allow time to pretreat the patient or the liver in such a way as to reduce or remove the likelihood of rejection. It would enable the scheduling of transplant operations and the transport of organs over considerable distances without loss of viability.

Dr. Debra Hullett, Principal Investigator
Alexander Pietsch, Research Associate
Dr. Bryan Becker, Collaborator

University of Wisconsin, Madison, USA

Effect of Vitamin D on Renal Allograft Function

Graft failure following transplantation results from either acute or chronic rejection. There are currently many immunosuppressive agents that are effective at preventing acute rejection. There are no such reagents for the prevention of chronic rejection. In the kidney, chronic rejection is characterized by the development of fibrotic changes, which include interstitial fibrosis, tubular atrophy, glomerulosclerosis, and concentric intimal hyperplasia. This process has been termed chronic allograft nephropathy (CAN).

1,25-Dihyroxyvitamin D_3 [1,25-$(OH)_2D_3$] is the active metabolite of vitamin D. Its long-known function is to regulate calcium metabolism. Recent evidence suggests that 1,25-$(OH)_2D_3$ also regulates the growth and differentiation of many cell types, and in particular that 1,25-$(OH)_2D_3$ regulates immune responses. We have shown that 1,25-$(OH)_2D_3$ treatment prolongs allograft survival. Importantly, we have shown in a retrospective study that renal transplant patients who received 1,25-$(OH)_2D_3$ supplementation because of renal insufficiency had stabilized graft function.

The mechanisms that lead to the development of CAN are unknown, but are thought to involve both immune and non-immune factors. The cytokine transforming growth factor beta (TGFβ) has been implicated in the pathogenesis of the fibrotic changes in CAN. We and others have shown an interaction between the TGFß and 1,25-$(OH)_2D_3$ signal transduction pathways. Thus, we hypothesize that 1,25-$(OH)_2D_3$ will be an effective agent in preventing CAN and that 1,25-$(OH)_2D_3$ may regulate TGFβ-mediated fibrosis. We intend to determine whether 1,25-$(OH)_2D_3$, when used in combination with low-dose standard immunosuppression, is effective in preventing CAN, and to determine the effect of 1,25-$(OH)_2D_3$ treatment on the expression of genes for proteins that regulate fibrotic changes, namely the matrix metallo-proteinases and tissue inhibitors of matrix metalloproteinases.

The experiments proposed in this application should lead to a greater understanding of the mechanisms underlying CAN and may identify new targets for therapy.

Prof. Peter Lonai, Principal Investigator
Dr. Marat Gorivodsky, Research Associate

Weizmann Institute of Science, Rehovot, Israel

Can ES Cells Form a Specific Complex Organ?

Stem cell technology is one of the greatest advances of recent biology. Its first step was cloning of farm animals by transfer of embryonic cell nuclei into oocytes. Later, in the famous Dolly experiment, a sheep was cloned from somatic cell nuclei. This meant that transgenic animals can be made to produce pharmaceutical and other agents using nuclei from cells that express genes important for industry. These results also meant that the differentiated state of our cells is less stable than we thought. Knowing that stem cells, such as embryonic stem (ES) cells, can form multiple cell types, schemes were worked out to produce stem cell lines from somatic cells via nuclear transplantation into oocytes. Most recently, the great flexibility of adult stem cells was discovered. The possibility of cloning higher animals and even man, and the prospect of patient-derived cell therapy drew little attention. These results also revived interest in basic questions, such as the flexibility of the differentiated state, stem cell biology, and regeneration.

It is generally expected that stem cell technology will greatly advance biotechnology and cell therapy. Its application to organogenesis and to the production of organ grafts has however yet to be addressed. The importance of this problem, in the face of a chronic graft shortage, requires little emphasis.

The proposed research asks whether stem cells can contribute to the development of specific complex organs. We will use a loss-of-function mutation of Fgfr2 (fibroblast growth factor receptor 2) to analyse this question. This mutation completely abrogates limb development in mouse embryos. In preliminary experiments, ES cells injected into the prospective limb fields of the mutant caused outgrowth and chondrogenesis. We believe that a careful analysis of this experimental system, even if it may not yield complete limbs immediately, should bring us a few steps closer to making it feasible.

Dr. Dianne B. McKay, Principal Investigator
Dr. Charles B. Carpenter, Co-Applicant
Dr. Edgar L. Milford, Co-Applicant
Dr. Steven Burakoff, Co-Applicant

Dana-Faber Cancer Institute, Boston, USA

Alterations in IL-2R Signaling Induced by Anti-IL-2R Antibodies

T cell proliferation is a key step in amplification of immune responses to alloantigens and thus is a prime target of immunosuppressive therapy in solid organ transplantation. Interleukin-2 (IL-2), a soluble cytokine released by activated T cells, plays a major role in this amplification by autocrine and paracrine mechanisms after binding to IL-2 receptors (IL-2R). The IL-2R structure is dependent on the activation state of T cells; two chains (β/γ) are expressed on the surface of resting T cells and three (α, β, γ) on activated T cells.

Recently, antibodies have been developed that specifically target activated T cells by binding to the inducible IL-2Rα chain. Anti-IL-2Rα antibodies reduce the incidence and severity of acute rejection, but in clinical practice require concomitant use of agents that decrease IL-2 expression (i.e. calcineurin inhibitors). We have found marked differences in the Jak/STAT signaling pathway of T cells activated in the presence or absence of the anti-IL-2Rα antibody daclizumab. The Jak/STAT pathway is one of three inter-related pathways involved in IL-2R-mediated signaling (Jak/STAT, Ras/MAPK, and c-myc/bcl-2). Blockade of IL-2R-mediated signaling has the potential to effect multiple pathways in the T cell, resulting in modification of a wide number of functions from proliferation to apoptosis.

Using biochemical and molecular technologies we will examine the impact of daclizumab on the Ras/MAPK and c-myc/bcl-2 signaling pathways. In addition, daclizumab causes internalization of IL-2Rα chains, leaving residual cell surface IL-2Rβ/γ chains. We will also examine signals generated through residual IL-2Rβ/γ chains. Information obtained from these studies will expand our understanding of IL-2-induced cell signaling and the interplay of the intracellular signaling pathways. We believe that defining molecular targets of IL-2Rα blockade will elucidate new strategies for the use of anti-IL-2Rα antibodies and may lead to the development of novel immunosuppressive agents.

Dr. Simon C. Robson, Principal Investigator
Dr. Imrana Qawi, Research Associate

Beth Israel Deaconess Medical Center, Boston, USA

Disordered Thromboregulation in Xenotransplantation

Clinical transplantation is limited by the availability of suitable human organ donors. The proposed use of an unlimited supply of animal organs in clinical practice (xenotransplantation) could provide a bridge to a later successful human graft in a critically ill patient, or more optimistically may even substitute for such a graft. Unfortunately, the clinical application of xenotransplantation has resulted in almost total failure to date.

Recent developments in the fields of xenotransplantation and vascular biology have greatly expanded our understanding of the mechanisms by which xenografts are rejected and have given new hope to the field. However, one additional novel barrier observed is the molecular incompatibility between natural anticoagulants expressed by blood vessels of pig organs and primate/human blood coagulation factors and platelets. This results in clotting within transplanted porcine xenografts in baboons, with rapid loss. To mitigate against these effects, we propose to derive triple transgenic animals overexpressing two human natural anticoagulants (tissue factor pathway inhibitor and thrombomodulin) and the thromboregulatory factor CD39 that profoundly inhibits the activation of platelets irrespective of the species of origin.

This project is a preliminary component of a long-term clinical xenotransplantation strategy ultimately involving the genetic modification of donor pigs to render them biologically more compatible. We will first investigate whether the respective human cDNAs can be functionally expressed in porcine vascular endothelium, either individually or in tandem. We then propose derivation of multi-transgenic mice, again checking that there are no deleterious effects of these combinations and testing the function of the overexpressed factors in relevant models of xenograft rejection.

This work will be judged successful if the data are of potential relevance for clinical transplantation and result in the testing of novel treatments to prolong xenograft survival.

Dr. Ana Maria Schor, Principal Investigator
Prof. Seth Lawrence Schor, Co-Applicant
Prof. Peter Downes, Collaborator

University of Dundee, Dundee, UK

The Control of Angiogenesis: Matrix Modulation of Cellular Responses to Cytokines

The ability of organ transplants to survive is dependent upon the establishment of an adequate blood supply. This is of paramount importance for artificial organ grafts, which lack their own vascular system and are therefore strictly dependent upon the ingrowth of new vessels from the host tissue, a process referred to as angiogenesis. Many soluble angiogenic factors have been identified that stimulate new blood vessel growth in various experimental models. Unfortunately, incorporation of these factors in tissue grafts has not consistently resulted in the expected induction of vessel growth.

Recent data suggest that this apparent discrepancy is due to the hitherto unrecognised role played by common connective tissue molecules (such as collagen), which form the scaffold to which tissue cells are attached. It is now clear that these molecules determine the precise manner by which cells respond to soluble angiogenic factors.

We have recently identified and cloned a novel and highly potent angiogenic factor. In accord with our current understanding of the control of cell responses to such factors, we find that the activity of this new molecule (referred to as MSF) is only manifested in cells attached to certain connective tissue molecules. The objectives of this study are concerned with understanding the molecular mechanisms responsible for this dependence and providing the basic scientific platform required to introduce MSF into clinical practice.

Prof. Jean-Paul Soulillou, Principal Investigator
Dr. Abdel Saoudhi, Research Associate
Dr. Marina Guillet, Research Associate
Dr. David Laplaud, Research Associate
Dr. Annaick Palier, Research Associate
Dr. Marc André Delsuc, Research Associate
Dr. Claire Usual, Research Associate
Dr. Gilles Benichou, Consultant
Dr. Samia Khoury, Consultant

ITERT, Nantes, France

Integrated TCR Vβ Transcriptome Analysis, a New Method to Follow Allo- or Auto-Immune Responses

Organ transplantation allows patients with end stage diseases of a vital organ to recover a normal life. Immune recognition of foreign tissues remains the main obstacle to transplantations that require long-term immunosuppression. The initial alloimmune responses proceed through the usual pathway of immunity in which allopeptides are presented by self-MHC. However, an unusually high proportion of naive T cells also directly recognises foreign MHC. This “direct” recognition is supposed to play a major role in acute rejection early after grafting. Normal recognition (self-APC) is supposed to operate in chronic rejection.

Our programme deals with a global representation of TCR usages in direct and indirect pathways *in vitro* and *in vivo*. So far, TCR biases were only studied through qualitative TCR alteration of the Vβ chain segment (CDR3) that interact with peptide/MHC. This is relevant for analysing TCR biases in the indirect pathway, but not in the direct pathway where CDR3 may not be involved. In addition, in all cases the possible relevance of qualitative alteration must benefit from the knowledge of the number of clones involved (i.e. reflected by the amount of mRNA). We propose another approach that links qualitative and quantitative parameters and allows a global assessment of TCR alterations in which qualitative alterations of Vβ mRNA are corrected by the amount of altered mRNA assessed by quantitative PCR.

Our first aim is to optimise this new method. The method will then be used to revisit allorecognition *in vitro* in direct-type MLR where pure T cells are confronted with allogeneic APC, and *in vivo* during acute rejection of heart allografts in rat and in an established model of experimental tolerance induction.

Dr. Adam Szewczyk, Principal Investigator
Anna Kicinska, Research Associate
Grazyna Debska, Research Associate

Nencki Institute of Experimental Biology, Warsaw, Poland

Improving the Preservation of Heart Transplants: the Role of Mitochondrial Ion Channels

Mitochondria play a central role in energy-generating processes within the cell. Despite this important function, mitochondria also seem to be involved in such complex processes as cardioprotection. Recently, ischaemic preconditioning has emerged as a new strategy for improving the preservation of heart transplants. Hence, there is a continuing effort to identify the mechanisms of the ischaemic preconditioning signalling pathways in an attempt to use similar mechanisms therapeutically during heart transplantation.

Mitochondria attract attention due to the identification of the ATP-regulated potassium channel in mitochondria and its possible role as an effector in ischaemic preconditioning in heart. It has been shown that mitochondrial potassium channel openers (e.g. diazoxide) can protect ischaemic myocardium. These findings support the concept that cardioprotective effects of ischaemic preconditioning can be simulated by mitochondrial potassium channel openers. Activation of mitochondrial channels could be an effective means of improving the preservation of ischaemic cold-stored hearts.

The aim of this project is to identify the mechanisms responsible for cardioprotective action of potassium channel openers acting on mitochondria in cardiac myocytes. The project may lead to the definition of new concepts concerning ischemic preconditioning via stimulation of mitochondrial channels by potassium channel openers and its application to improve the preservation of ischaemic cold-stored hearts, for example during cardiac transplantation.

Dr. Jiangping Wu, Principal Investigator

Notre-Dame Hospital, Montreal, Canada

Use of Proteosome Inhibitors to Control Rejection and to Induce Long-Term Tolerance of Organ Grafts

The proteosome is a protein complex, whose job is to degrade most of the proteins in a cell. It was considered as a "garbage collector" of cells and its role in cells was thought humble and insignificant. Our recent work shows that the function of the proteosome is tightly regulated and that it plays critical roles in controlling cell growth and death. Notably, we have found that if the activity of the proteosome is inhibited, cells that are vigorously growing will be easily killed, while cells that are not growing will be spared. This is a very interesting feature and we will take advantage of it to improve outcomes of organ transplantation.

Organ transplantation is an effective way to treat end-stage diseases of various organs. After organ transplantation, a graft will be rejected by a recipient because the immune system of the recipient recognises the graft as a foreign entity. T lymphocytes of the immune system play a major role in the graft rejection. Some T cells are specific to foreign molecules (alloantigens) of the graft and are stimulated by these molecules. These cells will start to grow and proliferate and finally cause damage to the graft, while other irrelevant T cells that might be protective against microbes remain in a resting status. If we kill the growing T cells, then we can create a hole in the immune system such that it cannot reject the graft but its protective functions against other microbes remain intact.

In this project, we will attempt to use novel proteosome inhibitors in an animal model to eliminate alloantigen-specific T cells after organ transplantation with a view to controlling graft rejection. The underlying mechanisms will be studied. Derivatives of the prototype proteosome inhibitors will also be developed to produce more effective and less toxic compounds. If the results are as expected, we will have a new category of drugs that can not only inhibit graft rejection but also induce long-term graft tolerance.

This project if a follow up of a one-year proof-of-concept study that was funded by the ROTRF in cycle II (see page 38 for the Final Report).

4. Progress Reports

Prof. Jonathan S. Bromberg, Principal Investigator

Mount Sinai School of Medicine, New York, USA

Chemokine Antagonists to Block Alloimmune Responses

The first specific aim of the project is to demonstrate that transfer of the vMIP-II and MC148 genes inhibits alloimmunity, and to evaluate the effects of these genes on T cell, B cell, and APC components of the alloresponse. The first part of this aim is to optimize the expression of the transferred genes. Significant progress towards achieving this goal has been made and presented in two papers that detail methods for optimizing dendrimer-based transfer of plasmid DNA to vascularized cardiac allografts and electroporation methods to enhance gene transfer. These methods will now be applied to vMIP-II and MC148 gene transfer. The third part of this specific aim is to evaluate the effects of chemokine inhibitor gene transfer on immunologic events *in vivo*. Much of this work, which provided the basis for the preliminary data of the grant application, has been completed using a non-optimized gene transfer system. The results show that gene transfer prolongs graft survival, decreases donor-specific CTL infiltration of the graft, and inhibits alloantibody production. With the optimized gene transfer systems, these experiments will be repeated and it is anticipated that immunosuppression will be enhanced.

Specific aim 2 is to co-transfer vMIP-II or MC148 with other immunosuppressive cytokine genes, such as IL-10, or with conventional immunosuppressive agents in order to determine whether there is a synergistic effect on prolongation of graft survival and inhibition of alloimmunity. Now that the gene transfer systems have been optimized, these experiments are planned for the coming year.

Specific aim 3 is to modify the vMIP-II and MC148 constructs by replacing the beta-sheet scaffolding of the carboxyl region of the molecule with the beta-sheet scaffolding of human IL-8, in order to produce clinically relevant chimeric molecules. These experiments are planned for the latter half of the second year and the third year of the grant.

Specific aim 4 is to create adenoviral vectors expressing vMIP-II and MC148 constructs. These constructs and vectors were created during the first year. The fidelity of these constructs has been confirmed by sequencing and RT-PCR analysis. Vascularized cardiac transplants have been performed with both adenoviral vectors. The results show prolongation of graft survival, which was better than that achieved with plasmid-mediated gene transfer. Immunologic analysis of these allografts is planned for the coming year. In addition, because of innate and adaptive immune responses triggered by adenoviral vectors, the combination of these vectors with immunosuppressive reagents, such as anti-CD40L mAb, may result in significant synergistic effects.

Publications

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Potentiation of Dendritic Cell Tolerogenicity with NF-κB Antagonist Phosphorothioate Oligonucleotide

Experimental efforts during the first year have focused on devising an effective strategy to deliver the NF-κB antagonist phosphorothioate oligonucleotide (NF-κB ODN) to dendritic cells (DC), and on determining the functional efficacy of NF-κB ODN in the modulation of surface molecule expression and bio-immunological function of bone marrow (BM)-derived DC *in vitro* and *ex vivo.*

We have shown that DC maturation and function can be inhibited by addition of NF-κB ODN to DC cultures. Since DNA can be toxic to cells, it is necessary to determine the therapeutic window of NF-κB ODN and the most appropriate dose that will yield the maximum effect with minimal toxicity. A significant elimination of the use of DC for tolerance induction in other models has been the delayed upregulation of cell surface costimulatory molecules (CM) after *in vivo* administration. The ability of NF-κB ODN to maintain prolonged suppression of DC function is therefore of considerable potential importance. However, the duration of action of NF-κB ODN on DC function is unknown. A series of experiments were conducted to answer these questions.

We devised an effective strategy to deliver NF-κB ODN to DC. DC propagated from mouse BM with GM-CSF or GM-CSF + IL-4 were exposed to different doses of FITC-conjugated NF-κB ODN for various time periods. The majority of DC (>80%) can take up double-stranded NF-κB ODN efficiently with optimal dose and exposure conditions determined by FACS analysis or fluorescence microscopy. Intracellular NF-κB ODN was detected for at least 14 days in culture. During this period, DC remained viable without evidence of toxicity. Uptake was detected as early as 2 h after incubation, with peak fluorescence noted after an 18-h exposure of DC to NF-κB ODN. It was determined that the most significant inhibitory effect of NF-κB ODN on CM expression of DC occurred when NF-κB ODN was added at the beginning of DC propagation, indicating that NF-κB ODN does not alter CM expression or the function of mature DC.

Even though we have shown that an NF-κB-specific ODN decoy effectively inhibits functional DC maturation, it is important to confirm the specific binding of the ODN decoy to NF-κB protein in DC. The specificity of the DNA-binding activity of NF-κB was assessed in electrophoretic mobility shift assays (EMSA) of the nuclear/cytosolic/membrane protein fractions of cultured DC using ^{32}P-labeled probes containing the κB binding site. Addition of excess unlabeled consensus NF-κB probe or excess unlabeled NF-κB ODN to the binding reaction competed for the binding to the labeled NF-κB probe. In contrast, no competition was observed when the binding reaction was performed in the presence of excess unlabeled control ODN. The addition of NF-κB ODN but not control ODN to the cultured DC completely inhibited NF-κB binding. These data strongly demonstrate that the addition of NF-κB ODN to DC specifically blocks NF-κB DNA-binding activity.

The specificity of NF-κB ODN was further confirmed by inhibition of NF-κB-dependent transcription in DC. Transient transfection of DC cultured with or without NF-κB ODN or control ODN was performed using the luciferase reporter gene fused to NF-κB binding sites and subsequently stimulated by LPS. Our results indicated that LPS stimulation increased luciferase activity in DC treated with control ODN but not in DC treated with NF-κB ODN. The requirement of NF-κB activation for iNOS expression is well documented. Thus, the effectiveness of NF-κB ODN to inhibit NF-κB transcription was also confirmed by detection of NO production in LPS-stimulated DC.

An important property of DC is their capacity to migrate from peripheral tissue to T cell areas of secondary lymphoid tissue, as occurs following organ transplantation. We observed that NF-κB ODN does not interfere with the migration pattern of DC as determined by immunocytochemical staining with anti-(donor MHC class II) mAb at various times after injection of DC treated with NF-κB ODN into allogenic recipients. In comparison with DC or DC treated with control ODN, there were significantly increased levels of NF-κB ODN DC in the recipients. We have shown previously that NF-κB ODN DC have markedly impaired ability to induce T cell proliferation and CTL generation. Taken together, these results demonstrate the potential of NF-κB ODN DC to induce T cell hyperresponsiveness and to survive in increased numbers in the MHC-mismatched allogenic recipients.

We consistently observed that NF-κB ODN effectively inhibits functional maturation of DC. Markedly inhibited allostimulatory function is associated with significant inhibition of CD80. CD86 expression was noted in DC treated with NF-κB ODN. Expression of MHC class I, II and CD11c, the DC marker, were not effected, and

CD40 expression was slightly impaired by NF-κB ODN treatment. Since a significant limitation to the use of CM-deficient DC (propagated with GM-CSF + TGFγ) for tolerance induction has been the activation of DC in allogeneic recipients, we performed parallel studies to compare the surface molecule expression and immunoregulatory function of DC propagated with GM-CSF + IL-4, GM-CSF + TGFγ, or GM-CSF + NF-κB ODN after contact with allogeneic T cells *in vitro* and *ex vivo.* The results indicated that NF-κB ODN potentiates the tolerogenicity of DC in the context of allotransplantation.

We observed that NF-κB ODN prevented activation of CM-deficient DC induced by adenoviral vector transfection without interfering with the expression of adenovirus-encoded genes. This finding will extend the proposed design of cell-based biological therapy of allograft rejection using NF-κB ODN and engineered DC with viral-vector-encoded genes of immunosuppressive molecules.

Our work during the upcoming second year of the award will focus on determining the influence of donor-derived NF-κB ODN DC on immune responses in normal allogeneic mice and in a mouse heart transplantation model. We plan to elucidate the mechanisms underlying the role of DC in the regulation of T cell responses by targeting nuclear transcription factors.

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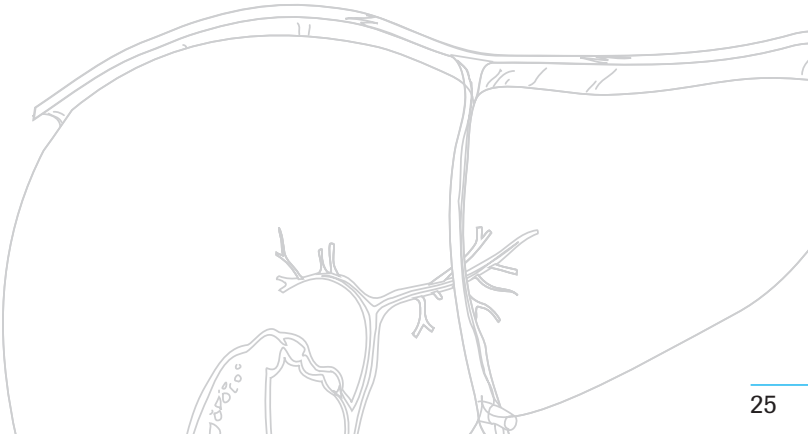

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Suppressing Clonotypic and Bulk Alloreactive CTL with Soluble Peptide-Loaded MHC Dimers

Despite numerous technical and medical advances made recently in organ transplantation, rejection of a graft by the immune response of the host continues to represent a critical clinical problem. Current therapies require life-long treatment and act by non-specifically suppressing the immune system. Furthermore, these therapies have cumulative toxic effects from the extensive drug intake and expose patients to higher risks of infection and cancer. Therefore, specific immunosuppression of alloreactive T cells in graft recipients has long been a major goal in transplant immunology.

In this work, we proposed to design and test a regimen that would be allo-specific by using soluble peptide-loaded MHC dimers. Funding from the ROTRF has enabled the principal investigator to demonstrate suppression of a specific CTL *in vivo* with the resultant survival of allografted tissue. Tests were performed using 2C transgenic T cells adoptively transferred into immunodeficient animals as proposed. By administering MHC dimer with peptide of the same allotype as the graft simultaneously with the graft, T-cell-mediated damage was markedly suppressed. It was thought that MHC dimers were mediating this suppressive effect by inducing a "suppressive" signal as in the case of altered peptide ligands. However, further analyses indicated that the dimers actually activate the T cell, as seen by activation marker up-regulation and TCR down-regulation. Activation was further indicated in western blot analyses, wherein phosphorylation patterns of the TCR zeta chain were observed that are typically induced upon TCR crosslinking with an agonist or anti-TCR antibody. Thus it is likely that the mechanism through which the suppression occurs is via activation-induced cell death. Studies are planned to confirm the mechanism.

The current work will now be extended to grafted tissues such as skin and intestine. In a previous preliminary experiment, the delay in onset of rejection was lengthened nearly twofold in the case of a single class-one disparate-mismatched skin graft.

Future Plans

For more effective immunosuppression, it was proposed to link anti-CTLA-4 to the dimers. This would allow co-engagement of the negatively regulating molecule CTLA-4. While a covalently linked MHC/anti-CTLA-4 construct is being made in collaboration with Dr. Bluestone's group, other means to link these two signals are being performed presently. This is being carried out both using beads and using a chemical cross-linker to validate the efficacy of the heteroconjugate in suppressing or deleting antigen-specific T cells. The conjugate will be tested *in vivo* and *in vitro.*

Overall, the work performed has demonstrated the efficacy of the dimer alone in suppressing effector functions of T cells. This work largely covers Specific Aim 1, i.e. to develop a model system for testing the efficacy of pepMHC-Ig dimers in prolonging graft survival. These results give a strong indication that the dimers will be effective in mediating suppression in more natural models of organ transplantation, as proposed in Specific Aim 2. They also indicate that improved immunosuppression may result from the combination of molecules that simultaneously engage the TCR and the negative regulatory marker CTLA-4 in a heteroconjugate format, as outlined in Specific Aim 3. It is anticipated that significant progress will be made on these aims over the next year.

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Thymic Transplantation to Achieve Tolerance

We have made significant progress over the past year in our efforts to demonstrate the ability of vascularized thymic grafts – in the form of composite thymokidneys – to reconstitute depleted T-cell populations and induce tolerance across allogeneic barriers. We have addressed Aims 1 and 2 of our proposal, and our current and future studies seek to make advances in the third aim of this proposal. The specific accomplishments made in the year 2000 are detailed in the following report.

Aim 1. Examine the ability of thymic tissue included as part of a thymus organ to support normal thymopoiesis following removal of the recipient's thymus and depletion of peripheral T cells.

We had initially intended to perform these studies using the anti-(pig CD3) diphtheria-based immunotoxin CRM9 to deplete the T-cell populations in thymectomized juvenile miniature swine. Unfortunately, limitations in the supply of the immunotoxin necessitated a change of plan. We decided to reserve the limited amount of CRM9 for the subsequent investigation of allogeneic thymokidney transplantation across fully mismatched barriers (Aim 2). Rather than use an immunotoxin-based T-cell depletion regimen in Aim 1 of our studies, we performed thymokidney transplantation into a recipient who had been thymectomized 3 to 4 years prior to transplantation. The T-cell populations in this animal were monitored in the period following thymectomy and the levels decreased markedly with time. Marked decreases in the absolute numbers of CD4+ and CD8+ T cells were seen in the thymectomized animal compared with the non-thymectomized age-matched controls, and the CD4+ T-cell count decreased earlier and more markedly than the CD8+ count.

We then sought to determine whether vascularized allogeneic thymic tissue, transplanted as part of a composite thymokidney, was capable of reconstituting the T-cell population to levels comparable to those seen in the control animals. Three

months prior to thymokidney transplantation, thymic tissue was transplanted autologously under the renal capsule of the prospective donor, which permits the creation of a vascularized thymokidney in the donor [1]. This thymokidney was then transplanted into the thymectomized animal (3.7 years post-thymectomy) across a class-I-mismatched barrier with a 12-day course of cyclosporin A (CyA). Confirming our results from previous thymokidney transplants across similar MHC barriers [2], the class-I-mismatched thymokidney was accepted with stable renal function in the thymectomized animal. The CD3+ T cells in the recipient showed a marked increase, beginning on POD 30, to a maximum number of 5324/mm^3, which is a level similar to that seen in an age-matched, naive animal (5800 mm^3). CD4 single-positive (SP) T cells also began to increase on POD 30, and reached levels similar to those of a naive animal by POD 80. No donor-type cells were detectable using FACS analysis by POD 30 or in any assay thereafter, indicating that the increases in T cells were not due to donor thymocytes.

CD45RA is a phenotypic marker of naive T cells [3,4]. The CD45RA/CD4 double-positive (DP) cell population was examined to determine whether the increased number of CD4 SP cells was a result of T-cell development in the thymokidney. The absolute number of CD45RA/CD4 DP cells increased by POD 30 and this pattern correlated with that seen in the analysis of total CD4 SP cells. In addition, high-intensity CD45RA (CD45RA high)/CD4 DP cells were analyzed, and were found to increase in a manner that paralleled the increase in total CD4 SP cells. These data suggested that the thymic component of the thymokidney graft was functional *in vivo.*

In order to assess recipient T-cell function *in vitro,* we utilized MLR studies to determine whether the increased numbers of T cells seen in our FACS analysis represented immunocompetent cells. We determined the ability of T cells isolated from the recipient prior to and following thymokidney transplantation to respond to allogeneic cells. The recipient animal MHC was of haplotype SLAdd (Class Id, Id) and SLAaa (Class Ia, Ia). Irradiated PBMCs were used as allogeneic stimulator cells to assess the immune response. The results demonstrated that anti-SLAaa cells evoked a strong recipient lymphocyte response 3 months after the thymectomy. The animal had lost the anti-SLAaa response by 3.7 years after the thymectomy, providing evidence that total thymectomy had eventually rendered the animal relatively immunoincompetent. However, the fully restored MLR response seen 3 months after the thymokidney graft indicated that the thymic graft had resulted in a return of immunocompetence.

These data indicate that the vascularized allogeneic thymic tissue reconstituted T cells and restored immune responses.

Aim 2. Apply the technique of thymokidney transplantation across fully mismatched allogeneic barriers in miniature swine.

We examined next whether a fully MHC-mismatched thymokidney could induce tolerance with the same immunosuppressive regimen in a thymectomized miniature swine (group 1) in a manner similar to the class-I-mismatched model that we have reported previously [2]. In contrast to the class-I-mismatched model, the thymokidney was rejected on POD 42. These data indicate that a 12-day course of CyA therapy was not sufficient to permit acceptance of a fully MHC-mismatched thymokidney.
Other studies in our research center have demonstrated that both thymectomy and complete T-cell depletion of the host are essential for the induction of tolerance in a pig-to-mouse xenogeneic thymic tissue transplant model [5]. Therefore, we tested the ability of the CRM9 immunotoxin to augment our immunosuppressive regimen (group 2). The immunotoxin was administered on days -3 and -2 (0.1 mg/kg/day i.v.), and a 12-day course of CyA (10 mg/kg/day) was begun on day 0. Host thymectomy was performed 3 weeks prior to the day of transplant. In this model, four recipient animals received thymokidney transplants and three control animals received renal transplants without thymic tissue. In these studies, we also addressed whether a lymphokidney that contains donor lymphocytes and stromal cells could induce tolerance similarly to the induction seen with a thymokidney, and whether the presence of host thymus interfered with the induction of tolerance by vascularized thymic grafts in the thymokidneys. Plasma creatinine levels served to monitor renal function. In contrast to recipients of renal allografts without thymic tissue, all four recipients of two-haplotype fully mismatched composite thymokidneys demonstrated stable renal function after treatment with the T-cell depletion agent CRM9 and a short course of CyA. Only minimal cell infiltrates were seen in the thymokidney biopsies taken on POD 20 and POD 150, and the sample from POD 150 demonstrated viable thymic tissue, indicating that no rejection of the composite thymokidney graft was occurring.
We then determined whether a lymphokidney had an effect similar to that of the thymokidney on the induction of tolerance. While thymic tissue showed evidence of growth following thymokidney creation, the lymph nodes in the lymphokidney offered no evidence of proliferation in the 3 months following creation. Nonetheless, lymph nodes were present when the composite lymphokidney was transplanted. However, the lymphokidney did not prolong graft survival in the recipient, and the graft was rejected on POD 17. These data indicate that vascularized donor thymic tissue, but not lymphoid tissue, induced transplantation tolerance across a fully

MHC-mismatched barrier. One year following thymokidney transplantation, a kidney that was MHC matched (minor antigens mismatched) to the thymokidney donor was transplanted without immunosuppression into the thymokidney recipient. This second graft was accepted, while the recipient rejected a third-party outbred kidney within 7 days of the transplant, indicating that the unresponsiveness was specific to the donor.

We also tested the role of the host thymus in our model. A non-thymectomized recipient rejected the fully MHC-mismatched thymokidney on POD 15. However, as we tested only a single animal, the experiment should be repeated to confirm this result.

Further Research

We plan to perform two additional fully mismatched thymokidney transplants into non-thymectomized recipients to confirm our findings. We will continue to examine the ability of allogeneic vascularized thymic tissue to induce transplantation tolerance in miniature swine, using the data generated to create a model that we intend to extend across xenogeneic barriers. In an effort to gain a further understanding of the mechanisms responsible for the induction of tolerance in our model, we will evaluate those animals that have demonstrated long-term tolerance. We plan to examine CTL killer restriction (TNP CTL assays) and MHC restriction (PPD assays) *in vitro* using these tolerant animals. We continue to strive to apply these techniques across xenogeneic barriers and intend to perform thymokidney transplants across a xenogeneic barrier (pig to baboon) in the coming year, thus addressing the final aim of our proposal.

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Induction of Transplant Tolerance by Antibody against CD45RB

Aim

To characterize the cellular and molecular mechanisms tolerance induced by monoclonal antibody against CD45RB (mAb).

Results

1. Donor MHC class II is required for tolerance induced by CD45RB mAb:

   To test whether tolerance induction with CD45RB mAb (MB23G2) requires donor MHC class II expression, BALB/c mice were transplanted with MHC class II deficient B6 (B6KO) kidneys, and treated with or without CD45RB mAb. Controls received wild type B6 donor kidneys (B6WT). Long-term survival was equivalent in B6WT and B6KO groups receiving CD45RB mAb (>100 days) but decreased in mice not given CD45RB mAb (26.8±9.4 days, $p<0.05$). Renal function was better in B6KO kidney recipients given CD45RB mAb (serum Cr=36.8 ± 4.3 mol/l) than untreated mice (100 ± 34.6 mmol/l, $p<0.05$). In contrast to the B6WT group tolerized with CD45RB mAb, prominent renal injury (glomerular and arteriolar fibrinoid necrosis) was present in all B6KO kidneys even with anti-CD45RB antibody. B6KO mice (>100 day survival) also rejected both third party C3H and B6KO skin transplants suggesting absence of tolerance. B6WT kidney recipients treated with CD45RB (>100 day survival) showed CD4+, CTLA-4+ cells with a predominance of $CD45RB^{low}$ isoform expression, cells which may have a regulatory function in CD45RB tolerized mice. In contrast, CTLA-4+ T cells from mAb-treated B6KO recipients displayed mostly the $CD45RB^{high}$ isoform. In conclusion, CD45RB mAb prolonged survival of both B6WT and B6KO allografts. However, the tolerizing effect of CD45RB mAb requires donor MHC class II expression and possibly the induction of $CD45RB^{low}$, CTLA-4+ T cells.

2. CD45 monoclonal antibody induces T cell apoptosis without DNA fragmentation and promotes CD45RB^{high} to CD45RB^{low} isoform shift.

   T cells from C57BL/6 (B6, H2b) mice were divided into 4 treatment groups: (1) cells alone, (2) dexamethasone (DEX) 20 mg/ml, (3) MB23G2 (effective CD45 mAb) and (4) MB4B4 (ineffective CD45 mAb). Apoptosis at 6 h was significantly higher in CD4+ cells treated with either MB23G2 (45%) or DEX (61%) compared with untreated (13%) and MB4B4-treated CD4+ cells (16%) ($p<0.05$). Apoptosis was also higher in CD8+ cells treated with MB23G2 (43%) compared with untreated (8%) and MB4B4-treated cells (12%) ($p<0.05$). In addition, MB23G2-mediated apoptosis was not associated with DNA fragmentation. Tolerance induction by CD45RB mAb has been shown to be related to changes in isoform expression. MB23G2 selectively induced apoptosis in CD45RB^{high} cells. As a result, 98% of viable CD45RB+ cells expressed the CD45RB^{low} isoform after MB23G2 treatment for 24 h, compared with 40% in untreated cells, and 53% in DEX-treated cells. We conclude that CD45RB mAb induces T cell apoptosis that is not associated with DNA fragmentation. As well, effector CD45RB^{high} cells appear to be selectively targeted for apoptosis compared with the potentially regulatory CD45RB^{low} cells.

Publications and Presentations

Two abstracts were submitted to AST 2001.

5. Final Reports

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Dr. Cristina Cuturi, Co-Applicant
Prof. Jean-Paul Soulillou, Co-Applicant
Cécile Voisine, Research Associate
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The Role of TRANCE/TRANCE-Receptor Interaction during Allogenic Immune Responses *in vivo*

Members of the tumour necrosis factor (TNF) and TNF-receptor (TNF-R) superfamilies both subserve and govern diverse cellular events during development and following insult or immunologic challenge. Biological effects include proliferation, differentiation, death and survival. An important role of these molecules in transplantation immunology has emerged recently. For instance, the CD40-CD154 pathway is critical during allogenic immune responses, as CD154-blocking reagents have been shown to prevent acute allograft rejection.

TRANCE (TNF-related activation-induced cytokine; also known as RANKL, OPGL and ODF) and its receptor RANK (receptor-activating NF-κB) are recently described members of the TNF and TNF-R superfamilies, respectively. TRANCE plays a major role in bone physiology by directly inducing osteoclast differentiation and activation. In the immune system, TRANCE is expressed by activated T cells, and RANK is mostly expressed by mature dendritic cells, which are antigen-presenting cells. Similarly to CD154, TRANCE induces survival and activation of mature dendritic cells. Despite the critical role of the CD154 pathway of costimulation in antigen-presenting cell functions, we have shown that the TRANCE pathway can mediate CD154-independent T helper cell activation *in vivo.* Thus, the use of CD154-blocking reagents may not be sufficient to fully inhibit DC-mediated allogenic T helper cell activation and therefore to completely prevent chronic graft rejection.

Our working hypothesis is that the TRANCE pathway might be involved in dendritic cell-mediated T cell priming during graft rejection. Specific aims of our project are to study the regulation of expression of TRANCE and RANK during acute and

chronic rejection and during tolerance in animal models and in humans, to assess the effect of TRANCE-blocking reagents on allograft survival and chronic rejection in rat models, and to analyse the immunological mechanisms of allograft enhancement by TRANCE-blocking reagents.

Using a rat model of heterotopic heart allograft we have shown that TRANCE and CD154 mRNA were strongly upregulated in acutely rejected grafts with a peak of expression on day 4 (D4) after transplantation. Whereas CD154 mRNA expression decreased rapidly thereafter, that of TRANCE was sustained for at least 3 days. Unlike CD40, RANK was barely detected in rejected allografts, a finding that correlates with the different pattern of expression of those molecules. We have also found that, unlike CD154 and CD40, TRANCE and to an even greater extent RANK are strongly expressed in heart allograft tolerised by pregraft donor-specific blood transfusion. To inhibit the TRANCE pathway *in vivo,* we used a soluble form of RANK fused to the Fc region of human IgG1 (RANK.Fc). Rats injected with RANK.Fc (1 mg i.v. on D0 and D2) exhibited a significant prolongation of allograft survival (26 $\pm$ 12 days vs. 6 $\pm$ 0.5 days in controls) similar to that observed with a blocking anti-CD154 mAb in the same combination. Similar graft prolongation was obtained with three post-graft injections of RANK.Fc (0.5 mg on D0, D2 and D4), whereas recipient animals that received only one injection of 1 mg RANK.Fc on D0 exhibited only a modest increase of graft survival (14.8$\pm$12 days).

We also generated a RANK.Fc-encoding recombinant adenovirus that will be used to directly transduce heart allograft before transplantation. Moreover, adenovirus-mediated local delivery of RANK.Fc will be useful to study the role of TRANCE in bone resorption during arthritis or bone metastasis, for instance. We have generated monoclonal antibodies specific against mouse RANK, and we have cloned rat TRANCE and RANK. We have produced a c-myc-TRANCE fusion molecule, and monoclonal antibodies will be generated against rat TRANCE by immunising mice with this fusion molecule.

These results suggest that the TRANCE-RANK pathway plays a role in the allogenic immune response *in vivo,* leading to acute rejection. Further studies are required to define the role of TRANCE in chronic rejection, the mechanisms of heart allograft enhancement by RANK.Fc, and the effect of blocking TRANCE and CD154 or TRANCE and CD28 on allograft survival. Potential side effects will be determined with an interest in bone metabolism, especially with adenoviral-mediated delivery of RANK.Fc.

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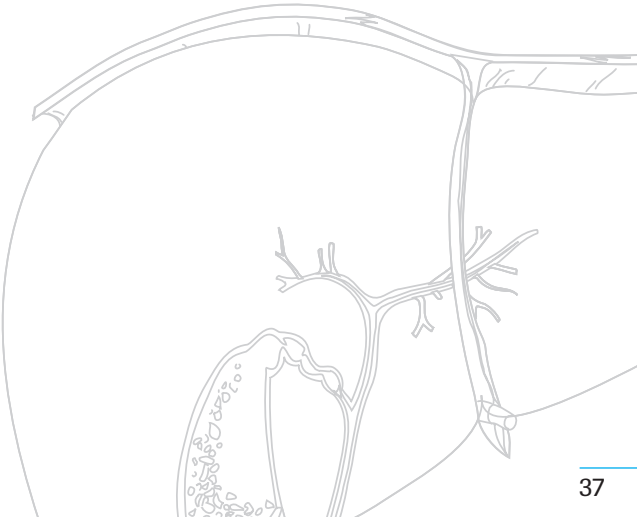
Anti-Rejection Therapy Inhibiting Fucose-Dependent Leukocyte Traffic

Acute inflammatory episodes, such as organ allograft rejections, are characterised by infiltration of lymphocytes into the tissue. This extravasation is initiated by interactions between selectin proteins and their endothelial ligands, such as CD34, provided that they are decorated properly. Prior work from our laboratory has identified endothelial fucosylated sLex glycans as key components of selectin ligands. We have characterised the synthesis pathways and expression profiles of these fucosylated glycans on vascular endothelium at sites of inflammation in experimental models, in human heart and kidney transplant recipients, and in bronchial asthma patients. Furthermore, we have used these glycans as inhibitors of leukocyte traffic in rats during transplant rejection.

Normally, L-selectin guides lymphocyte traffic to lymph nodes and tonsils, as postcapillary venules express sLex glycans only at these sites. CD34 is a ubiquitous protein expressed all along the blood vasculature, but under normal conditions it acts as a L-selectin ligand only in lymphatic tissue. We plan to analyse in detail the glycans on CD34 isolated from human lymphatic tissue (adenoids and tonsils) and kidneys removed during elective surgery. The tissue will be lysed, ultracentrifuged, and membrane fractions collected. This supernatant will then be subjected to several rounds of perfusion chromatography and affinity chromatography with anti-CD34 antibody to purify the relevant protein. Currently we have been able to isolate intact CD34 glycoprotein in extremely pure form (as analysed by mass spectrometry) and in sufficient quantities to allow further glycan analysis. The O-glycans were removed with high pH (>11) and the isolated glycans were analysed by mass spectrometry combined with enzymatic degradation, as described previously by our group. We have characterised novel sLex-containing glycans from lymph node endothelial CD34 that are putatively responsible for the L-selectin-dependent lymphocyte traffic. This information will help us to synthesise novel fucosylated glycans that potentially inhibit the L-selectin-dependent inflammations.

Along this line we have decided to clone all the enzymes required to generate such sLex glycans. So far we have identified and expressed functionally active enzymes required for the biosynthesis of GDP-fucose (the donor) and several novel fucosyltransferases. Currently we are applying novel bioinformatic techniques to identify from bacterial and eukaryotic gene banks novel enzymes crucial for these pathways. We are currently applying whole-genome analysis of the genetically engineered transformant yeast cells with the enzymes converting GDP-mannose to GDP-fucose. The first data identified several interesting novel glycosylation pathways that are regulated in these yeast transformants synthesising GDP-fucose, and we aim to use this information to metabolically engineer the cells to produce even higher amounts of this valuable sugar nucleotide.

We have provided evidence that the *de novo* expression of sLex-decorated L-selectin ligands perpetuates and accelerates ischaemia/reperfusion injuries linked to transplantation as well as graft rejection. To test the role of these fucosylated glycans we will perform heart transplantations to and from FucT knock-out mice. This approach will allow a direct comparison of a setting where the only variable is the presence or absence of L-selectin ligand decorated with sLex (i.e. fucosylated) glycans on the allograft endothelium. We will investigate the role of selectins and their glycosylated ligands in the initiation of inflammatory reactions and find new diagnostic applications, such as non-invasive confocal microscopy, with which we can analyse the leukocyte rolling on vascular endohelium and the number of tissue-infiltrating cells at sites of conjuctival inflammation. Finally, we will attempt to block inflammation by inhibiting leukocyte traffic with the fucosylated glycans in an organ-selective manner without the side-effects caused by current treatments.



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Use of Proteosome Inhibitors to Control Rejection and to Induce Long-Term Tolerance of Organ Grafts

Background

Proteosomes are large protease complexes located in both cytoplasm and nuclei, which degrade most cellular proteins. The proteosome has been regarded as a housekeeping enzyme and a "garbage collector" to dispose of spent proteins. However, through recent studies by us and other researchers, it is becoming increasingly clear that the proteosome plays critical and active roles in the regulation of many different cellular functions. This is achieved by its ability to timely, selectively and irreversibly destroy regulatory protein factors, and by its ability to process precursors of regulatory factors into active ones.

Our laboratory was the first to document the critical roles of the proteosome in lymphocyte activation and proliferation. We concluded from our *in vitro* results that proteosome inhibitors can effectively inhibit T cell activation and proliferation. This suggests that such inhibitors can be used as immunosuppressants in the induction phase of organ transplantation. In addition to inhibiting T cell proliferation, we have shown that proteosome inhibition causes death of activated but not of resting T cells. Our results suggest that by inhibiting proteosome activity, it is possible to clonally delete activated alloantigen-specific T cells *in vivo,* and thus achieve long-term graft tolerance.

Results

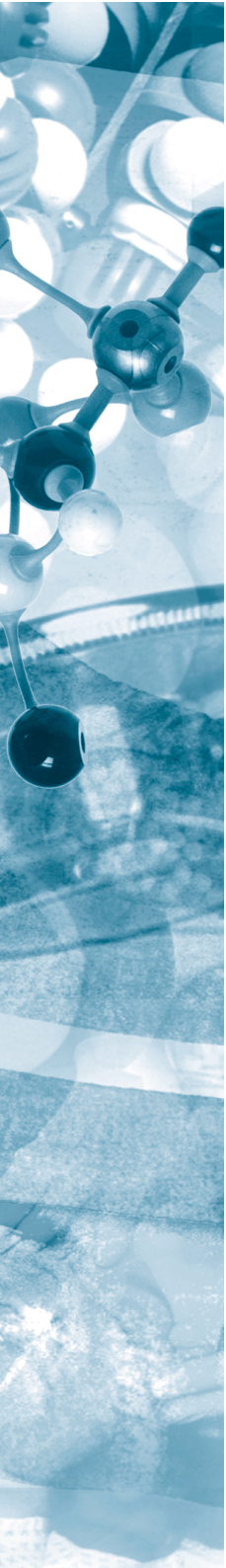
With the financial support from the ROTRF, we studied the efficacy and side-effects of proteosome inhibitors in a mouse heart transplantation model. We synthesised a new proteosome inhibitor, dipeptide boronic acid (DPBA), which is stable in aqueous solution. DPBA suppressed T cell proliferation and potently inhibited IL-2, IL-6, IL-10, IL-13 and IFN-γ production by anti-CD3-activated T cells. Given i.p. starting one day after transplantation at 0.66 mg/kg/day for 16 days, or at 1 mg/kg/day for 4 days followed by 0.5 mg/kg/day for 12 days, DPBA prolongs the heart allograft survival to 35.5 days (mean survival time, MST) and 36.2 days, respectively. The control group had a MST of 7.3 days. When administrated 72 h post operation at 1 mg/kg/day for 4 days, DPBA prolonged the graft survival to 19.8 days. During the course of these effective dosages, DPBA had no apparent toxicity in the liver, kidney, pancreas and heart according to analysis of blood chemistry. Thus, the proteosome inhibitor could repress allograft rejection in mice without apparent side-effects at the effective dosages.

Significance

We have for the first time successfully used a proteosome inhibitor to prevent allograft rejection. Proteosomes were previously thought to degrade cellular proteins in an unregulated way. We have raised a novel concept and proved that the proteosome plays critical roles in immune regulation and that proteosome inhibitors can be used as novel immunosuppressants in organ transplantation. We have proved that there is a therapeutic dose window for the proteosome inhibitors *in vivo,* and that the inhibitors are effective in treating ongoing graft rejection. Thus, the proteosome inhibitors are a new class of immunosuppressants. The usefulness of this class of immunosuppressants is in the following three aspects: they can be used alone, or in combination with other immunosuppressive drugs in allo or xeno organ transplantation; they are especially useful in controlling clinical rejection episodes, which are normally diagnosed when the T cells are already activated and are less responsive or even resistant to conventional immunosuppressants; and they could be used to induce long-term graft survival by clonal deletion of alloantigen- or xenoantigen-specific T cells when administrated after the activation of these cells.

Publication

Luo H, Qi S, Wu L, Wan X, Wu J. A preoteasome inhibitor dipeptide boronic acid effectively suppresses alloresponses *in vitro* and prolongs mouse heart allograft survival *in vivo.* Transplantation 2001; in press (Research article accepted for publication).



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