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Treatment with immunotoxin

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T-cell depletion prior to or beginning at the time of transplantation has been shown to be a valuable adjunct to the induction of immunological unresponsiveness. Both total lymphoid irradiation and anti-lymphocyte globulin have been used for this purpose in experimental models of transplantation as well as in human organ transplant recipients. However, these methods of T-cell depletion are limited in their ability to deplete T cells selectively due to non-specific targeting and limited efficacy. A new anti-CD3 immunotoxin has been developed with a far more potent ability to deplete T cells selectively as measured by flow cytometry analysis of peripheral blood T lymphocytes as well as lymph node lymphocytes. This immunotoxin is well tolerated by rhesus monkeys when administered *in vivo*. When administered as a single immunosuppressive agent pretransplant, it substantially promotes allograft survival, inducing tolerance in at least one-third of recipients as measured by subsequent acceptance of donor skin grafts and rejection of third-party skin grafts. When administered on the day of transplant in combination with steroid pretreatment and a brief course of deoxyspergualin or mycophenolate mofetil (4 to 14 days), long-term unresponsiveness is also produced and in a more reliable manner than using immunotoxin alone. A new immunotoxin directed at the human CD3ε has been developed with excellent potency in T-cell killing and lacking the Fc portion of the CD3 antibody. This construct may be useful for T-cell depletion in humans and has a potential application in tolerance induction in human organ transplantation. Lessons learned from anti-CD3 immunotoxin in the non-human primate model to date include (i) profound (2–3 log) depletion of T cells can be accomplished safely without inducing lymphoma or infection, (ii) such depletion is a useful adjunct for tolerance induction to allogeneic organ transplants, and (iii) tolerance to both allogeneic renal transplants and xenogeneic islet transplants has been accomplished using such strategies to date in non-human primates and in pigs. Immunotoxin may be useful for the induction of chimerism using strategies that include donor bone marrow infusion. Successful strategies for tolerance induction have also been developed using immunotoxin without the adjunct of donor bone marrow or stem cell infusion. Clinical application of immunotoxin will use a newly engineered construct with the potential for causing cytokine release, less susceptibility to neutralization by anti-diphtheria antibody and not dependent on chemical conjugation of an antibody and toxin. The usefulness of immunotoxin is directly related to its tremendous potency for depleting T cells. Based on results in non-human primates, it is anticipated that it will become a useful agent in tolerance induction in humans.

Keywords: immunotoxin; tolerance; T lymphocyte; depletion

1. EARLY EXPERIMENTS IN TOLERANCE

The idea that the immune system learns to distinguish between self and non-self was first suggested by Owen and colleagues in 1945 from observations of dizygotic cattle twins (Owen 1945). Owen found that twins were chimeric, containing red blood cells of their own genotype and that of their twin. Normally, an animal would make an immune response to mismatched blood cells from other members of the species (an allogeneic response). The haemopoietic chimerism demonstrated by the cattle twins suggested to Owen that a mature adult host would tolerate cells of another genotype if it was exposed to them during ontogeny (Owen 1945).

In subsequent experiments by Billingham and others, the hypothesis of self-learning was confirmed by injecting allogeneic murine spleen cells into developing foetuses or newborn. Subsequently, recipients accepted donor skin grafts (Billingham *et al.* 1953).

Subsequent experiments showed that tolerance to foreign antigens could be achieved in adult animals (Mitchison 1964; Klein 1982). Principles derived from these experiments include the importance of large doses of antigen, intravenous or oral rather than subcutaneous or intramuscular administration, and impairment of the immune response by irradiation, anti-lymphocyte globulin (ATG), or drugs, such as cyclophosphamide, at the time of antigen presentation.

The majority of the above experiments were performed in mice as a model for the human immune system. Many experimental models of tolerance have been achieved in rodent models, which for obvious reasons of cost, efficiency and experimentation are attractive models. Nevertheless, the relevance of these methods to inducing tolerance to large animals and humans is often absent as tolerance appears to be far more difficult to achieve in a large animal model and, in particular, in humans (Hamawy & Knechtle 1998; Cosimi 1999). This appears

to be particularly true of clinical transplantation where only a handful of approaches to transplantation tolerance have succeeded. The first of these is best described as serendipity where non-compliant patients on rare occasions have excellent long-term graft function off all medications (Uehling *et al.* 1976; Burlingham *et al.* 1995). The biological basis of most of these cases is of course unknown, although Burlingham *et al.* demonstrated the importance of donor-derived regulatory cells by the *in vitro* testing of one patient.

2. TRANSPLANT TOLERANCE IN HUMANS

Strober and colleagues reported the successful tolerance induction in three patients who received cadaveric renal transplants following a course of total lymphoid irradiation (TLI) (Strober *et al.* 1989). One of these patients remains tolerant more than ten years later (S. Strober, personal communication). The profound T-cell depletion accomplished by TLI as well as the subsequent inversion of the CD4-to-CD8 ratio may be related to the efficacy of TLI.

Mazariegos described the successful removal of more than 30 patients from immunosuppressive therapy following liver transplantation at the University of Pittsburgh, PA, USA (Mazariegos *et al.* 1997). Patients were carefully selected for stepwise withdrawal of immunosuppression based on long-term stable hepatic allograft function, a baseline biopsy demonstrating absence of rejection, absence of autoimmune liver disease, and compliance with immunosuppressive medication. Nevertheless, in this series, a substantial fraction of the patients were unable to be withdrawn completely from immunosuppression.

Most recently, induction of tolerance by combined renal transplantation and bone marrow transplantation has been accomplished by Cosimi's group in a patient who clinically required bone marrow transplantation as a cure of multiple myeloma associated with renal failure (Spitzer *et al.* 1999). This patient received a multimodality regimen for induction of tolerance including total body irradiation, thymic irradiation, ATG, splenectomy and a limited post-transplant course of cyclosporine. The patient discontinued all immunosuppressive drugs 70 days post-transplant and continues to do well more than six months later.

These clinical examples of transplantation tolerance demonstrate that, in principle, stable transplant tolerance can be achieved in adult humans using a variety of approaches. Unfortunately, such cases remain exceptional and spectacular rather than common.

3. DEVELOPMENT OF IMMUNOTOXIN

In order to test whether models of tolerance developed in rodents might have clinical use, my laboratory turned to the rhesus monkey renal allograft model that had been used extensively by the Thomas laboratory. The cynomolgus monkey model used by Cosimi and colleagues would also have been a possibility, but the University of Wisconsin Primate Center, USA had greater access to and familiarity with the rhesus monkey. In particular, David Watkins had expertise in the major histocompatibility complex (MHC) class I and class II of rhesus monkeys and was experienced in one-dimensional isoelectric focusing methodology to type the MHC class I antigens of rhesus monkey leucocytes. Not long after we started this work, his laboratory developed a DNA typing methodology for rhesus monkey MHC class II (Knapp *et al.* 1997*a*) and subsequently for MHC class I (Knapp *et al.* 1997*b*). Because of substantial sequence homology between the MHC of rhesus and humans, as well as a similar degree of polymorphism at class I and class II, we felt that the rhesus monkey model was likely to predict the immune response of humans to immunological manipulation related to transplantation tolerance.

Having carried out preliminary work in a rodent model to evaluate MHC class I gene transfer to prolong graft survival, we had found that thymic expression of donor-type MHC class I following cDNA transfer effectively prolonged hepatic allograft survival (Knechtle *et al.* 1997*b*). This work was a variation on the theme first developed by the laboratory in Philadelphia of Ali Naji and colleagues and generically termed 'thymic tolerance'. Despite the potentially cumbersome nature of thymic manipulation in humans, we sought to evaluate whether thymic manipulation in the primate model would have similar efficacy as in rodents. In most models of thymic tolerance, including work from the Naji laboratory (Posselt *et al.* 1990) and Wayne Flye's group in St Louis (Goss *et al.* 1992), thymic tolerance in rodents was generally accompanied by ATG pretreatment. Our initial experiments in rhesus monkey, therefore, involved ATG (kindly provided by Judy Thomas) combined with intrathymic injection of donor lymph node lymphocytes. Three experimental monkeys were treated with survival times of 24, 35 and more than 1227 days and rejection at 30 days in one ATG-alone control. Not enough ATG was available to increase the number of animals, and this work was never published. In need of another source of T-cell depleting agent, I turned to an immunotoxin from the laboratory of David Neville at the USA's National Institutes of Health.

4. CONSTRUCTION OF ANTI-CD3 IMMUNOTOXIN

A wide variety of immunotoxins have been created to maximize *in vivo* targeted cell killing (Chang & Neville 1977; Frankel 1998). The protein toxins such as diphtheria toxin (DT) exert their effector function by inhibiting protein synthesis and, hence, causing cell death. The toxins have their enzymatic functional domain separate from their receptor that mediates cell entry (Drazin *et al.* 1971; Collier 1975; Neville & Hudson 1986; Olsnes & Sandvig 1988). By splicing toxin domains containing the effector function with monoclonal antibodies to alter their cellular specificity, cell-specific toxins can be created.

Youle *et al.* (Greenfield *et al.* 1987; Johnson *et al.* 1988, 1989) developed the strategy of mutating DT in order to create immunotoxins with equal potency to those made with wild-type DT but reducing the toxicity associated with wild-type DT. Neville *et al.* (1989) showed that it was the intracellular routing of DT-based immunotoxins by intracellular DT receptors or alternative receptors that determine their efficacy. For instance, the CRM9 immunotoxin, lacking the DT-binding site, nevertheless routed

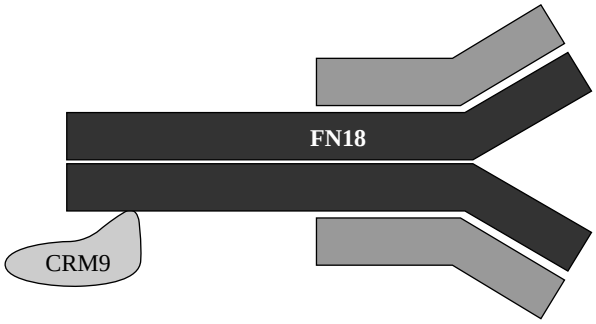

Figure 1. The FN18–CRM9 construct is shown. Specificity, cell entry and routing are provided by the FN18 monoclonal antibody specific for rhesus $CD3^+$ lymphocytes. CRM9 has its own translocation and toxicity domains without the cell entry and routing domains of wild-type DT (Neville *et al*. 1993).

appropriately, resulting in cell killing when directed at the CD3 or the transferrin receptor (Neville *et al*. 1989). Subsequently, Neville *et al*. evaluated an anti-human CD3–CRM9 immunotoxin to kill human T-cell subcutaneous tumours in N:NIH/bg/nu/xid mice which were deficient in their killer cell immune responsiveness. The model consisted of injecting 2.5×10^7 Jurkat cells (human T-cell leukaemia, $CD3^+$ $CD4^+$ $CD5^+$) subcutaneously. The immunotoxin was administered starting seven days after subcutaneous injection of Jurkat cells. Mice injected intraperitoneally with the anti-CD3–CRM9 at a dose of $25\,\mu g\,kg^{-1}$ on three consecutive days had tumour regression in five out of six cases (83% tumour regression rate). The anti-CD3 immunotoxin was consistently effective, whereas an anti-CD5–CRM9 immunotoxin was ineffective in causing tumour regression. The rate of tumour regression by the anti-CD3–CRM9 approximated the tumour regression rate achieved by 600 cGy of gamma irradiation, a non-specific lymphocytotoxic therapy. Marsh and others emphasized that high-affinity immunotoxins, such as anti-CD3–CRM9, would achieve fractional cell killing in a manner inversely proportional to the target cell number (Marsh & Neville 1987; Yan *et al*. 1991). They extrapolated that the anti-CD3–CRM9 could achieve in humans a 3 log depletion of a normal number of $CD3^+$ T cells (Neville *et al*. 1992). Application of this immunotoxin was considered for the treatment of graft-versus-host disease (GVHD) following bone marrow transplantation, treatment of human immunodeficiency virus (HIV) infection and treatment of T-cell leukaemia (Neville *et al*. 1992).

In order to develop a non-human primate model of the anti-CD3–CRM9, Neville *et al*. used a monoclonal antibody (FN18) specific for rhesus monkey CD3 cells to conjugate to CRM9 resulting in an immunotoxin specific for rhesus CD3 T cells (figure 1). *In vivo* testing of this construct revealed a highly potent immunotoxin capable of causing a 2–3 log depletion of rhesus $CD3^+$ T cells with minimal toxicity. Concerns had included cytokine release syndrome associated with capillary leak and development of lymphoproliferative disorders. Except for transient facial erythema, monkeys tolerated the immunotoxin well while maintaining appetite and weight. At doses of up to $0.05\,mg\,kg^{-1}$, the immunotoxin did not cause abnormal serum chemistries except for a transient elevation of lactate dehydrogenase (LDH) 1.4 times the normal upper limit at day 2 (Neville *et al*. 1996). Of considerable note was that in addition to causing peripheral blood T-cell depletion, lymph node T cells in the rhesus macaques were markedly depleted. For instance, at the highest dose of immunotoxin used ($0.2\,mg\,kg^{-1}$), lymph node $CD3^+$ cells decreased to 0.25% of the total number when evaluated two days after the last immunotoxin dose. The repopulation kinetics of T-cell recovery in peripheral blood demonstrated variable kinetics according to individual animals, but suppression was maintained for 28 days, the duration of the study. In two animals that had allogeneic skin grafts placed, skin graft survival was prolonged from 9.2 ± 0.2 days in ten saline-treated controls to 19 and 20 days. In contrast, two animals treated with antibody alone rejected their grafts at 12 days (Neville *et al*. 1996).

One of the principal concerns with a DT-based immunotoxin with regard to use in humans is that most humans are vaccinated to diphtheria and contain neutralizing antibody. In order to address this, Thompson *et al*. (1995) evaluated the susceptibility of the anti-human CD3 immunotoxin (UCHT1–CRM9) to neutralization by human serum. These authors found that human anti-DT antibodies inhibit the toxicity of UCHT1–CRM9 and that the inhibition correlated with the anti-DT titre. These sera did not inhibit endocytosis of the UCHT1–CRM9. An alternative DT mutant (CRM197) was capable of absorbing anti-DT antibody from human serum. CRM197 is a full-length DT-like construct containing both A and B subfragments of DT. The B subfragment was capable of complete absorption but 100-fold more was required. The A subfragment of DT had little or no effect on antibody absorption from serum (Thompson *et al*.). These results suggested that the pre-existing anti-DT antibodies responsible for neutralizing DT antigen were directed against the last 150 amino acids of DT.

Based on the above findings, Thompson *et al*. constructed a single-chain immunotoxin using the first 390 amino acids (out of 535) of DT. This construct (sFv-DT390) maintained specificity for the CD3 complex but was 16-fold less toxic than UCHT1–CRM9 to Jurkat cells. The sFv-DT390 was only partially inhibited by neutralizing antibody. In other words, this strategy partially evaded the obstacle of pre-existing anti-DT antibodies present in most human sera.

Hu *et al*. (1997) reported the use of FN18–CRM9 in a rhesus monkey model of experimental allergic encephalomyelitis (EAE) induced by myelin basic protein. Monkeys showing cerebrospinal fluid (CSF)-pleocytosis were treated with FN18–CRM9 alone or in combination with cranial irradiation. EAE in non-treated control monkeys progressed rapidly. Paralysis occurred four to six days after CSF-pleocytosis. Paralysis was either delayed or never occurred in monkeys treated with FN18–CRM9 and histopathology revealed few inflammatory plaques. When T cells repopulated in the peripheral blood following treatment, they were not present in the central nervous system (CNS) in large numbers, suggesting that the repopulated T cells had lost their CNS homing

Table 1. *Kidney allograft survival times*

FN18–CRM9 dose	intrathymic injection	graft survival time (days)
none	none	5, 7, 7, 7, 8, 9
0.2 mg kg^{-1} days −7 to −5	none	51[a], 84, 203, 302[a], 728
0.2 mg kg^{-1} days −7 to −5	normal saline	36, 68, 72, 887, 1038
0.2 mg kg^{-1} days −7 to −5	donor lymph node cells	41[a], 45, 181, 846[a], 1489

[a] Denotes that animal was sacrificed with a serum creatinine of less than 2.0 mg dl^{-1}.

capability. This study in an autoimmune model suggests a possible role for anti-CD3 immunotoxin in treating autoimmune diseases (Hu *et al.*).

5. IMMUNOTOXIN IN A RENAL TRANSPLANT RENAL ALLOGRAFT MODEL

Following the initial report by Neville of the rhesus anti-CD3 immunotoxin, my laboratory began a collaboration with the Neville laboratory to evaluate the immunotoxin in a rhesus monkey renal allograft model. Donor–recipient pairs were selected with the intention of mismatching MHC class I and MHC class II. Mixed lymphocyte culture (MLC) was performed to obtain a stimulation index, and cytotoxic T-lymphocyte (CTL) precursor frequency was determined between recipients and donors. However, stimulation index from MLC and CTL precursor frequency results correlated poorly with DRB (MHC class II) typing disparities.

Similar to results reported by Neville, FN18–CRM9 at a dose of 0.2 mg kg^{-1} on three consecutive days beginning seven days prior to the transplant caused profound T-cell depletion in the peripheral blood (2–3 logs) as well as 1–2 log depletion in lymph node lymphocytes. Immunotoxin administration was well tolerated with no evidence of cytokine-release syndrome. Eight out of 14 monkeys administered immunotoxin in this manner had marked prolongation of graft survival for greater than 100 days and greater than 200 days for 7 out of 14 (table 1). Histology of long-surviving renal allografts demonstrated an interstitial infiltrate but no evidence of glomerular or tubular injury (Knechtle *et al.* 1997*a*).

Skin grafts placed at least 140 days after the kidney transplant showed long-term survival in five out of six monkeys with the skin grafts surviving indefinitely (more than 140 days). However, one recipient rejected his renal allograft 40 days after the donor skin graft had been placed. Third-party skin grafts were promptly rejected by two weeks (table 2). The preliminary results were reported in Vienna in 1996 at the European Society of Organ Transplantation. Professor Roy Calne, on hearing these results, became a helpful advocate of immunotoxin for organ transplantation. In evaluating the alloimmune response of recipient monkeys to the donor, Fechner *et al.* reported that CTL precursor frequencies declined significantly after FN18–CRM9 treatment and renal transplantation (Fechner *et al.* 1997*a*,*b*). The initial decline was non-specific inasmuch as third-party CTL precursor frequencies declined equally. The duration of CTL precursor frequency suppression continued for six months in all of the five animals tested. However, following donor and third-party skin grafting, third-party CTL precursor frequency rebounded within one month of skin grafting, whereas the donor CTL precursor frequency remained suppressed following acceptance of the donor skin graft. Third-party skin grafts had been promptly rejected by two weeks. Anti-donor IgG was reduced in all animals tested despite the suppression of anti-donor CTL responsiveness. In addition, anti-donor mixed lymphocyte reaction (MLR) stimulation index was not significantly altered after renal transplantation and immunotoxin treatment relative to third-party MLR responses. In other words, although CTL responsiveness remains suppressed in immunotoxin-treated recipients, neither MLR responsiveness was altered nor was alloantibody production suppressed. There was poor correlation between length of survival and alloantibody production. Hence, Fechner *et al.* (1997*a*) described the model as another demonstration of split tolerance meaning a lack of a relationship between *in vitro* immune responses and allograft acceptance *in vivo*.

6. LONG-TERM HISTOLOGICAL CHANGES IN IMMUNOTOXIN-TREATED RENAL TRANSPLANT RECIPIENTS

Armstrong *et al.* (1998) reported on a population of rhesus monkeys that received post-transplant FN18–CRM9 starting immediately after renal transplantation. All treated monkeys had significant prolongation of renal allograft survival. However, in this group, rejection often occurred three to five months post-transplant and was characterized histologically by a significant interstitial T-cell infiltrate of the allograft without evidence of acute rejection such as tubulitis. Histological and serological data showed alloantibody-mediated glomerular and arterial damage. Intragraft cytokine analysis by polymerase chain reaction (PCR) duplication of graft biopsies showed expression of the co-inflammatory cytokines, interferon (IFN)-γ and interleukin (IL) 2. While FN18–CRM9 given on the day of transplant effectively ablated T-cell-mediated acute cellular rejection, early T-cell allosensitization might still occur and promote subsequent alloantibody-mediated graft damage and loss (Armstrong *et al.* 1998).

While long-surviving monkeys in the Armstrong report had minimal evidence of tubulitis and intimal arteritis—hallmarks of acute cellular rejection—by days 90 to 100 after transplantation and immunotoxin treatment, allograft biopsy specimens from five out of seven monkeys showed hyalinization of the mesangial matrix glomeruli and thickening of the glomerular capillary walls. Intimal hypertrophy of the renal arterioles was also seen. These changes were felt to be consistent with alloantibody-mediated endothelial damage. In evaluating interstitial infiltrates in long-surviving immunotoxin-treated monkeys, CD3$^+$, CD4$^+$ and CD20$^+$ lymphocytes were present in the interstitium with a remarkable absence of tubular evasion. Large number of plasma cells could be seen.

Observations in the rhesus monkey renal allograft model with immunotoxin administered on day 0 lends

Table 2. *Test for tolerance by skin graft*

(Abbreviations: GST, graft survival time; Cr, creatinine.)

animal	treatment	kidney	reason for sacrifice	final Cr (mg dl^{-1})	time of skin graft (days post-transplant)	donor skin GST (days)
X3J	none	203	severe weight loss, and acute and chronic rejection	3.8	189	> 14
WJ0	none	302	wasting	1.6	260	> 42
P1N	none	728	chronic rejection	7.0	194	> 534
P0F	saline	887	severe oedema and chronic rejection	3.9	147	82
P1P	saline	1038	acute and chronic rejection	7.6	176	> 862
92108	donor cell	181	acute rejection	6.5	140	41
93023	donor cell	846	wasting	1.8	182	> 664
P0J	donor cell	1489	acute and chronic rejection	6.6	188	> 1301

support to the concept that early allosensitization is an important causative factor for the future development of humorally mediated allograft rejection. This probably explains the differences in allograft survival and graft histology between monkeys pretreated at day 7 and monkeys treated beginning on the day of transplantation.

The presence of alloantibody in long-surviving monkeys accompanied by CD20$^+$ B cells and plasma cells in graft infiltrates suggests a role for B-cell-mediated chronic allograft injury. CD4$^+$ T cells presumably contribute to this process. The widespread detection of IFN-γ and IL-2 in experimental monkeys may either reflect a non-specific intragraft inflammation and lymphocytic activity or may reflect ongoing allograft rejection mediated by a mixed T- and B-cell response.

7. B-CELL IMMUNITY FOLLOWING IMMUNOTOXIN TREATMENT

In order to thoroughly evaluate the ability of monkeys with profound T-cell depletion following immunotoxin treatment and renal transplantation to mount an antibody response, the antibody response to CRM9, mouse IgG, tetanus antigen and alloantigen was systematically assessed.

(a) *Alloantibody measurements*

Using a flow cytometric cross-match test to measure antidonor IgG in recipient monkeys, anti-donor IgG increased in 8 out of 11 monkeys tested two to four months after transplantation. These monkeys had been treated beginning on the day of transplantation. Five out of these eight monkeys also subsequently developed chronic rejection histologically. Out of the 11 monkeys with anti-donor antibody present by flow cytometry cross-match, nine were evaluated for the presence of complement-fixing antibody and three out of nine had detectable titres of complement-fixing antibody.

(b) *Anti-tetanus antibody*

In order to measure primary and secondary responses to tetanus antigen as a measure of T-cell dependent B-cell responses after immunotoxin treatment, monkeys were immunized with tetanus toxoid between one and five months after immunotoxin treatment and boosted four weeks after initial exposure. Anti-tetanus IgG was measured by ELISA three weeks after initial immunization and one week after boosting. Six out of eight monkeys developed anti-tetanus IgG after primary immunization and an augmented titre after a second exposure.

(c) *Antibody response to FN18–CRM9*

Following exposure to FN18–CRM9, monkeys were evaluated for xenoantibody to mouse monoclonal antibody (FN18) and for antibody to the mutant DT (CRM9). A rhesus anti-mouse IgG response was detectable in four out of six monkeys at two weeks after immunotoxin treatment, all of the five monkeys tested at four weeks, and all nine tested at two months, or later after immunotoxin treatment. Eight out of nine monkeys had an increase in anti-CRM9 antibody when tested six to 60 weeks after immunotoxin treatment.

These data showed that the rhesus monkey is able to mount an antibody response to protein antigens following immunotoxin treatment.

8. T-CELL REPOPULATION

The kinetics of repopulation of rhesus monkeys undergoing renal transplantation and treatment with anti-CD3–CRM9 have paralleled the kinetic studies on immunotoxin alone performed by Neville *et al.* (1996). Briefly, CD3$^+$ T cells begin to return by two weeks post-treatment but take two to four months to achieve levels of 85% of baseline (figure 2). It may take as long as four to 12 months to reach 100% of baseline levels. In general, CD8 cells repopulate earlier than CD4 cells. CD8 cells may repopulate to baseline by four to ten weeks, whereas CD4 cells may require six to 12 months to repopulate. In humans, it is known that CD4 cell repopulation following ablation is age dependent (Mackall *et al.* 1995). The above description of rhesus T-cell repopulation was done in male monkeys two to three years old. The skewed CD4-to-CD8 ratio after repopulation may be a significant factor promoting graft prolongation.

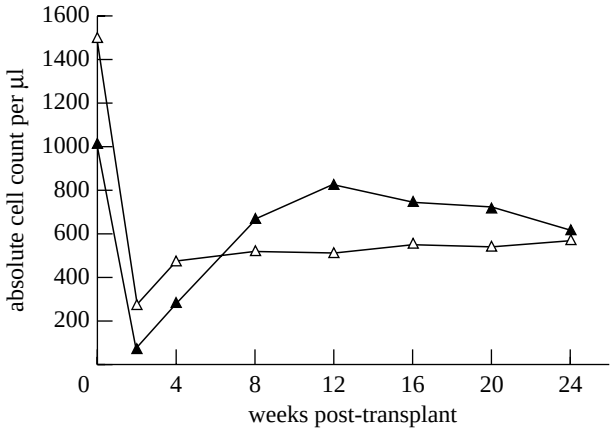

Figure 2. The kinetics of repopulation of T cells are shown for rhesus macaques following treatment with FN18–CRM9 and renal transplantation. Open triangles, CD3–CD4; closed triangles, CD3–CD8.

9. REVERSAL OF ACUTE ALLOGRAFT REJECTION

In order to evaluate the ability of FN18–CRM9 to reverse established acute rejection, three monkeys experiencing acute rejection four to six months post-transplant were re-treated with immunotoxin. All three monkeys had anti-CRM9 antibody levels twice the blank value at one to 100 dilution. Although the first monkey treated did not respond, this monkey had a creatinine level of $5.4\,\mathrm{mg\,dl^{-1}}$ at the time of re-treatment. Two other monkeys had a creatinine level of 2.3 and $2.2\,\mathrm{mg\,dl^{-1}}$ at the time of rejection and had reversal of acute rejection histologically with a fall in their creatinine and stabilization of graft function. Both of these monkeys, however, ultimately lost their grafts three to six months later from chronic rejection. These results demonstrated that prior exposure to FN18–CRM9 does not preclude effective T-cell depletion by subsequent administration (Knechtle *et al*. 1998*b*).

10. ACTIVATION OF LYMPHOCYTES FOR ADHESION AND CYTOKINE EXPRESSION

Hamawy *et al*. (1999*a*,*b*) showed that FN18–CRM9 incubated with purified normal rhesus monkey T cells increased protein tyrosine phosphorylation, cell adhesion to the extracellular matrix, and expression of IFN-γ and TNF-α. These effects were similar in magnitude to those induced by unconjugated antibody. However, immunotoxin (IT)-induced T-cell proliferation was far less than that caused by unconjugated antibody. Furthermore, the mitogenic agents IL-2 and anti-CD28 monoclonal antibody did not prevent IT-induced inhibition of cell proliferation. Those observations show that IT in addition to inhibitory protein synthesis in T cells causes activation of protein phosphorylation, adhesion and cytokine expression (Hamawy *et al*.).

11. ANTI-CD3–CRM9 PLUS DONOR BONE MARROW

Studies by J. M. Thomas and others, also in collaboration with the Neville laboratory, initially focused on combining the IT with donor bone marrow infusion (Thomas *et al*. 1997). This laboratory, with extensive experience in donor bone marrow infusion as an adjunct to tolerance induction (Thomas *et al*. 1987, 1989, 1992, 1994, 1995), found that FN18–CRM9 used alone at a lower dose than used by the Wisconsin group ($67\,\mu\mathrm{g\,kg^{-1}}$ 14–16 h pretransplant, $33\,\mu\mathrm{g\,kg^{-1}}$ at 4 h and 24 h post-transplant) resulted in acute rejection between 43 and 57 days post-transplant. The combination of this same dose of IT with a day 0 infusion of $\mathrm{DR^{-dim}CD3^{-dim}}$ donor bone marrow prolonged allograft survival to between 124 and greater than 600 days. The prolonged graft survival with combined IT and donor bone marrow correlated with later appearance or, in three cases, absence of alloantibody (IgG) in recipients. Microchimerism was observed in all recipients studied including those not given donor bone marrow, but levels of microchimerism did not correlate with graft survival. Despite the unclear role of chimerism in this study, donor bone marrow provided a clear benefit to allograft survival. This was in contrast to the Wisconsin group's findings that thymic injection of lymph node lymphocytes did not prolong graft survival more than IT alone.

12. FN18–CRM9 PLUS DEOXYSPERGUALIN

In order to develop a tolerance-inducing strategy based on IT administration at the time of renal transplantation, the Thomas laboratory focused on combining a brief course of deoxyspergualin (DSG) and methylprednisolone with FN18–CRM9. This work was largely driven by the clinical goal of establishing a regimen that would be relevant to cadaveric renal transplantation where preoperative administration of IT is not feasible (Contreras *et al*. 1998). FN18–CRM9 plus perioperative steroids and a 14 day course of DSG at $2.5\,\mathrm{mg\,kg^{-1}}$ daily substantially prolonged allograft survival in four out of eight recipients to greater than 100 days. Furthermore, FN18 antibody alone, plus steroids and DSG, was associated with acute rejection prior to 50 days in all three of the recipients studied. These latter data clearly support the superiority of IT over antibody alone.

DSG, reported to inhibit transcription factor NF-κB in human B cells (Hoeger *et al*. 1994; Nadler *et al*. 1995; Tepper *et al*. 1995) has also been reported to inhibit antigen presentation by dendritic cells (Hoeger *et al*. 1994). Furthermore, since NF-κB is an important regulator of pro-inflammatory cytokines (Baeuerle & Henkel 1994; Auphan *et al*. 1995; Barnes & Karin 1997), the Thomas group rationalized that the addition of DSG to FN18–CRM9 might suppress the pro-inflammatory cytokine production associated with immunotoxin administration. These two observations may underlie the beneficial effect of the addition of DSG to FN18–CRM9 in the rhesus monkey renal allograft model.

13. F(ab)$_2$–CRM9 PLUS DEOXYSPERGUALIN

In order to avoid the pro-inflammatory cytokine release associated with FN18 (a murine anti-rhesus CD3ε monoclonal antibody), the pepsin digest of the FN18 antibody was used by Neville *et al*. (1996) to create an F(ab)$_2$IT since the Fc portion of the intact IgG1 antibody

was thought to be largely responsible for cytokine release by binding to Fc receptors on monocytes. The Thomas laboratory used this construct in combination with DSG and found that plasma IFN-γ and IL-4 levels were substantially reduced compared to the intact IgG1 IT even when the latter was given with DSG. Graft survival in $F(ab)_2$ plus DSG-treated monkeys was greater than 200 days all of the three recipients. Monkeys receiving a 15-day course of DSG were noted to have a significant decrease of mature dendritic cells at day 4 post-transplant with repopulation by one month. The mature T cells were defined by expression of cytoplasmic RelB and membrane expression of DR, CD83, and CD86. In summary, the $F(ab)_2$IT was found to induce less pro-inflammatory cytokine release compared with the FN18–CRM9, and in combination with DSG was found to promote greater than 200-day renal allograft survival in a reliable manner (three out of three recipients). The long-term benefit of DSG was attributed to arrest of dendritic cell maturation (J. M. Thomas *et al.* 1999).

14. SIDE-EFFECTS OF FN18-CRM9

In addition to the cytokine activation mentioned above following administration of FN18–CRM9, this immunotoxin has been associated with an initial weight loss during the first two months following treatment (Contreras *et al.* 1999). Weight loss averages of 10–15% is maximal at one to two months, and monkeys generally regain baseline weight by three to four months. Although Neville reported a significant rise in LDH levels early after immunotoxin administration, neither the Thomas laboratory nor the Knechtle laboratory has noted significant alterations in aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, or γ-glutamyltransferase following immunotoxin administration. Both laboratories doing renal allografts in IT-treated monkeys have experienced severe wasting in a subgroup of animals surviving long term. This has been characterized by weight loss despite a good appetite, although eventually monkeys lose their appetite as well. In the Thomas laboratory, wasting was associated with co-administration of $CD34^+$ cells (50% of recipients requiring euthanasia for wasting), while in the Knechtle laboratory (Knechtle *et al.* 1998*a*), wasting of long-term monkeys has been sporadic, occurring in *ca.* 1 in 20 recipients surviving more than 200 days.

15. ISLET XENOGRAFT TRANSPLANTATION WITH FN18–CRM9

Using a spontaneous model of insulin-dependent diabetes mellitus in non-human primates (two *Macaca fascicularis* and one *Ceropithecus aethiops*), F. T. Thomas *et al.* (1999) reported on three xenogeneic pancreatic islet transplants from *Macaca mulatta* (rhesus) donors. Immunosuppression consisted of methylprednisolone 15 mg kg^{-1} day^{-1} for three days, cyclosporine 20 mg kg^{-1} on the day of transplantation and 60 mg kg^{-1} twice a day for three days post-transplant.

FN18–CRM9 was given intravenously at 100 μg kg^{-1} day^{-1} on the day of transplantation and the following day. All three diabetic monkeys became euglycaemic within one to two weeks of the transplant. They remained euglycaemic for 410, 255 and 100 days post-transplant at the time of report (F. T. Thomas *et al.* 1999). Plasma C-peptide levels normalized in all three recipients as did glycosylated haemoglobin. These dramatic results suggest the potential use of T-cell depletion by an anti-CD3 IT as a platform for tolerance induction for islet transplantation. Although concordant islet xenografts are not likely to be a significant source of islets for human transplantation, lessons learned from this study may be relevant to islet allotransplantation in humans (Contreras *et al.* 2000).

16. STABLE MIXED CHIMERISM USING BONE MARROW TRANSPLANTATION AND A NON-MYELOABLATIVE REGIMEN INCLUDING CD3 IMMUNOTOXIN

Using an inbred miniature swine model of skin allotransplantation, Huang *et al.* (2000) described the use of anti-porcine CD3–CRM9 as part of a non-myeloablative regimen to enable donor haemopoietic chimerism to be established. Recipient swine were conditioned with whole body and thymic irradiation followed by T-cell depletion using pCD3–CRM9 at a dose of 0.05 mg kg^{-1} 48 h before peripheral blood stem cell infusion and a second dose on day two. Cyclosporine (Neoral®) at a dose of 30 mg kg^{-1} was started one day prior to donor cell infusion. Peripheral blood T-cell depletion was accomplished using this regimen by the day of donor cell infusion. Some pigs experienced respiratory complications and polyneuropathy as a side-effect of the porcine CD3–CRM9. Skin grafts were placed on four long-term stable mixed chimeras at 60 days after donor stem cell infusion. All four had significantly prolonged skin-graft survival (45, 50, more than 50 and more than 235 days). Third-party skin grafts were rejected between one and four weeks. This report describes a successful establishment of stable mixed chimerism in miniature swine using T-cell depletion by pCD3–CRM9 in addition to irradiation and cyclosporine. Multilineage mixed chimerism persisted for at least 160 days in all of the four treated recipients. This alternative approach to tolerance based on stable mixed chimerism is dependent on profound T-cell depletion at the time of donor cell infusion. IT may have unique advantages when applied to this strategy (Huang *et al.* 2000).

17. FUTURE DIRECTIONS

In order to produce a T-cell depleting IT for potential clinical trials, it would be ideal to have a fusion protein not requiring chemical conjugation, to have an IT without an Fc portion with its attendant cytokine-activating properties, and to have an IT less susceptible to neutralization by anti-DT antibody. Such a construct would have to maintain the unique potency of the FN18–CRM9 and, of course, target the human T-cell receptor. Significant progress in this pursuit has been reported by Liu *et al.* (2001) in constructing a DT390–scFvUCHT1 construct produced in a hamster cell line, CHO K1 RE1.22c (Moehring & Moehring 1979). Following treatment with N-glycosidase F to remove N-linked oligosaccharides, this IT had an IC_{50} of 4×10^{-12} M towards $CD3^+$ Jurkat cells. Compared with the prokaryotic

Escherichia coli expression system, this eukaryotic expression system resulted in a 12-fold increase in toxicity (Liu *et al.* 2001). Development of a modified IT such as this will probably serve as the prototype for anti-CD3 ITs for clinical trials.

In anticipation of clinical trials in transplantation, the focus of work in the non-human primate renal transplant model is aimed at improving the reliability and long-term success of tolerance induction based on T-cell depletional strategy. Identification of adjunctive immunosuppressants such as DSG, which do not interfere with unresponsiveness associated with IT but instead downregulate the B-cell response, will be looked to for early clinical trials. The principle of profound T-cell depletion at the time of transplantation as a platform promoting long-term unresponsiveness to allografts has already been applied by Calne *et al.* using CAMPATH-1H to accomplish T-cell and monocyte depletion (Calne *et al.* 1998). Lessons learned from the ITs with regard to depletional strategies to tolerance are likely to lead to improved strategies towards clinical tolerance in organ transplantation. It is anticipated that clinical trials in transplantation will begin using IT in the near future.

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