Use of *in Vitro* Correlates for Selection of Candidate Immunosuppressive Antibodies Prior to a Primate Transplant Model

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Summary

The selection of possible candidate immunosuppressive antibodies to prevent graft rejection is performed in vitro. Additionally, due to the species specificity of these monoclonal antibodies (MABs), pre-clinical studies in non-human primates are necessary. If a positive correlation between the in vitro and in vivo findings would exist, these tests can act as a pre-screening before new reagents are tested in vivo. The correlation of the in vitro and in vivo efficacy of an anti-T-lymphocyte globulin (ATG) and an anti-CD80 MAB is evaluated in a rhesus monkey skin transplant model. The results show that lymphocytotoxic titers (NIH-test) do not predict the outcome of in vivo effectiveness of ATG in rhesus monkeys. Additionally, no evidence of tolerance to a skin allograft could be shown to correlate with inhibition of a secondary mixed lymphocyte culture (MLC) by anti-CD80 and cyclosporin A (CsA). Thus, these in vitro assay used can not predict the in vivo efficacy of new immunosuppressive antibodies.

Zusammenfassung: Korrelation von in vitro Tests mit einem Primaten-Transplantationsmodell zur Auswahl geeigneter immunsuppressiver Antikörper

Die Auswahl geeigneter immunsuppressiver Antikörper zur Verhinderung der Transplantat-Abstoßung wird in vitro durchgeführt. Zusätzlich sind, in Abhängigkeit von der Artspezifität dieser monoklonalen Antikörper, präklinische Studien an nichthumanen Primaten erforderlich. Sollte es eine positive Korrelation zwischen in vitro und in vivo Ergebissen geben, könnten diese Tests als Präscreening eingesetzt werden, bevor neue Antikörper in vivo getestet werden. Die Korrelation zwischen in vitro und in vivo Wirksamkeit eines Anti-T-Lymphozyten-Globulins (ATG) und eines anti-CD80 monoklonalen Antikörpers wird in einem Rhesusaffen Hauttransplantationsmodell geprüft. Die Ergebnisse zeigen, daß sich mit den lymphotoxischen Titern die Ergebnisse der in vivo Wirksamkeit des ATGs bei Rhesusaffen nicht voraussagen lassen. Darüberhinaus konnten keine Hinweise auf eine Toleranz gegenüber dem Hauttransplantat gefunden werden, die mit einer Hemmung der sekundären Mixed Lymphocyte Culture (MLC) durch Anti-CD80 und Zyklosporin A korrelieren. Damit kann mit diesem in vitro Test die in vivo Wirksamkeit neuer immunsuppressiver Antikörper nicht vorhergesagt werden.

Keywords: transplantation, antibodies, in vitro, primates

1 Introduction

Immunological rejection of an allograft through T cell dependent mechanisms is an evitable result after organ transplantation between genetically non-identical individuals. Therefore, administration of drugs suppressing the recipient's T cell function is required for successful transplantation of allogeneic organs. Nowadays calcineurin inhibitors and glucocorticosteriods are used clinically as a routine. Even though acute rejection can effectively be prevented with these drugs and rejection episodes can often be reversed by increase of the dose or administration of T-cell antibodies (ATG,OKT3), many transplant patients suffer late graft loss usually caused by chronic rejection. Although many cells participate in the process of transplant rejection, only T cells appear to be absolute required (Krensky et al., 1990). In a recent review, the role of T cell costimulatory activation pathways in transplant rejection has been described (Sayegh et al., 1998). The T cell requires beside triggering via the antigen-receptor, a costimulatory signal via CD154 or CD28, to become fully activated (Chamber et al., 1997; Springer et al., 1987). When the T cell encounters an antigen-specific stimulus in the absence of costimulation, the T cells becomes un-responsive or tolerant to that particular antigen (Mueller et al., 1989). Several strategies using different monoclonal antibodies directed against costimulatory molecules (anti-CD80, anti-CD86, anti-CD40L MABs and CTLA4-Ig), have proven to induce tolerance/non-responsiveness to allografts in rodents and non-human primates (Larsen et al., 1996; Lenschow et al., 1995; Kirk et al., 1997).

In organ transplantation new approaches are investigated that are aimed at the induction of complete non-responsiveness towards the allograft or at least at the reduction of standard immunosuppression. The selection of possible candidate antibodies is performed *in vitro*. Additionally, due to the species specificity of these

MABs, pre-clinical studies in non-human primates are necessary. To be able to predict the *in vivo* efficacy of the MABs, *in vitro* assays are developed. If a positive correlation between the *in vitro* and *in vivo* findings would exist, these tests can act as a pre-screening before new reagents are tested *in vivo*. The correlation of the *in vitro* and *in vivo* effectivity of ATG and an anti-CD80 MAB is evaluated in a rhesus monkey skin transplant model.

T-cell specific antibodies have been evaluated extensively, and a well known example is anti-T-lymphocyte globulin (ATG). To investigate the potency of rabbit ATG *in vitro*, lymphocytotoxic titers were determined in rhesus monkeys and were correlated to the *in vivo* effects of ATG on skin allograft survival time.

A recent valid approach for immune suppression after solid organ transplantation is the blockade of the costimulatory pathways, such as CD80-CD28 pathway. This costimulatory signal is relatively resistant to cyclosporin A (CsA). Therefore, it is suggested to use anti-CD80 MAB as a CsA-sparing agent. Additionally, *in vitro* a combination of anti-CD80 MAB with CsA can induce allo-antigen specific non-responsiveness in human mixed lymphocyte cultures (MLC), whereas reactivity to „third party" antigens remains intact (Comoli et al., 1995; Van Gool et al., 1994). These promising data initiated a study to explore the use of anti-CD80 in combination with a suboptimal dose of CsA as a prophylactic treatment to prevent skin transplant rejection in a rhesus monkey model. The *in vivo* effects of this treatment were correlated to the effect of this combination of drugs to mixed lymphocyte culture using rhesus monkey peripheral blood mononuclear cells (PBMC).

2 Animals, material and methods

2.1 Animals

Rhesus monkeys (*Macaca mulatta*) were born and raised at the Biomedical Primate Research Centre or were purchased from an outdoor station. The animals were between 4 and 10 years of age and typed for Mamu-A, B, DR antigens and ABO antigens. Recipients had no history of alloimmunisation (pregnancy, blood transfusion, allografts) and had not received murine antibodies. Male and female animals were used. The protocol of the study and the care was in accordance with guidelines of the Animal Care and Use Committee installed by Dutch law.

2.2 ATG skin transplant experiment

Prior to the transplantation experiment, each batch of ATG is tested *in vitro* in a lymphocytotoxicity assay using unseparated PBMC (NIH-test) and B- or T-cell enriched suspensions (B or T-test) of the rhesus monkey selected for the skin graft experiment (Mittal et al., 1968; Roger et al., 1976). Each batch of ATG is tested in one rhesus monkey for batch release purposes. ATG (60 mg/kg) is administered on days -2, 0, 2, 5 and 7. Two skin grafts from two donors each are transplanted on day 0. Skin graft survival is scored by visual inspection. The day on which complete crust formation of the skin grafts is observed, is taken as graft survival time. The mean survival time of the 4 grafts is averaged. During the experiment, leukocyte and lymphocyte counts are determined.

2.3 anti-CD80 MAB and CsA skin transplant experiment

To test the *in vitro* efficacy of anti-CD80 MAB in combination with CsA, a prima-

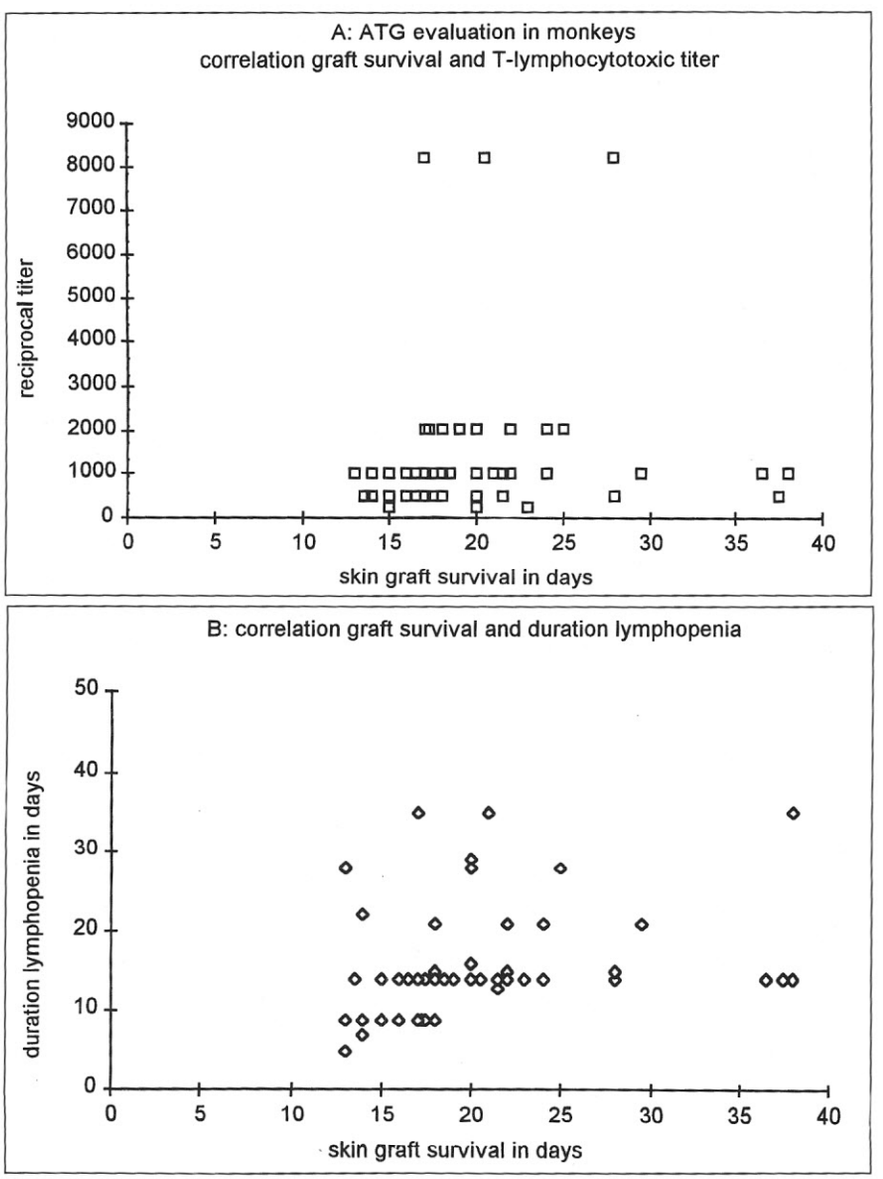

Figure 1: Evaluation of ATG batches in rhesus monkeys; each dot represents the mean skin graft survival time of one monkey and the lymphocytotoxicity titer of the ATG on T-cell enriched suspensions (A) or the duration of the lymphopenia (B)

ry one-way mixed lymphocyte culture (MLC) was performed (Ossevoort et al., in press). The rhesus monkey PBMC were stimulated with an irradiated rhesus monkey Herpes-B virus-transformed B cell line for 5 days in the presence of anti-CD80 MAB (10 µg/ml) and/or CsA (400 ng/ml). The proliferative response was measured as ^{3}H-thymidine incorporation. For the secondary MLC, the cells were cultured for 5 days in the presence of anti-CD80 MAB and CsA. Then, the cultures were washed to remove the reagents and left unstimulated for 2 days after which the cultures were restimulated with same stimulator cells or rIL-2. No reagents were added to the second culture. The secondary cultures were harvested after 3 days, the last 18 hours in the presence of ^{3}H-thymidine. The immunosuppressive potency of the combination of anti-CD80 MAB (0.5 mg/kg) and CsA (5 mg/kg) was tested in a rhesus monkey skin transplant model. The anti-CD80 MAB was given i.v. daily for 10 days starting at day -1. CsA was given i.m. daily from day -2 until rejection was scored (Ossevoort et al., in press).

3 Results

3.1 ATG skin transplant experiment

Untreated rhesus monkeys reject the grafted skin between day 9 and 12, with a mean survival time of day 10 post-transplantation. Figure 1 shows the results of the immunosuppressive efficacy of different batches of ATG displayed as skin graft survival times. The lymphocytotoxic titers were determined using T-cell enriched suspensions and the duration of lymphopenia (lymphocytes less than 50% of pre-treatment value)

No correlation between lymphocytotoxic titers on T-cell enriched suspensions and the survival was found (correlation coefficient = 0.11). Similar results were found for the lymphocytotoxic titers using B-cell enriched suspensions or unseparated lymphocytes (data not shown). The duration of the lymphopenia showed a weak correlation (correlation coefficient = 0.33) with the skin graft survival.

3.2 anti-CD80 MAB and CsA skin transplant experiment

In vitro results demonstrated that the induction of allo-antigen specific rhesus monkey T cells can be prevented using CsA in combination with anti-CD80 MAB. To this end primary MLC assays were performed in the presence or absence of anti-CD80 MAB and / or CsA. After 5 days tritiated thymidine incorporation was measured (Table 1). Addition of anti-CD80 MAB or CsA alone resulted in a mean inhibition of the proliferative response of 52 or 77 % respectively. Addition of both anti-CD80 MAB and CsA resulted in a complete abrogation of the alloantigen-specific response. These primary cultures were then washed, left unstimulated for 2 days and then restimulated with the same stimulator cells. No anti-CD80 MAB or CsA were added to the secondary cultures (table 1). Now the cultures that had previously been exposed to CsA in combination with anti-CD80 MAB did not proliferate, whereas the cultures previously exposed to medium, CsA or anti-CD80 MAB could be restimulated. The T cells in the primary cultures were still able to respond since restimulation with the same antigen in the presence of rIL-2 did result in proliferation (data not shown). This suggested that the T-cell had been silenced during the first phase of the experiment. The *in vivo* immunosuppressive effects of the combined treatment with a suboptimal doses of CsA (trough levels 100-150 ng/ml) and anti-CD80 MAB was examined in a skin allograft model (Ossevoort et al., 1998). The skin graft survival time of untreated or CsA-treated rhesus monkeys was 10 days. Treatment with CsA in combination with anti-CD80 MAB resulted in a significantly increased skin graft survival time to 14 days. Thus a short term immunosuppression, resulting in prolonged skin graft survival could be obtained using CsA and anti-CD80 MAB as a prophylactic treatment in a rhesus monkey skin transplant model.

Table 1: The immunosuppressive efficacy of anti-CD80 mAb (10 µg/ml) in combination with CsA (400 ng/ml) in a primary and secondary MLC using a B cell line stimulator cells

experiment 1	primary MLC	secondary MLC
medium	0*	0
anti-CD80	30	38
CsA	86	46
anti-CD80 + CsA	98	89
experiment 2	**primary MLC**	**secondary MLC**
medium	0	0
anti-CD80	54	32
CsA	61	0
anti-CD80 + CsA	89	51

*** % inhibition**

4 Conclusions

Since many of the anti-human MABs also react with lymphocytes of macaques, the rhesus monkey skin transplant model is a valid model to test the immunosuppressive efficacy of monoclonal antibodies specific for human T cell subsets (Jonker et al., 1983; Jonker et al., 1986; Nooij et al., 1987).

The application of a valid *in vitro* assay that predicts the immunosuppressive potential of a new reagent in organ transplantation, implies a significant reduction of the number of animals used for *in vivo* experiments. In this study, the *in vitro* and *in vivo* outcome of the immunosuppressive activity of ATG and an anti-CD80 MAB were compared. The results show that lymphocytotoxic titers do not predict the outcome of *in vivo* effectiveness of ATG in rhesus monkeys. Additionally, in this protocol, no evidence of tolerance to a skin allograft could be shown to correlate with inhibition of a secondary MLC. Thus, until now no *in vitro* assay has been established to predict the *in vivo* efficacy of new immunosuppressive antibodies to prevent graft rejection.

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