Chapter 3
Immunosuppresive Drugs Commonly Used in Transplantation Models

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Abstract The chapter describes the four most commonly used immunosuppressive drugs in transplantation rat models: polyclonal antibody, tacrolimus, mycophenolic acid and mTOR inhibitors. The aim of the chapter is to provide available information about the administration and doses of single drugs with appropriate trough levels or AUC curves that will enable the reader to better estimated therapy in his or her experiments. Furthermore, we outline available data about rejection times of different organ transplantations in various strain combinations.

Keywords Immunosuppression • Rat model • Kinetics • Dose

3.1 Monoclonal and Polyclonal Antibodies in Rat Organ Transplantation

In human transplant medicine, two antibodies are available for organ recipients: basiliximab (monoclonal antibody against receptor for interleukin-2) and ATG (polyclonal anti thymocyte or anti T-lymphocyte globulin). Only limited data are available about the effects of basiliximab in rat models. Therefore, this section will be focused on experiments done with ATG.

3.1.1 Anti Thymocyte Globulin

Currently, there are three commercially available preparations of ATG (ATG Fresenius – rabbit, Thymoglobuline Genzyme – rabbit, AtGam Roche – horse). Rabbit preparations are preferred to equine ATG due to better tolerance and a lower incidence of side effects. Generally, polyclonal antibodies are prepared by immunizing rabbit models with cell suspension from human thymic tissue. Then,

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polyclonal globulins are obtained from harvested rabbit serum and samples from several thousands of rabbits are pooled to obtain batch-to-batch consistency.

3.1.2 Mechanism of Action

The primary mechanism of immunosuppressive action is T-cell depletion. Depleting effects are mostly mediated through the complement based cell lysis though other mechanisms, including antibody mediated toxicity or activation of apoptosis, can be involved. Treatment with ATG decreases T-cells not only in peripheral blood but also in secondary lymphoid tissue, except for the thymus. Modulating effects are demonstrated with down-regulation of expression of CD2, 3, 4, 5 and 6 on the T-cell surface. Decreased expression of integrins and intercellular adhesion molecules results in decreased adhesion of leukocytes during the reperfusion period. Moreover, ATG binds to the number of receptors on the surface of B-lymphocytes and activates apoptotic pathway in these lymphocyte cell subsets [1, 2].

3.1.3 ATG in Prevention of Rejection

The effect of ATG on lymphocyte cell count was investigated by Olausson, et all in heterotopic heart transplantation in PVG to DA strain combination. Rabbit ATG was administered in two doses, 0.1 and 0.01 ml. Untreated recipients of the heart demonstrated decline in CD5, CD4 and CD8 cell counts. The number of cells return to their pre-transplant level in the first week post-surgery. Both doses of ATG effectively decreased numbers of CD4, 8 and 5 cells, while the count of B-cells remained unchanged. Animals treated with lower dose of ATG rejected their grafts and demonstrated higher counts of t-cell subsets in comparison to those recipients with functioning grafts. In another experiments, treatment with 25 mg/kg of ATG on day-3 and 5 mg/kg on days -2,-1 and 0 resulted in indefinite survival of PVG and LEW heart allografts transplanted in the DA recipient [2, 3]. The same regimen did not prolonged function of lung grafts in the same strain combinations [3]. In the PVG to DA strain, the combination 0.02 ml of ATG was ineffective in prolongation of heart allografts, while doses of 0.1 and 0.2 ml significantly increased survival of the graft to the median of 100 days. Friedmann et all demonstrated that 4 mg/kg of rabbit ATG prolonged the survival of allogeneic islets to 11 days in Wistar/Furth to LEW combination and to more than 100 days in Fischer to LEW model [4].

ATG has been widely used in human organ transplantation as a part of induction protocols and an anti-rejection treatment. The efficacy of the treatment may depend on the presence of antibody specificities. Popow et al. comprehensively described composition of commercially available ATG preparations and found out differences in the amount of specific antibodies. For instance, in ATG-Fresenius they did not detect antibodies against CD1a, CD27, CD68, CD80 and CD209, while in

Thymoglobulin they did not detect antibodies against CD 102, CD298, CD58 and CD68 [5]. In another experiment, Penack et al. demonstrated significantly toxigenic effects of ATG – Fresenius and Thymoglubuln Genzyme on NK cells in comparison to Lymphoglobulin [6]. At present, it seems that these differences among preparations have little or no impact in clinical settings. In any case, with increasing knowledge about tolerance mechanism and immune network, these differences may play a significant role in prolonging the graft survival.

3.2 Tacrolimus

3.2.1 Mechanism of Action

Tacrolimus binds to its intracellular receptor FKBP (FK binding protein). Once bound, it forms an inhibitory complex that blocks the enzymatic activity of calcineurin. Calcineurin inhibition results in complete blockage of the translocation of cytoplasmatic NF-ATc, which consequently leads to T-cell inactivation and inhibition of cytokine gene transcription.

3.2.2 Pharmacokinetics of Tacrolimus Rat Model

Tacrolimus is poorly absorbed drug with almost 50 % first- past elimination after absorption. The mean 24 h AUC were 16.2, 76.9 and 450.2 (ngxhr/ml) after oral administration of tacrolimus in solid dispersion formulation as demonstrated in experiment with fed Sprague- Dawley rats. For all dosing regimens, maximal concentrations were achieved 30 min after administration. The mean maximal blood concentrations of tacrolimus were 8.8 ± 4.9, 11.6 ± 5.3 and 40 ± 19.4 ng/ml after oral administration of 1, 3.2 and 10 mg/kg, respectively. Tacrolimus is stable in gastric juice and intestinal secretions. Studies of the absorption site were done using a close loop model at five different sites and show that tacrolimus is predominantly absorbed in the jejunum and duodenum. Significantly lower absorption was observed in the ileum and colon. Only a minimal amount of tacrolimus gets into the blood through the stomach. Almost 50 % of tacrolimus is eliminated in the liver [7].

Bioavailability after intraintestinal and intraportal administration of tacrolimus 1 mg/kg in Wistar rats were 26.2 % and 39.8 %, respectively. Hepatic extraction at a dose of 1 mg/kg was about 60 %. At the same dose, 34 % of tacrolimus was metabolized in the small intestine. Experiments with the everted sacs method showed that 23 % of tacrolimus disappear after 1 h incubation with the inhibitor of CPY3A, providing evidence that intestinal enzymes moderately participate in the metabolism of tacrolimus [8]. Jejunal or ileal segmental small bowel transplantation decreases bioavailability of tacrolimus by 40 % of 5 mg/kg in comparison to control non-transplanted group (LEW RTA[1]) [9].

Bioavailability of tacrolimus can be improved by rectal application. In experiment with Sprague-Dawley rats, 2 mg/kg of tacrolimus administered rectally in suppository form resulted in significant increase of 24 h AUC when compared to per oral administration of the same dose (707 ± 28.6 and 103 ± 6.7 ng.h/ml, respectively). Similarly, blood trough levels of tacrolimus were higher after rectal administration in comparison to oral administration (25.8 ± 4.8 and 1.6 ± 0.8 ng/ml, respectively). The tacrolimus was applied under general anesthesia [10].

In experiments with LEW rats, it has been established that the pharmacokinetics of tacrolimus was influenced by the circadian rhythm. Tacrolimus administered at 10 h p.m. resulted in a higher maximal concentration in comparison to a morning dose (46.4 ± 12.6 and 15.9 ± 4.1 ng/ml, respectively). Consequently, 24 h AUC was 2.9 times higher in rats treated with tacrolimus at 22 h p.m. [11].

Oral administration of tacrolimus in 4 mg/kg dose to Lewis rat concomitantly with MMF 20 mg/kg did not change availability of mycophenolic acid. The mean AUC 2–24 of MPA in controls and Tacro/MMF group were 79 ± 10 84 ± 26 mg/L, respectively [12].

3.2.3 Prevention of Allograft Rejection

Tacrolimus effectively prolongs graft survival from doses of 0.2 mg/kg given intramusculary [13–24]. Peroral doses are generally 10 times higher, which results from intensive metabolism of tacrolimus in the liver and small intestine. For practical use, intramuscular administration is the simplest approach. Intravenous, inhaled and peroral administration are more complicated, requiring general anesthesia (intravenous and inhaled) or a special technique (peroral – gastric feeding tube). In our experiments with islet transplantation in LEW to BN strain models, we administered tacrolimus in daily regimen at dose of 0.05 mg/kg intramusculary, which resulted in long term survival of the islet graft and trough levels of tacrolimus in the range of 5–10 ng/ml. Rejection times in various strain combination and tacrolimus doses are described in following tables.

Donor strain (heart)	Recipient strain	Tacrolimus dose (mg/kg)	The mean graft survival (days)	Administration of the drug	References
Brown-Norway $RT1^n$	Lewis $RT1^l$	1	10–15	Oral	Deuse et al. [13]
Brown-Norway $RT1^n$	Lewis $RT1^l$	2	15–20	Oral	Deuse et al. [13]
Brown-Norway $RT1^n$	Lewis $RT1^l$	8	20–25	Oral	Deuse et al. [13]
Brown-Norway $RT1^n$	Lewis $RT1^l$	0.3	7.8	Oral	Kinugasa et al. [14]
Brown-Norway $RT1^n$	Lewis $RT1^l$	0.6	9.8	Oral	Kinugasa et al. [14]

Donor strain (heart)	Recipient strain	Tacrolimus dose (mg/kg)	The mean graft survival (days)	Administration of the drug	References
Lewis RT1^{l}	Lewis RT1^{l}	0.7	17	Oral	Kinugasa et al. [14]
ACI	Lewis RT1^{l}	0.032	16	Intramuscular	Fang [15]
F344 (RTA^{lvl})	Lewis RT1^{l}	0.025	11	Intramuscular	Li et al. [16]
F344(RTA^{lvl})	Lewis RT1^{l}	0.05	13	Intramuscular	Li et al. [16]
F344(RTA^{lvl})	Lewis RT1^{l}	0.1	52	Intramuscular	Li et al. [16]
Lewis RT1^{l}	Lewis RT1^{l}	0.2	40	Intramuscular	Jeske [17]
DA RT1^{avl}	PVG RT1^{c}	2.4	13	Oral	Qi et al. [18]
DA RT1^{avl}	PVG RT1^{c}	4.8	18	Oral	Qi et al. [18]

Donor strain (islet)	Recipient strain	Tacrolimus mg/kg dose	The mean graft survival (days)	Administration of the drug	References
ACI RT1^{a}	Lewis RT1^{l}	5	30	Intramuscular	Rastellini [19]
ACI RT1^{a}	Lewis RT1^{l}	10	30	Intramuscular	Rastellini [19]
Wistar	ACI RT1^{a}	1	71	Intramuscular	
WKA RT1^{u} (renal subcapsular grafts)	Lewis RT1^{l}	0.32	13	Intramuscular	Yasunami [20]
WKA RT1^{u} (renal subcapsular grafts)	Lewis RT1^{l}	1	20	Intramuscular	Yasunami [20]
WKA RT1^{u} (intrahepatic grafts)	Lewis RT1^{l}	0.1	7	Intramuscular	Yasunami [20]
WKA RT1^{u} (Intrahepatic grafts)	Lewis RT1^{l}	0.32	42	Intramuscular	Yasunami [20]
WKA RT1^{u} (intrahepatic grafts)	Lewis RT1^{l}	1	45	Intramuscular	Yasunami [20]

Donor strain (lung)	Recipient strain	Tacrolimus dose	The mean graft survival (days)	Administration of the drug	References
Brown-Norway RT1^{n}	Lewis RT1^{l}	3 mg/kg	8.7	Intramuscular	Misao [21]

Donor strain (pancreas)	Recipient strain	Tacrolimus dose	The mean graft survival (days)	Administration of the drug	References
DA RT1^{avl}	Lewis RT1^{l}	1 mg/kg	16	Intramuscular	Sakuma [22]

Donor strain (kidney)	Recipient strain	Tacrolimus dose	The mean graft survival (days)	Administration of the drug	References
Brown-Norway RT1^{n}	Lewis RT1^{l}	0.32 mg/kg	10	Oral	Jiang et al. [23]
Brown-Norway RT1^{n}	Lewis RT1^{l}	1 mg/kg	23	Oral	Jiang et al. [23]
Brown-Norway RT1^{n}	Lewis RT1^{l}	3.2 mg/kg	More than 100	Oral	Jiang et al. [23]
WKAH	Lewis RT1^{l}	5 mg/kg	68 (tacro administered 4,5,6 poTx days)	Oral	Hayakawa et al. [24]

3.3 Mycophenolate Mofetil/Sodium

3.3.1 Mechanism of Action

Mycophenolic acid is a fermentation product of Penicillium species that effectively blocks purine synthesis through allosteric inhibition of inosine monophosphate dehydrogenase. Purine nucleotides are synthetized by two pathways. The primary one is a de novo synthesis of inosinemonophosphate (IMP) from PPRP. The IMP is then used for synthesis of adenosine and guanosine by inosinemonophosphate dehydrogenase and adenosine deaminase, respectively. The second pathway for synthesis of purine nucleotides is their recyclation from guanosine and adenosine mediated by hypoxanthine guanine phosphoribosyltransferase and adenosine deaminase. Both pathways used PRPP.

The amount of PRPP in T and B lymphocytes is significantly increased after antigenic stimulation of the cells and is proven to be an important step before cell proliferation. Adding mycophenolic acid into the mixed lymphocyte reaction has effectively inhibited cell proliferation. Analyzing the cells by flow cytometry proved that lymphocyte passed through the G1 phase and stopped their mitosis in the S-phase. Another mechanism of action included depletion of GTP that may result in inhibition of G-protein based transduction signals. In immunized rats, the GTP pool significantly decreased after 4 days treatment with 20 mg/kg of MPA. MPA was shown to have a unique dual activity, both immunosuppressive and antimicrobial. Rats infected with Pneumocystis carinii did not develop pneumonia if they were treated with MPA [25].

3.3.2 Pharmacokinetics and Dynamics

Pharmacodynamics effects of MMF were tested on Lewis recipients of BN hearts after an 8- day treatment at oral doses of 5, 10 and 20 mg/kg. The AUC values after 8 days of treatment were 8.2 ± 1.5, 24 ± 2.6 and 41.2 ± 5.1 for above mentioned doses, respectively. AUC values significantly correlated with inhibition of lymphocyte proliferation and rejection grading in heart allografts. In a dose dependent manner, MPA effectively inhibited CD25, CD134, 71, CD45 positive cells, with the strongest effect at 20 mg/kg. The rejection changes were significantly worse in lower doses of MPA treatment [26]. Therapeutic window of MPA was very narrow ranging from 10 to 30 mg/kg.

3.3.3 Tolerability and Safety

Side effects and tolerability of mycophenolic acid were tested in experiments with Lewis recipients of either BN kidneys and heart or DA aorta. A dose of 40 mg/kg was not tolerated and most of the animals were terminated due to adverse events. Mycophenolate mofetil or sodium were tolerated at doses from 10 to 30 mg/kg with only minor to moderate side effects observed. The most frequent events included a decrease in red and white blood cells counts, thymic atrophy and villous atrophy in the jejunum [27, 28].

3.3.4 Prevention of Allograft Rejection

Mycophenolic mofetil/sodium was shown to dose-dependently inhibit intima thickening of aortal allograft in DA to Lewis strain combination. However, the prevention of rejection changes in aorta grafts was incomplete, perhaps due to the lower effect of MPA on intimal myocytes. 5 mg/kg of MPS was shown to be the minimal dose for effective kidney survival in BN to the Lewis model. 10 mg/kg of MPS prolonged kidney graft survival in the same model indefinitely. The same dose was not effective in kidney allotransplantation of DA to the Lewis model resulting in rejection of the grafts. MPS administered at 20 mg/kg was not tolerated in Lewis recipients of DA kidney grafts. In DA to Lewis model of heart transplantation, a dose of 10 mg/kg of MPS prolonged the time of rejection for several days, while a dose 20 mg/kg seemed to be effective with survival more than 14 days in all animals. All Lewis recipients of BN hearts showed rejection changes after treatment with 20 mg/kg of MMF. The same dose did not prevent rejection in DA to Lewis heart transplantation. 40 mg/kg were not tolerated resulting in termination of almost half of the recipients due to serious adverse events [27]. Similar results in DA to Lewis heart transplantation were ascertained by Matsumuto et al. [29]. Experimental

studies with left lung transplantations showed that MMF at a dose 30 mg/kg blocks acute lung rejection in Fischer 344 to Wistar Kyoto strain combination, but only in grafts with no or minimal rejection changes. Administration of MMF in a later phase of rejection was not successful [30]. In the liver allotransplantation of PVG (Piebald Viral Glaxo) to Lewis strain combination, 5 days treatment with 40 mg/kg of MMF, if given subcutaneously, prolonged graft survival to more than 100 days [31]. Monotherapy with 20 mg/kg of MMF has resulted in median islet survival of 12 days when a strain combination Wistar to Lewis was used. Islet survival was similar to recipient treated with cyclosporine at a dose of 5 g/kg [32].

3.4 MTOR Inhibitors

Sirolimus and everolimus are two representative drugs that belong to the mTOR inhibitor group. Sirolimus is a macrocyclic lactone drug obtained from Streptomyces hygroscopicus. Everolimus is derived from sirolimus by chemical modification and has the same mechanism of action on cellular and molecular levels.

3.4.1 Mechanism of Action

Both drugs bind to intracellular binding protein called FKBP (FK binding protein) which is the substrate for tacrolimus as well. Molecular complex sirolimus/FKBP blocks pathway different from that blocked by tacrolimus/FKBP. Sirolimus/FKBP inhibits the so-called mTOR molecule (mammalian target of rapamycin). MTOR phosphorylates S6 kinase which became activated and further phosphorylates S40 subunit of ribosome. Activation of this pathway results in increased translation of mRNA transcripts. MTOR inhibitor effectively diminished these events. Another mechanism of action includes inhibition of CD28 mediated costimulating pathway through decreased translocation of c-REL protein to nucleus and consequent inhibition of lymphokine production. Furthermore, mTOR inhibitors blocks cdk2/cyclin E and cdk4/cyclin D complexes in G1 phase of cell cycle. Sirolimus acts synergistically with both cyclosporine and tacrolimus. The synergistic effects of cyclosporine and tacrolimus are not surprising as both drugs bind to a different molecule and acts at different pathways. On the other hand, tacrolimus binds to the same kind of intracellular protein as sirolimus; in spite of that, both drugs acts syngergically. The explanation seems to be in the excess amount of FKBP12 present in cells which is sufficient for both drugs. Accordingly, at therapeutic levels, the drug did not compete for the substrate. Competition can occur only in 50–1000 molar excess of tacrolimus [33].

3.4.2 Pharmacokinetics and Dynamics

Trough levels of sirolimus, if administered in therapeutical doses, range between 5 and 30 μg/l. Sirolimus is metabolized by P45O 3A enzyme group in rat liver and intestinal cells. Metabolite products usually have less than 10 % activity of the drug. Everolimus, a drug derived from sirolimus, seems to have similar pharmacokinetic properties. In a rat model, both drugs had been proved to have similar AUC values after oral dosing of 1.5, 5 and 15 mg/kg/day (435, 1468, 7076 and 228, 1104, 4071 ng.h/mL for everolimus and rapamycin, respectively). The slightly higher levels of AUC were reported for everolimus, perhaps due to increased bioavailability. Experiments with Lewis rats showed that whole blood levels correlated with graft survival. Oral therapy with sirolimus at doses 0.3, 0.8, 2 and 6 mg/kg resulted in whole blood levels of 0.47 ± 0.04, 1.55 ± 0.16, 7.13 ± 1.2 and 12.5 ± 1.4 ng/ml in Lewis rats, respectively [34].

3.4.3 Prevention of Allograft Rejection

14 days intravenous continuous therapy of sirolimus at doses 0.01 and 0.02 mg/kg was ineffective in prolonging cardiac survival of BUF (Buffalo) hearts transplanted into WF (Wistar Furth recipients). Sirolimus at 0.04 mg/kg increased survival of heart allograft in the previous model to 14 days. In the same strain, combination sirolimus doses 0.01, 0.02 and 0.04 effectively increased survival of kidney grafts from 11 days in untreated control animals to 21, 42 and 61 days [35]. In the same strain combination, sirolimus administered orally at doses 0.3, 0.8, 2 and 6 mg/kg prolong cardiac allograft survival to 13 ± 1, 15.5 ± 0.8, 32 ± 5 and 30 ± 4 days, respectively [34]. Continuous administration of sirolimus by osmotic pump at doses 0.08, 0.32 and 0.8 mg/kg extended heterotopic cardiac survival to 354, 55 and 74 days in BUFF to WF strain combination, respectively [36]. Sirolimus given orally at doses 0.5, 1.2 and 4 mg/kg provided dose dependent prolongation of cardiac allograft function to 12, 18, 52 and 90 days in the same strain combination [37]. Survival of pancreas allograft in above mentioned strain combination was prolonged from 9 days to 14.5 ± 1.4 and 15.3 ± 2.6 after 30 days oral therapy with sirolimus at doses 0.2 and 0.4 mg/kg. The same dosing regimen in the same strain combination prolonged kidney graft survival from 7.7 ± 0.8 in untreated controls to 10 ± 2.2 and 14.4 ± 7.5 days, respectively [38]. In the lung transplantation rat model (BN to LEW), oral therapy with 2.5 mg/kg of everolimus failed to prevent graft failure [39].

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