Transplant Immunology 39 (2016) 74–83

Contents lists available at ScienceDirect

Transplant Immunology

journal homepage: www.elsevier.com/locate/trim

Lymphodepletive effects of rabbit anti-pig thymocyte globulin in neonatal swines☆

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ARTICLE INFO

Article history:
Received 10 May 2016
Received in revised form 18 August 2016
Accepted 20 August 2016
Available online 22 August 2016

Keywords:
Anti-thymocyte globulin
T-cell depletion
Swines
Transplantation
Tolerance induction
Neonates

ABSTRACT

Lymphodepletive agents play important role in different clinical applications or experimental transplant studies. In order to facilitate preclinical pediatric transplant studies, we have developed the rabbit anti-pig thymocyte globulin (pATG) and studied its effects in neonatal swines. *In vitro* assays showed that pATG can bind to lymphocytes and neutrophils in a dose-dependent manner and lyse peripheral blood mononuclear cells by apoptosis and complement-dependent cytotoxicity. *In vivo*, pATG as a monotherapy was administered at different doses (2.5, 5, 20, 40 and 80 mg/kg) in newborn pigs. Results showed that pATG induced a dose-dependent but transient T-cell depletion in peripheral blood. Lymphodepletion was also observed in lymph nodes, spleen and thymus. Pharmacokinetic studies showed dose-related cell-bound pATG on lymphocytes, as well as the presence of free pATG in the serum. Both cell-bound and free pATG levels decreased gradually after administration. Interestingly, adjuvant mycophenolate mofetil (MMF) given at 1 g/m^2/day for 1 week successfully maintained pATG-induced T-cell depletion. In conclusion, pATG administration can cause transient T-cell depletion in neonatal pigs and this effect can be maintained by MMF. Therefore, we have developed an original immunosuppressive regimen that can be used for transplantation studies in swine model.

1. Introduction

T-cell depletive agents are routinely used in solid organ transplantation (SOT) [1], hematopoietic stem cell transplantation (HSCT) [2] and vascularized composite allotransplantation (VCA) [3]. Less-toxic lymphodepletion-based conditioning regimens plus HSCT have help to induce tolerance in HLA-identical renal transplantation [4,5] or have facilitated immunosuppression (IS) withdrawal in VCA [6]. One of the most widely used T-cell depleting agents in clinical transplantation is polyclonal rabbit anti-human thymocyte globulin (Thymoglobulin), for which mechanisms of action have been reviewed [7–9]. For laboratory studies of Thymoglobulin, a surrogate rabbit anti-murine thymocyte globulin (mATG) has been generated [10]. However, Thymoglobulin's counterparts in large-animal models have never been reported.

Swine is an excellent large-animal model for transplant research, due to its similarities to human on immunogenetic profiles and body size [11,12]. Tolerance induction protocols proved to be effective in swine model can also be justified by promising clinical outcome in clinical trials [13,14]. Although nowadays protocols of tolerance induction through HSCT have achieved success in clinical renal transplantation in adult [15–19], safe and efficient tolerance induction protocol for pediatric patients has not been developed. In order to develop a preclinical model for the research of tolerance induction protocol in pediatric patients, we have successfully established a hind-limb composite tissue allograft model in newborn swines [20]; subsequently we have studied the pharmacokinetic profiles of oral cyclosporine A (CsA) [21] and mycophenolate mofetil (MMF) [22] in newborn pigs. For T-cell depletion in pigs, previous studies used total lymphoid irradiation or thymic irradiation [13,14], which may not be used in infants for pediatric tolerance induction, considering their potential risk of tumors and destructive

☆ Financial Support: This research was supported by Fondation Centaure (France); Institut Mérieux, No. 42VAL0310 (France) and the National Natural Science Foundation, No. 30901365 (China).

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http://dx.doi.org/10.1016/j.trim.2016.08.005
0966-3274/

effects on the development of childhood immune system [23,24]. Besides, the dynamics of T-cell depletion and repopulation during and after the conditioning therapy in swines have never been evaluated.

We thus developed a surrogate of Thymoglobulin for swine model: rabbit anti-pig thymocyte globulin (pATG). This present study characterized the *in vitro* and *in vivo* effects of pATG; especially, it described the T-cell dynamics of neonatal swines undergone pATG conditioning. Investigation of this process could help to define a protocol for preclinical pediatric transplant study.

2. Materials and methods

2.1. Experimental animals

Newborn (5–6 days of age) Youna domestic piglets were housed with sows as previously described [20]. For *in vitro* studies, blood samples were collected from neonates or 3–6 months' old juvenile pigs. All animal experimentations were approved by the Ethical Committee of Vetagro Sup-Veterinary Campus of Lyon (Number – 2012/1205).

2.2. pATG production

pATG was produced by Genzyme Corporation (a Sanofi company, Marcy l'Etoile, France), following the same process used for Thymoglobulin® [10]. pATG was generated by immunizing rabbits with a mixture of thymocytes from 3–6 months' old Youna domestic pigs. Thymocyte suspensions were prepared from extracted thymuses and New Zealand white rabbits were immunized twice without adjuvant. The first one is subcutaneous along spinal column and the second one, intravenous in the ear. About 30 million cells were injected per rabbit per immunization, 2 weeks apart, and terminally bled 2 weeks following second immunization. Total rabbit IgG from the resulting serum was purified with a process similar to that used for human Thymoglobulin with main steps including decomplementation, hemadsorption, UF-DF 30KDa, chromatography, Na2SO4 precipitations, Nanofiltration, UF-DF 10KDa, formulation and pasteurization. At the end, a ready-to-use liquid pATG solution containing 5 mg/ml of purified IgG was prepared. Purified polyclonal rabbit IgG (AbD Serotec, France) was used as isotype control.

2.3. In vitro pATG binding and blocking assays

In vitro pATG binding assay was performed to evaluate pATG's affinity on blood lymphocytes and neutrophils, while pATG blocking assay was used to evaluate pATG's blocking capacities to the cell surface molecules CD3ε, CD4a, and CD21a on lymphocytes [25,26].

In binding assay, 25 μl of heparinized peripheral blood was collected from non-treated neonatal or juvenile pigs, incubated with different concentrations of pATG (or control rIgG) solution at 4 °C for 30 min, then stained with goat anti-rabbit IgG (GARIG) (AbD Serotec, France). Following red blood cell lysing, cell-bound pATG (or control rIgG) on lymphocytes or neutrophils was analyzed by BD Accuri C6 flow cytometer. The quantity of binding pATG and rIgG was expressed indirectly by value of Median Fluorescence Intensity (MedFI).

In blocking assay, peripheral blood mononuclear cells (PBMCs) were isolated from neonatal or juvenile pig blood, incubated with indicated concentrations of pATG (or control rIgG) at 4 °C for 30 min. Respectively, cell surface molecule staining was performed on before- and after-incubation cells, using anti-pig CD3ε FITC, CD4a PE-Cy7 or anti-human CD21 PE at 4 °C for 30 min, and then analyzed on Accuri C6. pATG's blocking capacities (or control rIgG) were evaluated by comparing the before- and after-incubation MedFI values of each molecule. All antibodies used for flow cytometry were listed in Table 1.

Table 1
Antibody combinations used for flow cytometry staining.

Specifity	Clone name	Ig isotype	Labelling	Provider	Cat. No.
Conjugated Abs					
Pig CD3ε	PPT 3	Mouse IgG1,κ	FITC	Clinisciences	4510-02
Isotype	N/A	Mouse IgG			0107-02
Pig CD4a	74-12-4	Mouse	PE-Cy7	BD	561473
Isotype	27-35	IgG2b, κ	PE-Cy5.5	Biosciences	558304
Pig CD8a	76-2-11	Mouse	Alexa	BD	561475
Isotype	G155-178	IgG2a, κ	647	Biosciences	557715
Human CD21	B-ly4	Mouse	PE	BD	555422
Isotype	MOPC-21	IgG1,κ		Biosciences	555749
Mouse/Rat Foxp3	FJK-16s	Rat IgG2a, κ	PE	eBioscience	72-5775-40
Isotype	eBR2a				12-4321
Non-conjugated Abs					
Pig CD25	K231.3B2	Mouse		AbD Serotec	MCA1736
Isotype	N/A	IgG1			MCA928
Mouse IgG1 label kit			Alexa 647	Life Technologies	Z25008

2.4. In vitro complement mediated cytotoxicity (CDC) and apoptosis assays

CDC and apoptosis are among the most important mechanisms of ATG induced lymphodepletion [27,28].

To compare the pATG-induced CDC between neonatal and juvenile pigs, PBMCs from neonatal or juvenile pigs were incubated with pATG (or control rIgG) at 4 °C for 30 min. Corresponding serum (the same origin with PBMCs) was used as source of complement components and added into the culture, incubated for 30 min in 37 °C. Afterwards, PBMCs were collected and stained with propidium iodide (PI) (BD Biosciences) and analyzed on Accuri C6. pATG-induced CDC was evaluated by percentage of lysed PBMCs (PI^+ cells).

To analyze pATG-induced apoptosis, PBMCs (end concentration of 5×10^5 cells/ml) from neonatal or juvenile pigs were incubated with pATG (or control rIgG) at 37 °C. Cells undergoing apoptosis were detected by Annexin-V FITC (BD Biosciences) staining at 4 h (h) of culture and expressed by percentage of Annexin-V^+ cells. In order to calculate total cell death (most of them are completely lysed and "disappeared" on flow cytometer), PBMCs were thoroughly resuspended at 6 h and stained by PI, residual non-lysed PBMCs were differentiated as PI^- cells and their concentration was measured by Accuri C6. Thus, the total ATG-induced cell death was determined with the equation: % of ATG-induced cell lysis = (1- PI^- cells concentration / 5×10^5 cells/ml) × 100%.

2.5. In vivo pATG administration and follow-up schedule

As administration of pATG has never been reported in previous study, in present study pATG doses were arranged according to (i) Thymoglobulin doses administered in clinical studies [29–32] and (ii) difference in body surface area *versus* body weight between neonatal normal pigs and adult human [33]. Besides, (iii) ATG doses in experimental studies of Thymoglobulin in cynomolgus monkeys [25] and murine ATG in mice [10] were also taken into account. Thus, piglets were randomized into 7 groups according to doses of pATG administered: very low dose (VLD, 5 piglets), administered with 0.25/1/1.25 mg/kg/day on days 0/1/2 respectively; low dose (LD, 5 piglets), administered with 0.5/2/2.5 mg/kg/day on days 0/1/2 respectively; median dose (MD, 4 piglets), administered with 10 mg/kg/day on days 0/1; high dose (HD, 4 piglets), administered with 20 mg/kg/day on days 0/1; and very high dose (VHD, 4 piglets), administered with 40 mg/kg/day on days 0/1; high dose pATG plus concomitant mycophenolate mofetil (HD + MMF, 4 piglets), administered with 20 mg/kg/day of pATG on