Chapter 2

Effects of immunosuppressive drugs on purified human B cells; evidence supporting the use of MMF and rapamycin

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ABSTRACT

Humoral immunity is increasingly recognized as an important factor in the rejection of organ transplants. In general, humoral rejection is treated with standard immunosuppressive drugs. The direct effect of these immunosuppressive drugs on B cells is not well known. Purified human B cells devoid of T cells were stimulated with either CD40L expressing L cells, or by anti-CD40 mAb with or without TLR triggering, all in the presence of B cell activating cytokines. These three protocols resulted in various degrees of B cell stimulation. We added four commonly used immunosuppressive drugs (tacrolimus, cyclosporin, MPA and rapamycin) to these cultures and tested a variety of parameters of B cell activity including proliferation, apoptosis induction and both IgM and IgG production. Tacrolimus and cyclosporin marginally inhibited B cell proliferation and immunoglobulin production, and the extent of inhibition depended on the degree of the B cell stimulation. In contrast, MPA and rapamycin profoundly inhibited both B cell proliferation and immunoglobulin production, which was independent of the degree of B cell stimulation. Both drugs induced B cell apoptosis. Moreover, rapamycin caused a reduction of the number of B cells capable of producing immunoglobulins. Our data show that MPA and rapamycin are capable of strongly inhibiting B cells responses. This provides a rationale for the use of both MPA and rapamycin to prevent and/or counteract humoral responses.
INTRODUCTION

When an organ is transplanted in a major histocompatibility complex noncompatible recipient, it can elicit an immune response. To achieve long-term graft survival with a fully functional graft, this immune reactivity needs to be suppressed. To this end potent immunosuppressive drugs have been developed. The downside of these non-specific immunosuppressants is their toxicity (1), the increased susceptibility to infections (2) and an increased tumor incidence (3). Therefore, minimizing the dose of immunosuppressive drugs is pivotal.

The impact of humoral immunity towards mismatched donor antigens was obvious from hyperacute rejection, where preformed anti-HLA or ABO antibodies destroyed the graft within minutes after transplantation (4). Such preformed antibodies are induced by pregnancies, blood transfusions or previous transplants. With the practice of pre-transplant serologic crossmatching, the occurrence of hyperacute rejection has become extremely rare (5). For a long time, T cells were considered the prime contributors to acute rejection. Therefore, immunosuppressive therapies for prevention or treatment of graft rejection so far have been mainly targeted towards T cells. However, both acute and chronic rejection can be antibody-mediated as well (6).

The only B cell specific agent that is currently in clinical use is Rituximab, a B cell depleting monoclonal antibody of which varying results in transplantation have been published (7, 8). Therefore, lacking B cell specific drugs with proven efficacy in transplantation, clinicians at the present time mostly treat antibody-mediated rejections with immunosuppressive therapies that are not specifically targeting the humoral immune system.

There are indications that standard immunosuppressive drugs have effects on humoral immunity. Cyclosporin was superior to azathioprine in suppressing the development of protective antibody titers following influenza vaccination post transplantation (9). Similar studies show a profound inhibitory effect of MMF and rapamycin on the formation of influenza-specific antibodies (10, 11). These studies provide information on the reduction of antibody production, but whether the drugs studied affect B cells directly is not known. The present study has been performed to gain insight on the direct effects of currently used immunosuppressive drugs on a variety of B cell parameters including proliferation, apoptosis and immunoglobulin production.
MATERIALS AND METHODS

Cells
Blood was obtained from blood bank donors after informed consent. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll Hypaque density gradient centrifugation. B cells were immunomagnetically isolated from PBMC by positive selection using Dynabeads CD19 pan B (Dynal, Oslo, Norway). The CD19-positive cells were released from the beads using Detach-a-Bead CD19 (Dynal). This typically yielded >98% pure B cells, as assessed by staining with a FITC-conjugated CD19 mAb (BD Biosciences, Breda, the Netherlands) followed by flow cytometric analysis (FCM).

Culture conditions
B cell cultures were done in culture medium consisting of IMDM (Gibco, Paisley, UK) supplemented with 10% FCS (Gibco), 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, the Netherlands) and ITS (insulin, transferrin and selenium, Sigma-Aldrich, 1000 fold diluted). B cells were cultured with 3 different stimuli at 37°C in a 5% CO₂ humidified incubator. The first stimulus consisted of irradiated (75 Gy) L-CD40L cells with 100 U/ml IL-2 (EuroCetus, Amsterdam, the Netherlands), 25 ng/ml IL-10 (R&D systems, Minneapolis, MN, USA) and 100 ng/ml IL-21 (Invitrogen, Leek, the Netherlands). The second stimulus consisted of an anti-CD40 mAb (1 µg/ml, R&D systems) combined with IL-2, IL-10, IL-21 (concentrations as above) and 2.5 µg/ml of the TLR9 ligand CpG ODN 2006 (Hycult Biotechnology, Uden, the Netherlands). Finally, the third stimulus was identical to the second, but lacking the ODN-2006 CpG.

Immunosuppressive drugs
Tacrolimus (Prograf, Astellas, Leiderdorp, the Netherlands, diluted in absolute ethanol) and cyclosporin (Sandimmune, obtained as a solution, was from Novartis, Arnhem, the Netherlands) were used at final concentration ranges of 0-1 ng/ml and 0-100 ng/ml, respectively. We based these concentration ranges on published plasma trough levels at 6 months post-transplantation (12-14). Calcineurin inhibitor concentrations reported for whole blood are considerably higher, but it should be noted that a substantial fraction of these drugs are bound to erythrocytes (15, 16), which are not present in our cultures. Mycophenolic acid (MPA, Sigma-Aldrich, Zwijndrecht, the Netherlands), the active metabolite of MMF, was dissolved in ethanol and used in concentrations up to 100 ng/ml, which is approximately 10-fold lower than used in the clinic. This concentration range was chosen because a maximal effect was already observed using 100 ng/ml in some experiments.
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and the limitation of low cell yields in other experiments. Rapamycin (Calbiochem, La Jolla, CA, USA) was dissolved in methanol and used in concentrations up to 8 ng/ml. Working solutions of all immunosuppressive drugs were made in culture medium.

**Proliferation assay**

B cells were seeded at $5 \times 10^3$ cells per well in 96-wells round bottom plates (BD Falcon, Breda, the Netherlands) and cultured with the stimuli described above (first stimulus with $10^3$ L-CD40L cells) in the presence of increasing concentrations of immunosuppressive drugs, either added directly or after 48 h. At day 6, supernatants were collected for immunoglobulin detection and 1 µCi $^3$H-TdR (Amersham International, Amersham, United Kingdom) was added per well for the last 18 h of culture. $^3$H-TdR incorporation was measured using a liquid scintillation counter (Wallac, Turku, Finland).

**Immunoglobulin Production**

Supernatants harvested from proliferation experiments were tested for IgM and IgG levels with a standard sandwich ELISA. Plates (Greiner, Alphen a/d Rijn, the Netherlands) were coated overnight with a goat anti-IgG or anti-IgM (Jackson Immunoresearch, Westgrove, PA, USA) diluted in 10 mM Tris pH 9.0, then were blocked with 2% bovine serum albumin (BSA, Sigma-Aldrich) in 0.025% Tween-20 (Sigma-Aldrich) in PBS (PBS-T). Fifty µl of supernatants or standard human serum (Sanquin, Amsterdam, the Netherlands) in a serial dilution were incubated for 60 min at 37°C. After washing with PBS-T, biotin labeled goat anti-IgM or anti-IgG (Biosource, Camarillo, CA, USA) diluted in 1% BSA/PBS-T was incubated for 60 min at 37°C. After extensive washing, streptavidin horseradish peroxidase (Pierce, Rockford, IL, USA), diluted in 1% BSA in PBS-T was added and incubated for 60 min at 37°C. A color reaction was obtained with 4.6 mM 2,2'-azine-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Sigma-Aldrich) in a citric acid / PBS buffer at pH 4.2. The reaction was stopped with 250 mM oxalic acid (Sigma-Aldrich) and measured at OD450nm in an ELISA reader (Bio-Rad, Veenendaal, the Netherlands). Data were analyzed using Microplate Manager software version 4 (Bio-Rad). Sensitivities of the ELISA were 10 ng/ml for IgM and 5 ng/ml for IgG.

**ELISPOT assay**

To determine the number of IgM and IgG producing cells, we developed an ELISPOT assay. B cells were cultured at $10^4$ cells per well in a 96-well round bottom plate (BD Falcon) with either $10^3$ L-CD40L cells/well or anti-CD40 mAb + CpG in the presence of 100 ng/ml cyclosporin, 1 ng/ml tacrolimus, 50 ng/ml MPA or 1 ng/ml rapamycin. These concentrations
were chosen to yield sufficient numbers of cells for subsequent analysis. ELISPOT plates (Millipore, Amsterdam, the Netherlands) were prepared by coating with either goat anti-IgM or anti-IgG (Jackson Immunoresearch) in PBS and blocked with 5% FCS/IMDM. At day 6, cells were harvested, washed, counted and seeded in the ELISPOT plates at $6 \times 10^3$, $3 \times 10^3$ and $1.5 \times 10^3$ cells/well in 5% FCS/IMDM. These fixed cell concentrations were used to eliminate the potential effect of the immunosuppressive drugs on cell numbers during the first culture. A more extensive dilution series of B cells cultured without immunosuppressive drugs was included for a standard dilution curve. After 6 h of incubation at 37°C, cells were lysed with water. Following washing, the plates were incubated with biotinylated anti-IgM or anti-IgG (both from Biosource) overnight at 4°C. Plates were washed and incubated with streptavidin-conjugated alkaline phosphatase (Sigma-Aldrich) for 1 h at RT followed by incubation of BCIP-NBT (Mabtech, Nacka Strand, Sweden) for 5 min. The reaction was stopped by rinsing with water. Plates were analyzed using a computer guided ELISPOT reader (Bioreader 3000, Bio-Sys GmbH, Karben, Germany). The cell number selected for analysis of drug effects was in the log phase of the standard dilution curve.

**Apoptosis Assay**

For determination of the percentage of apoptotic cells after culture, Propidium Iodide (PI) and Annexin-V-FITC (VPS Diagnostics, Hoeven, the Netherlands) were used following manufacturers’ descriptions. In brief, CD19+ cells were cultured at $5 \times 10^5$ cells per well in 24-well plates (Costar, Veenendaal, the Netherlands) with either $5 \times 10^4$ L-CD40L cells or anti-CD40 mAb + CpG, both in the presence of immunosuppressive drugs. After 3 days, cells were harvested and resuspended at $3 \times 10^5$ cells/ml. Annexin-V-FITC and PI were added and incubated for 10 min on ice. Cells were acquired using a FACS Calibur (BD Biosciences) and analyzed using CellQuest Pro software (BD Biosciences).

**Toxicity assay**

To test whether the immunosuppressive drugs were toxic to B cells, a lactate dehydrogenase (LDH) activity assay (Roche Diagnostics GmbH, Mannheim, Germany) was performed according to the manufacturer’s instructions. Briefly, $2 \times 10^5$ B cells were cultured in 96-well round bottom plates in the presence of the immunosuppressive drugs in concentrations used in the proliferation assays. The culture was done in RPMI 1640 (Gibco) supplemented with 5% human serum (Sanquin Blood Supply, Leiden, the Netherlands) and 0.05 mM 2-mercaptoethanol. After 16 h incubation, cells were spun and 100 µl of cell-free supernatants were incubated with 100 µl of the kit’s substrate for 30 min. The reaction was terminated with 1N HCl. Plates were read on the ELISA reader, and results were
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expressed as $\text{OD}_{490\text{nm}}$ minus $\text{OD}_{620\text{nm}}$.

Statistics

The paired T test was used for the analysis of the effects of single doses of immunosuppressive drugs on proliferation, immunoglobulin production and the number of immunoglobulin producing cells. Statistical level of significance was defined as $P<0.05$.

RESULTS

Effects of the different stimuli on purified B cells

We tested if CD40 ligation in combination with B cell activating cytokines (17) was sufficient for activating human B cells. In mice, B cell receptor signaling is not required when antigen specific T cell help is provided (18). Similarly, we observed that CD40 ligation using either anti-CD40 mAb or L-CD40L cells in combination with cytokines is sufficient to obtain proliferative, immunoglobulin producing B cells (Figure 1). As CpG was shown to have an important role in B cell activation (19, 20), we also developed a culture condition including the TLR9 ligand CpG ODN 2006 (Figure 1). B cells cultured with L-CD40L cells proliferated vigorously and produced large quantities of both IgM and IgG. Culturing with

![Stimuli:](image)

(a) L-CD40L cells, IL-2, IL-10, IL21

(b) anti-CD40 mAb, CpG, IL-2, IL-10, IL-21

3. anti-CD40 mAb, IL-2, IL-10, IL21

Figure 1. Proliferation, IgM and IgG production induced in three culture conditions. (a) Purified B cells were cultured for 7 days and $^{3}H\text{TdR}$ was added for the last 18 h for proliferation assessment. (b) Supernatants collected at day 6 were tested for the presence of IgM and IgG with ELISA. CPM: counts per minute.
anti-CD40 mAb + CpG resulted in intermediate levels of proliferation and immunoglobulin production. Stimulation with anti-CD40 mAb resulted in low B cell proliferation and immunoglobulin production. The latter stimulus was not used in some subsequent assays because of low cell yield. There was no correlation between the degree of responsiveness of individual donors and their susceptibility to the action of immunosuppressive drugs (data not shown).

**Figure 2.** Immunosuppressive drugs are not toxic and have differential effects on the proliferation of B cells. (a) The LDH activity was measured in supernatants of B cells that were incubated with serial dilutions of immunosuppressive drugs. The effects of the highest concentrations of drugs are shown of a representative experiment (n=3). (b) Purified B cells were stimulated with L-CD40L cells in the presence of graded concentrations of immunosuppressive drugs. The percentage of proliferation relative to the addition of no immunosuppressive drugs is shown. *p<0.05; **p<0.01; ***p<0.001 and ****p<0.0001. Horizontal bars indicate mean values. (c) Proliferation of anti-CD40 mAb + CpG stimulated B cells cultured with immunosuppressive drugs, legend as figure 2b. (d) Effect of immunosuppressive drugs on anti-CD40 mAb stimulated B cells, legend as figure 2b.
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**Effects of immunosuppressive drugs on B cell proliferation**
The immunosuppressive drugs were not toxic to B cells in the concentrations used, as tested by an LDH release assay (Figure 2a). Solvents of the immunosuppressive drugs did not affect B cells, warranting that effects observed are solely caused by the immunosuppressive drugs (data not shown).

Tacrolimus in concentrations up to 1 ng/ml had minimal effects on the proliferation of B cells regardless of the stimulus (Figure 2b-d). It was previously shown that Staphylococcus aureus induced B cell proliferation was susceptible to high doses of tacrolimus (21). We therefore tested tacrolimus up to 16 ng/ml and found that high concentrations did not result in a stronger inhibition (data not shown).

Cyclosporin had no effect on proliferation when B cells were cultured with L-CD40L cells (Figure 2b). However, we observed a moderate inhibition of proliferation with B cells stimulated with anti-CD40 mAb + CpG (Figure 2c). Cyclosporin strongly inhibited the proliferation of anti-CD40 stimulated B cells (Figure 2d). No effect of either tacrolimus or cyclosporin on proliferation was observed when the drugs were added 48 h after culture initiation (data not shown). This is in concordance with the mechanism of action of calcineurin inhibitors on T cells, which is early in activation (22).

In contrast with the calcineurin inhibitors, both MPA and rapamycin were extremely potent inhibitors of B cell proliferation. MPA as well as rapamycin almost completely inhibited proliferation of B cells, regardless of the stimulus (Figure 2b-d). The inhibition was dose-dependent. Moreover, MPA or rapamycin were still effective when added 48 h after stimulation (data not shown).

**MPA and Rapamycin induce apoptosis in B cells**
A possible explanation for the inhibition of proliferation is the induction of apoptosis. To test this, B cells were cultured for 3 days with L-CD40L cells or anti-CD40 mAb + CpG in the presence of graded concentrations of immunosuppressive drugs after which B cell apoptosis was determined by Annexin-V and PI staining followed by FCM analysis (exemplified in Figure 3a). Tacrolimus and cyclosporin did not induce higher levels of apoptosis in the B cells when compared to controls. In contrast, addition of both MPA and rapamycin caused an increase of the percentage of apoptosis and this effect was dose dependent (Figure 3b).

**Effects of immunosuppressive drugs on immunoglobulin production**
Tacrolimus was marginally effective in inhibiting the production of both IgM and IgG with all culture conditions (Figure 4a-f). The degree of inhibition was dependent on the
strength of the B cell stimulation. Cyclosporin inhibited IgG production in all stimulatory conditions, and IgM in all except the most potent (L-CD40L) conditions (Figure 4a-f).

**Figure 3.** Apoptosis induction by immunosuppressive drugs. B cells were stimulated with either anti-CD40 mAb + CpG or L-CD40L in the presence of two concentrations immunosuppressive drugs. After 3 days cells were stained with Annexin-V and PI and analyzed by flow cytometry. (a) Effect of immunosuppressive drugs on the percentage of apoptotic cells stimulated with anti-CD40 + CpG. Depicted are dot plots of B cells treated with the highest concentration immunosuppressive drugs. The lower left quadrant are live cells, the lower right quadrant are early apoptotic cells, the upper right quadrant are late apoptotic cells and the upper left quadrant represents dead cells. (b) Percentage of apoptotic cells stimulated with anti-CD40 mAb + CpG (left panel) or L-CD40L (right panel) in the presence of two concentrations immunosuppressive drugs. Data from a representative experiment are shown.

Effects on immunoglobulin production of anti-CD40 mAb stimulated B cells were profound. Concurrent with the effects on proliferation, both tacrolimus and cyclosporin added 48 h after culture initiation failed to inhibit IgM as well as IgG production (Figure 5). In line with proliferation inhibition, both MPA and rapamycin were very potent in inhibiting immunoglobulin production. Addition of these immunosuppressive drugs led to
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a virtually complete inhibition of IgM and IgG production in B cells cultured with any of the culture conditions (Figure 4a-f). These effects on immunoglobulin production were dose dependent. Addition of MPA and rapamycin 48 h after stimulation was equally effective in inhibiting IgM and IgG production compared to direct addition to the cultures (Figure 5).

Rapamycin decreases the number of immunoglobulin producing cells

To test whether the decrease in immunoglobulin production by the various immunosuppressive drugs was solely due to lower cell numbers caused by proliferation inhibition and/or induction of apoptosis, we developed an ELISPOT assay in which we enumerated the number of IgM and IgG producing cells. We normalized the effects on B cell division and apoptosis by adding fixed living cell numbers into ELISPOT plates, and therefore the effects of immunosuppressive drugs on the percentage of immunoglobulin producing cells reflected the effects within the live cell population only. Tacrolimus, cyclosporin and MPA did not decrease the number of immunoglobulin producing cells (data not shown and Figure 6). MPA marginally inhibited the number of IgG producing cells when B cells were stimulated with anti-CD40 mAb + CpG, but this was not dose-dependent. In contrast, rapamycin significantly and dose dependently decreased the number of IgM and, to a lesser extent, IgG producing B cells with either L-CD40L cells or anti-CD40 mAb + CpG stimulus (Figure 6).

DISCUSSION

The impact of humoral immunity on transplant outcome is increasingly recognized, as antibodies are associated with various types of rejection. The development of HLA antibodies was shown to precede all chronic rejections of kidney transplants (23). Moreover, post-transplant donor HLA-specific antibodies were described to have a strong predictive value for transplant outcome (24). Peritubular capillary (PTC) deposition of C4d was associated with circulating antibodies and both chronic and acute rejections (25, 26). This deposition was shown to be a predictor of inferior 12-month graft function (27). In as many as 60% of all patients with chronic rejection a positive PTC staining for C4d was observed (25).

A substantial number of in vitro studies have been performed on effects of immunosuppressive drugs on B cells with either tonsillar or peripheral blood B cells stimulated with a variety of agents (28-32). Diversity in study parameters makes comparison of results difficult. To our knowledge, this is the first study comparing the effects of four different immunosuppressive
Figure 4. Effects of immunosuppressive drugs on immunoglobulin production. Supernatants from 6-day B cell cultures with immunosuppressive drugs were examined for the amount of IgM and IgG produced. (a-c) Percentage of IgM production with graded concentrations immunosuppressive drugs relative to the addition of no immunosuppressive drugs for B cells stimulated with L-CD40L cells, anti-CD40 mAb + CpG and anti-CD40 mAb, respectively. *p<0.05; **p<0.01; ***p<0.001 and ****p<0.0001, horizontal bars indicate mean values. (d-f) Percentage of IgG production of L-CD40L cell, anti-CD40 mAb + CpG and anti-CD40 mAb stimulated B cells, respectively, treated with immunosuppressive drugs. Legend as figure 4a-c.
drugs on purified B cells in a well-defined experimental system. We made use of three methods to stimulate B cells into proliferating, immunoglobulin producing cells. In this manner, we were able to investigate whether the effect of immunosuppressive drugs depended on the strength and type of stimulus. These stimuli are polyclonal and can therefore not be extrapolated to antigen-specific humoral immune responses.

Calcineurin inhibitors are included in most immunosuppressive regimens, often combined with steroids. As a major proportion of chronic rejections in patients receiving Cyclosporin-based immunosuppression appears to be antibody-mediated, this drug clearly is not able to prevent humoral rejection (25). We tested the effect of both tacrolimus and cyclosporin on B cell responses. We have taken the binding of calcineurin inhibitors to erythrocytes into consideration for selecting drug concentrations to be tested. Our in vitro data support cyclosporin’s relative ineffectiveness in directly inhibiting B cell responses. Likewise, tacrolimus had only marginal effects on B cell parameters. While multiple reports showed that in case of acute or chronic humoral rejection rescue therapy with tacrolimus and MMF was effective in reverting the rejection episodes (33-35), our data suggest that MMF

Figure 5. Calcineurin inhibitors are not effective in inhibiting IgM and IgG production when added 48 h after the start of the stimulation whereas MPA and rapamycin are still effective. Shown are effects of the highest concentrations of immunosuppressive drugs added to anti-CD40 mAb + CpG stimulated B cells. The percentage of inhibition relative to no addition of immunosuppressive drugs is depicted.
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is likely to be the main drug responsible for the effect. In a multi-center study, MMF in combination with cyclosporin resulted in significantly lower frequencies of HLA antibodies as compared with azathioprine and cyclosporin treatment (36). Moreover, MMF was described to be very effective in inhibiting primary antigen-specific antibody responses in renal transplant patients (37).

In our experiments, MPA was extremely potent in inhibiting both proliferation and immunoglobulin production. Moreover, these effects persisted when MPA was added to already activated B cells, implying that an ongoing B cell response may be dampened by MPA, while calcineurin inhibitors are ineffective. MPA levels used are lower than levels that
are usually achieved physiologically. In the experiments describing proliferation and total IgG and IgM levels in the supernatant of B cell cultures, we found in preliminary experiments already a complete inhibition when using these suboptimal MPA concentrations. Any higher dose would not result in a dose-dependent effect and would therefore raise the question whether the effect would be biologically relevant. In subsequent experiments such as the apoptosis and ELISPOT tests, we needed sufficient numbers of cells to analyze. Since MPA profoundly induced apoptosis already at 100 ng/ml, we were not able to perform these tests with higher concentrations than 50 ng/ml. Therefore, the effects observed might therefore even be an underestimation of the suppression of B cell function in vivo. Furthermore, an effect of MPA, in clinically relevant concentrations, on the number of immunoglobulin producing cells cannot be excluded.

Rapamycin is generally not included in maintenance immunosuppressive protocols from the day of transplantation onwards because it causes delayed graft function (38), increases surgical complications and impairs wound healing (39). However, rapamycin, like MMF, was described to be extremely potent in inhibiting humoral responses. For example, in vivo data comparing cyclosporin in combination with either azathioprine, MMF or rapamycin on the formation of antibodies against equine antithymocyte globulin after renal transplantation show that both MMF and rapamycin are capable of reducing anti-equine antibody formation (40). In our in vitro experiments rapamycin was the most effective drug tested, as it inhibited not only B cell proliferation and immunoglobulin production, but also inhibited the number of immunoglobulin producing cells. None of the other drugs tested were capable of decreasing the number of immunoglobulin producing cells.

An important effector mechanism of immunosuppressive drugs is apoptosis induction. We found that MPA and rapamycin induced B cell apoptosis. Since rapamycin induced apoptosis in dendritic cells as well, it is likely that this is a general effect of rapamycin (41). Since we stimulated the B cells polyclonally, we were not able to identify specific populations of B cells susceptible or resistant to apoptosis induction.

To get more insight in the possible mechanism of action on B cells, we performed cytokine production analyses on supernatants of B cells cultured in the presence of immunosuppressive drugs, but no correlations with the outcome of the proliferation and immunoglobulin data were found (data not shown). The antigen presenting function of B cells may play an important role in the process of allograft rejection (42). We therefore performed phenotypic analysis of B cells stimulated with anti-CD40 mAb and CpG treated with immunosuppressive drugs, but observed no alterations in the expression levels of HLA-DR, CD80 and CD86 (data not shown), suggesting that antigen presentation is not altered. However, studies using antigen specific interactions between B cells and T cells
are needed to address the influence of immunosuppression on the APC function of B cells in more detail.

Besides the effects of rapamycin described here, there are additional effects that would argue in favor of the use of rapamycin. Data on favorable effects of rapamycin on regulatory T cell subsets are emerging. A selective survival of both CD4⁺CD25⁺Foxp3⁺ as well as CD103⁺CD8⁺ regulatory T cells was recently described (43, 44). These effects are not observed for other immunosuppressive drugs such as cyclosporin (45, 46). Furthermore, rapamycin has been described to inhibit angiogenesis and might therefore be anti-tumorigenic (47).

In the present study, the effects of immunosuppressive drugs on T cell help are not taken into account. Whereas the prime target of calcineurin inhibitors is the T cell, the effect of calcineurin inhibitors might be underestimated. However, baseline calcineurin inhibitor therapy appears not to be very effective to prevent humoral rejection (25). To get more insight into this, studies on the in vitro effects on T cell dependent B cell responses are currently performed in our laboratory.

Many clinical studies are currently focused on drug tapering and withdrawal. Tapering or sparing of calcineurin inhibitors is widely performed to minimize their side effects (reviewed in (48)). Our data suggest that tapering of calcineurin inhibitors will most likely not increase the risk of humoral rejection.

Taken together, evaluation of highly purified, polyclonally stimulated B cells shows that MPA and rapamycin were superior in inhibiting B cell functionality in vitro, which underscores the clinical usefulness of these drugs for respectively prevention and treatment of humoral rejection. However, to predict accurately which immunosuppressive regimen will be optimal for treating humoral rejections, in vitro studies with combinations of immunosuppressive drugs are essential. Studies of this kind are currently underway in our laboratory, and are intended to define a combination regimen that can be preferentially used to treat humoral rejection.

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