Adoptively Transferable Tolerance Induced by CD45RB Monoclonal Antibody

ZUHUA GAO, ROBERT ZHONG, JIFU JIANG, BERTHA GARCIA, JING JING XING, MARTIN J. WHITE, and ANDREW I. LAZAROVITS

London Health Sciences Centre, University Campus, John P. Roberts Research Institute and Departments of Medicine, Surgery, Pathology, and Microbiology and Immunology, University of Western Ontario, London, Ontario, Canada.

Abstract. The phenomenon of rejection remains the most serious problem in transplantation. The ultimate goal in transplant immunology is to develop therapeutic strategies that lead to tolerance. It has been shown that two injections of a monoclonal antibody to CD45RB leads to indefinite acceptance of renal allografts in mice. Moreover, the CD45RB monoclonal antibody reverses acute rejection and still induces tolerance. The purpose of this study was to assess mechanisms that could underlie this therapeutic benefit. It was shown that splenic lymphocytes from tolerant animals augmented proliferation in allogeneic mixed lymphocyte reactions against donor alloantigens, and the serum of tolerant mice contained donor-specific antibodies, mainly of the IgG1 isotype, suggesting the presence of TH2 cytokines. Tolerance could not be broken by interleukin-2 infusion, but tolerance could be adoptively transferred by transfusion of tolerant mouse CD4+ splenic lymphocytes into naive allografted animals. These data suggest that an active immunoregulatory mechanism is partly responsible for the therapeutic effect. CD45RB-directed therapy may find clinical application in organ transplantation in human patients.

The phenomenon of rejection remains the most serious problem in the field of transplantation (1). The ultimate goal in transplant immunology is to develop immunosuppressive strategies that promote indefinite acceptance of a transplanted organ in the absence of continued therapy, a state known as tolerance. The currently used forms of immunosuppression lead to a widespread nonspecific depression that broadly affects all immunologic and inflammatory responses. Rejection is largely orchestrated by T cell-mediated immunity; therefore, various strategies have been devised to aim immunosuppressive therapy primarily against this arm of the immune system (2). Antibodies, both polyclonal and monoclonal, represent an approach to this specific targeting, but many preparations have important side effects (3–6) and treatment with these antibodies has not been entirely successful (3–21). Current treatment strategies thus fall far short of our goal of achieving tolerance.

To identify novel T cell surface structures that may be important in specifically preventing rejection, we reviewed what is known about the binding specificities of polyclonal antilymphocyte globulin (ALG). Although there is measurable reactivity with many CD antigens, the target most strongly presented has been termed the leukocyte common antigen (22–26). Warr and Marchalonis (24) also observed that the major reactivity of ALG involves a series of high-molecular weight glycoproteins that we now recognize as being CD45. Motivated by these observations, we designed experiments to assess a possible immunosuppressive role for CD45. These studies demonstrated that a monoclonal antibody (mAb) directed against the RB isoform of CD45 substantially inhibits the alloreactivity of human CD4+ lymphocytes in vitro, suggesting a possible therapeutic role (27).

CD45 plays a crucial role in tyrosine phosphorylation events. Persuasive evidence includes reports of structural homology of the cytoplasmic domain of CD45 with the placental protein tyrosine phosphatase, the demonstration that CD45 has protein tyrosine phosphatase activity, and the finding that CD45 regulates the phosphorylation and activation of Src-family protein tyrosine kinases (28–31). CD45 is a member of a diverse family of intracellular and transmembrane enzymes that have been shown to play important roles in the activity of drugs such as cyclosporine and tacrolimus (32). Several isoforms of CD45 are produced by alternate splicing of the fourth, fifth, and sixth exons (also known as A, B, and C). The exons generate extracellular domains that differ in size, but all forms bear the same cytoplasmic domains, which are protein tyrosine phosphatases (31).

CD45 is expressed at high density on hematopoietic cells and generally is immunoprecipitated as four bands (220, 205, 190, and 180 kD). On the other hand, the restricted CD45 cluster of mAb (termed CD45R) recognizes various combinations of the four bands on subsets of T cells, B cells, and monocytes. CD45RA mAb recognizes the 220- and 205-kD bands, CD45RB primarily the 190-kD band, CD45RC the 220-, 205-, and 190-kD bands, and CD45RO only the 180-kD band (31,33,34).

Certain CD45 mAb demonstrate important functional char-
acteristics \textit{in vitro}, including inhibition of natural killer cell function (35), B cell activation (36), allospecific cytotoxicity (37), lectin-dependent cytotoxicity (37), autologous mixed lymphocyte reaction (MLR) generation of suppressor/inducer cells (38), neutrophil chemotaxis (39), and phytohemagglutinin-induced T cell proliferation (40). An absence of CD45 predicts an inability to proliferate in an antigen-specific manner (41).

The particular CD45 isoform expressed by T lymphocytes is important in thymic selection and development. Thymic cells that are destined for intrathymic destruction bear CD45RO, whereas the generative thymic lineage expresses CD45RA (42). Evidence has been presented that CD4$^+$ CD45RA$^+$ T cells are the suppressor-inducer subset, whereas CD4$^+$ CD45RA$^-$ T cells are the helper-inducer subset (43,44). Others have suggested that CD45RA$^+$ CD45RO$^-$ T cells are antigen inexperienced (naïve), whereas those that are CD45RA$^-$ CD45RO$^+$ are antigen-experienced (memory) (45–47). This is likely an oversimplification, because some reports have shown that activated T cells retain CD45RA and there may be interconversion between CD45RA$^-$ and CD45RA$^+$ T cells (48–51).

There are data that support the notion that a natural ligand for CD45 is CD22 (52,53). This has not been firmly established, however (54,55). CD45RB has been noted to be rapidly downmodulated on mouse T cells after activation (56). In human lymphoid tissue sections, CD45RB is differentially expressed on T and B cell subpopulations (57). On the basis of the intensity of CD45RB expression, CD4$^+$ T cells in mice can be divided into CD45RB$^{\text{high}}$ and CD45RB$^{\text{low}}$ populations. CD4$^+$ CD45RB$^{\text{high}}$ T cells produce interferon-γ (IFN-γ), whereas CD4$^+$ CD45RB$^{\text{low}}$ T cells produce interleukin-4 (IL-4), suggesting that the former are TH1-type T cells, whereas the latter are TH2-type T cells (58). TH1-type cytokines are often associated with transplant rejection, whereas TH2-type cytokines are often associated with the immunologic quiescence of transplants (59).

We reported that therapy directed toward CD45RB prevents and reverses renal allograft rejection in mice (60). This treatment induced indefinite renal allograft acceptance and subsequent tolerance to skin transplants in a subset of animals. In addition, CD45RB mAb could reverse acute rejection and still lead to tolerance. The mechanisms of this therapeutic benefit are not known. In this study, we demonstrate that tolerance can be adoptively transferred to a fully immunocompetent, naïve mouse by transfer of the CD4$^+$ subset from a tolerant mouse spleen, indicating that active immunoregulation may be involved.

Materials and Methods

Animals

Male inbred C57BL/6 (H2b) and BALB/c (H2k) mice (weight, 25 to 30 g) were supplied by Harlan Sprague Dawley (Indianapolis, IN) and housed under conventional conditions at the Animal Care Facility at the University of Western Ontario. Animals were cared for in accordance with the institutional guidelines for experimental animals.

Kidney Transplantation

Kidney transplantation from C57BL/6 to BALB/c mice was performed as described previously (61). The left donor kidney, attached to a segment of the aorta and the renal vein, and the ureter and bladder patch were removed en bloc. The two native kidneys of the recipient mice were removed before transplantation. The donor aorta and inferior vena cava were then anastomosed (end to side) to the abdominal aorta and inferior vena cava, respectively, of the recipient, below the level of the native renal vessels. Donor and recipient bladders were anastomosed dome to dome.

Kidney Function

After transplantation, serum creatinine levels were measured. The urine output and general physical condition of the animals were also monitored for any evidence of rejection.

Pathologic Analysis

Kidney grafts were collected when mice were euthanized at various times; the grafts were fixed in 10% buffered formaldehyde, embedded in paraffin, and then stained with hematoxylin and eosin. The microscopic sections were examined for evaluation of rejection by a pathologist (Dr. Garcia) blinded to the study.

Induction of Tolerance

Kidney-recipient mice were treated with purified CD45RB mAb MB23G2 (American Type Culture Collection, Rockville, MD), at 3 mg/kg per d, from day 0 to day 7 after surgery. No other immunosuppressive treatment was administered. Kidney-recipient mice without immunosuppression served as controls.

Proliferation Assays

Spleen cells from five normal and five tolerant mice were obtained 100 d after surgery, and spleen cells from five acutely rejecting mice were obtained 10 d after surgery. The spleen cells were stimulated with concanavalin A (con A) (Sigma Chemical Co., St. Louis, MO) at 5 µg/ml for 3 d, in complete medium consisting of RPMI 1640 (Life Technologies, Grand Island, NY), 10% fetal calf serum (Life Technologies), and 1% penicillin/streptomycin (Life Technologies), at 37°C in a humidified atmosphere containing 5% CO$_2$. The cells (1 × 10$^6$/ml) were cultured in triplicate in 200 µl of medium, in 96-well plates (Nunc, Roskilde, Denmark) for 3 d. The cells were pulsed with 1 µCi of [3H]thymidine for the final 8 h and collected with a Tomtec cell harvester (Orange, CA), and radioactivity was measured with a Wallac 1450 Microbeta counter (Turka, Finland). Allogeneic MLR were performed using BALB/c cells against C57BL/6 or CBA spleen cells that had been treated with 3000 rad of gamma irradiation. The responder cells were cultured at 1 × 10$^6$/ml and the stimulators at 5 × 10$^4$/ml, in 200-µl volumes, in triplicate wells in 96-well plates. The grafts were fixed in 10% buffered formaldehyde, embedded in paraffin, and then stained with hematoxylin and eosin. The microscopic sections were examined for evaluation of rejection by a pathologist (Dr. Garcia) blinded to the study.

Donor-Specific Antibody Measurement by Flow Cytometry

Donor-specific antibody IgG, IgM, and IgG subtype levels were measured, as described by Onodera et al. (63), in five normal mice, five untreated mice undergoing acute rejection, and five tolerant mice. Briefly, target cells (1 × 10$^6$) from C57BL/6 mouse thymus were prepared in phosphate-buffered saline containing 0.25% bovine serum.
albunion and 0.02% azide and were then incubated with serially diluted (1/5 to 1/125), heat-inactivated serum samples for 30 min at 4°C. The cells were then washed and stained with FITC-conjugated goat antibody specific for mouse IgG Fc fragment (Jackson Immunoresearch Laboratories, West Grove, PA), mouse IgG1, IgG2a, IgG2b, and IgG3 subtypes (Cedarlane, Hornby, Ontario, Canada), and phycoerythrin-conjugated goat antibody specific for mouse μ chain (Jackson Immunoresearch). After staining, the cells were washed twice and analyzed using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). The intensity of staining was expressed as the mean fluorescence channel that correlated with donor-specific antibody levels.

Cytokine Measurement by Enzyme-Linked Immunosorbent Assay

Supernatants from MLR were collected, IFN-γ and IL-4 levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (PerSeptive Diagnostics, Framingham, MA), according to the instructions provided by the manufacturer.

Attempts to Break Tolerance and Prevent Its Induction

At 100 d after surgery, the tolerant mice were injected with 6000 IU of IL-2 (a gift from Novartis, Montreal, Quebec, Canada) administered intraperitoneally or 1 × 10^8 naive spleen cells administered intravenously. To attempt to develop the tolerance of three mice were given transplants and treated with CD45RB mAb but at the same time were treated with IL-2 (1000 IU, intraperitoneally) for 7 d. Mice were monitored for another 30 d, or until rejection occurred, before being euthanized.

Purification of Lymphocyte Subpopulations

Purified B lymphocytes and CD4^+ and CD8^+ T lymphocytes were obtained by positive selection using Dynal beads (Dynal, Oslo, Norway), according to the instructions provided by the manufacturer. Briefly, M-450 magnetic beads coated with rat IgM mAb specific for B220 (pan-B), L3T4 (CD4), and Lyt-2 (CD8) membrane antigens were incubated with tolerant mouse spleen cells, at a concentration of 10^7 Dynabeads/ml and a ratio of five beads/target cell, at 4°C for 30 min. Rosetted cells were then isolated by placing the test tubes in a Dynal magnet for 3 min. Supernatants were removed while the rosetted cells were kept on the walls of the test tubes with the Dynal magnet. After six washes with buffer (phosphate-buffered saline, pH 7.4, with 0.1% bovine serum albumin), the rosetted cells were incubated for 45 min at room temperature with either Dynal Detachabead (for CD4^+ T lymphocytes) or a solution containing 2 parts fetal calf serum, 2 parts ethylenediaminetetra-acetate, and 1 part RPMI 1640 (for CD8^+ and B lymphocytes), to detach them from the Dynabeads, as described (64). Cell purity was tested by staining with CD3, CD4, CD8, and CD19 mAb (Jackson Immunoresearch Laboratories) and flow cytometric analysis. CD4^+ populations were >95% pure, whereas CD8^+ populations were contaminated with <5% CD4^+ cells. CD19^+ populations were >90% pure.

Transfer of Tolerance

Spleen cells (4 × 10^7), purified B lymphocytes (1 × 10^7), purified CD4^+ T lymphocytes (1 × 10^7), or purified CD8^+ T lymphocytes (1 × 10^7) from tolerant mouse spleens obtained 100 d after surgery were infused intravenously into naive mice. Kidney transplantation was performed within 1 wk after the adoptive transfer, using the same donor strain. No additional immunosuppressive treatment was administered. Recipient mice were observed for 60 d, or until rejection occurred, before euthanization.

Statistical Analyses

Data are reported as mean ± SEM. Survival data were compared using the log-rank test. MLR and antibody data were analyzed using the t test. Differences with a P value of <0.05 were considered significant.

Results

Association of Tolerance with an Increased Proliferative Response to Donor Alloantigens But Not to Third-Party Alloantigens

As shown in Figure 1, spleen cells from normal mice, >100-d tolerant mice, and untreated, acutely rejecting mice (10 d after surgery) were studied for proliferative responses induced by the lectin con A and by alloantigens. There was no evidence of spontaneous blastogenesis in the tolerant mice, and proliferative responses to con A were normal. However, proliferation in the kidney donor-specific allogeneic MLR was significantly greater (P < 0.05) than was proliferation in the third-party allogeneic MLR, and proliferation in the tolerant mouse allogeneic MLR with donor-specific antigens was significantly greater than was the proliferation of normal mouse spleen cells in response to C57BL/6 alloantigens (P < 0.01). Normal mice showed no differences in proliferative responses to the two sets of alloantigens. In contrast, spleen cells from mice undergoing acute rejection on day 10 demonstrated donor-specific proliferation that was significantly greater than that with third-party cells and was significantly less than that of cells from tolerant animals in the donor-specific MLR (P < 0.01).

Donor-Specific Antibody Responses in Tolerant Mice

Donor-specific antibodies were measured by flow cytometric analysis. As shown in Figure 2, tolerant mice had significantly more donor (C57BL/6)-specific IgG in their serum than did normal mice (P < 0.01). There was no difference in donor-specific IgM levels between the tolerant and normal mice, but rejecting mice exhibited high IgM levels. Interestingly, most of the donor-specific IgG in the tolerant animals was of the IgG1 subtype. There was no statistically significant difference between the tolerant and normal mice with respect to other IgG subtypes. In contrast, control mice with acute rejection demonstrated a significant increase in donor-specific IgG2a (P < 0.01).

Cytokines Expressed in MLR

Tolerant mice would be expected to demonstrate abundant TH2-type cytokines (such as IL-4) (59). IL-4 would preferentially drive B cells to produce IgG1, as opposed to IgG2a (which is seen in rejection). We therefore assessed cytokine production in the MLR using ELISA. As shown in Figure 3, levels of >15 pg/ml IL-4 were observed for the tolerant mice. These data are consistent with our recent observations obtained in an islet transplant model (65).
Figure 1. Association of tolerance with an increased proliferative response to donor alloantigens but not third-party alloantigens. Spleen cells were studied for proliferative responses to autoantigens (tissue culture medium [TCM] alone), concanavalin A, and kidney donor-specific (C57BL/6) and third-party donor-specific (CBA) alloantigens, as described in Materials and Methods. Each histogram represents the mean ± SEM of five separate experiments. *P < 0.01; **P < 0.05.

Figure 2. Detection of donor-specific IgG of the IgG1 subtype in tolerant mice. Serum samples from tolerant mice, normal mice, and control mice undergoing acute rejection were evaluated for anti-C57BL/6 antibody responses (donor) by flow cytometric analysis, as described in Materials and Methods. Each histogram represents the mean ± SEM of five separate experiments. *P < 0.01; **P < 0.05.
Lack of Ability of the Cytokine IL-2 or Naive Spleen Cells to Overcome Tolerance

Table 1 demonstrates that infusion of 6000 IU of IL-2 failed to trigger acute rejection of tolerant kidneys. IL-2 administered daily at the time of induction of tolerance by CD45RB mAb also failed to prevent the development of tolerance. Furthermore, the infusion of 10^8 naive spleen cells into tolerant mice failed to break tolerance in animals who had retained their kidneys for 100 d. The animals in both groups were monitored for 30 d after the attempts to break tolerance. When the kidneys were removed, there was no evidence of rejection.

Adoptive Transfer of Tolerance Induced by CD45RB mAb

Table 2 demonstrates the effects of infusion of unpurified spleen cells, CD4^+ T cells, CD8^+ T cells, or B cells into naive animals 1 wk before transplantation of C57BL/6 renal allografts. Spleen cells and purified CD4^+ T cells induced a robust state of tolerance in the untreated animals. Adoptive transfer of B cells was without therapeutic effect. Two of three CD8^+ T cell-transfused animals promptly lost their renal allografts, whereas one animal retained its kidney for 56 d, at which time it succumbed to rejection.

Discussion

Immunosuppression directed toward CD45RB is a new method for induction of transplant tolerance. The underlying mechanisms of this beneficial effect remain to be identified. CD45RB mAb may interfere with T cell signal transduction, because CD45 is the critical protein tyrosine phosphatase that activates the protein tyrosine kinases p56lk and p59fyn (31). This interference may occur because of prevention of aggregation of T cell receptor (TCR) components by a sterically hindering mAb. Components of TCR that could be affected include the CD3 polypeptides and CD7. These molecules have been targeted successfully to prevent acute rejection (9,19), and we have shown the physical coassociation of these molecules (66). A consequence of this altered signaling mediated through the CD3/TCR-CD7-CD45 oligomeric complex may be the induction of activation-induced cell death (apoptosis) (reviewed in reference 67).

We have shown (60) that the CD45RB mAb MB23G2 induces partial depletion of circulating peripheral blood lym-
phocytes. This property may be associated with the immunosuppressive potency of other mAb, such as OKT3. On the other hand, certain nondepleting CD4 and CD8 mAb induce tolerance in a number of animal models and thus depletion may not be required with the targeting of all T cell surface molecules (63,68).

We have also shown (60) that MB23G2 therapy is associated with decreased expression, within the allograft, of genes for cytokines such as tumor necrosis factor α and IFN-γ, as well as intercellular adhesion molecule-1. These observations suggest that there may be upregulation of TH2 cytokines in MB23G2-treated animals, an observation that may provide an explanation for the shift in donor-specific IgG to the IgG1 isotype that we observed in this study. The IgG2a isotype is preferentially associated with transplant rejection (59). Our findings in this study are consistent with observations we recently made in an islet transplant model (65).

The high levels of proliferation noted in the allogeneic MLR of MB23G2-treated tolerant mice is somewhat surprising; we expected decreased donor-specific proliferation. However, other models of tolerance have also demonstrated increased proliferation in the allogeneic MLR (69). This may be correlated with the apparent active immunoregulation induced by the CD4+ T cells of the tolerant animals. This T cell subset, but not others, can transfer tolerance to naive animals. This active immunoregulation may also be referred to as induction of suppression (68). CD45RB mAb-induced immunoregulation was recently studied by Basadonna et al. (65), and those studies in an islet transplant model demonstrated a shift in CD45 isoform expression from CD45RA+ to CD45RO+. Functionally, this may be correlated with an alteration of the T cells to a mature “memory” phenotype, with the cells maintaining their suppressive function after adoptive transfer (31). The active immunoregulation is also supported by our observation that cyclosporine therapy interferes with CD45RB mAb tolerance induction in heart transplants (70).

Another attractive possibility for induction of tolerance in MB23G2-treated animals was suggested by our previous study of tyrosine phosphorylation in T cells after MB23G2 stimulation (60). Those experiments showed increases in the tyrosine phosphorylation of phospholipase Cγ1 and p69yn with decreased tyrosine phosphorylation of p56lck. These are properties of anergic T cells, as reported by Gajewski et al. (71). On a functional level, however, we could not break tolerance in vivo with administration of IL-2 (72). Therefore, although we could demonstrate an anergic biochemical profile, the tolerance could not be broken with IL-2. On the other hand, anergic T cells have been reported to have suppressor cell qualities (73).

Regardless of the mechanisms involved, CD45RB is an important molecule in transplant rejection. It is known that CD45 contains 10% of the carbohydrates of the T lymphocyte membrane. It is therefore of considerable interest that the major reactivity of polyclonal ALG involves the leukocyte common antigen (CD45), an observation made immunochemically by two groups approximately 20 yr ago (22–24). ALG is still used today for the prevention and treatment of renal allograft rejection. Perhaps the reactivity to isoforms of CD45, most notably CD45RB, is important in the beneficial effects of ALG. CD45RB-directed therapy may find clinical application in organ transplantation in human patients.

Acknowledgments
This work was supported by grants from the Kidney Foundation of Canada and the Physicians’ Services Incorporated Foundation. We thank Ms. K. McCormick for excellent secretarial support.

References


45. Sanders ME, Magkoba MW, Sharrow SO, Stephany D, Springer TA, Young HA, Shaw S: Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-2) and three other molecules (UCHL1, CD29, and Pgp-1) and have enhanced IFN-γ production. J Immunol 140: 1401–1407, 1988

(Lp220) represents a post-thymic T cell differentiation event.  
*J Immunol* 140: 1435–1441, 1988


