CTLA4Ig Inhibits Alloantibody Responses to Repeated Blood Transfusions

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Allosensitization is a fundamental problem that limits the effectiveness of blood transfusions. Patients who receive multiple transfusions of blood or blood components frequently develop alloantibodies against donor alloantigens. Allosensitized patients are refractory to further transfusion and difficult to transplant successfully. CTLA4Ig fusion protein, which blocks the CD28-B7 costimulatory pathway in T-lymphocyte activation, was tested for its capacity to inhibit allosensitization to blood transfusions. Groups of LEW (RT1b) rats were transfused with ACI blood (RT1a) together with L6 (a human immunoglobulin G1 [IgG1] antibody as isotype control) or CTLA4Ig in different doses (0.004, 0.02, 0.1, and 0.5 mg). Rats were retransfused with ACI blood after 28 and 84 days without any additional CTLA4Ig therapy. Weekly sera samples were tested for alloantibody against donor leukocytes using flow cytometry. CTLA4Ig caused a dose-dependent decrease in the IgM alloantibody response against donor major histocompatibility complex (MHC) class I antigens. In addition, 0.02-, 0.1-, and 0.50-mg doses of CTLA4Ig totally inhibited the IgG responses to the first transfusion, and this immunosuppressive effect persisted for the second and third transfusions. To study the capacity of CTLA4Ig to prevent a secondary immune response, three groups of LEW rats were transfused with ACI blood with no accompanying treatment. Animals were retransfused 28 days later with ACI blood together with L6 control antibody or 0.5 or 2.5 mg CTLA4Ig. CTLA4Ig, but not L6, prevented an increase in IgG alloantibody response despite repeated transfusions. The effects of CTLA4Ig treatment on helper T-lymphocyte proliferation was tested by limiting dilution analysis (LDA). Peripheral blood cells taken 30 days after blood transfusion and CTLA4Ig treatment contained significantly decreased donor-specific T-lymphocyte precursors compared with L6-treated rats. These data support the idea that blocking the B7/CD28 signal of T-lymphocyte activation by CTLA4Ig treatment at the time of transfusion may be an important therapeutic tool to inhibit alloantibody responses to blood transfusions. © 1996 by The American Society of Hematology.
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MATERIALS AND METHODS

Animals. Nine- to 12-week-old (200 g) inbred male rats were maintained in our colony under local and National Institutes of Health (NIH) guidelines. LEW (RT1^l) and ACI (RT1^w) were obtained from Harlan Sprague Dawley (Indianapolis, IN).

Fusion proteins and antibodies. CTLA4Ig, the soluble genetic fusion protein composed of the extracellular domain of CTLA4 and human IgG1 IgCy chain, has been described. Fusion protein composed of the extracellular domain of CTLA4 and human IgG1 IgCy chain, has been described. L6 (isotype control CTLA4Ig MoAb) were kindly provided by Dr P. Linsley, Bristol Meyers Squibb Pharmaceutical Institute (Seattle, WA).

Blood transfusion. Donor ACI whole blood was collected by cardiac puncture in a heparinized syringe and diluted 1:1 with sterile phosphate-buffered saline (PBS). One milliliter was injected intravenously into the dorsal penile vein. In treated groups, CTLA4Ig was added to the PBS in the desired dose at the time of transfusion.

Serum samples. Blood was collected by tail bleed under anesthesia, allowed to clot for 60 minutes at 37°C, and then centrifuged at 1,000g at 4°C. Serum was aspirated, heat-inactivated at 56°C, and stored at -80°C until use.

Serum clearance of human CTLA4Ig in rats. Three LEW rats were treated with a single 0.5-mg intravenous injection of CTLA4Ig together with an ACI blood transfusion. Animals were bled, and sera were collected 1, 3, 5, 7, 14, and 21 days after treatment. Antigenic CTLA4Ig levels in the sera were measured using sandwich enzyme-linked immunosorbent assay (ELISA). In the ELISA method, immulon ELISA plates were coated overnight using 11D4 MoAb. After blocking the uncoated portions of the wells with bovine serum albumin (BSA), the rat serum samples were serially diluted. The bound CTLA4Ig was detected with 7F8 biotinylated MoAb. CTLA4Ig concentrations were quantitated against a control curve of known amounts of CTLA4Ig. Functional CTLA4Ig levels were assessed using flow cytometry as described later. The binding of serially diluted serum samples to B7-expressing CH0 cells was compared with the binding of a known amount of CTLA4Ig to these target cells.

Flow cytometric analysis. Flow cytometry assays were performed according to techniques described previously. Briefly, cervical lymph nodes from ACI rats were removed and placed in PBS containing 0.05% BSA (Sigma, St Louis, MO) and 0.02% NaN3 (PBA). A single-cell suspension was produced by teasing gently with 25-gauge needles. The leukocytes were washed three times in PBA and resuspended at a concentration of 3 x 10^6 cells/mL. Then, 50 mL of the cell suspension was placed in 12-mm x 75-mm plastic tubes and incubated with 50 mL of diluted sera (1:4, 1:16, 1:64, and 1:256). To stain for IgM and IgG alloantibodies, the washed cells were reacted with 50 mL of PBA that contained a mixture of fluorescent isothiocyanate (FITC)-conjugated goat antirat IgG antibody and phycoerythrin-conjugated goat antirat IgG 0.5 (mu)g of rat IgM (Jackson Immunoresearch, West Grove, PA).

MoAbs for IgG subclasses. To stain for IgG subclasses, hybridomas secreting mouse MoAb RG1/39.4 (IgG2b; hybridoma secreting mouse MoAb RG1/39.4 (IgG2b; hybridoma secreting mouse MoAb RG1/39.4 (IgG2b; hybridoma secreting mouse MoAb RG1/39.4 (IgG2b; hybridoma secreting mouse MoAb RG1/39.4 (IgG2b; hybridoma secreting mouse MoAb RG1/39.4 (IgG2b; hybridoma secreting mouse MoAb RG1/39.4 (IgG2b; hybridoma secreting mouse MoAb RG1/39.4 (IgG2b; hybridoma secreting mouse MoAb RG1/39.4 (IgG2b; hybridoma secreting mouse MoAb RG1/39.4 (IgG2b; hybridoma secreting mouse MoAb RG1/39.4 (IgG2b; hybridoma secreting mouse MoAb RG1/39.4 (IgG2b; hybridoma secreting mouse MoAb RG1/39.4 (IgG2b; hybridoma secreting mouse MoAb RG1/39.4 (IgG2b) purchased from Zymed (San Francisco, CA). After incubating with the sera for 30 minutes at 4°C, the cells were washed twice, and resuspended with 50 mL mouse MoAb against IgG subclass for 30 minutes at 4°C. The cells were then washed twice and resuspended with FITC-conjugated F(ab)2 fragments of rat antirat IgG (Jackson Immunoresearch). After staining for 30 minutes at 4°C, the cells were washed twice, resuspended in PBS containing 1% formalin, and analyzed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). All values are expressed as mode channel ± 1 SD. This method of analysis allows the staining on T lymphocytes that express MHC class I antigens, but not class II antigens, to be separated from the B lymphocytes that express both MHC class I and class II antigens.

Test for reactivity to MHC class I antigens. The specificity of alloantibody against donor MHC class I (RT1^t) was confirmed as reported previously by using target lymph node cells from congenic strains of rats that differ only at MHC class I locus. The PVG and PVG.R1 strains are genetically identical, except that the PVG.R1 strains are serologically matched with ACI at the MHC class I locus (RT1.A'), whereas the PVG is RT1.A'. Therefore, sera that bind PVG.R1 target cells, but not PVG.R1 target cells, are reacting with the RT1.A' MHC class I antigen.

LDA. LDA cultures were established in triplicate for assessment of proliferative responses as previously described. Peripheral blood was collected by heart puncture, leukocytes were separated using lymphocyte separation medium (Organon Teknika, Durham, NC), and cells were counted and incubated in graded numbers (semilog dilutions from 1 x 10^6 to 3 cells/well) in 48 round-bottom wells with irradiated (2,000 rad) stimulator splenocytes. The culture medium consisted of RPMI 1640 supplemented with 10% fetal calf serum (FCS), 1% glutamine, and 1% penicillin-streptomycin. After 7 days in culture, proliferation was assessed in cultures by labeling with 0.5 mCi of [3H]thymidine for 18 hours. Wells were harvested and assayed for [3H]thymidine uptake. Wells were considered positive if the cpm of [3H]thymidine exceeded 3 SD of the response recorded for unstimulated cultures.

RESULTS

CTLA4Ig clearance. As reported for mice, the majority of CTLA4Ig was cleared within 3 days (Fig 1). About 2 pg/mL of CTLA4Ig was detected by ELISA at day 7 after a dose of 0.5 mg was injected, and functionally active CTLA4Ig was still detectable in the serum 3 weeks after treatment by a binding assay with B7-expressing CHO cells (data not shown). This attenuated clearance is the result of fusing the CTLA4 receptor with the Fc of IgG1, which confers on the CTLA4Ig pharmacokinetics similar to IgG1. IgG has an initial equilibration period of 5 to 7 days with the extravascu-
lar compartment followed by a more prolonged catabolic clearance.24,25

Dose-response study. To identify the optimum dose of CTLA4Ig needed to inhibit the alloantibody responses elicited by blood transfusions, a dose-response study was performed. Groups of LEW rats (four each) were transfused with ACI blood together with a single dose of CTLA4Ig (0.004, 0.02, 0.1, or 0.5 mg) or L6 at the time of transfusion. Sera were tested for the amount of alloantibody produced at serial time points as shown in Fig 2. The suppression of IgM alloantibody response in the 4 weeks after the first blood transfusion correlated with the dose of CTLA4Ig treatment. This inhibition was not evident after the second transfusion, which stimulated IgM alloantibody responses in CTLA4Ig-treated groups similar to or higher than in control animals. The IgM alloantibody responses after the third transfusion were decreased in animals in the high-dose, but not the low-dose, CTLA4Ig treatment group. In contrast, although the L6-treated animals responded to blood transfusion by making increasing amounts of IgG alloantibody, only the animals treated with 0.004 mg of CTLA4Ig made IgG alloantibody. IgG alloantibody responses in animals treated with 0.02, 0.1, or 0.5 CTLA4Ig were undetectable. As expected, the animals initially treated with L6 or 0.004 mg CTLA4Ig had increased alloantibody responses after the second and third transfusions; however, the animals treated with higher doses of CTLA4Ig made little IgG alloantibody response to these repeated transfusions in the absence of further treatment with CTLA4Ig.

CTLA4Ig inhibits alloantibody production to MHC class I antigens. We have demonstrated previously that ACI blood transfusions elicit antibodies to MHC class I antigens in LEW recipients.26 Antigen specificity was demonstrated by using target cells from congeneric strains of rats that differ only at the MHC class I locus. As found previously, the antibodies elicited by an ACI transfusion bound to PVG.RI target cells, but not to PVG target cells. These antibodies bind to MHC class I antigens, because the PVG and PVG.RI strains are genetically identical, except that the PVG.RI strains are serologically matched with ACI at the MHC class I locus (RT1.A'), whereas the PVG is RT1.A'. The single treatment with 0.02, 0.1, or 0.5 mg of CTLA4Ig inhibited the IgG alloantibody response to MHC class I antigens totally. In these groups of recipients, no binding of IgG alloantibodies was detectable with PVG.RI targets after the first, second, or third transfusion.

Effects of CTLA4Ig on antibody responses in presensitized animals. Because CTLA4Ig was effective in preventing a primary immune response in naive animals, we tested if it was effective in suppressing a secondary immune response in presensitized animals. Three groups of LEW rats (four each) were transfused with ACI blood 4 weeks before they received a second ACI blood transfusion together with L6 antibody or 0.5 or 2.5 mg CTLA4Ig. Animals were retransfused with ACI blood without CTLA4Ig monthly thereafter. The IgM alloantibody response in the presensitized animals to the second ACI blood transfusion was inhibited with CTLA4Ig treatment. After the third transfusion, CTLA4Ig-treated animals produced a limited amount of IgM alloantibody, which further increased after the fourth transfusion. On the other hand, the IgG alloantibody response was minimally increased after the second transfusion, but never increased above the primary response seen after the first transfusion, despite repeated transfusions (Fig 3).
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Fig 3. Effects of CTLA4 Ig on IgM and IgG alloantibody responses in presensitized LEW rats to repeated ACI blood transfusions. Flow cytometric analysis of IgM and IgG alloantibodies to ACI blood transfusion in presensitized LEW rats is shown. Animals were presensitized with 1 transfusion and then transfused every 4 weeks thereafter. ACI lymph node cells were incubated with dilutions of sera followed by optimal dilution of mouse antirat IgM or IgG. Each point represents the average mode channel staining ± SD (n = 4 for each point).

IgG subclass composition of the alloantibody responses in naive animals. To examine whether CTLA4 Ig inhibited alloantibody production of all IgG subclasses uniformly, sera collected 14 days after each transfusion were examined for IgG1, 2a, 2b, and 2c subclass production. Minimal amounts of IgG2c alloantibody were detected regardless of treatment (data not shown). Compared with control animals, IgG1 subclass production was minimal and limited to animals treated with the smallest dose of CTLA4 Ig (Fig 4). IgG2a alloantibody production was limited in both control and treated animals after the first transfusion. It increased after the second transfusion in control animals and in animals treated with the lowest dose of CTLA4 Ig, but not in those treated with higher doses. IgG2b alloantibody production was similar in the control group and the group treated with the lowest dose of CTLA4 Ig after the first transfusion. After the second and third transfusion, most of the animals increased their IgG2b alloantibody production, except those treated with 0.01 and 0.5 mg of CTLA4 Ig.

IgG subclass composition of the alloantibody responses...
in presensitized animals. As shown in Fig 5, all subclasses of IgG alloantibodies in the presensitized animals were partially suppressed with treatment. IgG1 alloantibody production was lower in treated animals after the second transfusion compared with controls, but after the third and fourth transfusions this difference was eliminated, partially due to a decrease in IgG1 alloantibody production by the control. On the other hand, IgG2a and 2b alloantibody responses in treated animals were lower than in controls after the second, third, and fourth transfusions (Fig 5).

Effects of CTLA4Ig on T lymphocyte proliferation. LDA was performed to investigate the effects of CTLA4Ig treatment on T-lymphocyte proliferative precursors to donor antigens. As seen in Fig 6A, the number of T-lymphocyte precursors in peripheral blood obtained 28 days after a primary transfusion was decreased in CTLA4Ig-treated animals compared with controls. That the decrease in T-lymphocyte precursors was specific for ACI-stimulator cells was demonstrated in parallel assays in which T lymphocytes from these CTLA4Ig-treated animals responded like those from control animals to third-party stimulators from Wistar Furth rats (Fig 6B). A similar assay on blood cells obtained from presensitized animals at day 56 after the first blood transfusion (day 28 after the second blood transfusion and CTLA4Ig or L6 treatment) demonstrated a dose-dependent decrease of T-lymphocyte precursors in CTLA4Ig-treated animals (Fig 6C).

DISCUSSION

Our experiments had the dual aim of studying the effects of CTLA4Ig on alloantibody responses to blood transusions and extending our understanding of the underlying cellular interactions that lead to alloantibody production. CTLA4Ig has been shown to block T-lymphocyte–dependent antibody responses in mice to whole rat blood transfusion, sheep RBCs, and keyhole limpet hemocyanin.19,27,28 Our study extends these observations to a clinically relevant model of allogeneic blood transfusion. As with clinical transfusions, the antibody response to the allotransfusion was directed primarily at MHC class I antigens. The specificity of alloantibody against donor MHC class I was confirmed by using cells from PVG.RI rats, which share the MHC class I with ACI, but differ in all other antigens, as target cells in a flow cytometry assay.

In contrast to previous studies on xenogeneic antigens, we have shown that a single dose of CTLA4Ig at the time of an allogeneic transfusion was effective in blocking the antibody response to the first transfusion and induced unresponsiveness to further transfusions. Moreover, a single dose of CTLA4Ig was effective in preventing an increase of alloantibody levels in presensitized animals to the second and subsequent transfusions.

Our understanding of the signals required for optimal cellular interactions leading to antibody production is evolving, but costimulation of T lymphocytes through CD28 has been shown to be a critical component of T-lymphocyte activation.14 Because CTLA4Ig fusion protein has a high affinity for B7 expressed on APCs,20 it blocks stimulation of antigen-specific T-lymphocyte clones through CD28 by B7 on both donor and recipient APCs. Presentation of antigen to recipient antigen-specific T-lymphocyte clones through the TCR in absence of the costimulatory signal CD28 can induce T-lymphocyte unresponsiveness under appropriate conditions.15,29

In this set of experiments, CTLA4Ig was administered with the donor blood. This was not essential to block alloantibody production, because similar results were obtained by injecting the animals with CTLA4Ig 2 days after transfusion (data not shown). Similarly, we and others have reported19,27 that treating mice with a single dose of CTLA4Ig at day 2 or multiple doses at days 1, 2, and 3 after antigen exposure was effective in blocking IgG antibody production.
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Although CTLA4Ig inhibits antibody responses to sheep RBCs, this immunosuppressive effect did not prevent the antibody response to a second challenge. In contrast, the immunosuppressive effects of CTLA4Ig on antibody responses to allogeneic blood transfusions were long-lasting and not dependent on the presence of the drug in the circulation. IgG allograft responses to the second and third transfusions administered at days 28 and 84 (Fig 2) were inhibited despite the absence of detectable CTLA4Ig in the circulation by ELISA and flow cytometry. The long-term effects of CTLA4Ig were evident at the level of decreased T-lymphocyte responsiveness in our model. CTLA4Ig decreased the number of antigen-specific proliferative T lymphocytes, as shown by the LDA, and consequently inhibited IgG allograft production. This is in agreement with recent reports showing limited expansion of antigen-specific T-lymphocyte clones in CTLA4Ig-treated animals.

Pairing CTLA4Ig with the alloantigen partially inhibited the primary IgM alloantibody response and totally blocked the IgG response. This also reflects the effects of CTLA4Ig on T lymphocytes, because T-lymphocyte help is more critical for IgG than IgM allograft production.

Production of different subclasses of IgG antibody is stimulated by different cytokines secreted by helper T lymphocytes. In this study, the control animals responded to the first transfusion by producing allograft to MHC class I consisting primarily of IgG1 and 2b with limited IgG2a. With repeated transfusions, IgG1 decreased and IgG2a and 2b increased. CTLA4Ig effects on IgG allograft subclass production were dose-dependent (Fig 4). Doses of 0.1 and 0.5 mg inhibited IgG allograft responses in all subclasses to all three transfusions, whereas 0.004 mg caused little decrease in allograft responses in any subclass. The intermediate 0.02-mg dose inhibited the IgG1 and IgG2a responses to the three successive transfusions as completely as the higher doses of CTLA4Ig, but it only partially decreased the IgG2b response to the first transfusion and had little effect on the IgG2b to subsequent transfusions. This is consistent with the ability of CTLA4Ig to decrease the secretion of IFN-γ and to a lesser degree IL-4. We have found that IgG1 and IgG2a responses to allogeneic blood transfusions in rats are regulated by IFN-γ (Ibrahim et al, manuscript in preparation). Such differential changes in cytokine production could reflect a CTLA4Ig-induced alteration in the T-helper response in favor of Th2 responses.

In the presensitized animals (Fig 3), CTLA4Ig prevented an anamnestic increase in IgG allograft levels in response to the second, third, and fourth transfusions. IgG allograft levels at day 100 posttransfusion (2 weeks after the fourth transfusion) were similar to those at day 14 (2 weeks after the first blood transfusion). Because the half-life of polyclonal IgG in rats is 63 hours, this suggests that in the animals treated with CTLA4Ig, a limited population of B lymphocytes continued to secrete IgG allograft, but failed to expand upon further exposure to the antigen. Even large doses (2.5 mg) of CTLA4Ig failed to prevent this limited antibody production. LDA showed that the helper T-lymphocyte response was greatly decreased in these presensitized rats, but not eliminated. As with the primary response, IgG2b was the most resistant subclass to CTLA4Ig treatment in the presensitized rats.

In summary, our findings demonstrate that unresponsiveness to multiple allogeneic blood transfusions can be achieved using single-dose treatment with CTLA4Ig. This unresponsiveness was long-lasting and resistant to repeated exposure to the same antigens.

ACKNOWLEDGMENT

The authors would like to thank Dr Thomas Kickler for helpful discussion and Louis Horwitz for technical assistance.

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