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14. The 18*S* rRNA sequences determined range in length from 1769 (*Styela*) to 1959 (*Eptatretus*) nucleotides and consist of all but the extreme 3′ end [39 nucleotides in the human sequence (*15*)]. These sequences have been deposited in the GenBank database under accession numbers M97571 to M97577. All sequences were determined both by direct RNA sequencing with reverse transcriptase and by polymerase chain reaction (PCR) amplification and sequencing of rDNA on the opposite (noncoding) strand. Templates for DNA sequencing were produced by either asymmetric PCR [U. B. Gyllensten and H. A. Erlich, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7652 (1988)] or (in the case of the two hagfishes, where asymmetric PCR did not produce satisfactory results) by cloning PCR products into M13mp19. Ambiguities in DNA sequences were resolved by the use of deoxyinosine triphosphate (dITP). Methods for direct RNA sequencing and asymmetric PCR, as well as primer sequences, are as described (*16*). Amplification of two overlapping segments of hagfish rDNA for cloning was performed with primer pair 20F 5′-GCCGGAGCTCGGTACCTGGTTGATCCTGCCAG-3′ and 429R 5′-GCCGCTGCAGTCGACTTTCTCAGGCTCCCTCTCCGG-3′ and pair 366F 5′-GCCGGAGCTCGGTACCGTCTGCCCTATCAACT-3′ and 1830R 5′-GCCGCTGCAGTCGACACCTACGGAAACCTTGTT-3′, where underlined sequences represent restriction sites added to the primer, numbers indicate the position of the nucleotide at the 3′ end in the human sequence (*15*), and F and R refer to primers that bind to the noncoding and coding strands, respectively. For *Eptatretus*, preliminary sequencing of six clones revealed less than 0.6% sequence difference with the RNA (aside from a single aberrant clone that was 14% different). A consensus for each position was assembled from the RNA sequence and at least two clones. For *Myxine*, two clones of the 20FL-429RL amplification were identical to each other and did not differ from the unambiguous portions of the RNA sequence. Eleven clones of the 366FL-1830RL amplification, however, fell into two sequence classes. Ten of the clones had fewer than 0.7% differences among each other but differed from the RNA sequence by 3.6%, while the remaining clone differed from the RNA by 0.9%. To enrich for clones similar to the RNA, two new primers, 501R 5′-GCCGCTGCAGTTCGTCACTACCTCACCGTG-3′ and 502F 5′-GCCGGGTACCAAATTACCCACTCCCGACA-3′, were designed based on differences between the two classes of clones and used for the amplifications 20F-501R and 502F-1830R. Two clones most similar to the RNA from each amplification (all had <1% difference) were sequenced and, along with the clones from the 20F-429R and the direct RNA sequence, were used to assemble a consensus. The differences among clones in *Myxine* (presumably due to nontranscribed copies of rDNA genes) are not likely to affect phylogenetic analyses because the RNA sequence was ambiguous at 143 out of the 1849 positions reported, and only 8 of these 143 positions were variable among clones.
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30 March 1992; accepted 3 June 1992

Long-Term Survival of Xenogeneic Pancreatic Islet Grafts Induced by CTLA4Ig

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Antigen-specific T cell activation depends on T cell receptor-ligand interaction and costimulatory signals generated when accessory molecules bind to their ligands, such as CD28 to the B7 (also called BB1) molecule. A soluble fusion protein of human CTLA-4 (a protein homologous to CD28) and the immunoglobulin (Ig) G1 Fc region (CTLA4Ig) binds to human and murine B7 with high avidity and blocks T cell activation in vitro. CTLA4Ig therapy blocked human pancreatic islet rejection in mice by directly affecting T cell recognition of B7$^+$ antigen-presenting cells. In addition, CTLA4Ig induced long-term, donor-specific tolerance, which may have applications to human organ transplantation.

At present, the major therapies to prevent the rejection of organ transplants rely on panimmunosuppressive drugs, such as cyclosporine A or monoclonal antibodies (MAbs) to CD3. These drugs must frequently be taken for the life of the individual, depress the immune system, and often result in increased infections and cancer. We attempted to develop a treatment that affected only the transplant antigen-specific T cells, thus inducing donor-specific tolerance. The binding of CD28 by its ligand, B7/BB1 (B7), during T cell receptor engagement is critical for proper T cell signal-

ing in some systems (*1–4*). When the interaction of CD28 with its ligand is blocked, antigen-specific T cells are inappropriately induced into a state of antigen-specific T cell anergy (*1, 5*). Recent studies have shown that the CTLA-4 molecule, a CD28 homolog, also binds to B7 (*6*). These studies used a soluble chimeric CTLA-4 fusion protein between the variable domain of the human CTLA-4 gene and the hinge, CH2, and CH3 domains of the human IgG1 constant region, CTLA4Ig (*6–8*). This soluble receptor molecule binds to both human and murine B7 (with a 20-fold greater affinity than CD28), blocks the binding of CD28 to B7, inhibits T cell activation, and induces T cell unresponsiveness in vitro (*5, 6, 9*). Using a xenogeneic transplant model (*10*), we found that CTLA4Ig prevented rejection of xenogeneic pancreatic islet cells and induced donor-specific tolerance.

Initial studies showed that CTLA4Ig bound to both murine and human B7 and inhibited primary xenogeneic mixed lymphocyte reactions in vitro (*11*). Therefore, we examined the effects of blocking CD28-B7 interaction in vivo. C57BL/6 (B6) or C57BL/10 (B10) mice were treated with streptozotocin to eliminate mouse pancreatic islet β cell function. Diabetic animals were grafted under the kidney capsule, and treatment was started immediately after surgery. Survival of the islet grafts was monitored by the analysis of blood glucose concentrations. Transplanted control animals, treated with either phosphate-buffered saline (PBS) (n = 14) or L6 (a human IgG1 chimeric MAb; n = 8), had a mean graft survival of 5.6 and 6.4 days, respectively (Fig. 1A). In contrast, islet rejection was delayed in animals treated with CTLA4Ig (10 μg per day for 14 days), with four out of the seven animals exhibiting moderately prolonged mean graft survival (12.75 days), whereas the remaining three animals maintained normal glucose levels for >80 days (Fig. 1B). This eventual increase in glucose concentration may be a result of islet exhaustion because no evidence of active cellular rejection was observed. In the three mice that maintained long-term islet grafts, the transient increase in glucose concentrations around day 21 after the transplant may have represented a self-limited rejection episode [consistent with the pharmacokinetics of CTLA4Ig clearance after therapy (*12*)].

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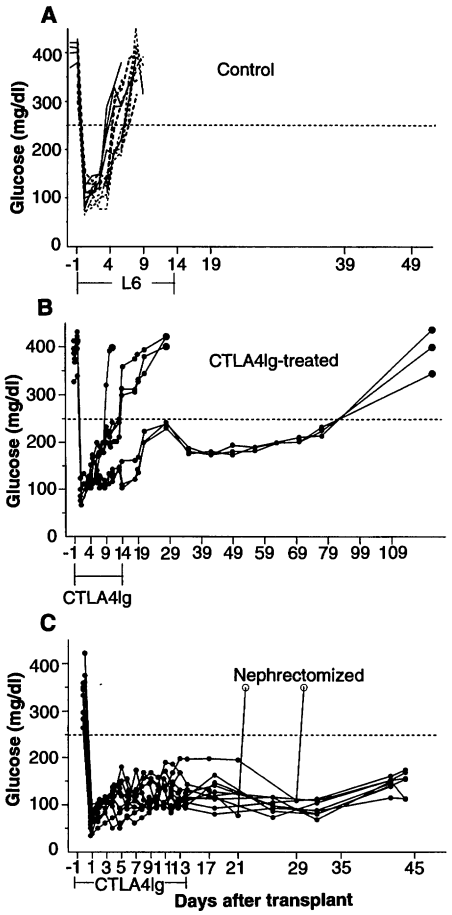
Fig. 1. Survival of human pancreatic islet xenografts. Human pancreatic islets cells were purified after collagenase digestion as described (*17*). B6 or B10 mice, treated with streptozotocin (175 mg per kilogram of body weight) 3 to 5 days before transplant and exhibiting nonfasting plasma glucose levels of greater than 280 mg/dl (with the majority over 300 mg/ml), were used as recipients. Each animal received approximately 800 fresh human islets of 150 μm in diameter beneath the left renal capsule (*10*). Treatment was started immediately after transplantation. (**A**) Control animals were treated with PBS (solid lines) or L6 (dotted lines) at 50 μg every other day for 14 days immediately after transplantation. Islet transplants were considered rejected when glucose levels were greater than 250 mg/dl for three consecutive days. Animals treated with PBS (n = 14) and L6 (n = 8) had mean graft survivals of 5.6 and 6.4 days, respectively. (**B**) Animals were treated with 10 μg of CTLA4Ig for 14 consecutive days immediately after transplant (n = 7). Three out of seven animals maintained their grafts for >80 days. The remaining four animals had a mean graft survival of 12.75 days. (**C**) Animals were treated with 50 μg of CTLA4Ig every other day for 14 days immediately after human islet transplantation. All animals (n = 12) treated with this dose maintained grafts throughout the analysis. Selected mice were nephrectomized on days 21 and 29 after the transplant to assess the graft's function.



Therefore, in subsequent experiments, the dose of CTLA4Ig was increased to 50 μg per animal every other day for 14 days. This treatment resulted in 100% of the animals maintaining normal islet function throughout the experiment with no signs of a rejection crisis (Fig. 1C). In order to confirm that insulin production originated from the transplanted islets and not from the native mouse pancreas, we nephrectomized selected animals at days 21 and 29 to remove the islet grafts (Fig. 1C). In these animals, glucose concentrations increased to above 350 mg/dl within 24 hours, which indicated that the islet xenograft was responsible for maintaining normal glucose levels. Thus, it appears that the blocking of the CD28-B7 interaction inhibits xenogeneic islet graft rejection. The effects of treatment with the soluble receptor were not a result of Fc binding (L6 did not affect graft rejection) or general effects on T cell or B cell function in vivo (*13*). The function of CTLA-4 on T cell surfaces as a potential costimulatory molecule is unknown. Therefore, the effects we observed might also reflect the importance of CTLA-4–B7 interactions or other, as yet unidentified B7 or CTLA-4 ligands.

Histological analyses of islet xenografts from control (PBS-treated) and CTLA4Ig-treated mice were done (Fig. 2). The islet tissue from the control animal demonstrated evidence of immune rejection, with a marked lymphocytic infiltrate into the graft and few remaining islets (Fig. 2A). Immunohistochemical staining showed that insulin-positive cells were present only rarely, and no somatostatin-positive cells (*13*) were present at all (Fig. 2B). In contrast, transplant tissue from the CTLA4Ig-treated mice was devoid of any lymphocytic infiltrate (Fig. 2C). The grafts were intact, with many islets visible. In addition, the β cells observed in the human islet tissue produced human insulin (Fig. 2D) and somatostatin (*13*).

Because the human CTLA4Ig used in this study reacts with both murine and human B7, the individual role of murine $B7^+$ and human $B7^+$ cells could not be distinguished (*6*). However, one advantage of the xenogeneic transplant model is the availability of a MAb to human B7 that does not react with mouse B7 (*14*). Thus, the role of human B7-bearing antigen-presenting cells (APCs) could be directly examined. The mice were transplanted as described and then treated with 50 μg of MAb to human B7 every other day for 14 days after transplant. This treatment prolonged graft survival in treated mice (9 to

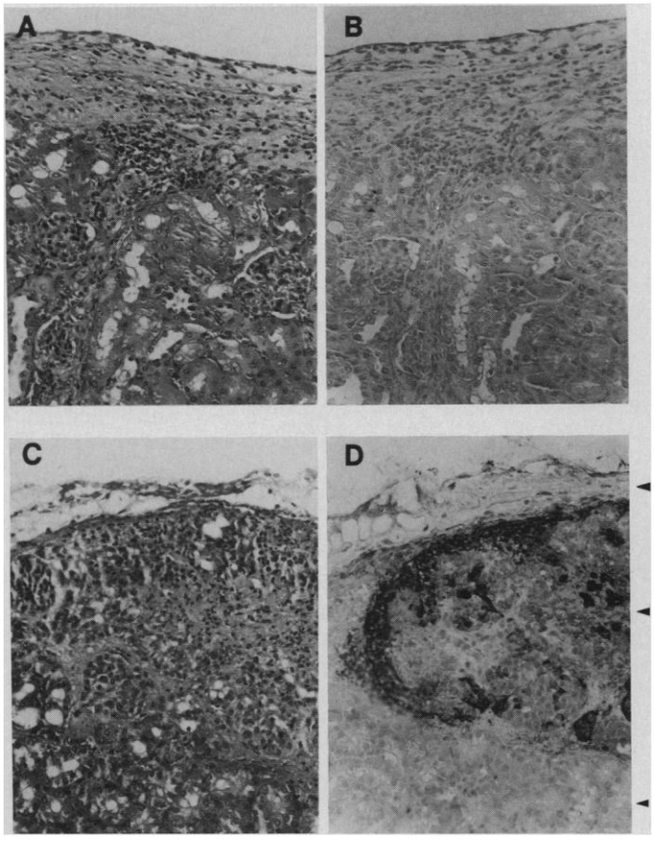


Fig. 2. Histological analysis of human islets transplanted under the kidney capsule of B10 mice. Histology was performed on kidneys transplanted with human islet cells. The slides were analyzed blindly. (**A**) Hematoxylin and eosin staining of a control human islet grafted mouse 29 days after transplantation showed a massive lymphocyte infiltration. (**B**) The same tissue, stained for insulin, showed no detectable insulin production. (**C**) Histological examination of tissue from a CTLA4Ig-treated mouse 21 days after transplant showed intact islets under the kidney capsule with very few lymphocytes infiltrating the transplanted tissue. The tissue was stained with hematoxylin and eosin. (**D**) The same tissue from the CTLA4Ig-treated mouse, stained for insulin, showed the production of insulin by the grafted islets. Similar results were observed in graft tissue examined at latter time points (*13*). The upper, middle, and lower arrowheads identify the kidney capsule, islet transplant, and kidney parenchyma, respectively. All tissues were fixed in 10% buffered formalin and processed, and 5-μm sections were stained either with hematoxylin and eosin or for insulin with the avidin-biotin-peroxidase method (*18*). Magnification is ×122.

>50 days) in comparison to that for control mice (Fig. 3). These results suggest that the immune response to the human islets involves direct presentation of human major histocompatibility complex (MHC)–restricted islet antigens by human APCs. This possibility contrasts with conclusions drawn in previous studies in which the predominant pathway for xenogeneic antigen presentation appeared to involve the processing and presentation of shed foreign proteins by syngeneic mouse APCs (*15*). However, other studies have noted the importance of carrier donor leukocytes in transplant rejection (*16*). The inability of the anti-B7 MAb to block rejection as effectively as CTLA4Ig may indicate that murine $B7^+$ APCs may also be involved in xenograft rejection. It is also possible that an inadequate dose of the anti-B7 MAb was used because it has a lower binding affinity to B7 than to CTLA4Ig (*6*). Further studies are needed to determine how the syngeneic and xenogeneic APCs interact to regulate graft rejection.

Although the CTLA4Ig therapy resulted in graft acceptance in the majority of mice, the animals may not be tolerant. Transient immunosuppression can lead to permanent islet graft acceptance because of graft adaptation (the loss of immunogenicity as a result of the loss of APC function) (*16*). In order to differentiate between these possibilities, we nephrectomized selected xenografted, CTLA4Ig-treated mice (day 40) and retransplanted them under the remaining kidney capsule with either the original donor islets (first party) or unrelated second party human islets (Fig. 4). Streptozotocin-treated control animals, having never received an islet graft, were also transplanted with either first or second party islets. No treatment after the transplant was given. Control animals rejected the first and second party islets by day 4. The CTLA4Ig-treated animals that had received the second party islets rejected these islets by day 5, whereas animals receiving first party donor islets maintained the grafts for >80 days (Fig. 4).

These results suggest that the CTLA4Ig treatment resulted in prolonged donor-specific unresponsiveness to the xenogeneic islets. The ability of the murine immune

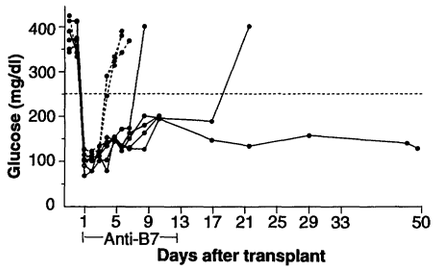


Fig. 3. Prolongation of islet graft survival with MAb to human B7. Streptozotocin-treated animals were transplanted as described (Fig. 1). The mice were treated either with PBS (dotted lines) or with MAb to human B7 (solid lines) at a dose of 50 μg every other day for 14 days. Control animals (treated with PBS) (n = 3) had a mean graft survival of 3.5 days, whereas anti-B7–treated animals (n = 5) maintained grafts from 9 to >50 days.

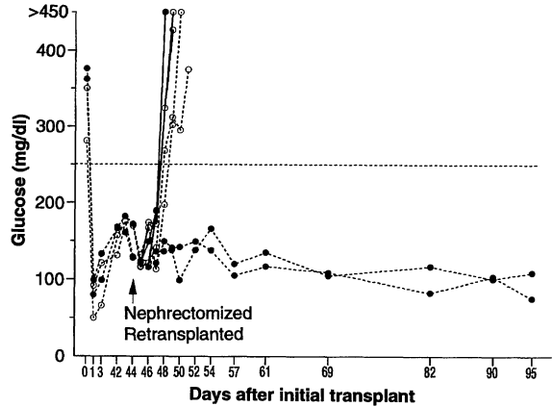


Fig. 4. Induction of donor-specific unresponsiveness to islet graft antigens by CTLA4Ig. Normal glycemic, CTLA4Ig-treated, transplanted mice (dotted lines) were nephrectomized on day 44 after transplant and immediately retransplanted with either 1000 first party donor islets (dotted lines, solid circles) or 1000 second party islets (dotted lines, open circles) beneath the remaining kidney capsule. These islets, frozen at the time of the first transplant, were thawed and cultured for 3 days before transplant to ensure islet function. B10 mice that had been treated with streptozotocin and exhibited nonfasting glucose levels of greater than 280 mg/dl were used as controls (solid lines). No treatment was given after transplantation. Control animals rejected both the first party (solid lines, closed circles) and the second party (solid lines, open circles) islet grafts by day 4 after transplant. The CTLA4Ig-treated mice retransplanted with second party islets had a mean graft survival of 4.5 days, whereas animals retransplanted with first party donor islets maintained grafts for as long as analyzed (>80 days).

response to distinguish differences among the human islet donors also supports the direct recognition of the polymorphic MHC products expressed on the human islet cells. The future of immunosuppressive therapies in transplantation and autoimmune disease depends on their ability to induce long-term, antigen-specific unresponsiveness. The capacity of CTLA4Ig to significantly prolong human islet graft survival in mice in a donor-specific manner suggests that blocking the interaction of costimulatory molecules such as CD28-B7 may provide an approach to immunosuppression.

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11 May 1992; accepted 2 July 1992

Immunosuppression in Vivo by a Soluble Form of the CTLA-4 T Cell Activation Molecule

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In vitro, when the B7 molecule on the surface of antigen-presenting cells binds to the T cell surface molecules CD28 and CTLA-4, a costimulatory signal for T cell activation is generated. CTLA4Ig is a soluble form of the extracellular domain of CTLA-4 and binds B7 with high avidity. CTLA4Ig treatment in vivo suppressed T cell–dependent antibody responses to sheep erythrocytes or keyhole limpet hemocyanin. Large doses of CTLA4Ig suppressed responses to a second immunization. Thus, costimulation by B7 is important for humoral immune responses in vivo, and interference with costimulation may be useful for treatment of antibody-mediated autoimmune disease.

Costimulatory signals delivered by antigen-presenting cells (APCs) have been proposed to control immune responses to transplanted tissues (*1*). Antigenic stimulation of T cells in vitro in the absence of costimulation leads to aborted T cell proliferation and the development of functional unresponsiveness or clonal anergy of T cells (*2*). Several molecules on APCs augment T cell proliferation (*3*, *4*) and regulate functional unresponsiveness in vitro (*4*). The B7 activation molecule binds CD28 (*5*) and delivers a costimulatory signal for T cell proliferation (*6*). T cell–dependent B cell differentiation requires the interaction of B7 with CD28 (*7*). CTLA-4, a T cell molecule homologous to CD28 (*8*), also binds the B7 counter-receptor (*9*). CTLA4Ig, a chimeric immunoglobulin C_γ fusion of CTLA-4, binds with high avidity (dissociation constant ~12 nM) to B7 and potently blocks T cell–dependent immune responses in vitro (*9*). CD28 probably participates in costimulation required to prevent anergy induction in T cell clones (*10*), in unresponsiveness in human mixed lymphocyte reactions (*11*), and in the costimulation of antigen-specific interleukin-2 production of human T cells (*12*). Despite data that indicate the importance of B7-CD28 interactions in the costimulation of in vitro T cell responses, the role of these interactions in regulating in vivo immune responses is unknown. Here, we show that CTLA4Ig is a potent suppressor of antibody responses in vivo.

Human CTLA4Ig [human CTLA-4 and human immunoglobulin (Ig)] binds to murine B7 and inhibits murine T cell responses in vitro (*13*). These findings led us to test the effects of human CTLA4Ig on murine immune responses in vivo. CTLA4Ig was purified to homogeneity by protein A chromatography from a serum-free culture medium of transfected mammalian cells (*14*). The chimeric monoclonal antibody (MAb) L6, which has a murine region and a human Fc region, was used as a control.

We first measured serum clearance of human CTLA4Ig in mice (Fig. 1). A plot of serum CTLA4Ig levels versus time was biphasic, giving a time of half-clearance ($t_{1/2}$) of ~4 hours and ~30 hours for the two components. Serum clearance after multiple injections of CTLA4Ig was more complex and appeared dose-related. The $t_{1/2}$ for the more slowly clearing component was increased to ~4 days after six daily intravenous injections of CTLA4Ig (200 μg per injection), and functionally active CTLA4Ig was detected in mouse serum for up to ~5 weeks after the last treatment with CTLA4Ig. No overt toxicity of CTLA4Ig was noted.

The ability of CTLA4Ig to suppress for-

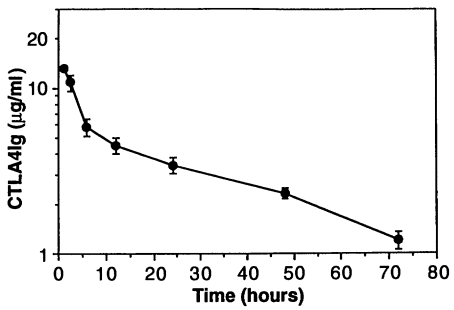

Fig. 1. Serum clearance of human CTLA4Ig in mice. BALB/c mice were each given a single intravenous injection of 50 μg of CTLA4Ig prepared from COS cells. At the indicated times, the mice were bled retro-orbitally. The binding of functional CTLA4Ig from sera to B7$^+$ CHO cells was measured by flow cytometry (*6*). CTLA4Ig concentrations were quantitated by comparison of the degree of binding with the binding of known concentrations of CTLA4Ig. Values represent mean concentrations ± SD from five mice.

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