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学 位 論 文

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(Cre/LoxP システムを用いた発現調節可能なアデノウィルスベクター

による同種移植片長期生着を目指した、CTLA4IgG 遺伝子療法)

北 海 道 大 学

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Running title: Gene therapy by a regulatable adenovirus vector

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Running title: a gene therapy by a regulatable adenovirus vector

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OVERVIEW SUMMARY

CTLA4IgG binds to B7-1 and B7-2, which are the ligands for T cell costimulatory molecules and effectively interferes with the ligand of CD28 and B7-1 or B7-2 to induce self- and allogeneic T cell activation. We previously demonstrated that adenovirus, Adeno-CTLA4IgG, could produce a high concentration of CTLA4IgG for a long time upon intravenous injection, which, however, rendered recipients susceptible to graft rejection. Therefore, we constructed a new type of adenovirus vector, Adeno-LoxP-CTLA4IgG, that was able to terminate the expression of CTLA4IgG by Cre recombinase *in vitro* and *in vivo*. Here, we demonstrate that the long acceptance of allografts was achieved after removal of CTLA4IgG expression, while the immune response against adenovirus was

ABSTRACT

CTLA4IgG was shown to inhibit the costimulatory signal for T cell activation by interfering with the ligation of CD28 and B7-1 or B7-2. To inhibit various immune responses including acute cellular rejection of allografts, a certain level of serum CTLA4IgG should be maintained for an appropriate period. We previously reported the adenovirus vector containing CTLA4IgG, which we designated as Adex1CACTLA4IgG.

Adex1CACTLA4IgG was able to maintain a significant level of serum CTLA4IgG for a long period upon intravenous injection, which in turn inhibited the various immune responses including protective immunity against insulting infectious agents. To overcome the inhibitory effect, we constructed a new type of adenovirus vector, Adex1CALoxCTLA4IgGlox by cloning CTLA4IgG cDNA between two lox P sequences under control of CAG promoter. We demonstrated that the administration of adenovirus vector containing Cre recombinase gene (Adex1CACre) at desired time induced Cre-mediated recombination within a gene derived from Adex1CALoxCTLA4IgGlox vector and the cDNA of CTLA4IgG was excised from the transduced gene, and terminated the expression of CTLA4IgG in vitro and in vivo. More importantly, we also demonstrated that the long acceptance of allografts was achieved after the termination of CTLA4IgG expression, while the immune response against adenovirus was restored.

OVERVIEW SUMMARY

CTLA4IgG binds to B7-1 and B7-2, which are the ligands for T cell costimulatory molecule, and effectively interferes with the ligation of CD28 and B7-1 or B7-2 to induce tolerance to alloantigens. We previously constructed an adenovirus vector, Adex1CACTLA4IgG, that produced a high concentration of CTLA4IgG for a long time upon intravenous injection, which, however, rendered recipients susceptible to many insulting agents. Therefore, we constructed a new type of adenovirus vector, Adex1CALoxCTLA4IgGlox, that was able to terminate the expression of CTLA4IgG by Cre recombinase in vitro and in vivo. Here, we demonstrate that the long acceptance of allografts was achieved after termination of CTLA4IgG expression, while the immune response against adenovirus was

restored.

INTRODUCTION

When an allogeneic organ transplantation between genetically non-identical donor/recipient invariably results in immunological rejection of the grafted organ through T cell-dependent mechanisms. Namely, the activation and expansion of alloantigen-specific T cells precede the cellular rejection in the recipient in allogeneic organ transplantation. Therefore, the target of the therapeutic intervention may be suppression of alloantigen-specific T cell activation/expansion, and this has been attempted by the use of calcineurin phosphatase inhibitors and glycosylated steroids. Both reagents act by interfering T cell receptor (TCR) signal transduction and act on all recipient T cells including alloantigen-specific T cells. To avoid rejection, the long use of these reagents is necessary, which inevitably induces problems including infection, malignancy and drug toxicity in the recipient.

Recently, it has been shown that T cell activation requires not only TCR-mediated signals but also costimulatory signals delivered through interaction of complementary molecules such as B7-1 and B7-2 on antigen-presenting cells and CD28 on T cells (Schwartz, 1990, 1992; Jenkins et al., 1991; Bonifant et al., 1993). Thus, the second target of therapeutic intervention should be blockade of the costimulatory signals. Cytotoxic T lymphocyte antigen 4 (CTLA4) shares its ligands, B7-1 and B7-2, with CD28, and the binding affinity to B7-1 or B7-2 is significantly higher than that of CD28. A soluble form of CTLA4 molecule, CTLA4IgG, effectively inhibits the interaction between CD28 and B7-1 or B7-2 and induces both antigen-specific T cell unresponsiveness and long-term acceptance of allogeneic and xenogeneic grafts (Lanzhew et al., 1992; Linsley et al., 1992; Tan et al., 1993; Wu et al., 1993; Baliga et al., 1994; Pearson et al., 1994; Kirk et al., 1995; Tarami et al., 1999). The effectiveness of CTLA4IgG is dependent on TCR engagement, and thus the specificity of T cell suppression can be expected rather than pan-T cell suppression. Two approaches have been employed to achieve the maintenance of a certain level of CTLA4IgG in recipients to avoid rejection; namely, CTLA4IgG protein administration versus CTLA4IgG gene therapy. However, repeated administration of CTLA4IgG protein was necessary due to the too short half-life time of CTLA4IgG to achieve long-term acceptance of allografts or xenografts (Lanzhew et al., 1992; Baliga et al., 1994; Pearson et al., 1994; Kirk et al., 1995; Tarami et al., 1999). The alternative approach was to

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deliver CTLA4IgG gene by the use of adenovirus vector (Kay et al., 1995; Gainer et al., 1997; Schowalter et al., 1997; Nakagawa et al., 1998; Feng et al., 1999). We generated an adenovirus vector carrying CTLA4IgG, which was designated as Adex1CACTLA4IgG (Nakagawa et al., 1998). Upon systemic administration, Adex1CACTLA4IgG produced a high titer of CTLA4IgG for a long period in vivo. Thus, it was conceivable that in vivo gene transfer using Adex1CACTLA4IgG could render the recipients vulnerable to various infectious agents during a gene therapy. Therefore, we constructed a new type of adenovirus vector, Adex1CALoxCTLA4IgGlox that could be manipulated to terminate in vivo CTLA4IgG expression at desired time point by using Adex1CACre virus (Kanegae et al., 1995, 1996). cDNA of CTLA4IgG was cloned between two lox P sequences in an adenovirus vector so that CTLA4IgG gene could be excised from the transduced gene of Adex1CALoxCTLA4IgGlox by Cre-mediated recombination. This termination system may minimize the adverse effects of CTLA4IgG gene therapy. Here, we demonstrate that (i) CTLA4IgG expression from Adex1CALoxCTLA4IgGlox was persistent and efficient; (ii) in vivo gene expression was completely terminated after administration of Adex1CACre virus; (iii) long-term acceptance of allografts (pancreatic islets and skins) was achieved even after the termination of CTLA4IgG expression, while immune responses against adenovirus were preserved.

MATERIALS AND METHODS

Animals

C57BL/6, BALB/C and C3H/He female mice, 8-10 weeks of age, were purchased from Japan SLC inc., Shizuoka, Japan. CD40 ligand knock-out mice (B6,129-*Cd40l*^{tm1Imx}) were purchased from the Jackson Laboratory, Bar Harbor, ME.

Virus preparation

The Adex1CALoxCTLA4IgGlox was prepared as follows: cDNA fragment of CTLA4IgG was excised from the expression vector CDM8-CTLA4IgG (Yamada et al., 1996), cloned between the lox P sequences under CAG promoter in pCALwL (Niwa et al., 1991) and then an expression unit, CAG promoter-lox P-CTLA4IgG-lox P-poly A, was isolated. The resultant expression unit was cloned into the cosmid vector, pAdex1CA that contained an entire adenovirus type 5 genome except for the E1 and E3 regions (pAdex1CALoxCTLA4IgGlox). Recombinant adenoviruses were constructed by in vitro homologous recombination in 293 cells by using pAdex1CALoxCTLA4IgGlox and the adenovirus DNA terminal-protein complex. The desired recombinant adenoviruses, termed as Adex1CALoxCTLA4IgGlox, and Adex1CACre consisting of Cre gene under control of CAG promoter, were propagated in 293 cells and purified by two rounds of cesium chloride density centrifugation as described previously (Yang et al., 1994; Nakagawa et al., 1998). The concentrated viruses were dialyzed against phosphate-buffered saline/10% glycerol. The titer of the virus stock was assessed by a plaque formation assay that used the 293 cells.

Nature of CTLA4IgG derived from Adex1CALoxCTLA4IgGlox

Five $\times 10^6$ COS7 cells were infected with either Adex1CALoxCTLA4IgGlox or Adex1CACTLA4IgG (1.0×10^7 PFU/ml) for 3 days. CTLA4IgG in 1 ml of culture supernatant was purified by protein A and was subjected to SDS-PAGE analysis as described previously (Murakami et al., 1996).

Furthermore, ligand binding ability of the purified CTLA4IgG was

determined by flowcytometry of B7-1-CHO cells (Murakami et al., 1996). The cells were treated with 5 μ g CTLA4IgG, followed by FITC-anti-human IgG. After washing, the cells were applied to FACScan (Beckton Dickinson Co., Mountain View, CA).

CTLA4IgG assay

Serum concentrations of mouse CTLA4IgG were assayed by ELISA as described previously (Nakagawa et al., 1998).

Islet transplantation

BALB/C and C57BL/6 male mice, weighing 20-30g, were maintained under the condition approved by the Institutional Animal Care and Use Committee (IACUC) at the Fukuoka University, School of Medicine. C57BL6 mice had been rendered diabetic by a single i.v. injection of streptozotocin (200 mg/kg; Sigma Chemical Co.) 7 days before transplantation. Seven hundred and fifty BALB/C islets were used as donors for single transplantation. The islets were transplanted into the liver via the portal vein as described previously (Yasunami et al., 1983). Functional survival of allografted islets was determined by the serum level of blood sugar in recipients. Only those mice with blood sugar levels (BSL) >20 mmol/L on the day of transplantation were used. The mice were divided into four experimental groups. Group I was not treated. Group II and III were treated with i.v. 1×10^7 PFU AdexLacZ and Adex1CALoxCTLA4IgGlox at day 0, respectively. Group IV was treated with i.v. 1×10^7 PFU Adex1CALoxCTLA4IgGlox at day 0, followed by i.v. 2×10^8 PFU Adex1CACre at day 8. Graft function was monitored by twice-weekly BSL measurements using Glycemic stix and Reflux II blood monitor system (Boehringer Mannheim Corp.). After the transplantation, successful islet engraftment was defined as a BSL <10 mmol/L, and graft rejection defined by a BSL >16 mmol/L on two consecutive occasions (Tran et al., 1997).

Skin grafting

Skin grafting was performed by the method of Barks and Billingham with

minor modification (Barker and Billingham, 1973). The mice were divided into three experimental groups. Group I was treated i.v. with 1×10^8 PFU AdexLacZ alone at day 0. Group II was treated i.v. with 1×10^8 PFU Adex1CALoxCTLA4IgGlox at day 0, followed by 3×10^9 PFU AdexLacZ i.v. at day 21 and group III with 1×10^8 PFU Adex1CALoxCTLA4IgGlox i.v. at day 0, followed by 3×10^9 PFU Adex1CACre at day 21. The grafts were inspected daily and rejection was considered complete when no residual viable graft epithelium could be identified.

In some experiments, group II and III recipient mice were grafted with donor-type (BALB/C) and third party (C3H) skins at day 50 and the grafts were inspected up to day 71.

PCR assay

The mice was given i.v. injection of 1×10^7 PFU Adex1CALoxCTLA4IgGlox at day 0, followed by 3×10^8 PFU Adex1CACre i.v. at day 14. Liver tissues were obtained at 4 weeks and DNA was isolated from the liver tissues. As a control, DNA was isolated from normal mouse liver. To detect deletion of CTLA4IgG sequence between lox P sites by Cre recombinase in vivo, we performed nested PCR analysis. Briefly, first PCR was performed with the use a set of primers (forward: 5'-GAATTGATTAATTTCGAGCGAACGC-3', reverse: 5'-CCTGAGGAGTGAATTGATTTGATCC-3') under the condition of 94°C for 1 min and 58°C for 1 min, followed by 72°C for 2 min for 35 cycles. The reaction mixture was applied to Chroma spin TE-100 column (Clontech, Palo Alto, CA) to remove the primers. Then, second nested PCR was performed using another set of primers (forward: 5'-TCTGCTAACCATGTTTCATGC-CTTC-3', reverse: 5'-GGCAGAGGGAAAAAGATCTCAGTG-3') under the condition of 94°C for 1 min and 55°C for 1 min, followed by 72°C for 2 min for 30 cycles. The final reaction mixture was analyzed by 2 % agarose gel electrophoresis. These primers were complementary to vector sequences located outside the two lox p sites. The expected sizes of non-deleted and deleted forms of vectors are approximately 2000 and 200 base pair (bp), respectively.

Histology

Immunohistochemical detection of CTLA4IgG staining was carried out on the liver tissue that had been fixed in 10% formaldehyde/PBS and sectioned. Since CTLA4IgG consisted of murine CTLA4 and Fc portion of human IgG, sections were stained with biotinylated anti-human immunoglobulin G (Jackson ImmunoResearch LABORATORIES, INC., USA) and followed by avidin horseradish peroxidase detection reagent (DAKO Japan, Kyoto). Some liver tissues were fixed in Bouin's solution and sections were stained with aldehyde fuchsin, and hematoxylin and eosin. Aldehyde fuchsin staining detected beta cell granules that contained insulin (Yasunami et al., 1983).

Anti-adenovirus antibody in sera

Serum concentrations of anti-adenovirus antibody were assayed by ELISA as described previously (Nakagawa et al., 1998).

Anti-keyhole limpet hemocyanin (KLH) antibody in sera

Mice were immunized subcutaneously with 50 µg KLH in 100 µl complete Freund's adjuvant (CFA) at day 4 after skin grafting and re-immunized with 50 µg KLH in 100 µl CFA at 4 weeks. The treatment of those mice with adenoviral vectors was the same as described in the section of skin grafting. Anti-KLH antibody titers in serum samples were detected at 5 and 7 weeks by ELISA. In brief, 96-well plates were precoated with KLH (10 µg/ml) and subsequently blocked with 1% BSA for 2 hr at room temperature. Then 100 µl serum samples were applied to the plates at 4° C for overnight. After washing, alkaline phosphatase-conjugated goat anti-mouse IgG was added and incubated for 1 hr. Finally, the colour reaction was developed with phosphatase substrate in diethanolamine and the plates were read at 405 nm. All serum samples were tested in duplicate at 1/20 dilution and absorbance for the duplicate wells was averaged.

RESULTS

Expression of CTLA4IgG by Adex1CALoxCTLA4IgGLox

CTLA4IgG was purified from culture supernatants of COS7 cells transfected with Adex1CALoxCTLA4IgGLox. Ligand binding ability of CTLA4IgG was comparable to that derived from previously described Adex1CACTLA4IgG (Figure 1A). However, SDS-PAGE analysis revealed that the two CTLA4IgG migrated slightly differently, possibly due to the difference in post-translational modification. After intravenous (i.v.) injection of Adex1CALoxCTLA4IgGLox, serum concentration of CTLA4IgG molecule was measured by ELISA at various time points. The maximum concentration was obtained at day 7 after injection of Adex1CALoxCTLA4IgGLox (4×10^8 PFU/body) into C57BL/6 mice (mean 10 mg/ml) (Figure 1B). Serum CTLA4IgG concentration sharply decreased, which marked approximately 1.8 mg/ml at 4 weeks after injection. Thereafter, a considerable amount of serum CTLA4IgG was detected up to 18 weeks. The maximum serum concentration of CTLA4IgG positively correlated with the dose of Adex1CALoxCTLA4IgGLox. When the titer of Adex1CALoxCTLA4IgGLox decreased to 4×10^7 PFU/body, the peak concentration decreased to 2 mg/ml at day 7 and gradually decreased thereafter. In all the mice tested, serum CTLA4IgG concentration was detectable for at least 18 weeks.

Termination of CTLA4IgG expression in vivo

Next, we examined whether termination of CTLA4IgG expression could be achieved in vivo. C57BL/6 mice were injected with 1×10^7 PFU Adex1CALoxCTLA4IgGLox via tail vein, which was followed by Adex1CACre at various multiplicities of infection (MOI) ratios 8 weeks after Adex1CALoxCTLA4IgGLox injection. Then serum CTLA4IgG concentration was measured. It was about 40-50 μ g/ml at 8 weeks after Adex1CALoxCTLA4IgGLox injection; then it gradually decreased and marked 25 μ g/ml of CTLA4IgG at 12 weeks. On the other hand, 1×10^9 PFU Adex1CACre injection resulted in the complete termination of CTLA4IgG expression within 3 weeks. Even 5×10^8 PFU Adex1CACre injection led to a significant decrease of serum CTLA4IgG concentration and CTLA4IgG was no

longer detected in the sera within 3 to 4 weeks (Figure 1C). As a control, mice were injected with 1×10^7 PFU Adex1CACTLA4IgG, which was followed by Adex1CACre at 50 MOI at 8 weeks. As shown in Figure 1D, the serum CTLA4IgG was not significantly reduced after administration of Adex1CACre. Cre-mediated recombination of Adex1CALoxCTLA4IgGlox in the liver was determined by nested PCR analysis, which demonstrated complete disappearance of CTLA4IgG gene after administration of Adex1CACre (Figure 2A). Additionally, immunohistochemical analysis confirmed the disappearance of CTLA4IgG protein expression in the liver after Adex1CACre injection (Figure 2B and C). Taken together, CTLA4IgG expression from Adex1CALoxCTLA4IgGlox was efficiently terminated by Adex1CACre treatment in vivo.

Long-term allograft survival after termination of CTLA4IgG

After streptozotocin treatment of recipients at day -7, all mice (n=20) showed blood sugar levels above 20 mmol/L (Figure 3 and data not shown) and received allogeneic islets at day 0. These diabetic mice were divided into four experimental groups (Table 1A). Survival of mouse islet allografts in group II ranged from 7 to 11 days (mean $\pm$ SD, 9.4 ± 2.2 days). Those in group I also ranged from 7 to 11 days. After islet cell transplantation, the levels of blood sugar sharply declined in all the mice of group III and in 4 out of 5 mice the blood sugar level remained below 16 mmol/L throughout experiment (Figure 3A). These four mice were sacrificed around 90 days after transplantation and viability of allografted islet cells in the recipient liver was histologically confirmed. At 70 days, serum concentration of CTLA4IgG was still over 50 μ g/ml in group III (Figure 3C). After Adex1CACre treatment, the serum CTLA4IgG level declined sharply and was not detectable at day 50 after transplation in group IV (Figure 3D); blood sugar level remained below 16 mmol/L in three mice and allografted islet cells were viable in the recipient liver at the time of sacrifice. Representative viable islets in the recipient liver was shown; islets cells were heavily infiltrated by lymphocytes and almost no beta cell granule was detected in group II (Figure 3E; day 10 sample), while the islets were viable with beta cell granules, intact, clearly stained with aldehyde fuchsin, and no obvious inflammation was detected in group IV (Figure 3 F; day 96 sample). These results demonstrated that long-

term allograft survival was achieved even after the termination of CTLA4IgG production.

Survival of mouse skin allografts from BALB/C to CD40 ligand (CD40L) knock-out mice was also studied (Table 1B). Survival of allografted skin ranged from 8 to 10 days (mean $\pm$ SD, 8.7 ± 0.6 days) in group I. In sharp contrast, group II and III exhibited prolonged acceptance of allografted skin (Table 1B). Nevertheless, serum CTLA4IgG was still over 100 μ g/ml in group II at day 42, whereas it was not detectable in group III at day 42 (Figure 4E and F). In order to rule out the possibility that low levels of CTLA4IgG expression, which were not detectable with ELISA, could prolong the graft survival, we treated mice (each group; $n=3$) i.v. with 1×10^8 PFU Adex1CALoxCTLA4IgGlox at day 0, followed by i.v. 3×10^9 PFU Adex1CACre at day 21. At day 28, allogeneic skin grafts were made. At the time of skin graft, serum CTLA4IgG was undetectable with ELISA. Skin grafts were rejected at day 9, 9, and 10 as expected.

We then examined whether group III (experiment 2) in Table 1B were tolerant to the donor-type skin graft after the termination of CTLA4IgG expression. Three mice from each group received secondary donor-type and third party skin grafts at day 50, and the grafts were inspected up to day 71. In group II, both donor-type and third party skin grafts survived up to 3 weeks. In contrast, group III rejected the third party skin grafts at day 7, while the donor-type secondary skin grafts were still viable at 3 weeks.

Antibody production after termination of the expression of CTLA4IgG molecules

By using the serum samples obtained from the same mice as those used in experiment 2 of Table 1B, we examined whether the mice which accepted the allografted skin could mount immune responses against adenovirus. After in vivo injection of Adex1CALoxCTLA4IgGlox (group II in Table 1B), the production of IgM class anti-adenovirus antibodies was detected at 1 week and then gradually decreased (Figure 4A); however, IgG class anti-adenovirus antibody production was minimal and was not augmented by the injection of AdexLacZ at day 21 (Figure 4C), indicating that serum CTLA4IgG suppressed anti-adenovirus antibody production (Figure 4E). In contrast, after injection of Adex1CACre at day 21 (group III in Table 1B), serum CTLA4IgG gradually

disappeared and IgG class anti-adenovirus antibody production increased in 3 out of 6 mice tested, which indicated that at least 50% of mice were able to mount secondary immune responses against adenovirus (Figure 4D). In addition, we tested whether recipient mice were able to mount immune responses against KLH. Skin grafts and adenoviral vector treatments were carried out (each group; n=3) as shown in Table 1B. Those mice were sensitized with KLH at 4 days and re-immunized with KLH at 4 weeks after skin graft. Serum samples were obtained at 5 and 7 weeks. In this setting, we tried to test whether group II was anergic to KLH since those mice had been sensitized with KLH while their serum CTLA4IgG was high and re-immunized with KLH after the termination of CTLA4IgG expression. As shown in Fig. 5, group I was able to mount IgG anti-KLH antibody response. Group III mice were also able to mount IgG anti-KLH antibody response. In contrast, group II mice were unable to mount IgG anti-KLH response. Nevertheless, the survival of allografted skin was not different from results shown in Table 1B. In addition, serum CTLA4IgG was not detectable in group III, whereas that in group II was still over 100 µg/ml at day 35.

DISCUSSION

Great advances have been made in organ transplantation. Among newly developed promising anti-rejection agents, CTLA4IgG has been shown to efficiently prolong graft survival in several transplantation models (Lenshow et al., 1992; Baliga et al., 1994; Pearson et al., 1994; Yamada et al., 1996; Gainer et al., 1997; Kirk et al., 1997; Levisetti et al., 1997; Feng et al., 1999; Tarumi et al., 1999). CTLA4IgG interferes with the interaction of CD28 on T cells and B7 on antigen-presenting cells and blocks the costimulatory signal essential for T cell activation and proliferation (Linsley et al., 1992; Tan et al., 1993; Wu et al., 1993). Since the effectiveness of CTLA4IgG is dependent on the engagement of TCR, theoretically the specificity of T cell suppression can be exploited well as compared to the effects of currently used immunosuppressive agents which lead to pan-T cell suppression. Two approaches have been attempted for prolongation of grafted organ with CTLA4IgG. One approach was the administration of purified CTLA4IgG proteins (both systemic and local application). Systemic administration of purified CTLA4IgG proteins generally required repeated injection of proteins (Lenshow et al., 1992; Baliga et al., 1994; Pearson et al., 1994; Kirk et al., 1997; Levisetti et al., 1997; Tarumi et al., 1999). In the case of human pancreatic islet xenograft into mouse recipients, treatment with 10 μ g CTLA4IgG per day for 14 days achieved the mean graft survival of 12.75 days. When the dose was increased to 50 μ g per animal every other day for 14 days, 100% of recipients mice maintained normal islet cell function at least up to 45 days after transplantation (Lenshow et al., 1992). In a recent study of non-human primate model of allogeneic islet transplantation, it was shown that only two of five systemically CTLA4IgG-treated primates had significant prolongation of graft survival (survival was approximately 80 days) (Levisetti et al., 1997). Those primates were treated with 12.5 mg/kg CTLA4IgG i.v. daily on days 1-7, followed by 12.5 mg/kg i.v. twice daily on day 8-14. It was shown that serum level of CTLA4IgG varied among primates and survival of islet cells depended on the serum levels of CTLA4IgG. In a preliminary study, we also found that the survival of allografted islet cells was less than 16 days in streptozotocin-treated mice that received 50 μ g CTLA4IgG at day 0, 2, and 4. On the other hand, blockade of CD28/B7 pathway by ex vivo coating of allogeneic islet cells with purified CTLA4IgG proteins was able to achieve

the mean graft survival of 37 days and long survival of allografted islet cells in 42% of recipients (Steurer et al., 1995). Taken together, to achieve long-term survival of allografted islet cells, the repeated injection of CTLA4IgG proteins and the maintenance of serum CTLA4IgG over critical level were required.

Alternatively, CTLA4IgG gene therapy was developed (Chahine et al., 1995; Gainer et al., 1997; Feng et al., 1999). The gene therapy for islet transplantation involved the following; islets in vitro infected with adenovirus vector containing CTLA4IgG (the mean survival with the highest MOI of 10 was 39 ± 8.7 days) (Feng et al., 1999) and islets in vitro transfected with CTLA4IgG gene by gene gun method (the mean survival was 66.8 ± 61.5 days; a long term-survival rate being 50%) (Gainer et al., 1997). While those therapies were designed to allow the production of CTLA4IgG locally, intramuscular injection of adenovirus vector containing CTLA4IgG (Feng et al., 1999) and transplantation of syngeneic myoblasts transfected in vitro with CTLA4IgG gene (Chahine et al., 1995) were also designed to allow the production of CTLA4IgG systemically. It is premature to conclude which treatment (CTLA4IgG protein versus gene therapy or systemic versus local therapy) is more beneficial to recipients only from those studies. The efficacy of CTLA4IgG in prolongation of graft survival is dose-dependent (Lenschow et al., 1992). While the larger doses of CTLA4IgG proteins or genes are more effective for preventing rejection, they render the recipients to be immunosuppressive state (Linsley et al., 1992).

We previously reported the generation of an adenovirus vector containing CTLA4IgG, Adex1CACTLA4IgG (Nakagawa et al., 1998). Contrary to the fact that in general transgene expression by adenoviral vector is in a limited period, a high concentration of serum CTLA4IgG was achieved for a long time upon in vivo administration of Adex1CACTLA4IgG, which resulted in the inhibition of antibody production including anti-adenovirus antibody. The result indicated that the recipients were unable to mount immune responses against insulting agents as long as the serum level of CTLA4IgG remained over critical level (Nakagawa et al., 1998; Takiguchi et al., 2000). To overcome this dilemma, we generated an adenovirus vector containing CTLA4IgG using Cre-lox P system. cDNA of CTLA4IgG was cloned between two lox P sequences under control of CAG promoter in an adenovirus vector. We had expected that the administration of

Adex1CACre at desired time would induce Cre-mediated recombination within a gene derived from Adex1CALoxCTLA4IgGlox vector and that the cDNA of CTLA4IgG would be excised from the transduced gene. Indeed, we demonstrated by nested PCR analysis, immunohistology and ELISA assay that CTLA4IgG gene was eliminated in vivo and production of CTLA4IgG was terminated. By using this new vector, we examined whether the long-term acceptance of allogeneic islet cells and skins can be achieved in recipients even after termination of CTLA4IgG production in vivo by Cre recombinase.

We demonstrated that the long acceptance of allografted islet cells was achieved by a single i.v. gene therapy with Adex1CALoxCTLA4IgGlox vector even after the termination of CTLA4IgG expression by Adex1CACre injection. The long-term islet cell allografts (group III mice in Table 1B) were histologically intact with no obvious inflammation accompanied as shown in Figure 3. It was previously shown that long-term islet cell allografts were accompanied by dense mononuclear cell peri-infiltrates in CTLA4IgG-treated mice (Steurer et al., 1995; Gainer et al., 1997). The discrepancy between our results and others may be due to the difference of the site of transplantation. We inoculated islet cells into the liver via a portal vein, while others injected islet cells under the renal capsule. Other possibility is due to the difference in the serum CTLA4IgG concentration. Thus, we demonstrated that the new vector was useful for the induction of long-term survival of allografted cells. We next tried to demonstrate that this vector was also useful for allografted tissues. Thus, we used alloskin graft model. It was already known that the blockade of B7/CD28 pathway by CTLA4IgG was not satisfactory for the prolongation of allografted skin survival (Pearson et al., 1994) and both CD40/CD40L and CD28/B7 pathways were involved in the generation of alloimmune responses (Larsen et al., 1996). Since our aim was to demonstrate the efficacy of the new vector, Adex1CALoxCTLA4IgGlox, we used the CD40L knock-out mice in which CD40/CD40L pathway was deficient. As expected, we found that the long-term acceptance of allografted skin was achieved with Adex1CALoxCTLA4IgGlox vector even after the termination of CTLA4IgG expression by Adex1CACre injection. Nevertheless, allografted skins were vigorously rejected in untreated control mice at day 7, while allografted skins were rejected at approximately 13 days in control mice treated with Adex1CALoxCTLA4IgGlox (data not shown).

To analyze the underlying immunological mechanisms that might explain the long-term acceptance of allografts after termination of CTLA4IgG expression, we performed the secondary skin graft experiment. Secondary grafts of donor type skin in Group III mice in Table 1B survived up to 3 weeks, whereas those of third party were rejected at day 7, indicating the possibility that Group III mice were tolerant to the allografts. However, the long-term evaluation of allografts is necessary to confirm that group III mice were really tolerant to alloantigens. In this regard, it was recently shown that combined blockade of the CD40 and CD28 costimulatory pathways does not adequately inhibit CD8-mediated allograft rejection in certain strains of mice (Trambley et al., 1999). It is possible that other interactions such as 41BB/41BB ligand and Fas/Fas ligand can provide costimulation for CD8 T cells (Trambley et al., 1999).

We then analyzed the immunological status of allogeneic skin graft recipients. In Adex1CALoxCTLA4IgGLox-treated mice (group II in Table 1B), IgG class anti-adenovirus antibody production was minimal, suggesting that these mice were susceptible to adenovirus infection. The low IgG antibody production even after AdexLacZ injection may be due to the blocking of T cell-dependent antibody responses by serum CTLA4IgG or induction of T cell anergy by interfering with the B7/CD28-mediated costimulatory signal by serum CTLA4IgG. It is also possible that naive T cells recognizing an antigen in the absence of costimulatory signals undergo apoptosis (Liu and Janeway, 1990) and are thus clonally deleted. In this regard, it should be pointed out that at least 50% of group III mice were able to mount secondary IgG antibody responses against adenovirus upon Adex1CACre injection, indicating that T cells were not anergic to adenovirus and T cells recognizing adenovirus were not clonally deleted. We confirmed that mice were not anergic to one of exogenous proteins, KLH. Currently we have not known how the host immune system decides tolerance to allografted tissues, but not to a virus antigen or an exogenous protein. It is possible that the passenger leukocytes that are strong antigen-presenting cells migrate out from the grafts, thus remaining allografted tissues might become less immunogenic. In contrast, exogenous and virus antigens are presented by recipient antigen-presenting cells. Finally, it should be pointed out, however, that secondary adenoviral transgene may not be expressed in recipients after Adex1CACre treatment since they are not anergic to adenoviral antigen and are able to

mount IgG anti-adenovirus antibody responses. In any event, we demonstrated that our new vector minimizes the adverse effects of CTLA4IgG gene therapy by terminating the expression of CTLA4IgG at desired time point, while it induces tolerance to alloantigens.

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Legends for Figures

Figure 1. Expression of CTLA4IgG and its termination. A; SDS-PAGE analysis of protein A-purified CTLA4IgG derived from 1 ml culture supernatant of 1×10^7 PFU Adex1CACTLA4IgG- (lane 2), mock- (lane 3), or Adex1CALoxCTLA4IgGlox- (lane 4) transfected COS cells (5×10^6 cells). Ligand-binding ability of purified CTLA4IgG was determined by FACS analysis with B7-1-transfected CHO cells. a; Adex1CACTLA4IgG. b; mock transfected. c; Adex1CALoxCTLA4IgGlox. B; Persistent expression of CTLA4IgG after in vivo injection of Adex1CALoxCTLA4IgGlox. ELISA values represent the mean of serum samples derived from 5 mice. C; Termination of CTLA4IgG expression after in vivo injection of various MOI doses of Adex1CACre at 8 week. Adex1CALoxCTLA4IgGlox (1×10^7 PFU/body) was given at 0. D; Adex1CACre does not terminate the expression of CTLA4IgG induced by classical Adex1CACTLA4IgG vector. 1×10^7 PFU Adex1CACTLA4IgG was given at day 0 followed by 5×10^8 PFU Adex1CACre at 8 weeks or no treatment. Each group consisted of 5 mice (3 mice in Figure 1D) and the ELISA values represent the mean of serum samples derived from 5 mice.

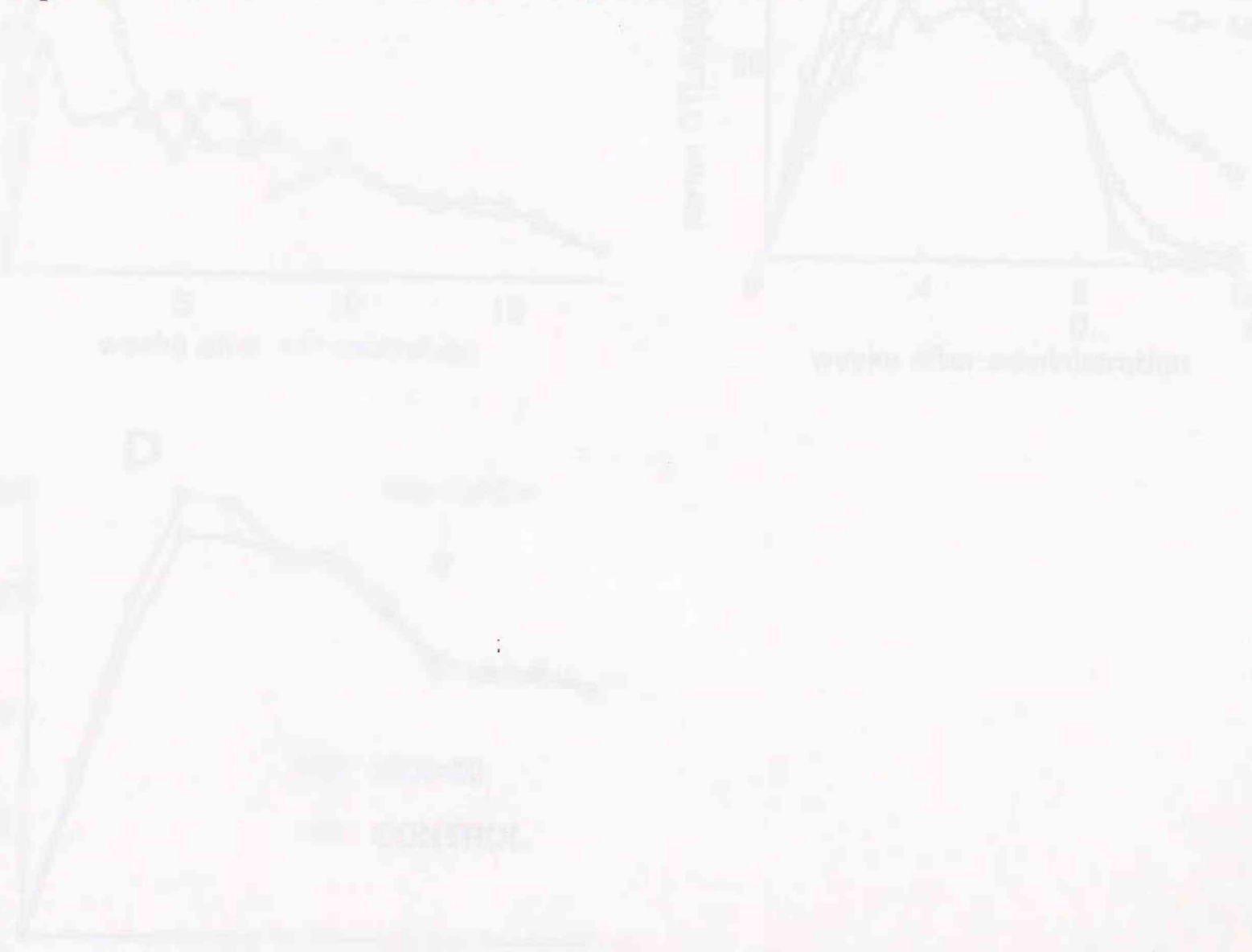
Figure 2. Termination of CTLA4IgG expression by Cre recombinase in vivo. A; Detection of vector sequence by nested PCR. Liver tissues were obtained from normal (lane 2), Adex1CALoxCTLA4IgGlox-injected mice (lane 3), and Adex1CALoxCTLA4IgGlox plus Adex1CACre-injected mice (lane 4). Primer settings were schematically shown. Arrows indicate the position of vector sequence. B and C; Liver tissues were also subjected to the immunohistochemical detection of CTLA4IgG expression as indicated by arrow heads and the representative results were shown (B; Adex1CALoxCTLA4IgGlox-injected mice and C; Adex1CALoxCTLA4IgGlox plus Adex1CACre-injected mice. $\times 170$).

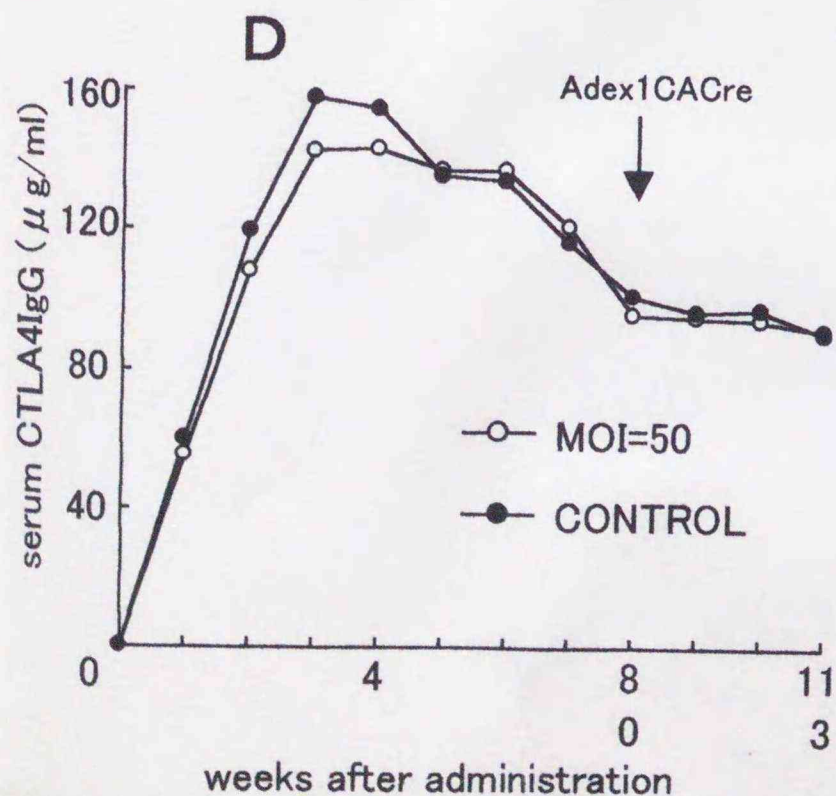
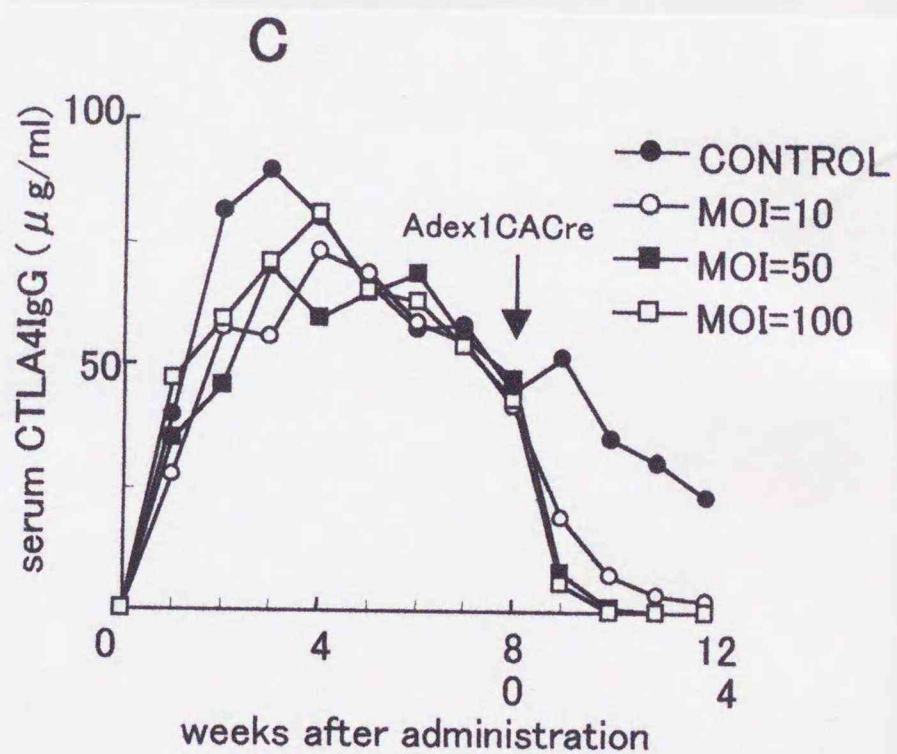
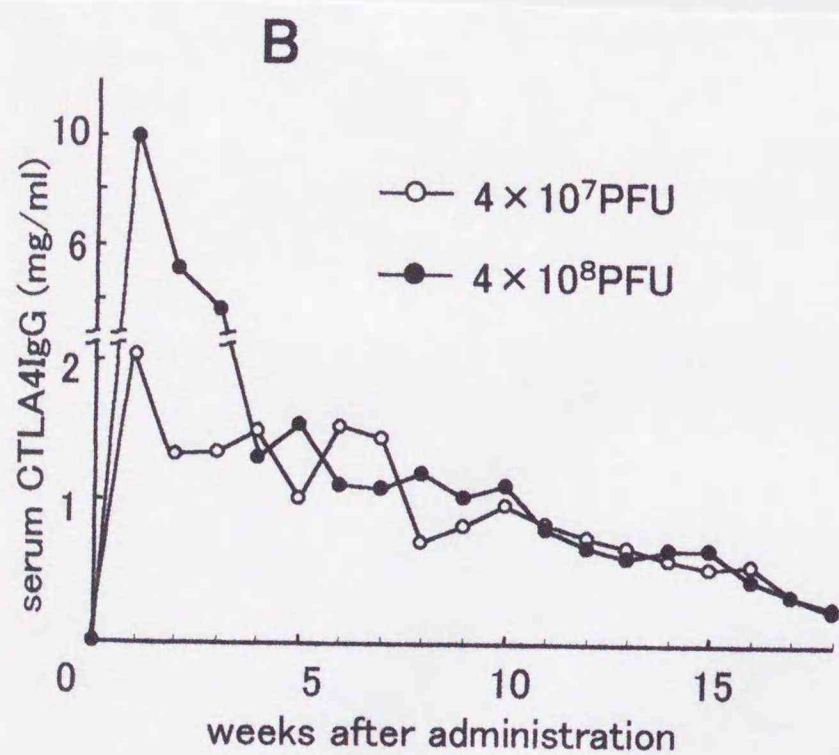
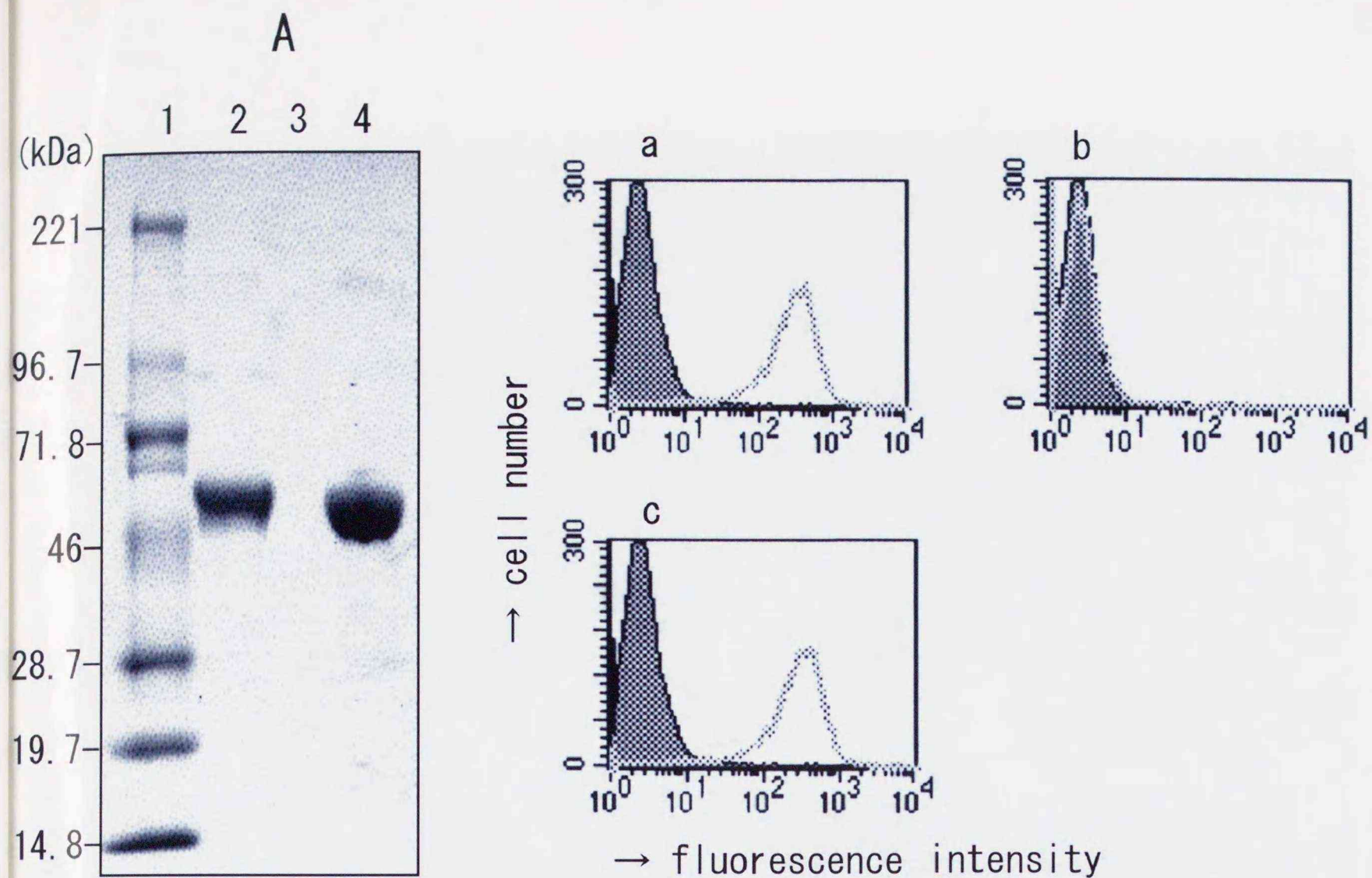
Figure 3. Long-term survival of allografted islets. Blood sugar (A; $n=5$ and B; $n=3$) and serum CTLA4IgG (C; $n=5$ and D; $n=3$) were examined in Adex1CALoxCTLA4IgGlox-injected (A and C) and Adex1CALoxCTLA4IgGlox plus Adex1CACre-injected (B and D) mice as shown in Table 1A. Presence of viable islets in the recipient liver was confirmed histologically at the time of sacrifice and the representative results are shown (E; mice in group II of Table 1A. Strong inflammatory cell

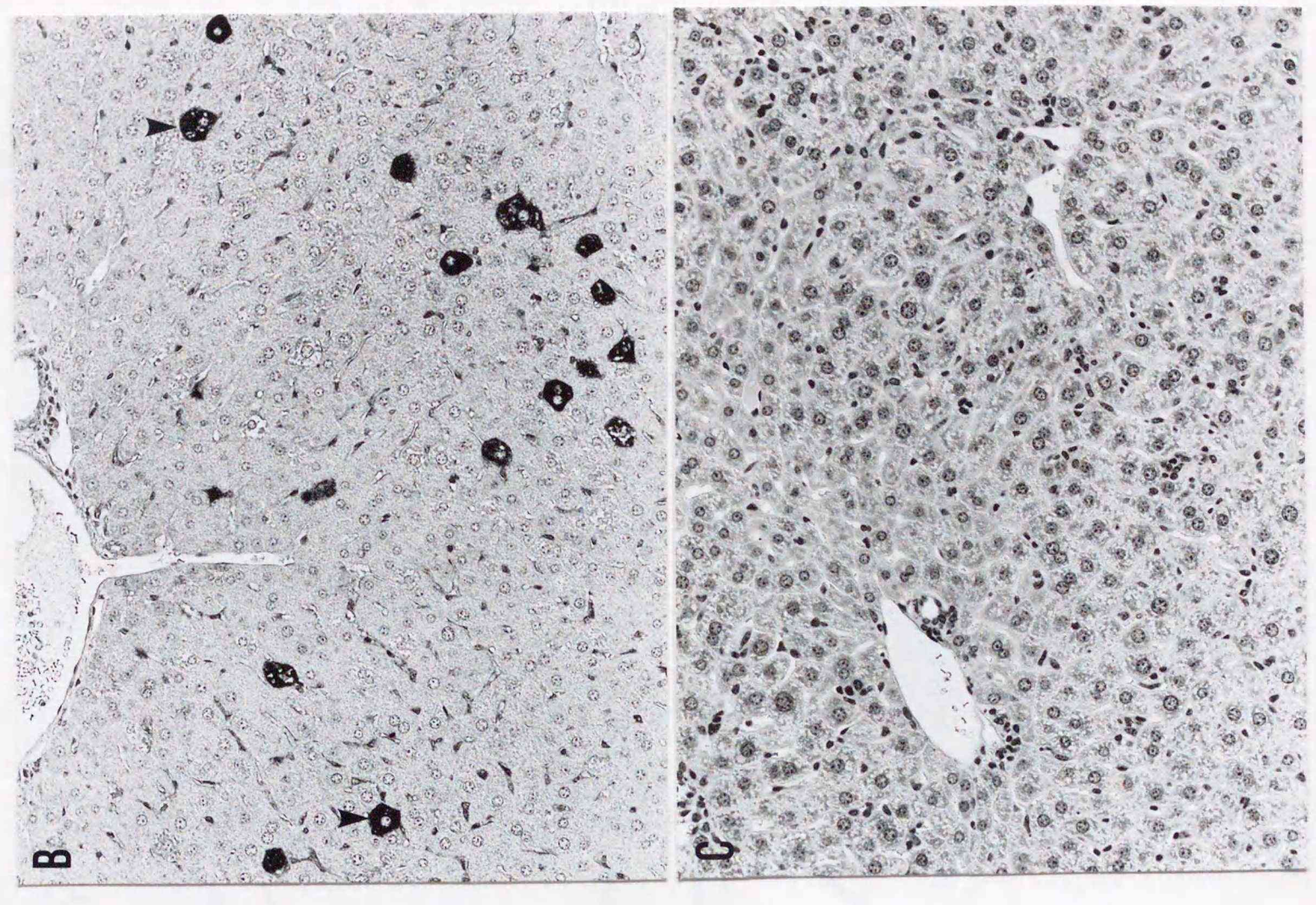
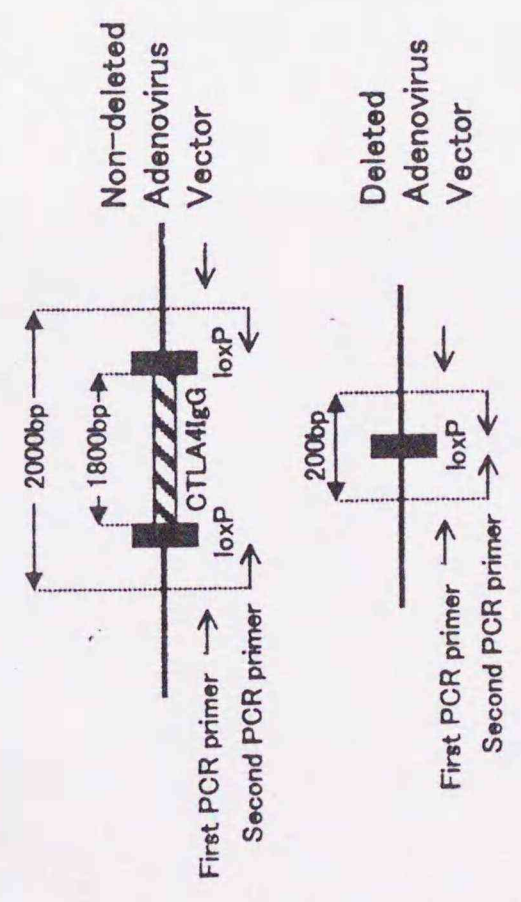
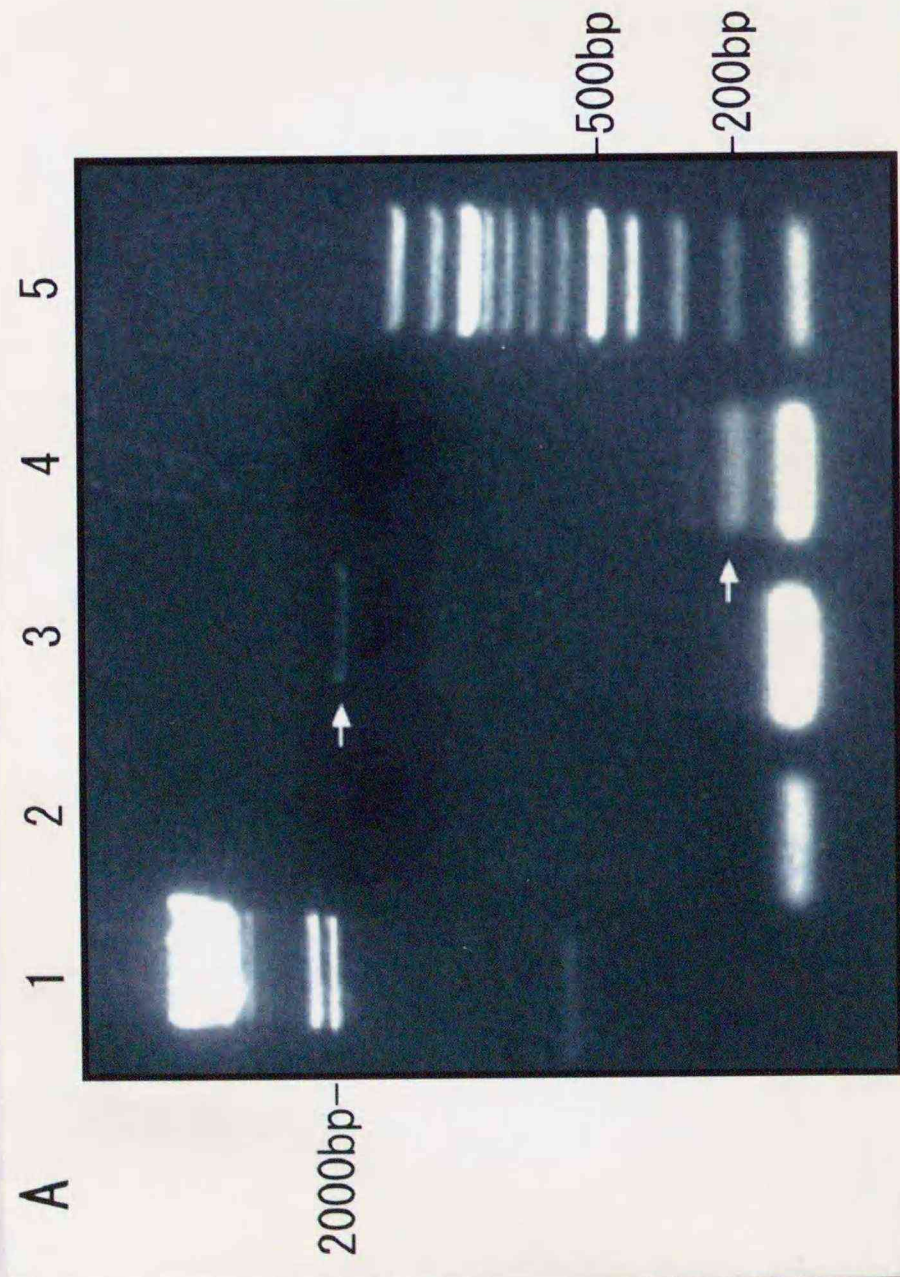
infiltration into islet cell clusters (1) and lack of beta cell granule staining by aldehyde fuchsin staining (2). F; mice in group IV shown in Table 1A. Intact islet cells (1) and strong beta cell granule staining (2). No obvious inflammation. Hematoxylin-eosin staining (1) and aldehyde fuchsin staining (2); $\times 340$). The mice were individually marked with different symbols such as $\bigcirc$, $\blacksquare$, and etc.

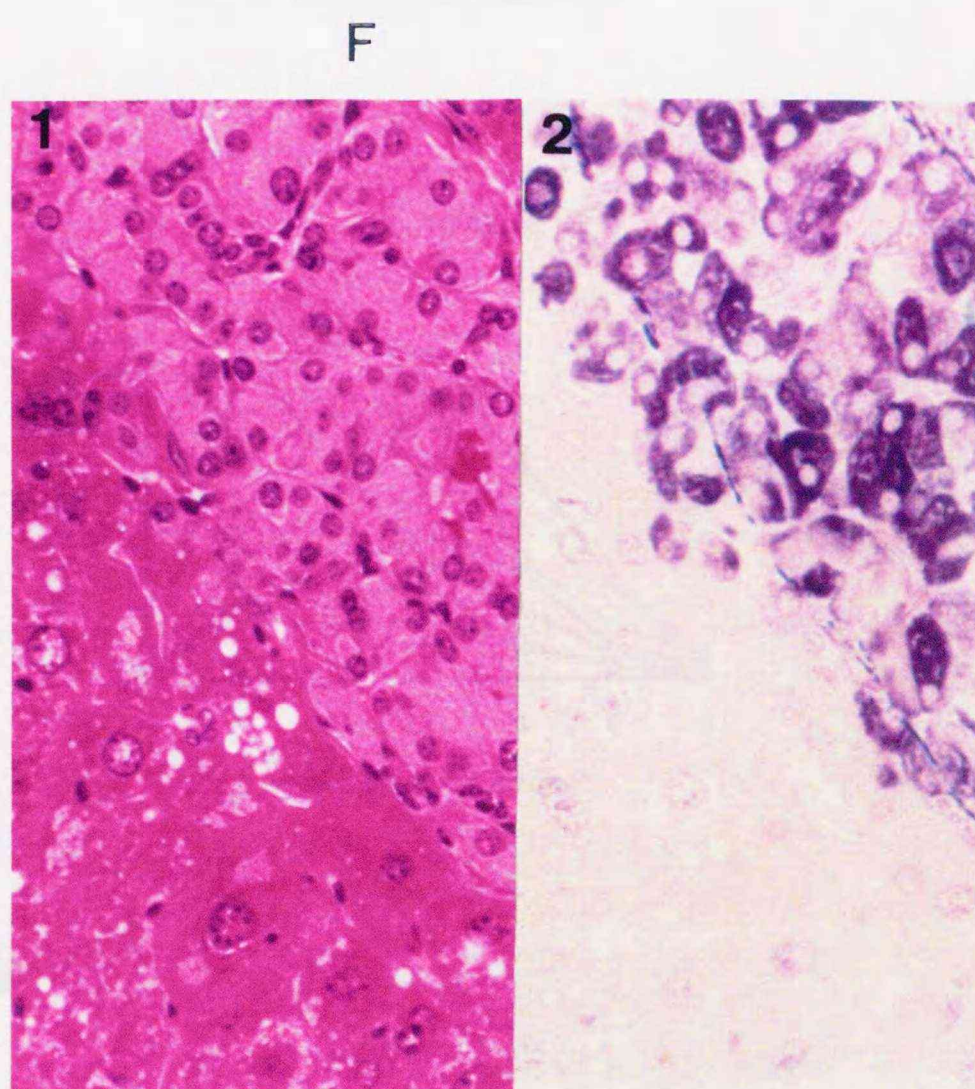
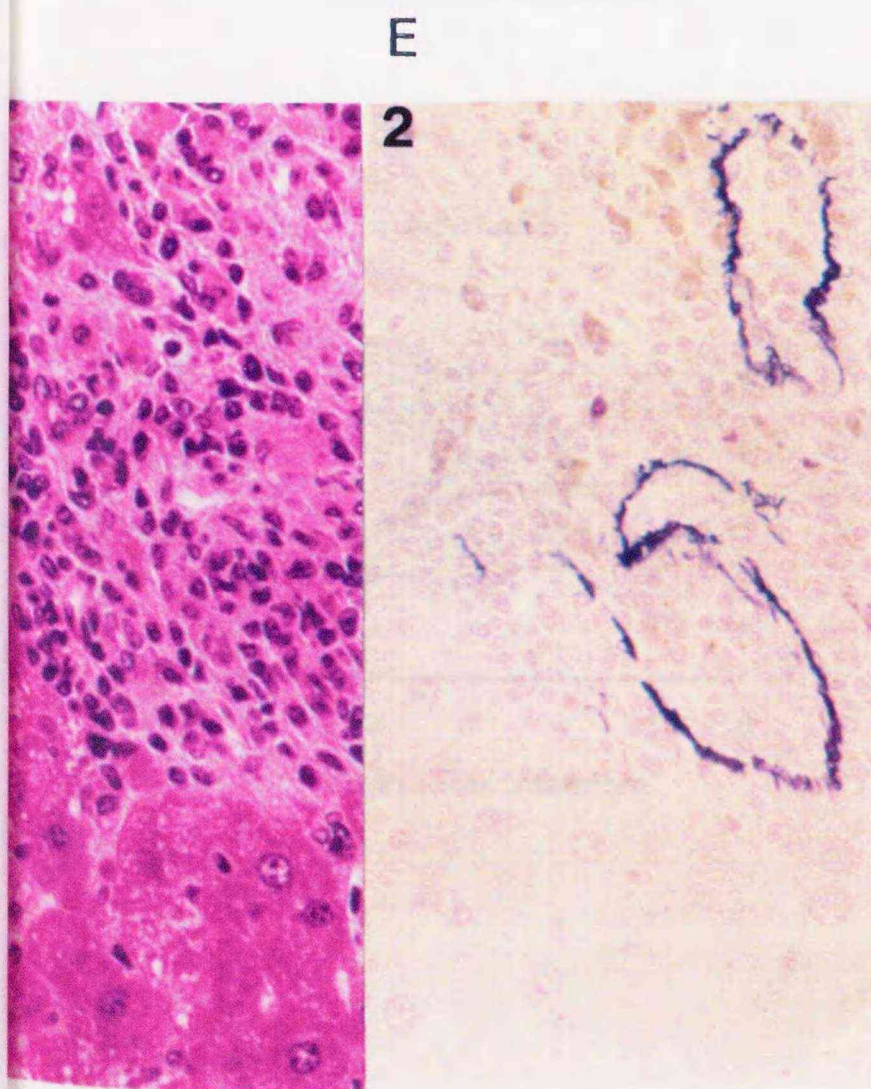
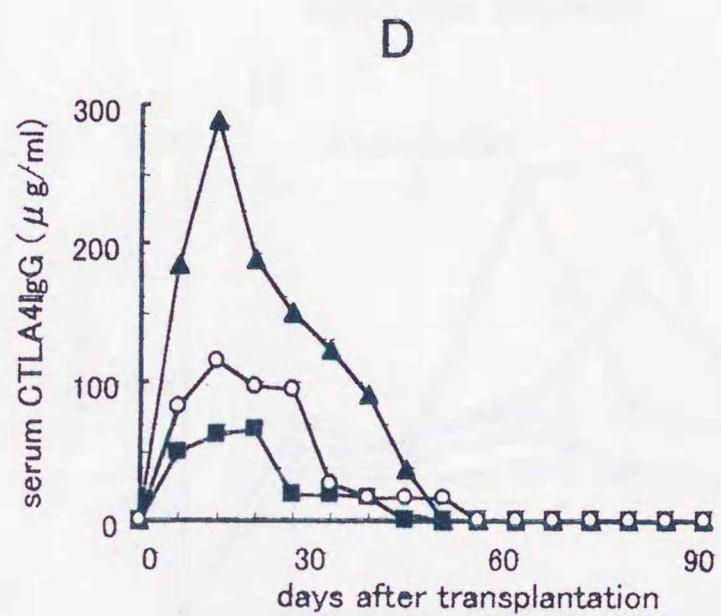
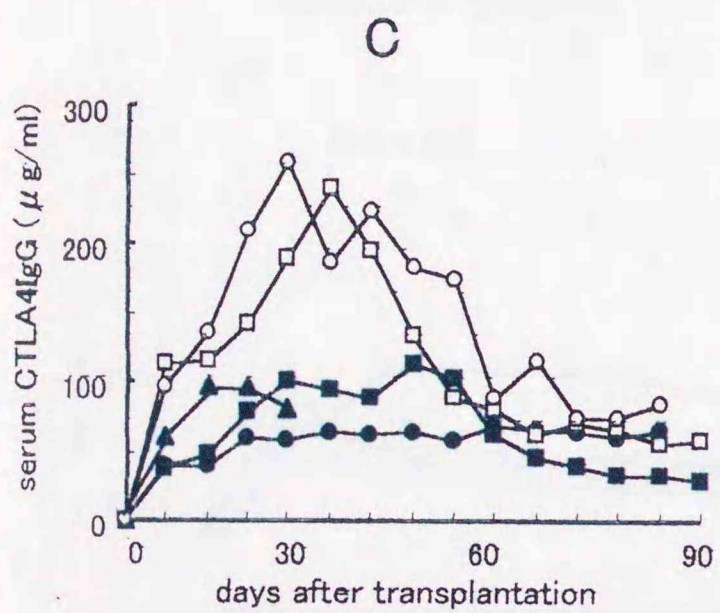
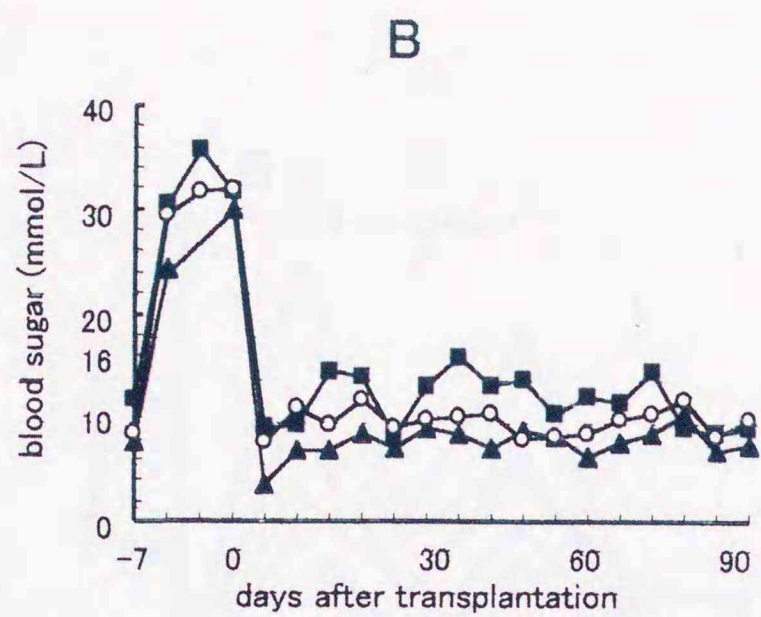
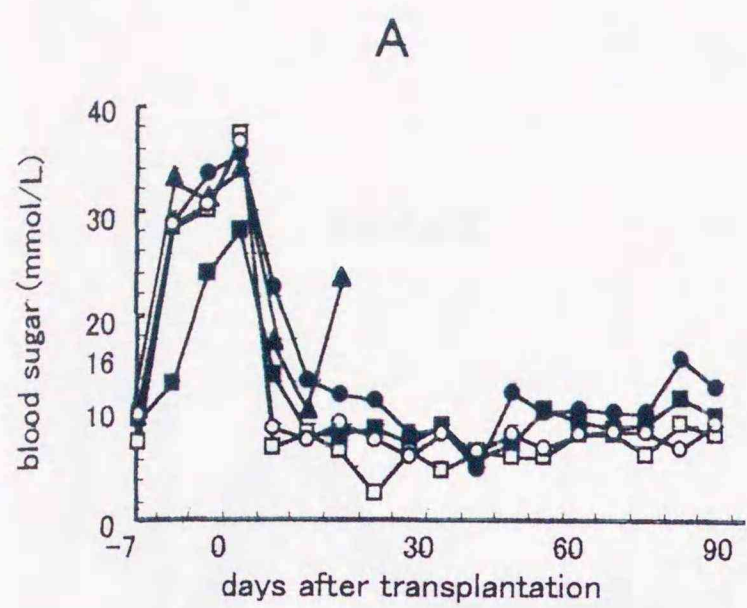
Figure 4. Appearance of IgG class anti-adenovirus antibody after the termination of CTLA4IgG expression in mice accepting allografted tissues. Serum were obtained from the same mice as those used in the experiment 2 shown in Table 1B. IgM (A and B) and IgG (C and D) class anti-adenovirus titer and serum CTLA4IgG (E and F) were examined in group II (A, C, and E; $n=6$) and group III (B, D, and F; $n=6$) mice. The mice were individually marked with different symbols such as $\bullet$, $\bigcirc$, $\blacksquare$, $\square$, and etc.

Figure 5. Mice sensitized with KLH while serum CTLA4IgG level was high were not anergic to KLH. Skin grafts were carried out as described in Materials and Methods. Those mice were treated with adenoviral vectors by the protocol used in the experiment 2 shown in Table 1B. Sera were obtained from mice at 5 and 7 weeks. IgG class anti-KLH antibody responses were examined in group I, group II and group III mice (each group $n=3$). Data represented the mean of serum samples derived from 3 mice.









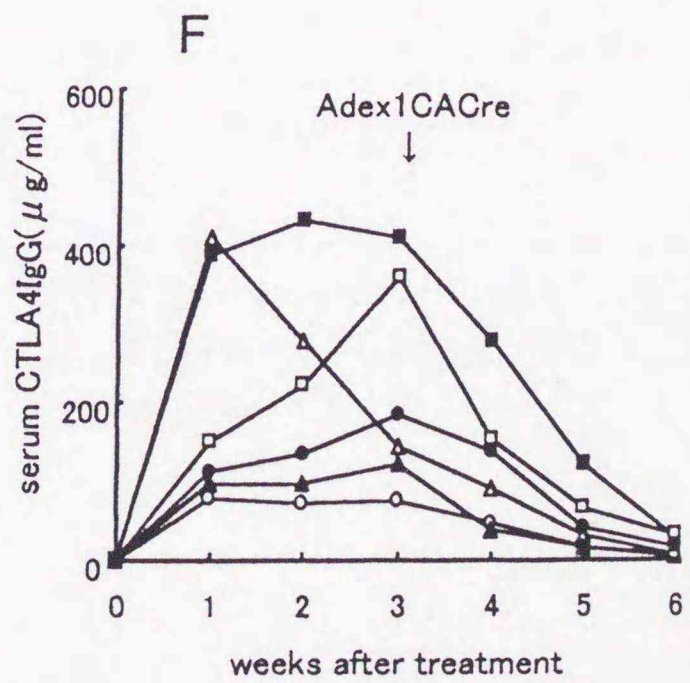
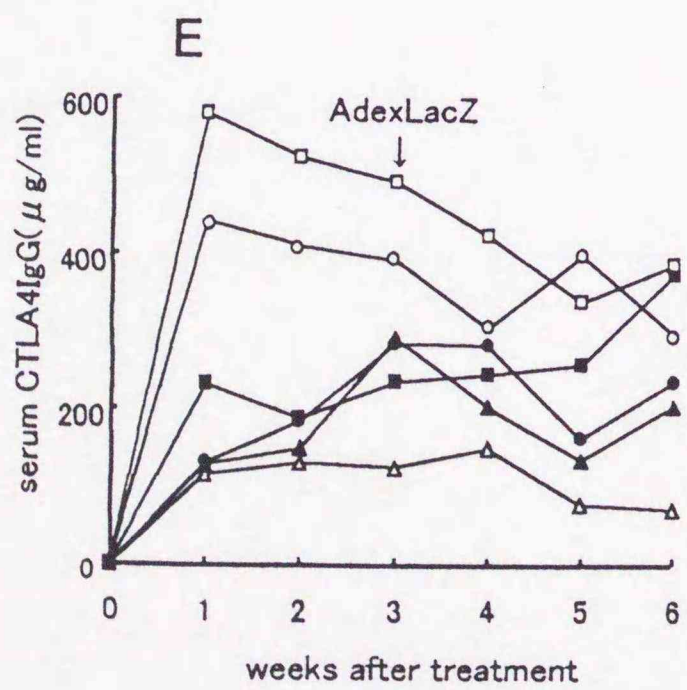
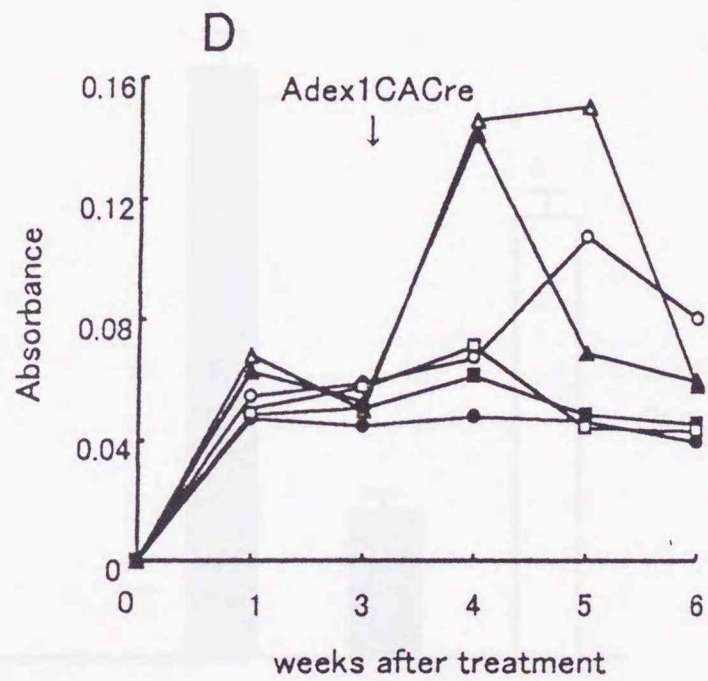
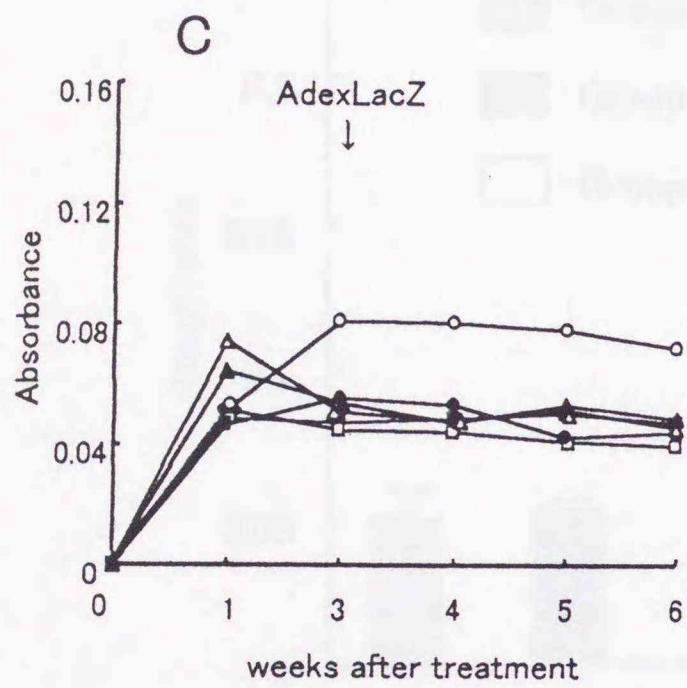
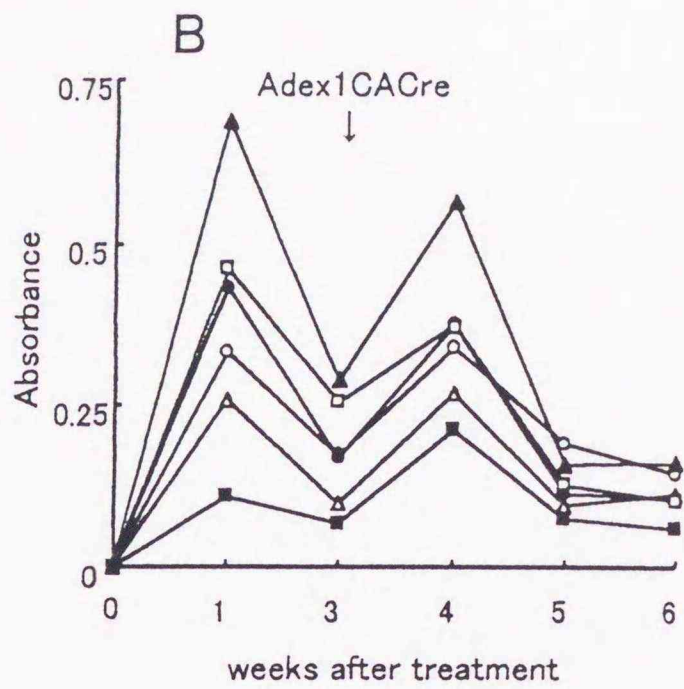
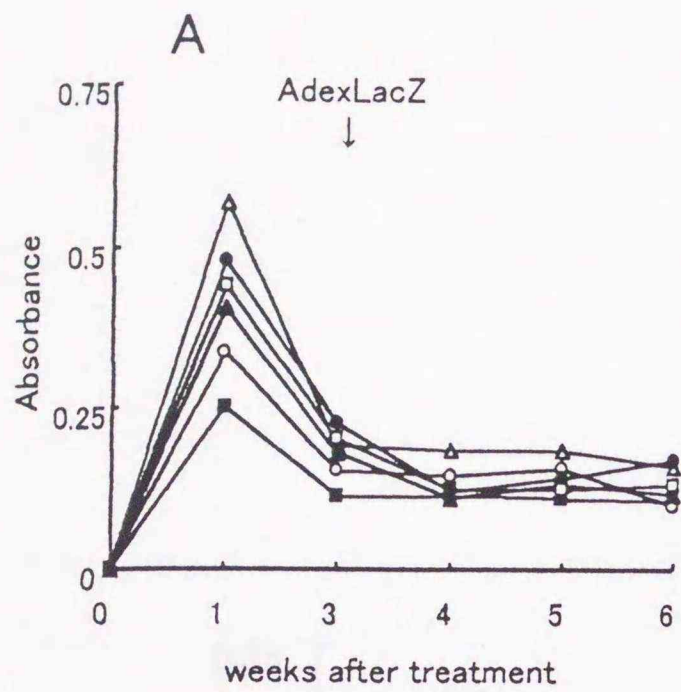


Fig. 4

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Table 1. Allograft survival

group	treatment	n	survival	mean $\pm$ SD	p < 0.05
I	none	2	7, 7, 8, 9, 10, 11, 12	8.0 $\pm$ 1.7	-
II	Adex100	5	7, 7, 11, 11, 12	8.4 $\pm$ 1.2	NS vs I
III	Adex100 + CTLA4Ig	5	7, 7, 8, 9, 10, 11, 12	8.0 $\pm$ 1.7	NS vs I
IV	Adex100 + CTLA4Ig + Ab	5	7, 7, 8, 9, 10, 11, 12	8.0 $\pm$ 1.7	NS vs I

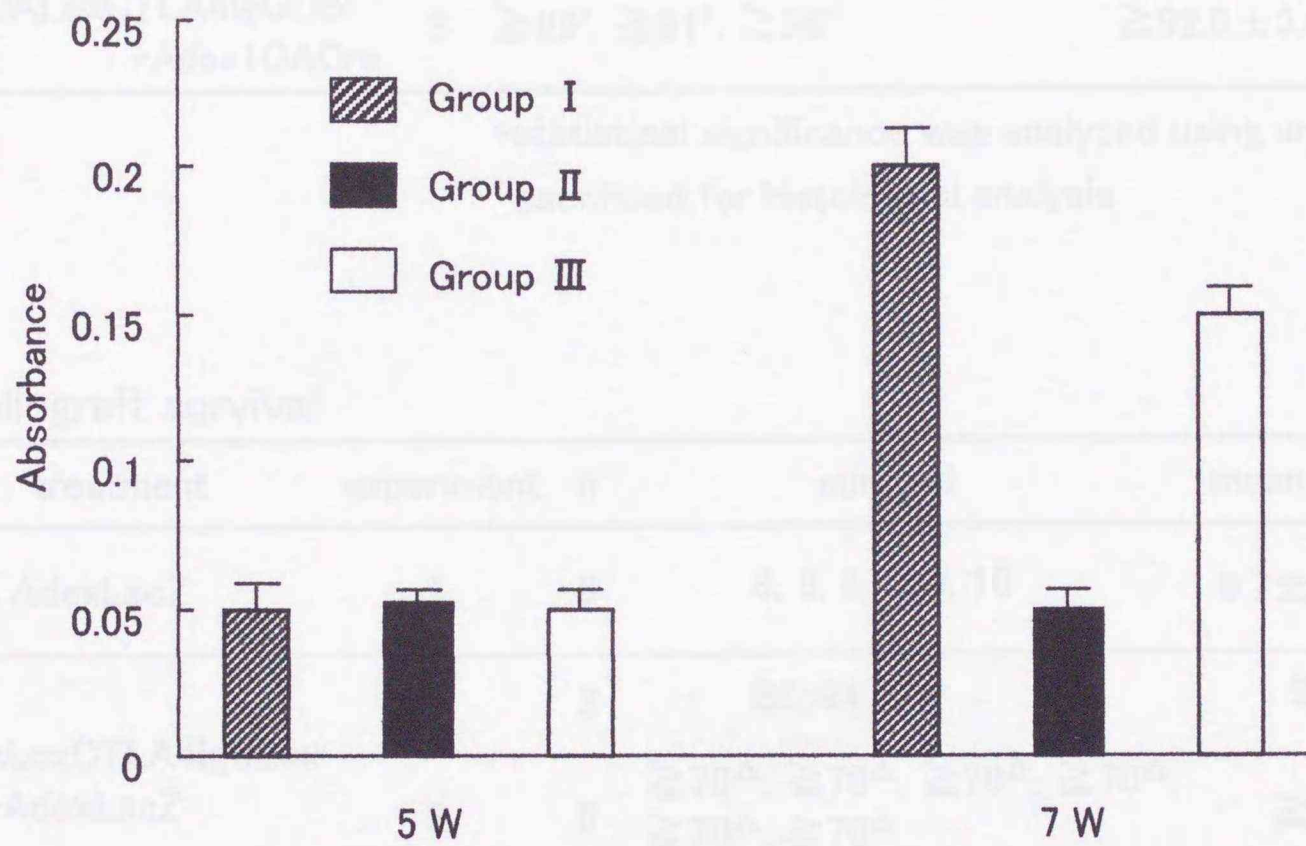
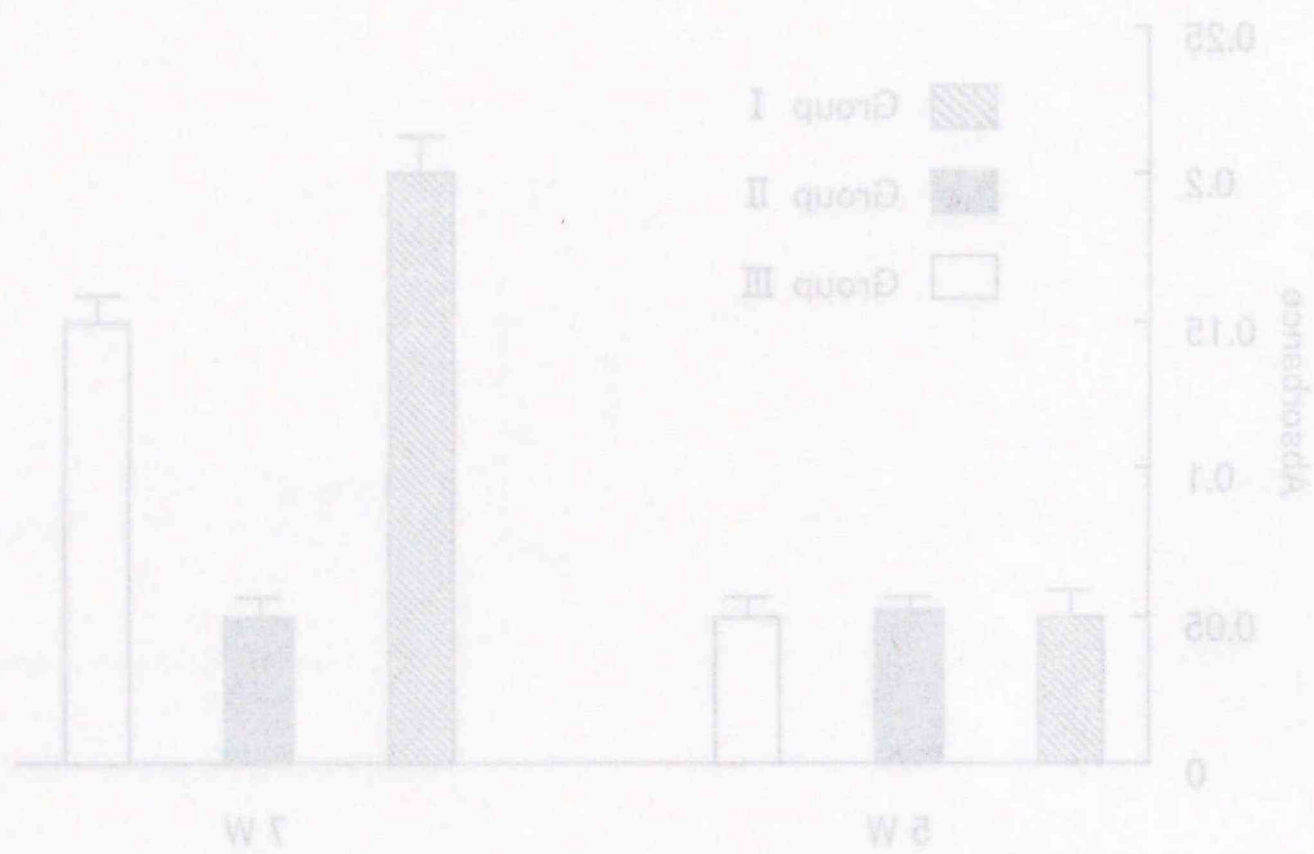


Fig 5
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Table

A.
group

I

II

III

IV

B.
group

I

II Ad

III Ad

Table 1: Allograft survival

A. islet cell allograft survival

group	treatment	n	survival	mean $\pm$ SD	*P < 0.05
I	none	7	7, 7, 8, 9, 10, 11, 11	9.0 $\pm$ 1.7	—
II	AdexLacZ	5	7, 7, 11, 11, 11	9.4 $\pm$ 2.2	N.S. vs I
III	Adex1CALoxCTLA4IgGLox	5	13, $\geq 91^\dagger$, $\geq 96^\dagger$, $\geq 98^\dagger$, $\geq 117^\dagger$	$\geq 83.0 \pm 40.4$	vs I, II
IV	Adex1CALoxCTLA4IgGLox +Adex1CACre	3	$\geq 89^\dagger$, $\geq 91^\dagger$, $\geq 96^\dagger$	$\geq 92.0 \pm 3.6$	vs I, II

*statistical significance was analyzed using unpaired U-test.

† sacrificed for histological analysis.

B. skin allograft survival

group	treatment	experiment	n	survival	mean $\pm$ SD	*P < 0.05
I	AdexLacZ	1	6	8, 8, 8, 9, 9, 10	8.7 $\pm$ 0.6	—
II	Adex1CALoxCTLA4IgGLox +AdexLacZ	1	2	86, 94	90	vs I
		2	6	$\geq 70^\Delta$, $\geq 70^\Delta$, $\geq 70^\Delta$, $\geq 70^\Delta$, $\geq 70^\Delta$, $\geq 70^\Delta$	≥ 70	vs I
III	Adex1CALoxCTLA4IgGLox +Adex1CACre	1	3	80, 90, 117	95.7 $\pm$ 19.1	vs I
		2	6	$\geq 70^\Delta$, $\geq 70^\Delta$, $\geq 70^\Delta$, $\geq 70^\Delta$, $\geq 70^\Delta$, $\geq 70^\Delta$	≥ 70	vs I

*statistical significance was analyzed using unpaired U-test.

$^\Delta$ serum CTLA4IgG and anti-adenovirus antibody titer were determined (See Figure 4).

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Table 1: Allogeneic survival

Group	Treatment	n	Survival	mean $\pm$ SD	95% CI
I	None	1	1, 1, 0, 0, 1, 1	1.1 $\pm$ 0.4	—
II	Subtotal	5	1, 1, 1, 1, 1, 1	1.4 $\pm$ 0.4	0.8-2.0
III	Subtotal (CTLA-4 IgG)	3	1, 1, 0, 1, 0, 1	1.0 $\pm$ 0.3	0.4-1.6
IV	Subtotal (CTLA-4 IgG)	3	1, 0, 1, 0, 1, 0	0.7 $\pm$ 0.3	0.1-1.3

* Statistical significance was assessed using Wilcoxon U-test.
 † Assessed for histological analysis.

Table 2: Allogeneic survival

Group	Treatment	n	Survival	mean $\pm$ SD	95% CI
I	Subtotal	1	1, 1, 0, 0, 1, 1	1.1 $\pm$ 0.4	—
II	Subtotal (CTLA-4 IgG)	3	1, 1, 0, 1, 0, 1	1.0 $\pm$ 0.3	0.4-1.6
III	Subtotal (CTLA-4 IgG)	3	1, 0, 1, 0, 1, 0	0.7 $\pm$ 0.3	0.1-1.3
IV	Subtotal (CTLA-4 IgG)	3	1, 1, 0, 1, 0, 1	1.0 $\pm$ 0.3	0.4-1.6

* Statistical significance was assessed using Wilcoxon U-test.
 † Assessed for histological analysis.

