J. Biochem. **142,** 105–112 (2007)
doi:10.1093/jb/mvm115

Transgenic Mice Expressing a Fully Nontoxic Diphtheria Toxin Mutant, not CRM197 Mutant, Acquire Immune Tolerance against Diphtheria Toxin

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Received April 3, 2007; accepted May 6, 2007; published online May 23, 2007

We previously developed a method termed "toxin receptor-mediated cell knockout" (TRECK). By the TRECK method, a single or repeated shot(s) of diphtheria toxin (DT) conditionally ablates a specific cell population from transgenic mice expressing the DT receptor transgene under the control of a cell type-specific promoter. In some cases of TRECK, frequent and high-dose administration of DT is required, raising the concern that these frequent injections of DT could cause production of anti-DT antibody, which would neutralize further DT administration. To solve this problem, we aimed to generate transgenic mice genetically expressing a nontoxic DT mutant, with the expectation that they may naturally acquire immune tolerance to DT. Unexpectedly, the G52E DT mutant, which is well known as the nontoxic DT variant cross reacting material 197 (CRM197), exhibited cytotoxicity in yeast and mammalian cells. Cytotoxicity of CRM197 was abrogated in cells mutated for elongation factor 2 (EF-2), indicating that CRM197 exerts its toxic effects through EF-2, similar to wild-type DT. On the other hand, the K51E/E148K DT mutant exhibited no detectable cytotoxicity. This led us to successfully obtain DT gene transgenic mice, which exhibited no histological abnormalities, and indeed acquired immune tolerance to DT.

Key words: ADP-ribosylation, diphtheria toxin, elongation factor 2, HB-EGF, immune tolerance.

Abbreviations: ADP, adenosine diphosphate; CRM, cross reacting material; DT, diphtheria toxin; DTA, diphtheria toxin fragment A; DTB, diphtheria toxin fragment B; EF-2, eukaryotic elongation factor 2; ELISA, enzyme-linked immunosorbent assay; HB-EGF, heparin-binding epidermal growth factor-like growth factor; TRECK, toxin receptor-mediated cell knockout.

Diphtheria Toxin (DT) is secreted as a single polypeptide chain of 58 kDa from *Corynebacterium diphtheriae* and consists of two major domains that can be separated by mild treatment with trypsin and reduction: the N-terminal fragment A (DTA, 21 kDa) and C-terminal fragment B (DTB, 37 kDa) (*1*). DT binds to the surface of cells expressing the DT receptor via the binding domain contained within DTB. Subsequently, DT is incorporated into the cells by receptor-mediated endocytosis, translocated into the cytosol using the translocation domain of DTB, after which DTA is cleaved and released into the cytosol. The target molecule of DTA is elongation factor 2 (EF-2), which is an essential component of the eukaryotic protein-synthesis machinery (*1–3*). One histidine residue in EF-2 (His715 in mammalian EF-2) is posttranslationally modified to a unique amino acid residue named diphthamide (*4*, *5*). DTA catalyzes the ADP-ribosylation of this diphthamide residue, resulting in the inactivation of EF-2, and consequent inhibition of protein synthesis and induction of cell death (*3*). It is notable that DTA can target EF-2 in all eukaryotic cells (*1*, *4*).

Naglich *et al.* (*6*) previously reported that the DT receptor is the membrane-bound precursor form of heparin-binding epidermal growth factor-like growth factor (HB-EGF). Unlike primates, mice and rats hardly suffer from DT, because their HB-EGF has little detectable affinity to DT. If human HB-EGF (hHB-EGF) is heterologously expressed in murine cells as a DT receptor, cells are highly susceptible to DT. This led us to develop a method named toxin receptor-mediated cell knockout (TRECK) (*7*). The first step of TRECK is to generate transgenic mice expressing the hHB-EGF transgene under the control of a cell type-specific promoter. Subsequently, DT is injected into the transgenic mice at a desired time point(s) to ablate those cells in which the promoter is active. We and others have demonstrated that the TRECK is a versatile and highly reproducible method for ablation, or partial incapacitation of specific cell types in mice and rats, which are otherwise grown healthy and normal (*7–13*). We aimed to refine the TERCK into a more useful and valuable method by solving residual problems. For example,

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we generated mutant hHB-EGFs that lost growth factor activity but retained DT receptor function (*14*). This mutant can be used instead of wild-type hHB-EGF to avoid possible problems with hHB-EGF acting as a growth factor in mice.

Another possible problem is that multiple injections of high-dose DT may produce anti-DT antibodies that could neutralize freshly injected DT. If so, it may be useful to generate mice having immune tolerance to DT. Expression of a heterologous protein in transgenic mice typically induces immune tolerance to the protein, which is recognized as self-protein (*15–18*). To develop transgenic mice with immune tolerance to DT, we used a transgene expressing a nontoxic DT mutant. CRM197 is a mutant of DT that carries a G52E mutation, and was selected as a nontoxic variant from a stock of randomly mutagenized *diphtheriae* bacteriophage carrying the DT gene (*1, 19, 20*). This mutant DT has been investigated for potential clinical applications as detailed later. In this paper, we tested two nontoxic DT mutants, including those that are expressed in the cytosol, and found that the K51E/E148K mutant is completely nontoxic, whereas CRM197 shows cytotoxicity. Thus we generated transgenic mice using the K51E/E148K DT transgene, and found that, unlike wild-type mice, these transgenic mice indeed acquired immune tolerance to DT.

MATERIALS AND METHODS

Diphtheria toxin—Diphtheria toxin was purified from conditioned medium of the *C. diphtheria* strain PW8 as described previously (*7*).

Plasmids—DNA fragments carrying wild-type and K51E/E148K DTA genes were amplified, respectively, from pBS-DTA [the *Eco*RI fragment of wild-type DTA from pDT201 (*21*) was cloned into pBluescript II SK(+)(Stratagene)] and pDTA-K51E/E148K (a generous gift from Dr. R. John Collier, Harvard Medical School, Boston) using 5-X-E-DTA; 5′-CCG *CTC GAG GAA TTC* ACC ATG GGC GCT GAT GAT GTT GTT GAT TCT TCT AAA TAT TTT GTG ATG G-3′ and DTA(−); 5′-CCC *AAG CTT CGA ATT CGG ATC C*TC ATC GCC TG-3′ (italic sequences show restriction enzyme sites).

Oligonucleotide primers used in the generation of G52E single mutant (CRM197) plasmids were as follows (mismatched bases and restriction enzyme sites are indicated by the underlined and italic, respectively); 5′-CCC *CTC GAG GAA TTC* ACC ATG GGC GCT GAT GAT GTT GTT GAT TCT TCT AAA TAT TTT GTG ATG G-3′; 5′-TCG GTA CTA TAA AAC TCT TTC CAA TC-3′. The sequences of all amplified genes were confirmed by direct sequence. Genes for full-length DT variants were constructed by *Eco*RI–*Bal* I digestion/ligation of the DTA genes and pβ176 (GenBank Accession No. M19546). For expression in mammalian cultured and yeast cells, the expression vectors pCAG [ref. (*22*); CMV/β actin promoter] or pGMU10 [ref. (*23*); *GAL1* promoter] were used. Genes for DTA or full-length DT variants were subjected to *Eco*RI digestion/ligation with these vectors to generate pCAG-DTA, pGMU10-DTA (for DTA) or pCAG-DTA + B (for full-length DT). CRM197 and K51E/E148K mutant versions of these expression plasmids were similarly constructed. To obtain the luciferase expression plasmid pCAG-GL3, the luciferase cDNA from pGL3 basic (Promega) was cloned into pCAG.

Mammalian Cell Cultures and Related Methods—Ltk- (*24*), HeLa (HeLa tet off cells, Clontech), COS7, Vero, CHO-K1 and its DT-resistant mutant KEE1 (*24*, *25*) cells were cultured in α-MEM or DMEM containing 10% fetal bovine serum, at 37°C in 5% CO_2. Incorporation of radioactivity from ^{35}S-labelled methionine/cysteine into Vero cells was measured as described (*7*). Transfection was performed in 24-well dishes (#3526, Corning) using Effectene (QIAGEN). Cellular luciferase activity was measured using the Luciferase TM Reporter Assay System (Promega) and a luminometer ATTO AB-2200-R.

Yeast Strains and Growth Conditions—The yeast FY24 strain (*MAT*α *ura3 trp1 leu2 GAL2*) was used as a wild-type EF-2 strain. Strains MKK-M [*MATa ura3 leu2 his3 trp1 eft1::LEU2 eft2::HIS3* (pRS314-EFT2 (H699M))] and SE2-GR [*MATa ura3 leu2 his3 trp1 eft1::LEU2 eft2::HIS3* (pRS314-EFT2 (G701R))] were described previously (*26*, *27*). Yeast strains were grown on agar-solidified synthetic minimal glucose medium (SD) and synthetic minimal galactose medium (SG; contained 2% galactose instead of glucose) at 30°C.

Transgenic Mice—A 3.8 kbp *Sal*I–*Pst*I fragment from the K51E/E148K mutant version of pCAG-DTA + B (Fig. 3A) was microinjected into fertilized (C57B/6J Jcl × C57B/6J Jcl) mouse eggs. The manipulated eggs were then cultured to the two-cell stage and transferred into oviducts of pseudopregnant foster females (jcl:ICR). Integration of the transgene was checked by Southern blot analysis of tail DNA. All animal experiments were carried out in accordance with the policies of the Committee on Animal Research at NAIST.

RNA Isolation and RT-PCR—In order to check transgene expression, total RNA was extracted from blood cells or tissues samples using ISOGEN (Nippon Gene) to check transgene expression. RT-PCR was performed using Superscript II (Invitorogen) according to manufacturer's instructions. The DT cDNA was amplified for 35 cycles by PCR using the following primers: forward, 5′-ACT AAA AGT GGA TAA TGC CG -3′ and reverse, 5′-TTT TTG ATA GGG CCA TGC TC-3′. GAPDH was amplified for 30 cycles using the following primers: forward, 5′-GCC GAA TTC ATG GTG AAG GTC GGT GTG A-3′ and reverse, 5′-GCC GAA TTA TTG CTC AGT GTC CTT GCT GG-3′ and was used as a control.

Northern Blot Analysis—10 μg of total RNA was electrophoresed on 1% denaturing agarose gels and transferred onto Hybond-N membranes (Amersham Biosciences). DT probe was amplified with PCR using the following primers: forward, 5′-CCC TGC AGG GCG CTG ATG ATG TTG TTG ATT C-3′ and reverse, 5′-CCC AAG CTT CGA ATT CGG ATC CTC ATC GCC TG-3′. Probe labelled by [α-^{32}P] dCTP for DT was synthesized using a random primer DNA labeling kit ver. 2.0 (TaKaRa). Hybridization was performed with a ^{32}P-labelled probe in Church solution (1 M $Na_2HPO_4{\cdot}12H_2O$, 1 mM EDTA, 7% SDS pH 7.2) overnight at 65°C. Hybridization signals were detected using the BAS 2500 system (Fuji Film).

Analysis of Sera from Immunized Mice by ELISA—Five C57B/6J mice were immunized with purified DT at 4 weeks of age. Each mouse received 0.5, 5 and 50 μg/kg DT without complete Freund's adjuvant intraperitoneally every 3 days. Blood was collected tail bleed to measure antibodies to DT by ELISA every 5 days. The well of 96-well microtiter plates were coated overnight at 4°C, 4 μg/ml purified DT in PBS. The wells were washed four times with PBS, and then blocked for 1 h at room temperature with 1% BSA/PBS. After washing three times with PBS, 50 μl of 100-fold dilutions of mouse sera in 1% BSA/PBS were added to the wells and incubated at room temperature for 1 h. The wells were washed four times with PBS, then 50 μl of PBS containing 1: 2,000 dilutions of HRP-conjugated goat anti-mouse IgG (#P0447, DAKO) was added to the wells and incubated at room temperature for 1 h. The wells were washed four times with PBS. One milligram per millilitre of *O*-phenylene diamide (WaKo) in 0.1 M citrate buffer (pH 4.5) containing 0.012% H_2O_2 was added and plates were incubated at room temperature for 20 min.

Protein Analyses—Extracts of tissue samples were obtained by homogenization in PBS followed by centrifugation. These extracts (50 μg protein) or cultured-cell lysates (30 μg protein) were analysed by Western blotting using anti-toxin serum prepared from horse (1:100 dilutions) as the primary antibody and HRP-conjugated rabbit anti-horse IgG (L + H) (#67-415, ICN; 1:2,000 dilutions) as the secondary antibody. For detection of mouse IgG, HRP-conjugated goat anti-mouse IgG (#P0447, DAKO) was used at 1:2,000 dilutions for Western blotting in Fig. 5B. Mouse monoclonal antibody against GAPDH (#ab9484, Abcam) was used at 1:5,000 dilutions.

RESULTS

K51E/E148K DT mutant is not cytotoxic, whereas CRM197 is cytotoxic, when expressed in cytosol—Expression of heterologous proteins in transgenic mouse naturally induces immune tolerance to the protein (*15–18*). In order to avoid competition with injected DT in the TRECK-transgenic mice, we decided to utilize cytosolic expression rather than secretory protein. The first step was to select a DT mutant that does not show any detectable cytotoxicity even when cytosolically expressed. We checked two variants of DT, CRM197 and K51E/E148K. The latter was also selected as a nontoxic mutant from a library of randomly mutated DT gene (*28*). As shown in Fig. 1A, wild-type or mutant DTA was expressed in yeast cells from the *GAL1* promoter. When DT expression was induced by inoculating cells on a galactose plate, CRM197 DTA, as well as wild-type DTA was found to significantly inhibit cell growth, while the K51E/E148K mutant did not. In Fig. 1B, mammalian cells were transfected with plasmids expressing DTA together with a luciferase-expression plasmid, and toxicity of the DT mutants was evaluated by inhibition of luciferase synthesis (*29*). In all three mammalian cell lines tested here, protein synthesis was almost completely blocked by co-transfection of the wild-type DTA-expression plasmid. Co-transfection of the CRM197 DTA-expression plasmid also inhibited protein synthesis, but more moderately than wild-type. On the other hand, no inhibition of protein synthesis was observed with the K51E/E148K DTA mutant. Western blot analysis of the detection of DTA in each of the lysates showed that the K51E/E148K DT mutant was efficiently expressed, whereas wild-type DT and CRM197 were undetectable (Fig. 1C). This indicates that CRM197 is also sufficiently toxic in mammalian cells as well as yeast.

The Target of CRM197 is EF-2, Similar to Wild-type DT—To examine whether the cytotoxicity of CRM197 is caused by residual ability to ADP-ribosylate EF-2, *in vitro* ADP-ribosylation assay was performed using crude cell extracts prepared from CHO-K1 cells, which is a standard method to measure the activity of ADP-ribosylation of DT (*25*). However, it was so weak to detect ADP-ribosylated EF-2 in this system, as other researchers reported (*19*), that we chose another method. KEE1 is a CHO-K1 cell line mutant carrying the G717R mutation in the EF-2 structure gene, which renders it virtually resistant to DT fragment A (*24*, *25*). In Fig. 2A, we transiently co-transfected luciferase and DTA expression plasmids into the KEE1 cells. Contrasting with the results in Fig. 1B, expression of CRM197 in KEE1 cells did not attenuate protein synthesis (Fig. 2A) and CRM197 were fully detectable as well as K51E/E148K (Fig. 2B). This observation indicates that CRM197 has residual activity sufficient to inhibit EF-2 when it is expressed in the cytosol. As for wild-type DT, its expression was undetectable by Western blot analysis (Fig. 2B), and it was found to significantly attenuate protein synthesis even in KEE1 cells (Fig. 2A). There are two possible explanations to interpret these results. The first one is that His715 in the G717R EF-2 mutant could be a substrate for DT when wild-type DTA is overexpressed in the cytosol. The other possibility is that DT has a target(s) other than EF-2. To clarify which one is correct, we tested this using yeast EF-2 mutant. The yeast strain SE2-GR carries an EF-2 gene mutated to carry the G701R substitution, which corresponds to the G717R mutation in mammalian EF-2 (*27*). Consistent with the results from the hamster KEE1 cells, yeast SE2-GR cells were fully resistant to CRM197 and K51E/E148K mutant DTA, whereas strong expression of wild-type DTA from the *GAL1* promoter retarded the growth of these cells (Fig. 2C, upper right panels, the second row). Histidine 699 in yeast EF-2 is posttranslationally modified to diphthamide, which is targeted by DT for ADP-ribosylation. In a yeast strain MKK-M, the EF-2 gene is mutated to replace this His 699 with Met (*26*). Therefore H699M EF-2 could not be a substrate for DT because diphthamide residue could not be formed due to this amino acid substitution. As shown in the lower panels of Fig. 2C, MKK-M cells were fully resistant even against wild-type DT. These findings support the former interpretation: wild-type DT partially attacks yeast G701R EF-2 or mammalian G717R EF-2 mutant when overexpressed in the cytosol, and the only target of wild-type DT is EF-2.

Generation of K51E/E148K DT-Mutant Transgenic mice—Because of the K51E/E148K mutant is completely nontoxic, we decided to generate transgenic mice

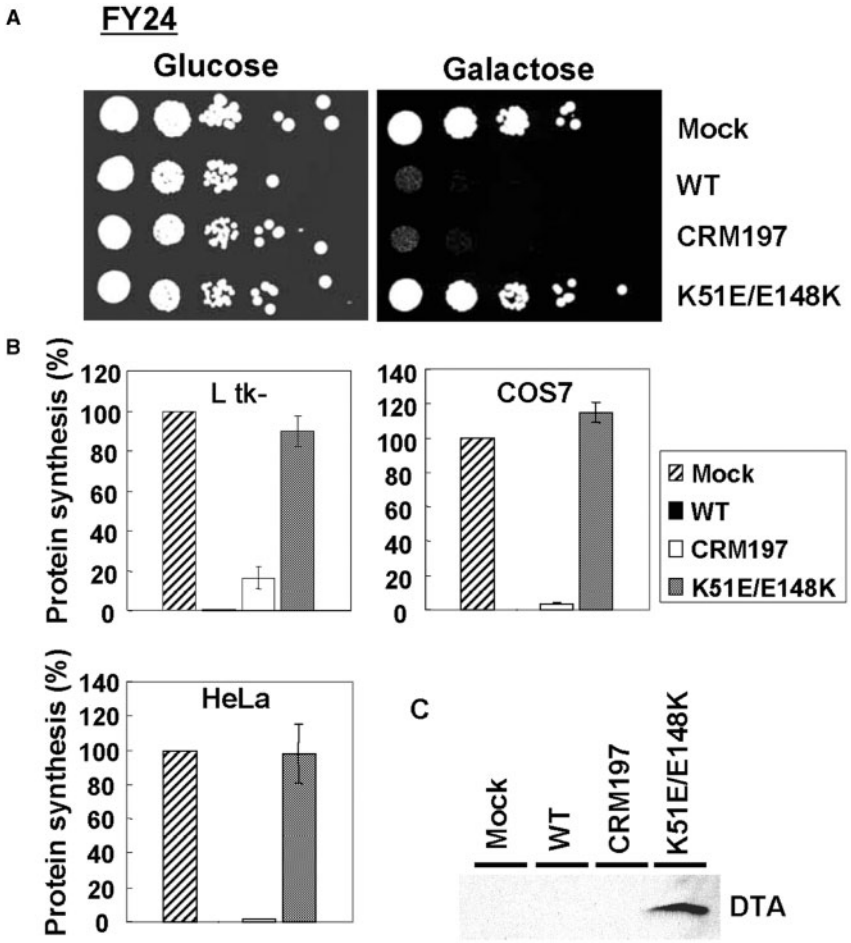

Fig. 1. **Toxicity of cytosolically expressed DT mutants**. (A) Cultures of yeast FY24 cells carrying a DTA expression plasmid pGMU10-DTA or its mutant versions were spotted onto SD or SG plates in a 10-fold dilution series. The plates were incubated for 3 days and imaged. (B) The indicated mammalian cell lines were co-transfected with DTA expression plasmid (pCAG-DTA or its mutant versions; 0.16 μg; pCAG vector for the mock control) and a luciferase reporter plasmid pCAG-GL3 (0.04 μg), and 24 h later, luciferase production was measured. (C) Lysates from L cells transfected with pCAG-DTA (or its mutant versions; pCAG vector for the mock control) were analysed by anti-DT Western blotting.

carrying the full-length K51E/E148K DT gene. As illustrated in Fig. 3A, the transgene is a 3.8 kbp fragment consisting of the CMV/β actin promoter that provides ubiquitous high-level expression, the mutant DT gene and a polyadenylation signal. Pronuclear microinjection of this construct into fertilized mouse eggs generated five founder mice (1F1, 1M1, 3M3, 4F7 and 4M6). To detect expression of transgene RNA, we carried out RT-PCR analysis of total RNA prepared from blood cells. Two transgenic lines (3M3 and 4F7) expressed mutant DT mRNA (Fig. 3B). Because transgene expression of the 4F7 line did not inherit, we used the 3M3 line in the following analysis. Tissue expression of the transgene was almost ubiquitous, both at the mRNA and protein levels (Fig. 3C). The 3M3 mice appeared normal, grew indistinguishably from wild-type mice, and were fertile (data not shown). This again indicates that expression of the K51E/E148K mutant results in little or no cytotoxicity.

Repeated Administration of DT to Mice Results in Production of Anti-DT Antibody Capable of Neutralizing Freshly Injected DT—To examine how much dose of DT repeated-injections would cause the production of antibody against DT, various amounts of DT were intraperitoneally injected every 3 days for 1 month into inbred C57BL/6 mice. The titer of anti-DT antibody in the sera was monitored by ELISA analysis (Fig. 4A). Production of anti-DT antibody was observed when the amount of injected DT was above 5 μg/kg weight. A similar result was obtained by in Balb/c mice (data not shown). We next checked whether freshly injected DT is neutralized *in vivo* by the anti-DT antibody. After the experiment shown in Fig. 4A, the used C57BL/6 mice were subjected to a final and single shot of 50 μg/kg of DT, and after 18 h, their blood was sampled for measurement of residual DT activity. As a control, we used mice that had been repeatedly mock-treated (PBS injection) before the final injection of 50 μg/kg of DT. In Fig. 4B, sera samples were added into cultures of DT-sensitive Vero cells and protein synthesis was monitored. We found that sera from mice that had been repeatedly injected with 5 or 50 μg/kg of DT contained

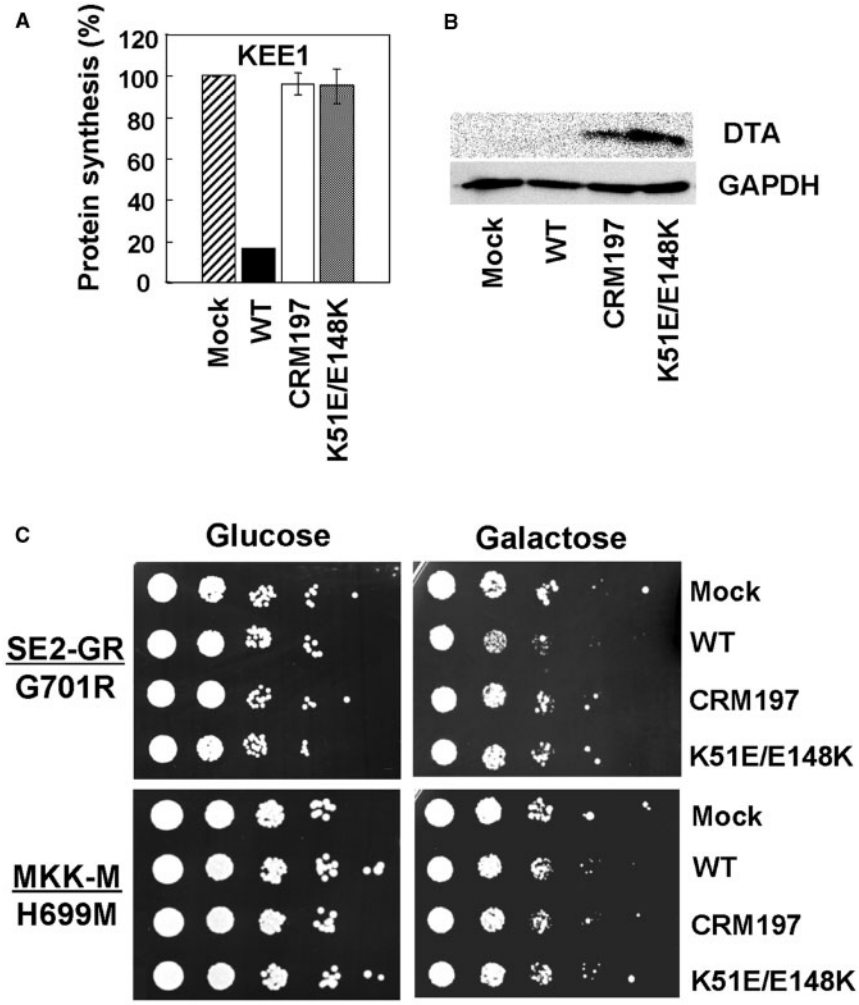

Fig. 2. **Cytotoxicity of wild-type and CRM197 DT is reduced in EF-2 structural gene-mutant cells**. (A) The same experiment as in Fig. 1B was performed using DT-resistant mutant KEE1. (B) The same experiment as in Fig. 1C was performed using the KEE1 cells. (C) The same experiment as in Fig. 1A was performed using the DT-resistant yeast mutant strains SE2-GR (G701R) and MKK-M (H699M).

detectable amounts of anti-DT antibody and exhibited no inhibitory effect on Vero cell protein synthesis. These observations indicate that repeated injections of DT at above 5 μg/kg lead to production of anti-DT antibody capable of neutralizing freshly injected DT.

K51E/E148K DT-Mutant Transgenic mice are Immune Tolerant of DT—Finally, we tested whether the K51E/E148 DT mutant-transgenic mice had actually acquired immune tolerance to DT. DT of 50 μg/kg was injected into the 3M3 line and littermates together with complete Freund's adjuvant, and 2 week later, another dose of 50 μg/kg of DT was administered without adjuvant. Next, serum samples prepared from those mice were added to cultures of Vero cells as a bioassay to detect the presence of DT in the sera. Strong inhibition of protein synthesis was observed when sera from the 3M3 line were added, while sera from the littermate showed no such inhibitory effect (Fig. 5A). This finding indicates that there is residual DT activity in the 3M3 line, and that the 3M3 line was not immunized by DT, in contrast to its littermate. Indeed, sera from DT-injected littermates, but not those from the 3M3 mice, tested positive when used as antibody probe against DT in Western blots (Fig. 5B).

DISCUSSION

In order for the TRECK method to be effective, especially when the target cells are not in close proximity to the vascular system, for example brain, multiple injections of large amounts of DT are sometimes required (*11*, *12*). DT may also have to be repeatedly administered in order to provide chronic damage to regenerative cells (*8*, *9*). Here we demonstrate that injection of live DT leads to the production of anti-DT antibody, which attenuates the activity of freshly injected DT. We therefore generated a transgenic mouse line in which a nontoxic DT mutant was cytosolically expressed and found that it acquired immune tolerance to DT. Expression of a nontoxic DT mutant as a cytosolic, rather than secretory protein,

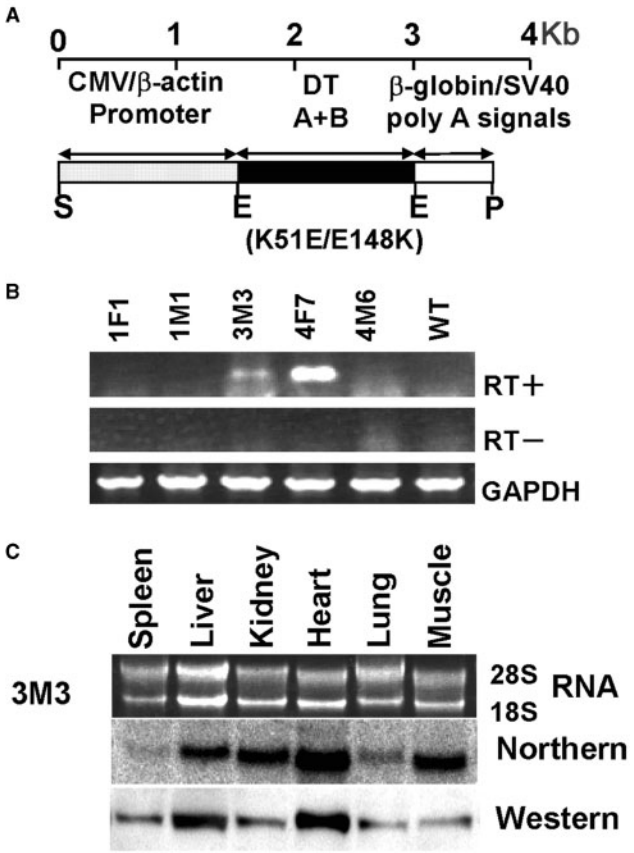

Fig. 3. **Transgenic mouse lines of the K51E/E148K mutant-DT gene**. (A) The transgene construct. E, *Eco*RI; S, *Sal*I; P, *Pst*I. (B) DT cDNA was RT-PCR amplified from total RNA of blood samples of the transgenic mice (+RT). Control experiments in which reverse transcriptase was not added (−RT) or GAPDH cDNA was amplified were also performed. (C) Total RNA (10 μg) and protein extracts (50 μg) were obtained from the indicated tissues of 3M3 transgenic mice and monitored for DT expression by Northern and Western blot analysis.

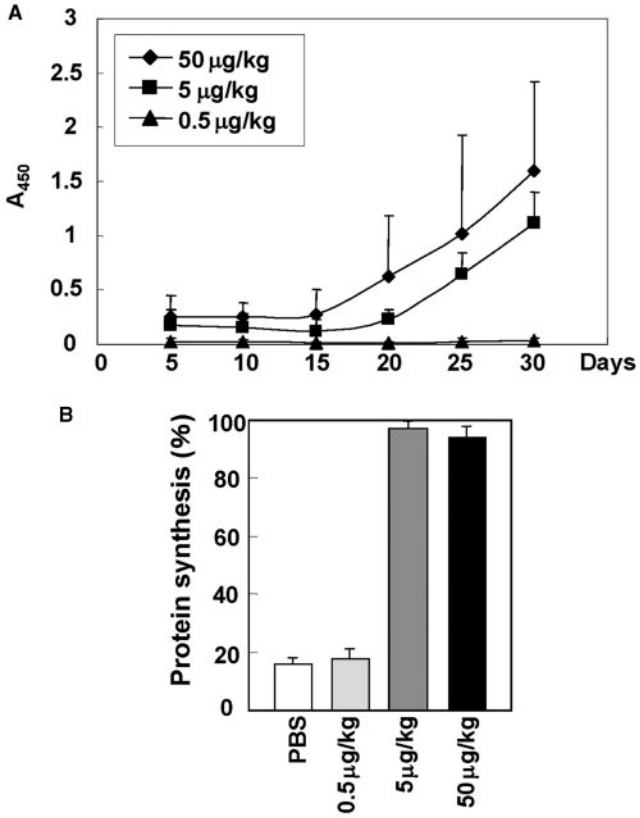

Fig. 4. **Effect of consecutive injections of DT to inbred mouse**. (A) DT was intraperitoneally injected to C57BL/6 mice at 0.5, 5 or 50 μg/kg every 3 days ($n = 3$), and sera were collected every 5 days (At day 0, the first DT injection and serum collection were performed). Titer of anti-DT antibody in the sera was monitored by ELISA using DT-coated plates. C57BL/6 sera were diluted to 1:100. The results are presented as absorbance values at 450 nm (A_{450}) after termination of the colour reaction. (B) C57BL/6 mice that had undergone 11 periodical injections of the indicated amounts of DT in Fig. 4A or mock-treated (injection of PBS at the same time points) were subjected to a single DT injection at 50 μg/kg. 18 h after this final injection, blood samples were collected, and the sera were added to Vero cell cultures at 1:100 dilutions. Then incorporation of radioactivity from ^{35}S-labelled methionine/cysteine into Vero cells was monitored, and the averages and standard deviations from triplicate assays using sera from different mice were calculated as an index of "protein synthesis". This index was normalized to a value of 100 in a control assay performed without addition of murine sera.

is also important to avoid competition with injected DT in the TRECK-transgenic mice. We believe that our DT immune tolerant mice will be highly useful for the TRECK method when multiple injections of DT are required, and this phenotype could be easily introduced into TRECK-transgenic mice by genetic crossing.

This paper also demonstrates that the CRM197 mutation (G52E) does not completely abolish the activity of DT against EF-2, whereas the K51E/E148K mutation does. This finding is consistent with previous biochemical and structural analyses of DT. According to photoaffinity labeling experiments by Carroll *et al*. (*30*), the ADP-ribose donor NAD is positioned in close proximity to Glu148 of DT. Moreover, an amino acid substitution in Glu148 drastically reduces the ADP-ribosylating activity of DTA (*31*), and this Glu residue is conserved in all ADP-ribosylating toxins (*32*). Thus Glu148 is considered to be a constituent of the catalytic centre of the DTA. Furthermore, Lys51 could form a salt bridge with Asp46, which is probably important for DT ternary structure. Therefore it is quite reasonable that amino acid substitutions of these positions would completely abolish activity of DTA.

Importantly, nontoxicity of the K51E/E148K DT suggests some possible clinical applications of this mutant. Full-length CRM197 that carries the intact hHB-EGF-binding DTB sequence exhibits anti-tumour activity, probably not as a toxin but as an hHB-EGF binding protein (33). Considering the weak but detectable toxicity of CRM197, use of the K51E/E148K DT may be preferable to CRM197 for safer therapeutic application. This nontoxic mutant may also be useful as a vaccine. In the case of pertussis toxin, another ADP-ribosylating toxin, double mutation of amino acids in the

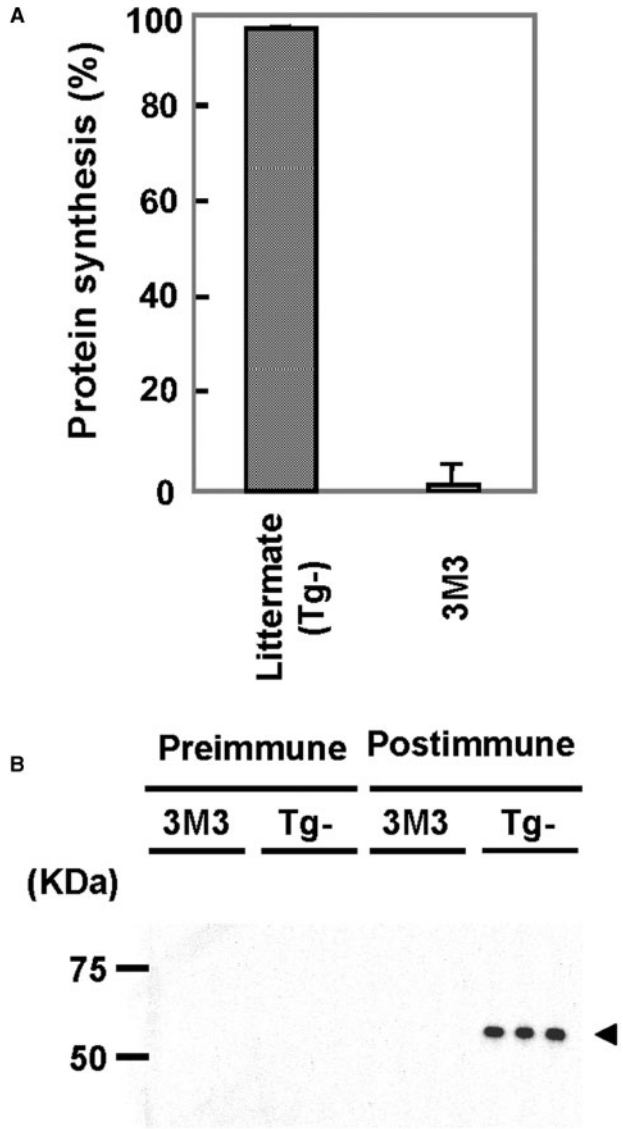

Fig. 5. **DT-immune tolerance of the 3M3 mice**. 3M3 or littermate mice were injected intraperitoneally with 50 μg/kg DT emulsified in Freund's complete adjuvant containing *Mycobacterium butyricum* at 0.05% (DIFCO, Detroit, MI) and 2 week later the mice were subjected to the following assays. (A) Mice were injected with another 50 μg/ml DT, and residual activity of DT in blood was assayed as in Fig. 4B. (B) Serum samples were prepared from three independent mice and used for Western blot detection of purified DT at 1:25 dilutions.

ADP-ribosylation catalytic domain, one of which corresponds to Glu148 of DT, has indeed been shown to possess higher safety and immunogenicity than pertussis toxin toxoid in animal models and clinical trials (34). Moreover, the genes of these completely nontoxic mutant toxins could be candidates for DNA or RNA vaccines, since those mutants will be harmless even when cytosolically expressed. We anticipate that completely nontoxic mutant toxins, including the K51E/E148K DT, will be powerful tools for novel methodologies in basic and medical biology.

We thank Dr. Eisuke Mekada (Osaka University) for exchanging and discussion about the data prior to publication. We also thank Dr. John Collier (Harvard Medical School) for plasmid and Dr. Chojiro Kojima (NAIST) for discussion about the structure of DT. Kazuaki Takahashi for micromanipulation work to make the transgenic mice, and Miki Matsumura, Misato Chiba and Junko Iida for technical assistance.

This work was supported by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN), for 21st Century COE Program from MEXT, Uehara Memorial Foundation and special support from Maruho Co. Ltd.

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