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Diphtheria Toxin Mutant CRM197 Possesses Weak EF2-ADP-ribosyl Activity that Potentiates its Anti-tumorigenic Activity

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CRM197, a mutated diphtheria toxin (DT), has long been recognized to be a non-toxic protein. Based on its non-toxic feature, this protein has been utilized for various purposes, including as an inhibitor of heparin-binding EGF-like growth factor (HB-EGF) and as an immunological adjuvant for vaccination. Here we show evidence that CRM197 has a weak toxicity. This toxicity was observed in cells over-expressing the DT receptor/proHB-EGF, but not in parental cells, indicating that the toxicity was mediated through DT receptor. CRM197 did not show any toxicity toward DT-resistant cells, which have a mutation in elongation factor 2, and a cell-free assay revealed the existence of weak EF-2-ADP ribosylation activity in fragment A of CRM197. Thus, the present study indicates a requirement for specific care in the use of CRM197 at a high dosage, although the toxicity of CRM197 is about 10^6 times less than that of wild-type DT. We found that a monoclonal antibody to DT inhibited CRM197 toxicity, but did not affect the inhibitory activity of CRM197 toward HB-EGF-induced mitogenic activity. CRM197 strongly inhibits tumour growth in nude mice. The anti-DT monoclonal antibody administered with CRM197 reduced the anti- tumourigenic effect of CRM197, indicating that the toxicity of CRM197 potentiates its anti-tumourigenic effect.

Key words: ADP ribosylation, CRM197, diphtheria toxin, EF-2, HB-EGF.

Abbreviations: ADPR, ADP-ribosylation; DT, diphtheria toxin; DTR, diphtheria toxin receptor; EGF, epidermal growth factor; EGFR, EGF receptor; HB-EGF, heparin-binding EGF-like growth factor; proHB-EGF, the membrane-anchored form of HB-EGF; TCA, trichloracetic acid.

Diphtheria toxin (DT)[4] (Mr = 58, 342), produced by toxigenic strains of *Corynebacterium diphtheriae* (*1*, *2*), inhibits cellular protein synthesis in eukaryotes by inactivating elongation factor 2 (EF-2) through ADP-ribosylation (ADPR) in the presence of NAD. DT is composed of two fragments, A and B, which are covalently linked by a disulphide bond. While the ADPR activity is located entirely in fragment A, fragment B is further divided by two functionally and structurally different domains (*3*): the transmembrane domain (T-domain), which is essential for the translocation of fragment A into the cytoplasm, and the receptor-binding domain (R-domain), which binds to the DT receptor (DTR) (*4*). Entry of the fragment A into the cytoplasm is requisite for the cytotoxic action of DT (*5*). After the binding of DT to a DTR on the cell surface through its R domain, DT is internalized by receptor-mediated endocytosis (*6*). A conformational change in DT takes place in an acidic compartment, resulting in the insertion of DT into the endosomal membrane by certain regions in the T-domain, and finally, the enzymatically active fragment A is translocated to the cytosol (*7–11*).

As well as playing a critical role in DT intoxication (*4*, *12*), the DTR is known to be physiologically essential for the functioning of our bodies: the DTR is identical to the precursor form of heparin-binding EGF-like growth factor (proHB-EGF) (*13*, *14*). Heparin-binding EGF-like growth factor (HB-EGF), a member of the EGF family of growth factors, exerts its biological activity through activation of the EGF receptor (EGFR) (*15*) and other ErbB receptors (*16*). The soluble form of HB-EGF is produced by proteolytic cleavage of proHB-EGF through a mechanism known as 'ectodomain shedding' on the cell surface (*17*), following which a significant amount of proHB-EGF is left uncleaved on the cell surface where it acts as the DTR. HB-EGF participates in diverse biological processes, including heart muscle maintenance, heart valve development, skin wound healing, eye lid formation, blastocyst implantation and the progression of atherosclerosis, *via* activation of signalling molecules downstream of ErbB receptors and interactions with molecules associated with HB-EGF (*18–32*). In addition, recent studies have indicated that HB-EGF gene expression is significantly elevated in many human cancers and its expression level in a number of cancer-derived cell lines is much higher than the expression

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levels of other EGFR ligands (*33–36*). Several lines of evidence have indicated that HB-EGF plays a key role in the acquisition of malignant phenotypes and that HB-EGF expression is essential for tumour formation in cancer-derived cell lines (*37–39*).

CRM197 is the product of a single missense mutation (Gly^{52} to Glu) within the fragment A region of DT (*40*, *41*). The mutation leads to an enzymatically inactive product, yielding a non-toxic mutant of DT (*42*). Based on its non-toxic feature, CRM197 has been utilized in various studies including structure-function analysis of DT (*43*), identification of the DTR (*4*) and as an immunological adjuvant or a carrier protein for vaccination against bacterial infection (*44–46*). More recently, an anti- tumourigenic effect of CRM197 has been highlighted. CRM197 binds to DTR/proHB-EGF at an affinity similar to or slightly higher than that of native DT (*47*). CRM197 binds to the soluble form of HB-EGF, as well as to proHB-EGF, and inhibits the mitogenic action of HB-EGF by inhibiting its binding to ErbB receptors (*48*). Since CRM197 does not inhibit the mitogenic activity of other EGFR ligands, this protein has been utilized as a specific inhibitor of HB-EGF (*48*, *49*). Experiments in which CRM197 was administered to mice bearing xenografted tumours showed a remarkable suppression of tumour growth (*36*). A clinical trial of CRM197 in patients with advanced cancer was also carried out, showing a promising anti-tumour effect (*50*). Thus, the development of CRM197 as a therapeutic for ovarian and other cancers is now underway (*39*).

In this study, we show evidence that CRM197 still has a weak EF2-ADPR activity and, thus, is toxic when added to cells expressing high levels of DTR/proHB-EGF. The present study also indicates that anti- tumuorigenic activity of CRM197 is potentiated by this ADPR activity in addition to its inhibitory activity towards HB-EGF mitogenic activity.

EXPERIMENTAL PROCEDURES

Materials—DT was prepared as described previously (*42*). For preparation of CRM197, single colonies of *C. diphtheriae* strain C7hm723 (β197) (*51*) were picked and cultured as described previously (*42*). CRM197 was purified from the culture medium as described (*42*). In this study, CRM197 was further purified using a Sephacryl S-200 gel filtration column, and LPS-like materials contaminating the CRM197 preparations were removed using Detoxi Gel (Pierce Biotechnology, Rockford, IL, USA). It is noted that specific care was taken to avoid contamination with DT during the preparation and purification of CRM197. Nicked forms of DT and CRM proteins were prepared as described previously (*52*). Anti-DT monoclonal antibodies (mAb) #2 and #7 were prepared as described previously (*53*).

Plasmids—Retrovirus vector **pCX4pur** (*54*) was provided by T. Akagi (Osaka Bioscience Institute, Osaka Japan). FLAG-tagged EF-2 cDNA was constructed as follows: oligonucleotide coding FLAG epitope sequence was ligated into XbaI–NotI sites of pBluescript-SK(+) (Stratagene, La Jolla, CA, USA). Human EF-2 cDNA was amplified by PCR with primers (5′–CTGGATCCACCATGGTGAACTTCACGGTAG–3′, where the underlining indicates an BamHI site, and 5′–GCTCTAGACAATTTGTCCAGGAAGTTGTCCAG–3′, where the underlining indicates a XbaI site) and cloned into BamHI–XbaI sites of the FLAG-tagging vector, and FLAG-tagged EF2 cDNA was subcloned into BamHI–NotI sites of **pCX4pur** vector.

Cell Culture—Vero cells, Vero-H cells (*17*) and Vdtr (DT-resistant Vero)-H cells were grown in Eagle's minimum essential medium containing non-essential amino acids (MEM-NEAA), supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and 100 μg/ml streptomycin. SKOV-H cells (*36*) were grown in RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and 100 μg/ml streptomycin. DER cells were maintained in RPMI1640 supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin, 10% fetal bovine serum and 5% WEHI-3 cell conditioned medium (*55*). WEHI-3 cells conditioned medium, a source of IL-3, was prepared as described (*56*). Buffalo rat liver (BRL) cells (provided by K. Miyazaki, Yokohama City University, Yokohama, Japan) and retrovirus packaging cell line Plat-E (*57*) were maintained with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, streptomycin and penicillin.

Isolation of Vdtr-H Cells—Vdtr cells were selected from a population of Vero cells by mutagenesis with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine followed by selection with DT. Vdtr-H cells were generated by transfection of Vdtr cells with human HB-EGF cDNA, as described previously (*17*).

Cytotoxicity of CRM197 by Protein Synthesis Inhibition—Cells were seeded into a 24-well tissue culture plates (1×10^5 cells/well) and cultured for 16 h at 37°C. After washing once with PBS(−), cells were incubated with 0.5 ml of HAM's F12 medium supplemented with 10% fetal bovine serum in the presence of various concentrations of CRM197 or DT, for 24–36 h at 37°C, followed by further incubation with 37 kBq/ml [^{3}H]-leucine (Leu) for 1 h at 37°C. The amount of radioactivity incorporated into protein was measured (*58*). The rate of protein synthesis in each culture was expressed as a percentage of that in cultures without CRM197 or DT.

Cytotoxicity of CRM197 by Colony Formation—Cells were seeded into 6-well plates at a density of 300 cells/well. Various concentrations of CRM197 or DT were added to wells and cells were incubated for 6–8 days at 37°C. Cells were washed once with PBS(−) and fixed with 100% methanol. Colonies were stained with Giemsa and colony numbers were counted under a microscope. Only colonies containing >50 cells were counted as a colony.

Binding of ^{125}I-labelled DT to Cells—Binding of ^{125}I-labelled DT (^{125}I-DT) to cells was measured as described previously (*13*) except for the binding medium. The binding medium used in this study was Ham's F-12 containing 20 mM HEPES, 1 mg/ml BSA and 3 mM NaN_3, pH 7.2. Non-specific binding of ^{125}I-DT was assessed in the presence of a 100-fold excess of unlabelled DT. Specific binding was determined by

subtracting non-specific binding from the total binding obtained using ^{125}I-DT alone.

Inhibition of HB-EGF Mitogenic Activity by CRM197—DER cells were incubated with HB-EGF (10 ng/ml) for 40 h at 37°C. The indicated concentrations of CRM197 or a mixture of CRM197 and anti-DT mAb #2 were also added to DER cell cultures. The number of live DER cells was measured by an MTT assay using Cell Count Reagent SF (Nacalai). Values represent the average of three independent experiments.

Tumour Growth in Nude Mice—Sub-confluent cell cultures of SKOV-H cells were detached from plates using trypsin-EDTA. A total volume of 250 µl, containing 5×10^6 cells suspended in serum-free RPMI1640, was injected into female BALB/c nu/nu mice at 5 weeks of age (Charles River Laboratories). Injected mice were examined every week for tumour apparition. Tumour volume was calculated as described previously (*36*). To assess the effect of CRM197 and anti-DT mAb #2, these proteins, each dissolved in 1 ml of 20 mM HEPES, 0.15 M NaCl, pH 7.2, were injected intraperitoneally into tumour-bearing mice.

Purification of EF-2—BRL cells expressing FLAG-tagged EF-2 (5×10^9 cells) were trypsinized and lysed with lysis buffer (50 mM Tris–HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 10 mM *O*-phenanthroline, 0.2 mM PMSF, protease inhibitor cocktail (Nacarai) incubating at 4°C for 20 min. Cell lysates were clarified by centrifugation at 39,000*g* and loaded to 1 ml of Anti-FLAG M2 affinity gel (Sigma) according to the manufacturer's instructions. The anti-FLAG M2-agarose gel (Sigma) columns were washed by 20-bed volumes of TBS (20 mM Tris–HCl, pH 7.2, 0.15 M NaCl). Bounded material was eluted with 5-bed volume of TBS containing 150 µg/ml FLAG peptide. FLAG-EF2 were dialysed against 20 mM Tris–HCl, pH7.2, 0.1 mM EDTA, 0.1 M KCl, 10% Glycerol, 1 mM DTT.

Cell-free ADPR Assay—NAD-dependent ADPR of FLAG-tagged EF-2 was performed as described in a previous report (*59*). FLAG tagged EF-2 (600 ng) were incubated with indicated amounts of nicked DT or nicked CRMs for 15 min at 37°C in 50 ml of reaction mixture containing 10 nM [^{32}P]-NAD (1000 Ci/mmol; Amersham GE Healthcare), 40 mM Tris–HCl, pH 8.0, 50 mM DTT, 1 mg/ml BSA and 5% glycerol. The reaction was terminated by the addition of 500 ml of 5% trichloracetic acid (TCA) and the TCA-insoluble radioactivity was counted by a liquid scintillation counter. The radio-labelled proteins were solubilized in 67.5 mM Tris–HCl, pH 6.8, containing 1% SDS, 25 mM DTT and 20% glycerol and subjected to SDS–PAGE. Radioactive bands were detected by an imaging plate (Fuji Film Co., Tokyo, Japan) and analysed with FLA-7000 image analyzer and Multi Gauge Ver3.0 software (Fuji Film).

RESULTS

Cytotoxicity of CRM197 Towards Vero-H Cells—In preliminary studies, we observed a weak cytotoxicity of CRM197, even though CRM197 has been thought to be a non-toxic mutated DT (*40*, *42*, *43*). To examine this cytotoxicity more clearly we performed a colony formation assay using Vero cells and Vero-H cells which are a transfected cell line derived from Vero cells over-expressing human proHB-EGF (*17*). Vero cells or Vero-H cells seeded at a density of 300 cells/plate were cultured with the indicated concentrations of CRM197 for 5–7 days, and colony numbers were counted. Although no significant loss of colonies was detected in Vero cell cultures incubated with CRM197, even at a concentration of 10 µg/ml, CRM197 inhibited colony formation by Vero-H cells in a dose-dependent manner (Fig. 1A). The toxicity of CRM197 was also detected using a protein synthesis assay in Vero-H cells, but not in Vero cells, when these cells were cultured with CRM197 for 36 h (Fig. 1B). Since CRM197 showed toxicity toward Vero-H cells, but not towards Vero cells, by both colony formation assay and protein synthesis assay, it is suggested that the toxicity of CRM197 is mediated by DTR/proHB-EGF. The toxicity of CRM197 towards Vero-H cells was completely blocked by the addition of anti-DT monoclonal antibodies (Fig. 1C), negating the possibility that impurities in the CRM197 preparation caused the observed toxicity.

The toxicity of CRM197 was compared with that of wild-type DT by colony formation and protein synthesis assays using Vero-H cells. Figure 1D shows that the toxicity of CRM197 is about 10^6 times less than that of DT when comparing the concentrations at which colony numbers are decreased by 50% (ID_{50}). The protein synthesis assay showed a similar result (Fig. 1E). The sensitivity of cells to DT is largely affected by the expression level of DTR/proHB-EGF (*12*). Even though Vero cells are known to be one of the most sensitive cell lines to DT (*12*), the toxicity of CRM197 was too low to detect using Vero cells. Vero-H cells express an ~7-fold higher level of DTR/proHB-EGF on the cell surface than Vero cells (*17*), which enabled us to detect the toxicity of CRM197.

CRM197 Did Not Show Toxicity Towards Vdtr-H Cells With a DT-resistant EF2—To examine whether the toxicity of CRM197 is due to the EF2-ADPR activity of CRM197, we next tested the toxicity of CRM197 towards Vdtr cells. Vdtr cells were selected from a DT-resistant Vero cell clone after selection with a lethal dose of DT. Vdtr cells were classified as class II DT-resistant mutants; that is, translational mutants at the level of EF-2 (*60*). Class II DT-resistant mutants are further classified into two groups: one has mutations in an EF-2 structural gene, while the other one is deficient in post-translational biosynthesis of diphthamide (*61*), which is essential for toxin-catalysed ADPR (*62*). The former group of mutants shows dominant DT resistance, whereas the later group behaves recessively (*63*). In dominance hybridization studies, Vdtr cells showed dominant DT resistance, indicating that Vdtr cells are DT resistant owing to a mutation in an EF-2 gene. Vdtr cells were transfected with human proHB-EGF and cells over-expressing DTR/proHB-EGF were isolated (Vdtr-H cells). The expression level of proHB-EGF on the cell surface of Vdtr-H cells was comparable with that of Vero-H cells, as indicated by a ^{125}I-DT binding assay (Fig. 2A). However, CRM197 did not inhibit the colony formation (Fig. 2B) and protein synthesis

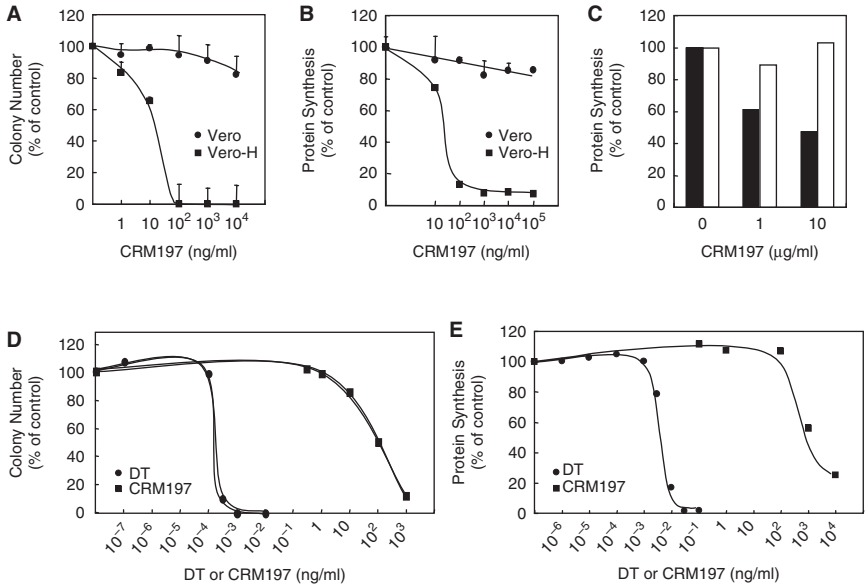

Fig. 1. **Cytotoxicity of CRM197.** (**A**) Colony formation assay showing the toxicity of CRM197. Vero cells and Vero-H cells were seeded at 300 cells/well and cultured with the indicated concentrations of CRM197 for 7 days. Colonies were stained with Giemsa and the colony number was counted. Data are shown as means ± SD of triplicate samples. (**B**) Protein synthesis assay showing the toxicity of CRM197. Vero cells and Vero-H cells were incubated for 36 h at 37°C with various concentrations of CRM197, and then incubated with [^{3}H]-Leu for 1 h at 37°C. The amount of radioactivity incorporated into protein was determined. Data are expressed as percent of control without CRM197. (**C**) Neutralization of CRM197 toxicity with anti-DT monoclonal antibodies. A mixture of CRM197, anti-DT mAb #2 and anti-DT mAb #7 (1: 5: 5 in molecular ratio) was pre-incubated for 1 h at 37°C. Vero-H cells were incubated for 24 h at 37°C with 1 μg/ml or 10 μg/ml of CRM197 alone (closed bar), or with the mixture of CRM197 and anti-DT mAbs (open bar), and then incubated with [^{3}H]-Leu for 1 h at 37°C. The amount of radioactivity incorporated into protein was determined. Similar results were obtained in two separate experiments. (**D**) Comparison of CRM197 cytotoxicity with that of wild-type DT by colony formation assay. Vero-H cells were cultured with the indicated concentration of DT or CRM197 for 5 days and the colony number was counted. Similar results were obtained in three separate experiments. (**E**) Comparison of CRM197 cytotoxicity with that of wild-type DT by protein synthesis assay. Vero-H cells were incubated for 24 h at 37°C with various concentrations of DT or CRM197, and then incubated with [^{3}H]-Leu for 1 h at 37°C. The radioactivity incorporated into protein was determined. Similar results were obtained in three separate experiments.

(data not shown) of Vdtr-H cells, indicating that the toxicity of CRM197 is due to the EF2-ADPR activity of CRM197.

Cell-free EF-2-ADPR Assay—To demonstrate the EF-2-ADPR activity of CRM197 more directly, a cell-free ADPR assay was performed using purified EF-2 and [^{32}P]-NAD, as described in the Experimental Procedures. Nicked forms of DT, CRM176 and CRM197 were added to the mixtures of FLAG-tagged EF-2 and [^{32}P]-NAD, incubated for 15 min at 37°C, and the TCA-insoluble radioactivity was determined. Although the TCA-insoluble radioactivity was <400 c.p.m. when DT, CRM176 or CRM197 were not added, the addition of nicked DT at a concentration from 0.4 ng/ml to 400 μg/ml resulted in 3.6×10^3 to 7.5×10^5 c.p.m. of TCA-insoluble radioactivity (Fig. 3A). CRM176 is another mutated DT with reduced ADPR activity (*42*). The ADPR activity of CRM176 is about one-tenth that of the wild-type toxin (*42*, *64*). Consistently, CRM176 induced ADPR of EF-2, but it required an amount ~10 times higher than that required by DT, to obtain a similar level of radioactivity. CRM197 also induced ADPR of EF2, but its activity was much lower than that of DT (Fig. 3A, inset). SDS–PAGE and autoradiography of the reaction products confirmed that a protein band the same size as EF-2 (96 kDa) was a major product labelled with [^{32}P]-NAD when EF-2 was incubated with CRM197 (Fig. 3B and C). Consistent with the toxicity assay, Fig. 3B also indicated that CRM197 was required an amount $\sim 1 \times 10^6$ times higher than that required by DT to obtain similar level of the ADPR. These results confirm that CRM197 still possesses EF2-ADPR activity, and that this catalytic activity underlies the weak toxicity of CRM197.

Anti-DT Monoclonal Antibody Inhibits the Toxicity of CRM197 But Does Not Prevent the Inhibition of HB-EGF by CRM197—CRM197 is widely used as an inhibitor of HB-EGF mitogenic activity (*36*, *49*, *65*). The present study revealed a weak but a certain toxicity of CRM197. Next, we explored the conditions in which the toxicity of CRM197 was neutralized, but its inhibitory activity towards HB-EGF-induced mitogenic action was retained. For this purpose, we used a previously isolated monoclonal antibody to DT (*53*). Anti-DT mAb #2 binds to fragment B and inhibits the toxicity of DT, probably due to blocking the internalization or translocation step of DT intoxication. Since this antibody does not inhibit the binding of DT to DTR (*53*), it is expected that the inhibitory effect of CRM197 towards HB-EGF-induced mitogenic activity might not be affected by this antibody. CRM197 was pre-incubated with #2 anti-DT

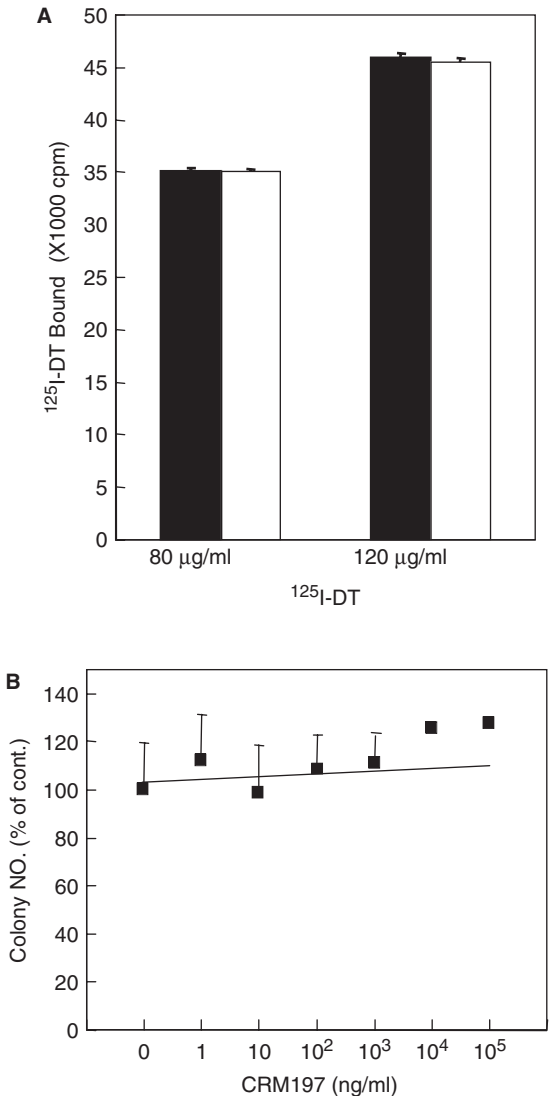

Fig. 2. **Effect of CRM197 on DT-resistant Vdtr-H cells.** (**A**) DT-binding assay showing the amount of cell-surface proHB-EGF. Vero-H cells (closed bar) and Vdtr-H cells (open bar) were incubated with 80 ng/ml or 120 ng/ml of ^{125}I-DT in the presence or absence of excess unlabeled DT for 6 h at 4°C. The amount of ^{125}I-DT bound to the cell surface was determined. Data are expressed as specific binding and means ± SE of duplicate samples are shown. (**B**) Colony formation assay showing the toxicity of CRM197 towards Vdtr-H cells. Vdtr-H cells were seeded at 300 cells/well and cultured with the indicated concentrations of CRM197 for 7 days. Colonies were stained with Giemsa and colony number was counted. Data are shown as means ± SD of triplicate samples.

mAb, and then the mixture was used in a toxicity assay. Figure 4 shows the effect of #2 anti-DT mAb on the toxicity of CRM197 as determined by a colony formation assay using Vero-H cells. This antibody completely inhibited the toxicity of CRM197. A colony formation assay was also performed in the presence of #2 anti-DT mAb alone. This antibody itself did not affect colony formation of Vero-H cells (data not shown).

Next, we tested whether the inhibitory activity of CRM197 towards HB-EGF-induced mitogenic activity is affected by the #2 antibody in the DER cell line, which is a stable transformant of IL-3-dependent 32D cells that expresses human EGFR (*55*). DER cell growth is promoted by HB-EGF in the absence of IL-3. The addition of CRM197 inhibited DER cell growth in a dose-dependent manner; partial inhibition of DER cell growth was observed with CRM197 at 100 ng/ml and complete inhibition was seen at concentrations >1 μg/ml (Fig. 4B). Even in the presence of the #2 antibody, CRM197 inhibited mitogenic activity induced by HB-EGF. The #2 antibody itself did not inhibit DER cell growth. These results indicate that the #2 antibody inhibits the toxicity of CRM197, but not the inhibitory action of CRM197 towards HB-EGF-induced mitogenic activity.

The Anti-tumourigenic Activity of CRM197 Is Partially Inhibited by the Anti-DT Monoclonal Antibody 2—HB-EGF expression is critically important for tumour formation by ovarian cancer cells injected into nude mice, and CRM197 administered into the mouse peritoneum strongly inhibited tumour formation by these cells (*36*). To examine whether the anti- tumourigenic activity of CRM197 is due to its inhibitory activity towards HB-EGF or its cytotoxicity based on EF2-ADPR activity, we tested the effect of the anti-DT mAb #2 on tumour formation. SKOV-H cells which are a transfected cell line derived from SKOV cells over-expressing human proHB-EGF (*36*) were injected subcutaneously into nude mice. One week later, CRM197 (1 mg/kg/week) or a mixture of CRM197 (1 mg/kg/week) and the anti-DT antibody #2 (8 mg/kg) was administered by injection into the peritoneum, four times in total. Tumour volume was measured weekly. SKOV-H cells formed large tumours rapidly in the absence of CRM197 injection. While CRM197 alone completely inhibited tumour formation, the mixture of CRM197 and the anti-DT antibody #2 only partially inhibited tumour formation by SKOV-H cells (Fig. 5). Since antibody #2 inhibits the cytotoxicity of CRM197, this result indicates that both activities of CRM197, its inhibitory activity towards HB-EGF and its cytotoxicity based on EF2-ADPR activity, contribute to the anti- tumourigenic effect of CRM197.

DISCUSSION

Although CRM197 has been known to be a non-toxic mutant of DT, the present study indicates that CRM197 has weak toxicity towards cells expressing high levels of DTR/proHB-EGF. The toxicity of CRM197 was detected using DTR-over-expressing Vero-H cells, but was hardly detected in the parental Vero cells. This could be a reason why the toxicity of CRM197 had not been documented until now. Cell-free ADPR reactions using [^{32}P]-NAD revealed that fragment A of CRM197, that is, the mutant with a Gly^{52} to Glu substitution, possesses a faint EF2-ADPR activity that was not detected in an earlier study using [^{14}C]-NAD (*43*). The specific activity of [^{32}P]-NAD is usually 1,000 times higher than that of [^{14}C]-NAD. The use of [^{32}P]-NAD in ADPR reactions

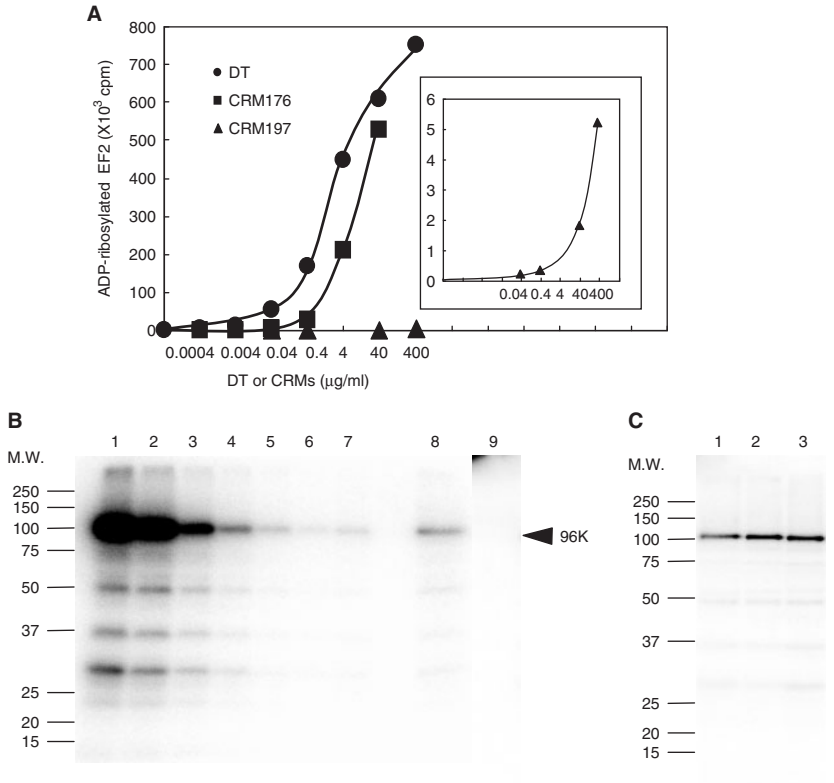

Fig. 3. **EF-2-ADPR activity of CRM197 fragment A.** (**A**) Cell-free ADPR reactions were performed in the presence of DT, CRM176 or CRM197. FLAG-tagged EF-2 and [^{32}P]-NAD were incubated with a nicked form of DT, CRM176 or CRM197 at the indicated concentrations, and the amount of TCA-insoluble radioactivity was determined. The inset is an enlarged figure of the data of CRM197. The data indicates dose-dependent ADPR activity of CRM197. (**B**) SDS–PAGE analysis of ADPR reaction products. The products of cell-free ADPR with DT, CRM176 and CRM197 in the presence of [^{32}P]-NAD were analysed by SDS–PAGE and autoradiography. Major radioactive bands of 96 kDa were seen in the reaction products. Lane 1, 40 μg/ml DT; lane 2, 4 μg/ml DT; lane 3, 400 ng/ml DT; lane 4, 40 ng/ml DT; lane 5, 4 ng/ml DT; lane 6, 400 pg/ml DT; lane 7, 400 μg/ml CRM197; lane 8, 400 ng/ml CRM176; lane 9, control without toxin. (**C**) SDS–PAGE analysis of ADPR reaction products. ADPR products of incubated with DT, CRM176 and CRM197 containing equal radioactivity (2000 c.p.m./lane) were subjected by SDS–PAGE and autoradiography. Lane 1, DT; lane 2, CRM176; lane 3, CRM197.

would enable us to detect the ADPR activity. Since CRM197 was used at high doses in this study, it is possible that impurities in the CRM197 preparation might have caused the toxic action of CRM197. However, the following results negate this possibility: (i) the toxicity of CRM197 was dependent on the expression level of the DTR; (ii) the anti-DT mAb inhibited CRM197 toxicity; (iii) CRM197 was not toxic to Class II DT-resistant mutant Vdtr-H cells; and (iv) the ADPR activity of nicked CRM197 was detected in a cell-free reaction. In addition, we took specific care to avoid contamination from wild-type DT during the culture of cells and purification of CRM197: (a) CRM197 cultures were started from single colonies to avoid contamination from wild-type or pseudo-wild-type back-mutants; (b) glass-ware and other equipment for culture and purification were kept separate from that used for DT. However, the toxicity of CRM197 was similarly observed in separate preparations. In addition to the above evidence, Kimura *et al*. (*66*) recently found that over-expression of fragment A of CRM197 (G52E) in yeast and mammalian cells resulted in cytotoxicity when expressed in the cytosol, but not in cells expressing mutant EF-2 (Dr Kenji, K., Nara Institute of Science and Technology, Nara, Japan, personal communication). Therefore, we conclude here that the toxicity of CRM197 is due to its ADPR activity.

CRM197 has been used as an inhibitor of HB-EGF in research (*36*, *48*, *49*, *65*) and as an immunological adjuvant for vaccination (*44–46*). It is also expected to be a useful therapeutic for the treatment of ovarian and other cancers (*39*, *50*). The toxicity of CRM197 is 10^6 times less than that of DT as shown in this study; therefore, no toxicity was observed when used in the short term. However, under conditions in which cells are exposed to CRM197 over a prolonged period, CRM197 would be toxic. Indeed, when DT-susceptible animals were injected with a large amount of CRM197 (at 50 mg/kg body weight, for example), those animals died with signs similar to cases injected with DT (Mekada, E., unpublished observation). Therefore, the toxicity of CRM197 should be remembered when it is used in the human body. It should also be noted that the toxicity of CRM197 could not be detected in mice and rats, because CRM197 does not bind to proHB-EGF of these animals (*48*, *67*). We showed here that anti-DT

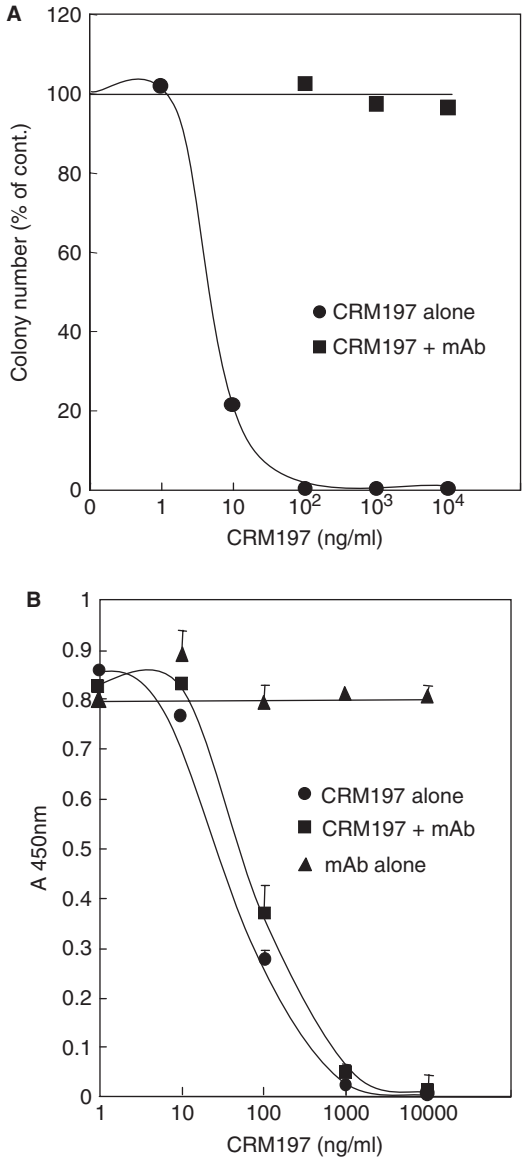

Fig. 4. **Anti-DT monoclonal antibody #2 neutralizes the toxicity of CRM197 but not its inhibition of HB-EGF.** (**A**) Effect of anti-DT mAb #2 on the toxicity of CRM197. CRM197 (0.5 mg) was pre-incubated with anti-DT mAb #2 (5 mg) at 37°C for 30 min. A colony formation assay was performed using Vero-H cells in the presence of CRM197 alone or a mixture of CRM197 and mAb #2, at the concentrations indicated. (**B**) Effect of anti-DT mAb #2 on the inhibitory activity of CRM197 towards HB-EGF-induced mitogenic activity. DER cells were incubated with HB-EGF in the presence of various concentrations of CRM197 alone, anti-DT mAb #2 alone, or a mixture of CRM197 and mAb #2, for 40 h. An MTT assay was then performed to measure live cell number.

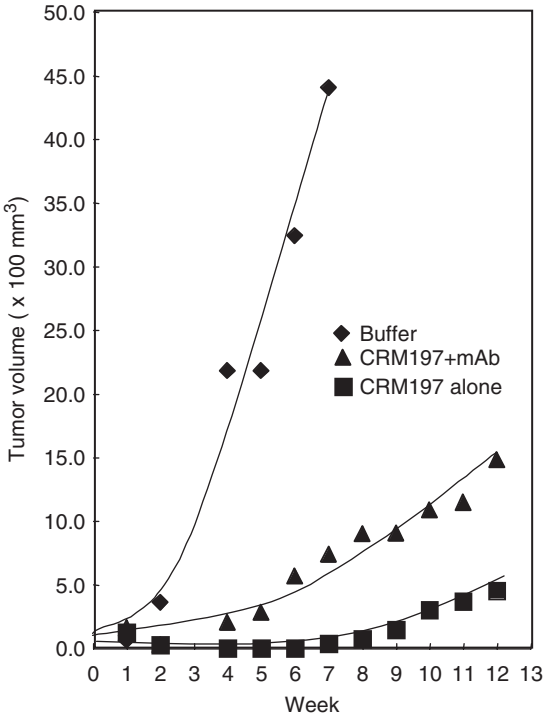

Fig. 5. **Effect of anti-DT mAb #2 on the inhibitory activity of CRM197 towards tumour formation by ovarian cancer cells.** SKOV-H cells were inoculated subcutaneously into nude mice. One week after injection, control saline, CRM197 (1 mg) alone, or a mixture of CRM197 (1 mg) and mAb #2 (8 mg), was injected intraperitoneally each week for 4 weeks. Each week, beginning 1 week after the subcutaneous injection of cells, tumour size was measured at the injection sites. Tumour volume was calculated as described in the MATERIALS AND METHODS section. Data are shown as means ± SE of duplicate samples.

mAb #2 inhibited the toxicity of CRM197, but not its HB-EGF blocking activity. Thus, the use of this antibody with CRM197 facilitates the ability to observe the inhibitory effect of CRM197 towards HB-EGF-induced mitogenic activity without its toxicity. When CRM197 is used as a neutralizing substance, both in research and in clinic, this antibody could be used in combination with CRM197.

The present study indicated that the toxicity of CRM197, that is, its EF-2-ADPR activity, contributed to its anti- tumuorigenic activity when administered into xenografted tumour-bearing mice. Ovarian cancer cells not only secrete the soluble form of HB-EGF, but also produce a high level of proHB-EGF (*36–38*). CRM197 is expected to show a cytotoxic effect towards cancer cells expressing a high level of proHB-EGF, in addition to blocking the mitogenic activity of secreted HB-EGF. A certain level of the toxicity of CRM197, if adequately controlled, would enhance the efficacy of cancer treatments. Further studies to produce mutated DT variants with different cytotoxicities could be useful to create an effective therapeutic agent.

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