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Lowering of Trichosanthin Immunogenicity by Site-Specific Coupling to Dextran

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ABSTRACT. Trichosanthin is a type I ribosome-inactivating protein possessing a broad spectrum of biological and pharmacological activities. Therapeutic use of this compound is hampered by its immunogenicity. It was shown earlier that coupling of dextran to trichosanthin can increase plasma half-life and reduce antigenicity. However, the site where dextran attaches to trichosanthin cannot be controlled; ideally, it should be at or near the antigenic determinant. The present study attempted to couple dextran to trichosanthin at a potential antigenic site. By site-directed mutagenesis, two sites, R29 and K173, were replaced by cysteine, and dextran was coupled to the newly created cysteine residues. The dextran–trichosanthin complex retained 50% of abortifacient activity and had a mean residence time in rats 27-fold longer than natural trichosanthin. Acute hypersensitivity reaction in guinea pigs was reduced greatly after coupling of K173C (a trichosanthin mutant with lysine-173 replaced by cysteine) to dextran. Compared with natural trichosanthin, dextran-K173C had a decrease in IgG and IgE response, whereas the coupling of R29C (a trichosanthin mutant with arginine-29 replaced by cysteine) to dextran did not show significant reduction of immunogenicity. This suggests that K173 but not R29 is located at or near an antigenic determinant. This study has demonstrated an alternative approach for mapping of antigenic determinants. The information obtained is also useful in producing an improved trichosanthin derivative for therapeutic use. BIOCHEM PHARMACOL **57**;8:927–934, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. trichosanthin; ribosome-inactivating protein; site-directed mutagenesis; dextran modification; immunogenicity; IgE

TCS§ is a type I ribosome-inactivating protein extracted from the root tuber of *Trichosanthes kirilowii*. It is a single polypeptide chain with a molecular mass of 27,000 Da. This compound has a broad spectrum of biological and pharmacological activities, including abortifacient [1–3] and antitumour [1, 4–9] activities. Recently, it was shown that TCS inhibited the replication of HIV-1 *in vitro* [10] and reduced HIV markers *in vivo* [11]. Regarding the therapeutic use of TCS, immunogenicity is one of the major concerns. Acute anaphylactic reaction has been observed in abortifacient practice [12] and after administration of TCS to some HIV-infected patients in clinical trials [13, 14]. As a protein foreign to a host, TCS also elicits antibodies such as IgG and IgE [15], and this imposes limitations on multiple uses of this compound as a therapeutic agent. The immunogenicity of TCS can be reduced by coupling it to dextran T40, which sterically masks the antigenic site. A dialdehyde method was used, which linked up activated dextran to the amino groups of TCS [16–18]. However, this method does not allow precise positioning of dextran to the antigenic determinant of TCS that can afford the maximum steric masking effect.

The objective of this study was to describe a method that allows coupling of dextran to TCS at any selected potential antigenic site so as to achieve maximum reduction in antigenicity and minimum reduction in biological activities. TCS is a polypeptide without cysteine residues. By site-directed mutagenesis, a cysteine residue can be introduced at a potential antigenic site of the protein. Dextran T40 then can be coupled to the cysteine residue by an alkylation method [19]. The combined use of site-directed mutagenesis and coupling is useful in producing a better therapeutic product and at the same time providing information on the antigenic mapping of the TCS molecule.

MATERIALS AND METHODS

Selection of Mutation Sites

Two approaches were used to analyze the degree of immunogenicity on each area of the TCS molecule. The command PEPTIDESTRUCTURE of the GCG computer pro-

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§ *Abbreviations*: TCS, trichosanthin; nTCS, natural trichosanthin; R29C, TCS mutant with arginine-29 replaced by cysteine; PCR, polymerase chain reaction; K173C, TCS mutant with lysine-173 replaced by cysteine; DX, dextran; IgG, immunoglobulin G; IgE, immunoglobulin E; PEG, polyethylene glycol; HIV-1, human immunodeficiency virus type I; and OPD, *o*-phenylenediamine dihydrochloride.

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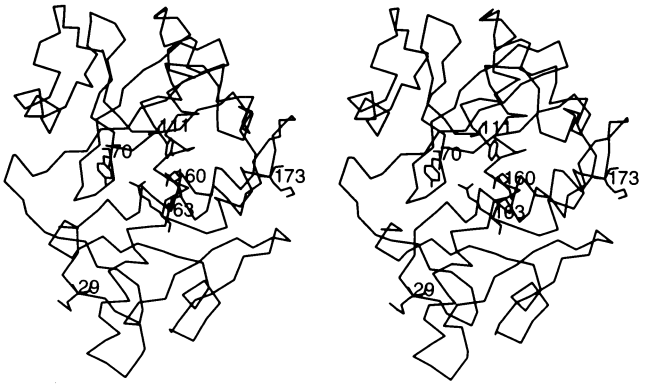

FIG. 1. Stereo-image of trichosanthin indicating the location of R29, K173, and the active site residues E160 and R163.

gram [20] performed calculations of the antigenic index by summing up several weighted parameters of the protein's secondary structure. With reference to the three-dimensional structure of TCS [21, 22], five parameters of a particular region were considered: (1) exposure on the surface, (2) hydrophilicity, (3) chain flexibility, (4) formation of loops or turns, and (5) direction and interaction of the amino acid side chains. Two sites, R29 and K173, with the highest probability to elicit antibody response were selected (Fig. 1).

Site-Directed Mutagenesis of TCS

Two mutants, R29C and K173C, were made by PCR using the overlapping primer method [23]. DNA fragments generated by PCR were sequenced using the T7 sequencing kit from Pharmacia to ensure that no secondary mutation had occurred. The mutated TCS was subcloned into expression vector pET-8c [24].

Protein Expression and Purification

Expression of mutated TCS genes was performed as previously described with some modification of the purification scheme [25]. In brief, after an expression culture was lysed by sonication, the lysate was applied to a CM-Sepharose CL-6B (Pharmacia) column (1.6 × 26 cm). After washing with buffer A (20 mM sodium phosphate, pH 6.5), the protein was eluted with 0.1 M NaCl in buffer A. The eluate was dialyzed against buffer A and applied to a Mono-S (HR 5/5) column (Pharmacia). After washing the column with 5 mL buffer A, a 60-mL linear gradient of NaCl (0–1 M) in buffer A was applied to elute the modified TCS protein.

Coupling of Dextran T40 to Cys-TCS

An alkylation method [19] previously described for coupling of dextran to the sulfhydryl of hemoglobin was adopted. In brief, dextran T40 (Pharmacia) was activated by cyanogen bromide (CNBr, Sigma) and then derivatized to form bromoacetyl function, which can then be coupled to the sulfhydryl group of the mutated TCS. Reaction of TCS with dextran T40 was done at a TCS/dextran molar ratio of 1:50 for 48 hr. The reaction mixture was dialyzed in 0.01 M Tris buffer, pH 7.5. Coupled and uncoupled TCS were separated by gel filtration chromatography on a Sephadex G-200 (Pharmacia) column. Protein content was determined by measuring UV absorbance at 280 nm. The concentration of TCS was determined by radioimmunoassay as described previously [16]. Completeness of coupling also was confirmed by SDS–PAGE.

Assay for Biological Activity

Biological activities of nTCS, cys-TCS, and conjugated TCS were assessed by measuring their respective ribosome-inactivating activity [26] using a rabbit reticulocyte lysate cell-free translation system (Promega) as a source of ribosome, mRNA, and other endogenous factors. Protein synthesis was assessed by [^{35}S]methionine (Amersham) uptake. Ribosome-inactivating activity was also determined on DX-TCS with the dextran moiety digested by incubating the complex with dextranase at pH 7.6 and 36° for 20 hr. The activity of cys-TCS and its conjugates was assessed *in vivo* by measuring the mid-term abortifacient effect. Mature female ICR mice weighing 25–35 g were caged with fertile males to produce pregnant mice. The presence of a copulation plug the following morning was designated as day 1 of pregnancy. Different doses (0.01 to 0.1 mg/25 g) of nTCS, R29C, K173C, and their respective conjugates were injected i.p. into mice on day 12 of pregnancy. Control mice were injected with saline solution. The mice were autopsied on day 14 of pregnancy. The numbers of uterine implantation sites, live fetuses, and dead fetuses were recorded. The mice were considered aborted when the number of dead fetuses exceeded 50% of the total implantation sites.

Assay for Immunogenicity: IgG and IgE Responses

C57BL/6N inbred mice (6- to 8-weeks-old) were used. Mice in groups of five were immunized s.c. at the back with 10 μg of various forms of TCS in complete Freund's adjuvant (Sigma) on day 0 (the 10 μg of DX-R29C or DX-K173C refers to the conjugate containing 10 μg of TCS). Booster injections were given with incomplete Freund's adjuvant (Sigma) on day 21. Blood samples were collected 7 days after booster injection by retrobulbar puncture under light ether anesthesia. Sera from mice were stored at −20° until used.

Specific IgG and IgE antibodies against nTCS were detected by conventional ELISA. ELISA plates (Immunoplate, Maxisorp, Nunc) were coated overnight at 4° with 100 μL of antigen (5 μg/mL of nTCS) in 0.05 M sodium carbonate-bicarbonate buffer (pH 9.6) with 0.1% sodium azide. Then each plate was rinsed with a washing buffer (PBS with 0.05% Tween 20). Serum samples (diluted with washing buffer and 1% BSA) were added in duplicate to the

ELISA plates at 100 μL/well. After incubation and washing, 100 μL of detecting secondary antibody (sheep anti-mouse IgG-horseradish peroxidase conjugate for measuring IgG or sheep anti-mouse IgE for measuring IgE, Serotec) was added. Finally, 100 μL of substrate solution (OPD; Sigma) was added to each well. The reaction was stopped by adding 50 μL/well of 2.5 M H_2SO_4 after incubating at 37° for 30 min. The absorbance was read on an ELISA plate reader (Dynatech MR5000) at 490 nm. For IgE measurement, a tertiary antibody, donkey anti-sheep IgG-horseradish peroxidase conjugate (Sigma), was added before adding the substrate solution.

The relative amounts of TCS-specific IgG or IgE were estimated by comparing the respective dilution (expressed as mean ± SEM) at the linear region of the dilution curve with the largest difference in absorbance. Statistical analysis was performed by one-way analysis of variance followed by the Scheffe multiple comparison test with a significance level of $P < 0.05$.

Assay for Acute Hypersensitivity in Guinea Pigs

Guinea pigs (300–400 g) were divided into groups of five and were sensitized i.p. with 0.1 mg/kg of test antigens on days 1, 3, and 5. Two weeks after the last injection, the guinea pigs were challenged i.v. through the ear vein with 0.2 mg/kg of the respective antigens. Signs of acute hypersensitivity were observed for 30 min immediately after injection, and the severity was scored accordingly: (−) no sign; (±) dubious signs but not obvious; (+) nose-rubbing or scratching; (++) coughing or gasping respiration; and (+++) cyanosis, convulsions, or prostration [27, 28].

Pharmacokinetics

The pharmacokinetics of a single i.v. bolus of various forms of TCS in Sprague–Dawley rats were examined as described in a previous study [16].

RESULTS

Mutation, Expression, and Purification of TCS

Codons at R29 and K173 on the surface of TCS were changed to that for cysteine by mutagenic PCR primers. PCR-generated fragments were sequenced to ensure that there was no secondary mutation. The cys-TCS was expressed at a high level in *Escherichia coli*. The typical yield of modified TCS protein after purification was 10–15 mg/L of bacterial culture. R29C and K173C were eluted at 100 and 130 mM NaCl from the Mono S column, respectively. Purified protein appeared as a single band as analyzed by 12% SDS–PAGE (Fig. 2).

Coupling of DX-T40 to Modified TCS Protein

After coupling cys-TCS to dextran T40, the molecular masses of R29C and K173C became larger, enabling them to be separated from uncoupled TCS by Sephadex G-200 column chromatography (Fig. 3).

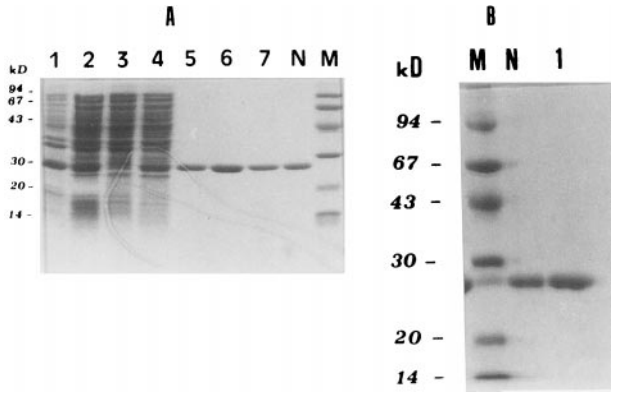

FIG. 2. Purification of R29C (A) and K173C (B) as analyzed by 12% SDS–PAGE. (A) lane 1: pellet of crude bacterial lysate; lane 2: supernatant of crude bacterial lysate; lanes 3 and 4: eluate during washing of CM-Sepharose column by buffer A; lanes 5 and 6: CM-Sepharose eluate of R29C; lane 7: Mono S eluate of R29C; lane N: nTCS; and lane M: low molecular weight marker (Pharmacia). (B) lane 1: Mono S eluate of K173C; lane M: low molecular weight marker; and lane N: nTCS.

Inhibition of In Vitro *Protein Synthesis*

The inhibitory effect of various forms of TCS on protein synthesis was shown in Fig. 4. Activity of both cys-TCS forms was not different from nTCS ($P > 0.05$). On the other hand, the IC_{50} was reduced by 20-fold after coupling ($P < 0.05$). Digestion of the dextran moiety restored activity to that of the parent compound, indicating that the reduction in activity was due to the steric hindrance of dextran.

Midterm Abortifacient Activity

The abortifacient activities of various forms of TCS are summarized in Table 1. The dose of nTCS required to produce 100% abortion was very much the same as those of R29C and K173C. After coupling to dextran T40, the abortifacient activity of the conjugates was only half that of nTCS and the respective cys-TCS forms.

Immunogenicity of DX-TCS Conjugates

The IgG responses against TCS and the derivatives in mice are shown in Fig. 5. It appeared that DX-K173C produced a lower IgG response than K173C and nTCS, while the IgG response of DX-R29C was not much different from the parent compound. Similar effects also were found with the IgE response (Fig. 6). This suggests that position 173 represents a stronger immunogenic site than position 29. In a separate experiment, the dextran moiety was digested before immunization. The IgG and IgE dilution curves all shifted toward those of nTCS after dextranase digestion,

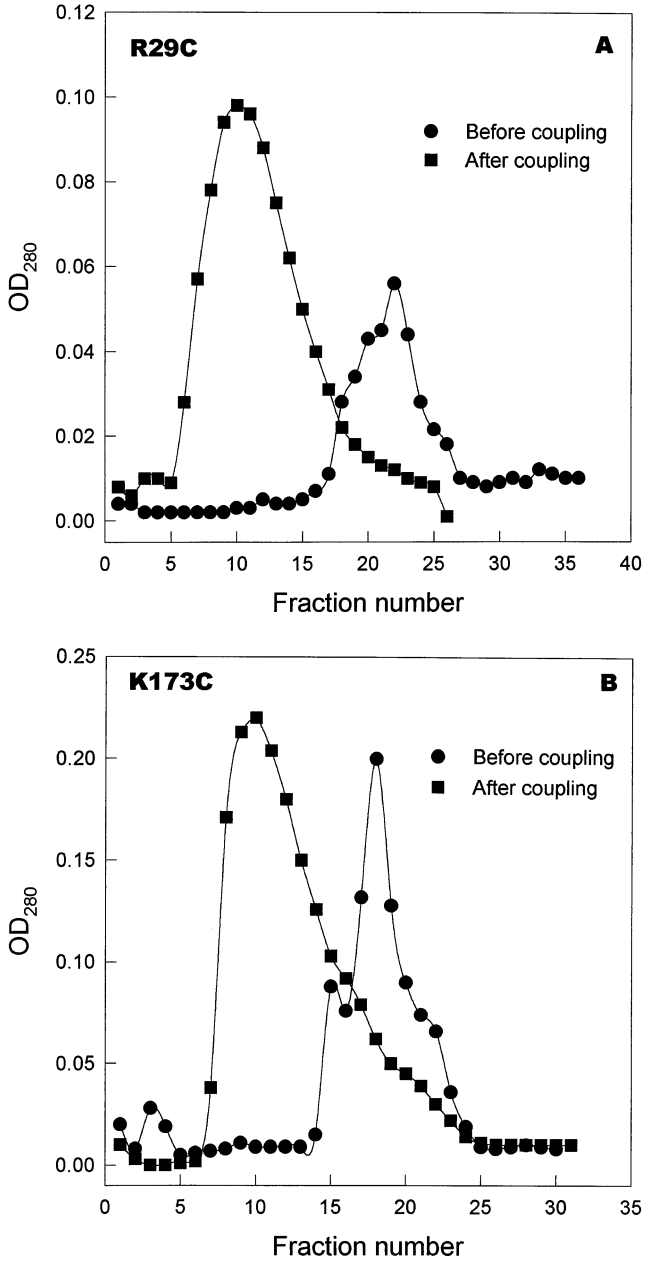

FIG. 3. Separation elution profile of (A) DX-R29C from R29C, and (B) DX-K173C from K173C on a Sephadex G-200 column.

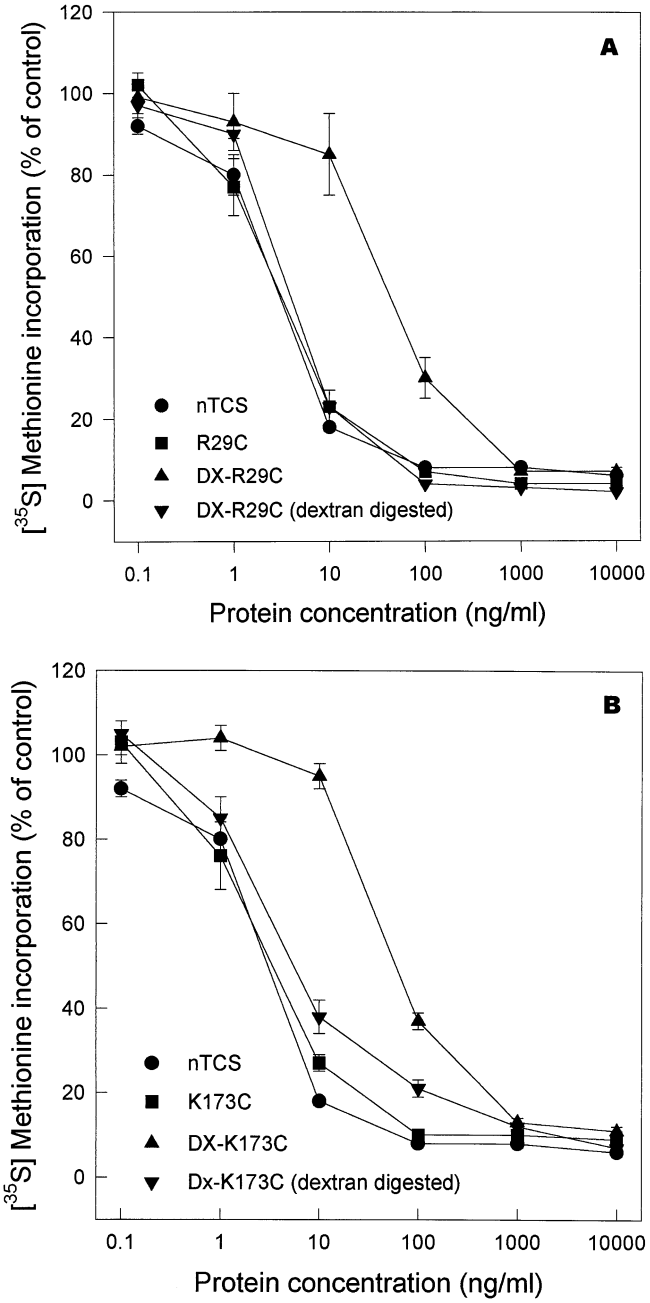

FIG. 4. Ribosome-inactivating activity of various forms of (A) R29C and (B) K173C. Inhibition of protein synthesis was assessed by [^{35}S]methionine uptake in a rabbit reticulocyte lysate cell-free translation system. Dextranase was used to digest the dextran moiety of the conjugate in order to remove the effect of dextran. Values are means ± SEM (N = 3). The value of the control from one typical experiment was 49,340 ± 1,592 cpm.

indicating that the dextran moiety of the conjugates played a significant role in reducing the IgG and IgE titer. All mice showed negative TCS-specific IgG and IgE toward dextran immunization.

Acute Hypersensitivity

Acute hypersensitivity reactions to the various forms of TCS are shown in Table 2. All guinea pigs sensitized with native TCS or modified TCS (K173C) died within 5 min after challenged with appropriate antigens. This suggested that TCS produced the most severe form of acute hypersensitivity. Coupling of K173C to dextran reduced the severity of acute hypersensitivity, and none of the animals died. Dextran T40 alone did not elicit any acute hypersensitive reaction.

DISCUSSION

There are several advantages of coupling dextran or other inert macromolecules to a biologically active protein. For example, the plasma half-life can be increased because of

TABLE 1. Midterm abortifacient activities of various forms of TCS in ICR mice

Dose (mg/25 g)	Number of mice	Number of dead fetuses/Number of implantation sites	% of Aborted mice*
nTCS			
0.01	11	67/163 (41%)	45
0.02	7	76/94 (81%)	100
0.05	6	53/59 (90%)	100
0.10	7	111/111 (100%)	100
R29C			
0.01	4	25/52 (48%)	50
0.02	9	128/139 (92%)	100
0.05	10	112/116 (97%)	100
0.10	9	92/94 (98%)	100
K173C			
0.01	6	46/85 (54%)	50
0.02	6	97/97 (100%)	100
0.05	6	75/75 (100%)	100
0.10	7	85/85 (100%)	100
DX–R29C			
0.01	10	25/75 (33%)	10
0.02	9	68/136 (50%)	55
0.05	8	92/97 (95%)	100
0.10	7	108/108 (100%)	100
DX–K173C			
0.01	11	49/150 (33%)	27
0.02	8	57/122 (47%)	38
0.05	5	66/66 (100%)	100
0.10	6	82/86 (95%)	100
Control			
Normal saline (0.3 mL/mouse)	29	6/413 (1%)	0
Bromo-dextran (0.016 g/mouse)	7	4/100 (4%)	0

*Mice were considered aborted when the number of dead fetuses exceeded 50% of the total implantation sites on day 14 of pregnancy.

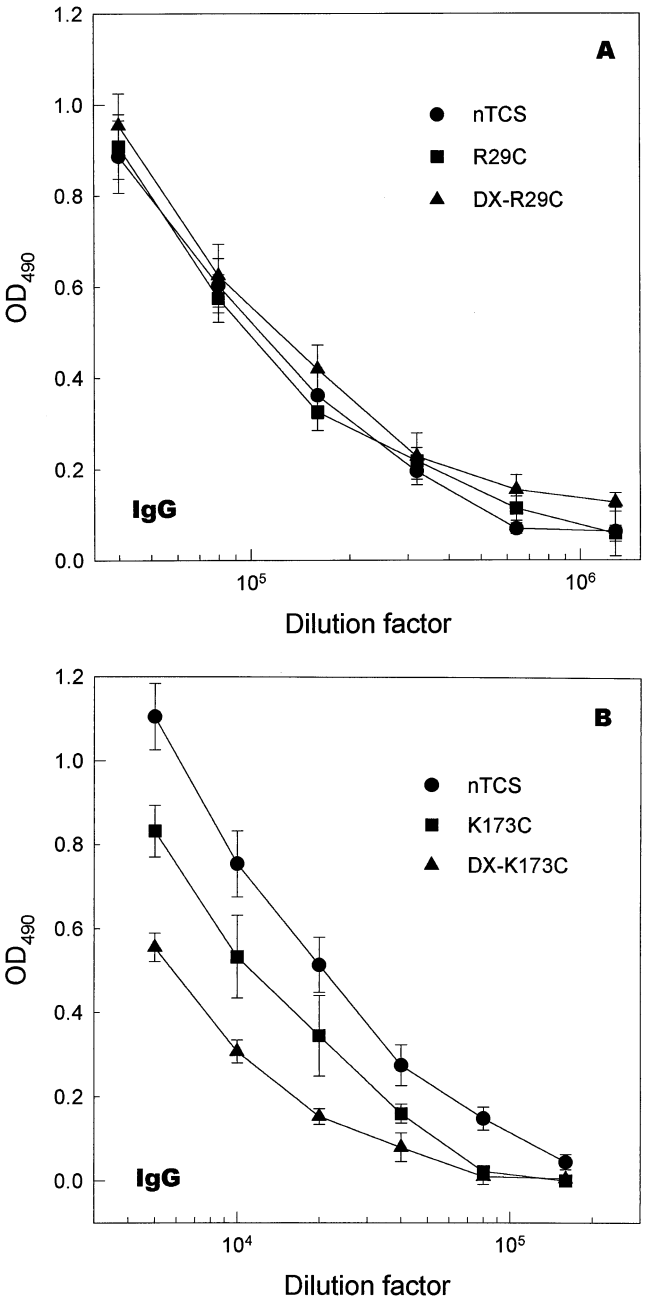

FIG. 5. TCS-specific IgG titer in mice immunized with (A) nTCS, R29C, and DX-R29C, and (B) nTCS, K173C, and DX-K173C. The IgG titer was measured by conventional ELISA on plates coated with nTCS and expressed as O.D.$_{490}$. A secondary antibody was used that was conjugated to horseradish peroxidase, and OPD was used as substrate. Values are means ± SEM (N = 5).

exclusion from renal clearance due to the enlarged molecular size; the antigenicity can be reduced because of steric masking of the antigenic determinants from exposure to the immune system of the host [16, 17, 19, 29]. There are also disadvantages such as decrease in biological activity due to steric influence on the active site of the protein. Therefore, the balance must be carefully controlled so that reduction in antigenicity will not be compromised drastically by decrease in activity. This is only possible if the dextran molecule can be placed on the protein molecule at specific desirable sites. The present study describes a method of attaching dextran at specific sites of a protein. TCS is a single polypeptide without cysteine residues. By site-directed mutagenesis, a cysteine residue was introduced to TCS at a specific site, allowing coupling of dextran to it by alkylation. The coupling reaction is highly specific to sulfhydryl groups. No coupling product was observed under the same condition when nTCS reacted with bromoacetyl dextran (data not shown).

There are four antigenic determinants on the TCS molecule as mapped by monoclonal antibodies [30]. Nevertheless, the exact location is not known. From its three-dimensional structure and by theoretical calculation, one can speculate on the whereabouts of the antigenic sites. With the method described above, a dextran molecule can be placed above a potential antigenic site so as to shield it from exposure to the immune system of the host. Immunogenic activity of the conjugate is expected to decrease if the site of attachment is at or near the antigenic determinants. This provides an alternative approach for mapping the antigenic determinants of the TCS molecule. With this information, ideally one can eventually design a dextran-

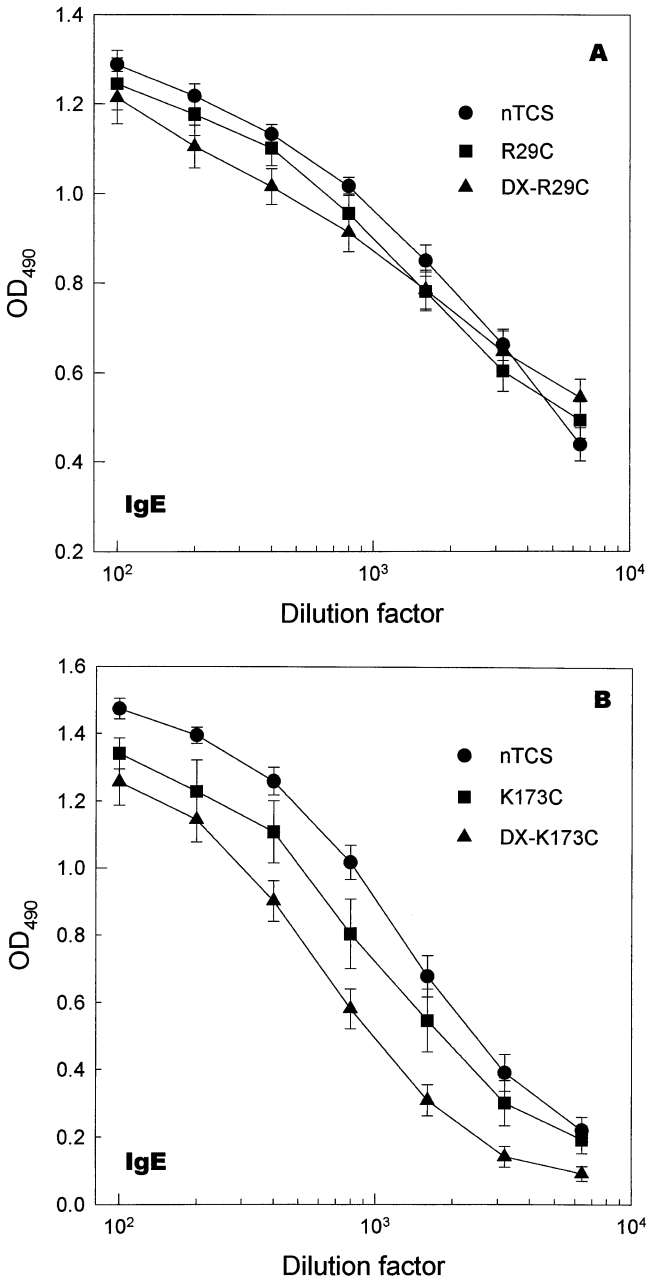

FIG. 6. TCS-specific IgE titer in mice immunized with (A) nTCS, R29C, and DX-R29C, and (B) nTCS, K173C, and DX-K173C. The IgE titer was measured by conventional ELISA on plates coated with nTCS and expressed as O.D.$_{490}$. A tertiary antibody was used that was conjugated to horseradish peroxidase, and OPD was used as substrate. Values are means ± SEM (N = 5).

TCS conjugate with maximum activity and minimum immunogenic activity. In this study, two potential antigenic sites, R29 and K173, were replaced by cysteine.

The ribosome-inactivating activities of the two cys-TCS forms were almost the same as nTCS and decreased 20 times after coupling to dextran T40. It is strongly suggested that this may be due to steric influence of the dextran moiety because its digestion by dextranase normalizes activity. With respect to the *in vivo* abortifacient activity, nTCS and the two cys-TCS forms were identical, and activity was reduced by 2-fold after conjugation to dextran. The conjugates produced by this method showed great improvement in activity over a previous preparation of DX-nTCS produced by non-specific coupling, of which the ribosome-inactivating activity was reduced by 200-fold and the abortifacient activity decreased by 20-fold [16, 18]. Such a large discrepancy in activity between the two preparations may be related to the site of dextran attachment. In the present preparation, the site at which dextran attached to TCS is remote from the active site, whereas in the former preparation, the site of attachment is not known and cannot be controlled. It might well be very close to the active site, causing a bigger reduction in activity.

There are numerous examples indicating that the antigenicity of a protein can be reduced after coupling to a macromolecule such as dextran [17, 27]. The decrease in antigenicity appears to be related to the size of the dextran molecule. Dextran with a molecular mass of 40 kDa is better in reducing the antigenicity than the smaller dextrans with sizes ranging from 1 to 4 kDa [31]. In general, low molecular weight dextrans are not very antigenic [32]. Dextran T40 and T70 (MW 40,000 and 70,000) are widely used clinically for plasma volume expansion, improvement of blood flow, and thromboprophylaxis. Although dextran is relatively safe, in a small percentage of patients receiving dextran various degrees of anaphylactic reactions were observed [33, 34]; the incidence can be reduced significantly by hapten inhibition [35]. In the present experiments, dextran-specific IgG and IgE were not detected in mice, and dextran did not cause any observable acute hypersensitivity in guinea pigs. Nevertheless, there is always the possibility that the response in humans will be different from that in mice or guinea pigs. It should be emphasized that the present study has described an approach rather than a final product. There are variables that can be manipulated to suit human needs. For example, the size of dextran can be reduced to minimize hypersensitivity. Alternatively, TCS can be conjugated to PEG instead of dextran. Using the method described in this study, other potential antigenic sites on the TCS molecule can be mapped and tested.

The present study showed that nTCS-specific IgG and IgE induced by R29C and DX-R29C were almost the same as nTCS. In contrast, there were substantial differences in the IgG and IgE titer induced by nTCS, K173C, and DX-K173C in the following order: nTCS was the most antigenic and DX-K173C was 3-fold less. In line with this finding, nTCS and K173C produced the most severe form of acute hypersensitivity in guinea pigs, while coupling of dextran to K173C substantially reduced such reaction. This suggests that the region centered at K173 is an antigenic determinant, while R29 is not. Increase in antigenic activity toward that of K173C after removal of the dextran moiety supports the masking role of dextran on the antigenic determinant of TCS. This is consistent with other

TABLE 2. Acute hypersensitivity reaction of various forms of TCS in guinea pigs

Antigen	Total number of guinea pigs	Number of animals with different degrees of acute hypersensitivity					
		−	±	+	++	+++	Death
No treatment	5	5	0	0	0	0	0
nTCS	4	0	0	0	0	0	4
K173C	5	0	0	0	0	0	5
Dx–K173C	5	0	1	1	2	1	0
Dextran	5	5	0	0	0	0	0

Signs of acute hypersensitivity were observed within 30 min after i.v. injection of antigens and scored semi-quantitatively as follows: (−) no sign; (±) dubious signs but not obvious; (+) nose-rubbing or scratching; (++) coughing or gasping respiration; and (+++) cyanosis, convulsions, or prostration.

findings on the location of TCS epitopes between positions 107 and 234 [36].

The abortifacient activity of DX-K173C was reduced only slightly, and therefore the reduction in TCS-specific IgG and IgE levels was unlikely to be due to the destruction of the TCS molecule during coupling. Compared with previous findings on DX-nTCS produced by nonspecific coupling [17], the decrease in immunogenicity is close to 8-fold, which is a larger reduction in antigenicity. This is probably due to the fact that the non-specific method coupled more than one dextran (1.8 dextran per TCS on average) to TCS, and therefore more than one epitope can be masked by dextran. In the present method, there was only one dextran per TCS molecule. By combining site-directed mutagenesis and PEG modification, Hershfield and colleagues have been able to further decrease the antigenicity of purine nucleoside phosphorylase by increasing the number of PEG molecules binding [29].

The DX-K173C conjugate can also be regarded as a more superior therapeutic agent than its parent compound or nTCS. Not only is the antigenicity reduced, but the mean residence time increased from 10 ± 1 to 275 ± 30 min, similar to a previous preparation of DX-nTCS [16]. This is probably due to the increase in molecular size leading to reduced renal clearance. Since the *in vivo* activity is reduced by only 50%, the 27-fold increase in mean residence time definitely allows administration of a smaller dosage of DX-K173C to achieve similar biological effects.

In summary, a method is described in this study that can couple dextran to TCS at any specific position. This can afford a masking effect on antigenic sites and, hence, reduce antigenicity of the molecule. By repeatedly examining the different sites on the TCS molecule, one can map the antigenic determinants of the molecule, and this information can be useful in designing a better therapeutic agent. In this study, two potential antigenic sites were tested. Results suggested that K173 is near or at an antigenic determinant, whereas R29 is not. Therefore, DX-K173C is a more superior therapeutic agent than nTCS or DX-R29C.

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