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Effect of Chemical Modification of Histidine and Tyrosine Residues in Toxic Shock Syndrome Toxin 1 on the Serologic and Mitogenic Activities of the Toxin

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Modification of three or four of the five histidine residues in the toxic shock syndrome toxin 1 (TSST-1) with diethylpyrocarbonate did not inhibit the precipitin reaction of the modified TSST-1 with polyvalent antisera to the toxin. Monoclonal antibody 7T did not react with the modified TSST-1, but monoclonal antibody 8T did react with the toxin. Up to 50% of the mitogenic reaction of TSST-1 was inhibited by the histidine modification. Modification of one or two of the nine tyrosine residues in TSST-1 did not inhibit the precipitin reaction with polyclonal antisera to the toxin but did inhibit 85% of the mitogenic reaction.

Toxic shock syndrome toxin 1 (TSST-1), the primary toxin responsible for toxic shock syndrome (1, 18), is a relatively simple protein with a molecular mass of 22,049 daltons (Da) and an isoelectric point of 7.0 (2, 14). It consists of a single chain lacking any half-cystine residues (10). TSST-1 is a very active molecule, and its mitogenic activity may play an important role in toxic shock syndrome. The biologically active sites in the toxin molecule have not been fully elucidated, although in previous studies they have been associated with the 15-kDa cyanogen bromide-generated middle fragment (3, 7, 9).

The purpose of this study was to determine whether the histidine or tyrosine residues are involved in the mitogenic and serological activity of TSST-1 and whether there is a relationship between the two activities. To accomplish this, the effect of chemical modification of the histidine and tyrosine residues in TSST-1 on its serological and mitogenic activities was studied.

MATERIALS AND METHODS

Modification of amino acids. Histidine residues in TSST-1 were modified by using diethylpyrocarbonate (DEPC; Sigma Chemical Co., St. Louis, Mo.) by the procedure of Miles (11). Purified TSST-1 (14) (0.8 mg/ml) dissolved in 0.05 M sodium phosphate buffer (PB), pH 6.0, was reacted with DEPC at room temperature for 60 min. The protein-to-reagent (P:R) molar ratios were 1:0 (control), 1:100, 1:500, and 1:1,000. After 60 min, the reaction was stopped by the addition of 250 mM L-histidine solution, and the reaction mixture was dialyzed against a large excess of 0.05 M PB (pH 7.0) with stirring for 24 h, with several changes of buffer.

The procedure of Riordan and Vallee (15) was used for modification of tyrosine residues. TSST-1 (0.6 mg/ml) was dissolved in 0.05 M Tris hydrochloride buffer, and tetranitromethane (Sigma), diluted 1:10 in 95% ethanol to give a 0.84 M solution (15), was added. The P:R molar ratios were 1:0 (control), 1:100, and 1:200. The reaction was allowed to proceed for 60 min at room temperature; the reaction products were dialyzed against 0.01 M PB containing 0.85% sodium chloride, pH 6.8.

Protein concentration determinations. The protein concentration of the modified toxins was determined by the Bradford assay (4) with a kit from Bio-Rad Laboratories. A standard curve for the assay was prepared by using purified TSST-1.

Absorbance spectra. Absorbance measurements were made on a Beckman DU-7 spectrophotometer at room temperature (25°C). Absorbance values in the UV regions were obtained with the use of a quartz cuvette of 1 cm path length.

CD spectra. Circular dichroism (CD) spectra were recorded on a JASCO 20A CD/ORD spectropolarimeter with a wavelength expansion of 10 nm/cm and a recording speed of 2 cm/min at 25°C (19). For the recording of CD spectra of histidine- and tyrosine-modified TSST-1, 0.05 M PB (pH 7.0) and 0.1 M PB containing 0.85% NaCl, pH 6.8, were used, respectively. All spectral recordings were done in triplicate, and the entire experiment was repeated at least once. Concentrations of control and modified TSST-1 were determined by the Bradford assay (4).

Serological activity. The serological activity of control and modified TSST-1 was determined by the Ouchterlony double-gel diffusion method (17). Polyvalent antiserum to TSST-1 was raised in New Zealand White rabbits by the procedure of Robbins and Bergdoll (16). The serological reactivity of monoclonal antibodies (MAbs) with control and modified toxins was tested by the competition radioimmunoassay (RIA) of Miller et al. (12). Labeled [^{125}I]TSST-1 was used in competition with control and modified toxins.

ELISA. An enzyme-linked immunosorbent assay (ELISA) (8) was also used for determining the concentrations of TSST-1.

Mitogenic activity. All mitogenic activity determinations were carried out in a 4-day murine splenocyte proliferation assay (9). Concentrations of the control and modified toxins were determined by the Bradford assay (4). Results of incorporation of [^{3}H]thymidine into cellular DNA are expressed as the average (mean) counts per minute of quadruplicate wells ± standard error of the mean. The stimulation index was calculated by dividing the cpm in test wells by the background cpm.

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TABLE 1. Calculation of the number of histidine residues modified by DEPC[a]

P:R	A_{240}		N-Carbethoxy histidine (mol/mol of TSST-1)
	Before modification	After modification	
1:0	0.3819	0.3819	0.00
1:100	0.3909	0.4780	1.53
1:500	0.3628	0.5069	2.73
1:1,000	0.3554	0.5537	3.83

[a] The number of histidine residues modified was calculated with an ε_{240} of 3,200 cm^{-1} M^{-1} for N-carbethoxy histidine (Oádi et al. [13]).

RESULTS

Modification of histidine. DEPC reacts with histidine residues in proteins to yield the N-carbethoxy histidyl derivative, whose formation can be followed spectrophotometrically at 240 nm. Modification of histidine residues was evidenced by a concomitant increase in A_{240}. Time versus increase in A_{240} indicated that the reaction of DEPC with histidyl residues was virtually complete within 10 min of incubation of the reagent with the protein (data not shown). A side reaction of DEPC with proteins, the O-carbethoxylation of tyrosine residues (5, 13), did not occur, as evidenced by no decrease in the A_{277} or no major decrease at wavelengths below 240 nm (data not shown) (5). Simultaneous modification of histidyl and tyrosyl residues results in an undetectably small change in the difference in the A_{240} due to N-carbethoxy histidyl residues (5). The extent of the reaction of DEPC with proteins and consequently the number of residues of histidine modified were determined by measuring the increase in A_{240} by using a molar absorption difference extinction coefficient for N-carbethoxy histidine at 240 nm of 3,200 cm^{-1} M^{-1} (14). The calculation of the number of histidine residues modified after treatment with DEPC is shown in Table 1. Caution was used in interpreting the number of histidine residues modified because excess DEPC can react to form a disubstituted histidyl derivative which has a higher A_{242} value than the monosubstituted histidyl derivative (13). The formation of a disubstituted histidine can lead to overestimation of the number of modified histidine residues, more than are present in the protein.

Modification of tyrosine. The degree of nitration of tyrosine was determined by the absorption of the nitrotyrosyl residue at 428 nm and the molar extinction coefficient, ε, of 4,200 at pH 9.0 (15). This absorption maximum shifts to 360 nm in acid with an isobestic point at 381 nm and ε of 2,200, which can be used for quantitation independent of the pH of the solution being measured. The calculation of the number of residues of nitrotyrosine formed as a result of nitration of tyrosine with tetranitromethane is shown in Table 2.

Conformational studies. The results of the secondary-structure analysis of the CD spectra of control and histidine- and tyrosine-modified TSST-1 are presented in Table 3. The CD spectra recorded with control and histidine-modified TSST-1 had very similar shapes (spectra not shown). The spectra for control (1:0) and modified TSST-1 had a negative minimum at 205 ± 1 nm, with a shoulder at 225 ± 1 nm. However, the shoulder at 225 ± 1 nm decreased with increasing modification of histidine residues in TSST-1. The mean residue ellipticity increased with increases in modification of the toxin. Secondary-structure analysis of these spectra showed a gradual increase in the α-helical content of the toxin from 4.5 to 7.0%, with a corresponding molar excess of DEPC (Table 3). The remainder of the secondary-structure parameters, including β-pleated sheets, β-turn, and random coil, were essentially unchanged. The reason for the two very different β-turn values obtained, 1.0 and 3.0 to 3.5%, was probably due to the program used for secondary-structure calculations. This program is known to be erratic for calculations of β-turn structures (7).

TABLE 2. Calculation of the number of tyrosine residues modified by TNM[a]

P:R	A_{428}	Nitrotyrosine (mol/mol of TSST-1)
1:0	0.000	0.00
1:100	0.150	1.38
1:200	0.195	1.79

[a] The number of tyrosine residues modified was calculated with an ε_{428} of 4,200 cm^{-1} M^{-1} for nitrotyrosine (Riordan and Vallee [15]).

TABLE 3. Secondary-structure analysis[a] of control and histidine- and tyrosine-modified TSST-1

TSST-1 and P:R	% of total structures			
	α-Helix	β-Sheet	β-Turn	Random coil
Histidine modified				
1:0 (control)	4.5	61.5	3.5	30.5
1:100	5.5	60.5	1.0	33.0
1:500	6.0	60.0	3.0	31.0
1:1,000	7.0	60.5	1.0	31.5
Tyrosine modified				
1:0 (control)	4.0	64.5	3.5	28.0
1:200	6.0	56.0	7.0	31.0

[a] Calculations for the secondary structure were done by the method of Chang et al. (6). Ellipticity data used for the calculations were taken at 1-nm intervals in the wavelength range from 240 to 200 nm.

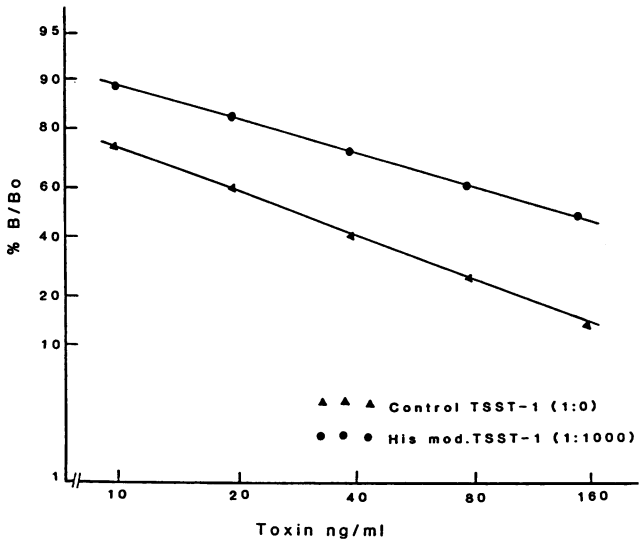

FIG. 1. Logit-log plot of competitive RIA of control (1:0) and histidine-modified (His mod., 1:1,000) TSST-1 with MAb 8T. A dilution of MAb 8T was reacted with constant amount of ^{125}I-labeled TSST-1 which was in competition with the indicated amount of unlabeled control (1:0) or histidine-modified (1:1,000) TSST-1. %B/B_0 represents the ^{125}I-labeled TSST-1 bound when in competition with the specified amount of unlabeled toxin divided by the ^{125}I-labeled toxin bound when no unlabeled toxin was present, multiplied by 100.

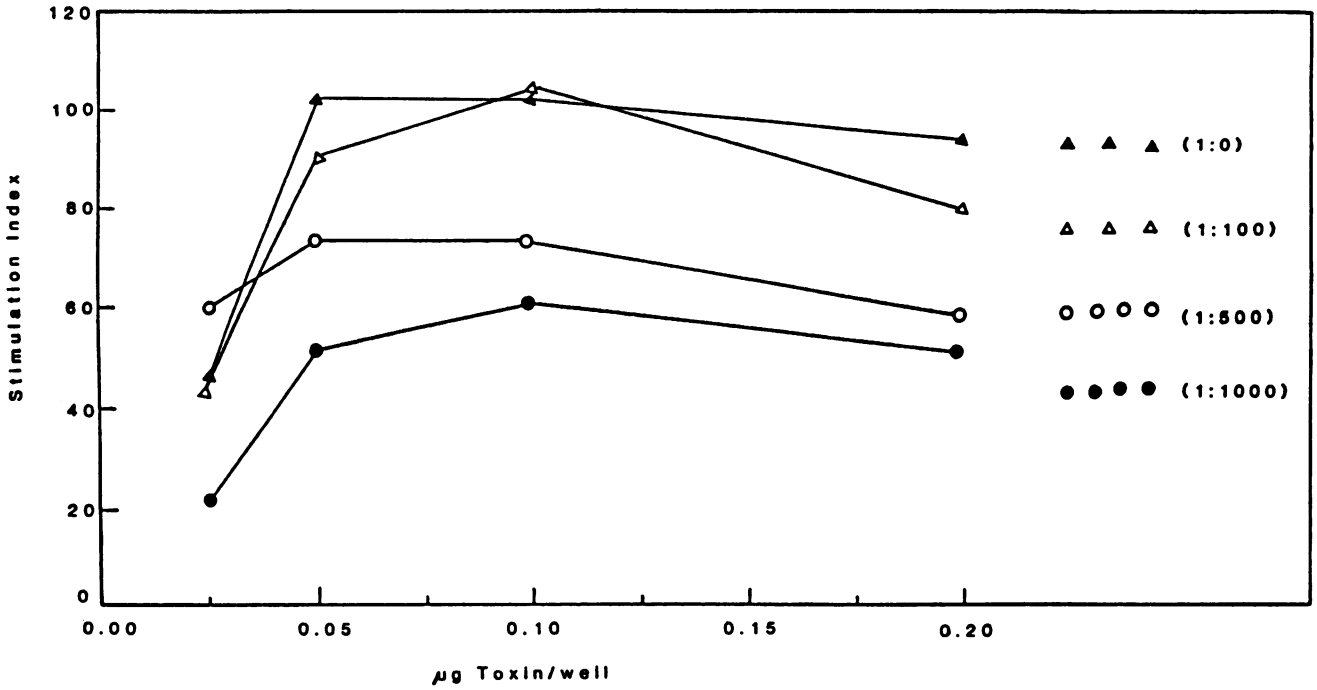

FIG. 2. In vitro proliferation of murine splenocytes in response to control (1:0) and histidine-modified (1:100, 1:500, and 1:1,000) TSST-1. The spleen cells were cultured in a 4-day proliferation assay, and each well contained 5×10^5 cells. Proliferation was indicated as counts of [^{3}H]thymidine incorporated per minute into cellular DNA. The stimulation index was calculated by dividing cpm in test wells by cpm in unstimulated wells. The standard error of the mean for quadruplicate wells was between 10 and 15%. Background or unstimulated wells had proliferation values of 1,093 ± 423 cpm.

As mentioned above under histidine modification, the spectra for control and modified toxins had a minimum at 205 ± 1 nm with a shoulder at 225 ± 1 nm. The shoulder at 225 ± 1 nm decreased with an increase in modification of the toxin. Inspection of the CD spectra produced by control and tyrosine-modified TSST-1 indicated that tyrosine modification did not alter the shape of the CD spectra. However, the mean residue ellipticity increased significantly upon tyrosine modification. The secondary-structure analysis for control and tyrosine-modified toxins showed a change in all the secondary-structure parameters (Table 3). There was an increase in the α-helical content from 4.0 to 6.0%, an increase in β-turn from 3.5 to 7.0%, an increase in random coil from 28.0 to 31.0%, and a decrease in the β-pleated sheets from 64.5 to 56.0%.

Serological activity. The serological activity of TSST-1, determined by Ouchterlony double-gel diffusion, was not affected by modification of the amino acid residues under investigation. Modification of histidine or tyrosine residues in TSST-1 did not result in any significant change in the toxin's reactivity with its antiserum in double-diffusion gels. The modified toxins formed complete lines of identity with the control toxin. The conclusion that there was no change was confirmed by ELISA.

MAbs7T and 8T were reacted with histidine-modified TSST-1 (P:R of 1:100, 1:500, and 1:1,000) and control (1:0) TSST-1 in a competition RIA. The reaction of 1:100 and 1:500 histidine-modified TSST-1 was not very much affected; however, MAb 7T failed to react with the 1:1,000 histidine-modified TSST-1. The reaction of MAb 8T with control (1:0) and modified (1:1,000) TSST-1 is presented in Fig. 1. Results of the reaction of MAb T7 with TSST-1 revealed that the 1:1,000-modified toxin failed to compete with the labeled TSST-1, whereas the reaction of MAb 8T with the modified toxin was not affected. The reaction of tyrosine-modified toxins was not tested with the MAbs.

Mitogenic activity. The result of modification of histidine on the proliferation of murine splenocytes is shown in Fig. 2. The modification of histidine residues at a P:R of 1:100 did not affect the proliferative response of the splenocytes. The stimulation index (S.I.) of 1:100-modified TSST-1 was comparable to that of control TSST-1 (1:0). There was approximately 30% reduction in the proliferation induced by 1:500-modified TSST-1 and up to 50% reduction in the proliferative response by 1:1,000-modified TSST-1.

Modification of tyrosine residues in TSST-1 had a drastic effect on the mitogenic activity of TSST-1. When tyrosine residues in TSST-1 were modified at 1:200, the mitogenic response was suppressed by 85% (Fig. 3). The effect of 1:100 modification was not tested.

DISCUSSION

The serological activity of TSST-1, as determined by Ouchterlony immunodiffusion, was not observably affected by modification of either its histidine or tyrosine residues. Polyclonal antiserum contains a mixture of antibodies produced in response to several antigenic determinants on a protein molecule; thus, insufficient antigenic determinants were affected to inhibit the precipitin reaction.

The majority of the amino acid residues that were chemically modified are located on the 15,000-Da internal fragment of TSST-1. This fragment was found to be highly immunogenic, as all of the MAbs isolated in this laboratory reacted strongly with this portion of the toxin molecule (9). The lack of reaction of histidine-modified (1:1,000) TSST-1 with MAb 7T in the competitive RIA could have resulted from a specific change brought about by the modification in

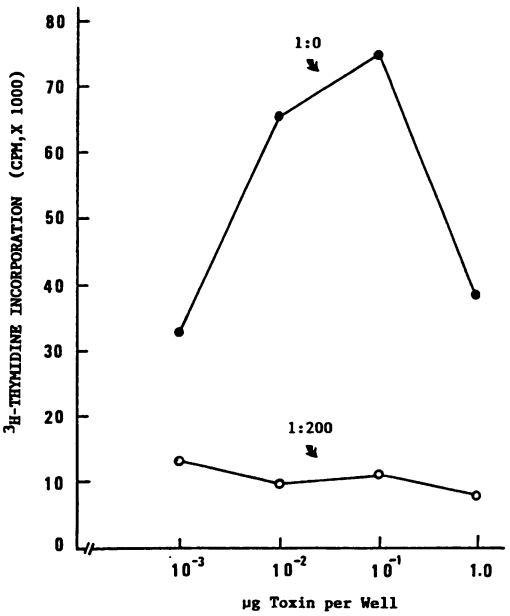

FIG. 3. In vitro proliferation of murine splenocytes in response to control (1:0) and tyrosine-modified (1:200) TSST-1. The spleen cells were cultured in a 4-day proliferation assay, and each well contained 5×10^5 cells. Each data point represents the mean cpm of quadruplicate wells. The standard error of the mean for quadruplicate wells was between 10 and 15%. Background or unstimulated well proliferation values were 2,753 ± 526 cpm.

the epitope recognized by this MAb, possibly due to a localized conformation change in the epitope, as there was no gross conformation change in the histidine-modified toxins. However, the reaction of MAb 8T with histidine-modified (1:1,000) toxin remained essentially unchanged (Fig. 1). The results of the mitogenic neutralization assay indicated that both of these MAbs neutralized mitogenicity, indicating that they recognize epitopes in proximity to each other on the toxin molecule (9). The reason for the difference in reactivity of MAbs 7T and 8T with histidine-modified TSST-1 (1:1,000) could be due to these antibodies having different affinities. The antibody affinity constant, K, for 8T was calculated to be twice as high as that for 7T.

Histidine- and tyrosine-modified TSST-1 molecules were further analyzed for secondary-structure parameters by far-UV CD measurements. For histidine-modified TSST-1, there was a gradual increase in the α-helical content, with all other parameters staying constant. Thus, it appears that a local change in the protein matrix is occurring with increased modification of histidine residues. Since other secondary-structure parameters were constant (Table 3), it is concluded that no gross conformational change in the histidine-modified TSST-1 occurred as a result of modification of histidine residues. Modification of tyrosine residues in TSST-1, however, resulted in alteration of all the secondary parameters. This indicates a change in the conformation of TSST-1 as a result of modification of tyrosine residues.

Histidine residues are known to be involved in the active sites of many proteins. Stelma and Bergdoll (20) showed that chemical modification of five to six histidine residues in staphylococcal enterotoxin A resulted in a loss in the emesis-causing activity of the toxin with only a slight reduction in the reaction with its specific antibody. Amino acid analysis of the modified toxins is the only quantitative way of determining the number of residues modified in the protein. Amino acid analysis, however, does not aid in locating the residues that are modified in the protein molecule. In this study, semiquantitative determination of the number of histidine residues modified by DEPC indicated that three and possibly four residues of histidine were substituted (Table 3), with a 50% loss in mitogenic activity of the toxin (Fig. 2). The loss in the mitogenic response of histidine-modified TSST-1, in the absence of a gross conformational change, could result from modification of a histidine residue located within or in proximity to the mitogenic site. If histidine is involved in the mitogenic activity, it would appear that at least one histidine residue involved might not have reacted with DEPC or the MAbs used in these experiments.

Modification of tyrosine residues in TSST-1 resulted in a significant loss of mitogenic activity of the toxin (Fig. 3). Determination of the number of tyrosine residues substituted by tetranitromethane (Table 2) indicated that at least one and possibly two tyrosine residues were modified, which resulted in a change in the conformation of the toxin (Table 3). It is concluded that the loss in mitogenic activity of tyrosine-modified TSST-1 may have resulted from this conformation change.

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