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Aldehyde modification of peptide immunogen enhances protein-reactive antibody response to toxic shock syndrome toxin-1

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Abstract: Introduction of aldehyde groups into protein conjugates enhanced the immune response to a coupled peptide without the use of strong adjuvants. Synthetic peptides representing the *N*-terminal (residues 1–16) and internal (residues 53–65) epitopes of toxic shock syndrome toxin-1 (TSST-1) were coupled to carrier protein, and carbonyl tags were introduced by Amadori reaction with glycolaldehyde. Modified and unmodified antigens in alum were used to immunize rabbits and the reactivities of antisera were compared. Aldehyde modification augmented the response detected by ELISA, which included enhanced binding to peptides and to native TSST-1. In western blot, TSST-1 was detected by antiserum elicited to the *N*-terminal peptide, but not that generated to the peptide representing the internal sequence. The same antiserum also neutralized TSST-1 activity in a lymphocyte proliferation assay. The circular dichroism spectrum of the *N*-terminal peptide indicated a propensity for helical conformation, similar to the structure at the corresponding sequence of the native protein. These data suggest that aldehyde modification can boost immunogenicity of peptide-based vaccines, generating epitope-specific immune responses against the cognate protein antigens without using potent adjuvants. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: peptide vaccine; Amadori reaction; aldehyde; adjuvant; innate immunity

INTRODUCTION

Experimental vaccines composed of synthetic or recombinant polypeptide antigens hold significant promise for use against infectious agents and their constituent proteins. However, simple peptides are poor mimics for conformational epitopes, which may be important for neutralizing antibody (Ab) responses. Furthermore, molecular vaccines typically require the use of potent adjuvants, which may themselves be toxic and thus unsuitable for human or veterinary applications. The development of alternative nontoxic synthetic immunogens that can overcome these limitations while conferring protective immunity is the central challenge in strategies to improve candidate peptide-based vaccines.

Certain chemical modifications of protein or peptide antigens can enhance the immune response by providing intrinsic adjuvant activity. Recent examples include the use of lipoyl modification of peptides [1–3] and aldehyde modification of foreign and self proteins [4,5]. Various modifications, including maleylation, acetylation, formylation and carbonylation, are known to identify protein ligands for uptake by macrophages or other antigen presenting cells [6,7], providing specific entry to the adaptive immune response [8,9]. Specific Abs elicited to the modified protein antigens can include high-titer IgG that are reactive with the unmodified native proteins. This suggests the potential for diversified responses to simpler polypeptides representing protein determinants, which could include Abs that react effectively with the native proteins.

TSST-1, a 194-residue single chain polypeptide of 22 kDa molecular mass, is one of several pyrogenic superantigen exotoxins produced by *Staphylococcus aureus*. It is associated with life-threatening staphylococcal toxic shock syndrome (TSS) in humans [10,11,] and is also implicated in other pathologies such as Kawasaki syndrome [12], atopic dermatitis [13,] neonatal TSS-like exanthematous disease [14] and arthritis [15]. In farm animals, *Staphylococcus aureus* is the major etiological agent of mastitis. Natural Abs to TSST-1 have been observed in infected animals [16] as well as in humans. Furthermore, reports indicate that most TSS patients have lower anti-TSST-1 Ab levels than

Abbreviations: TSST-1, toxic shock syndrome toxin-1; TT1–16C, peptide STNDNIKDLLDWYSSGC-amide representing the terminal 16 residues of TSST-1; TT1–16C-BSA, the terminal peptide conjugated to bovine albumin; gTT1–16C-BSA, the glycolaldehyde-modified *N*-terminal peptide conjugate; TT53–65C, peptide SPAFTKGEKVDLNC-amide representing an internal 13-residue sequence of TSST-1; TT53–65C-BSA, the internal peptide conjugated to bovine albumin; gTT53–65C-BSA, the glycolaldehyde-modified internal peptide conjugate; MSR, macrophage scavenger receptor.

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individuals who did not develop TSS [17,18]. Acute toxicity precludes the use of native TSST-1 directly in a subunit vaccine. Efforts to generate a suitable antigen have explored chemical modification or mutation to attenuate or eliminate toxicity of the whole TSST-1 [19–21]. This system is particularly attractive for approaches utilizing synthetic or recombinant polypeptides representing epitopes of the native protein to produce a relevant neutralizing immune response. Epitopes identified by mapping of the neutralizing polyclonal Abs elicited against toxin or toxoid suggest potentially useful sequences for presentation as peptides [22]. For example, the TSST-1 sequence spanning residues 47–64 was fused to glutathione-S-transferase to create a recombinant protein capable of eliciting the neutralizing anti-TSST-1 Abs in mice [23]. In each case, the antigen was administered in complete Freund's adjuvant for an adequate immune response.

In the present study, the aldehyde modification procedure was applied to two peptides representing epitopes of TSST-1. Immune responses induced to the peptide conjugates administered in alum were evaluated against both the unmodified peptides and the native toxin. Toxin neutralization in the presence of the antisera provided a more direct measure of the potential to elicit relevant cross-reactive Abs by this strategy.

MATERIALS AND METHODS

Reagents, Proteins and Animals

Bovine albumin, mouse albumin, TSST-1, Con A, nonfat powdered milk (NFM), glycolaldehyde and fluorescein isothiocyanate (FITC) were obtained from Sigma-Aldrich Chemical Co (St. Louis, MO). HRP-conjugated goat anti-rabbit Ab was purchased from Cappel Laboratories (Cochranville, PA). Protein concentrations were determined by modified Bradford assay (Bio-Rad Inc., Hercules, CA). Male New Zealand white rabbits were obtained from Morini (Reggio Emilia, Italy). BALB/c mice at 6 weeks of age were purchased from Charles River Laboratories (Wilmington, MA, USA). Animals were boarded at approved vivarium facilities maintained by dedicated staff and veterinary care provided in accordance with institutional animal welfare assurances. Rabbits were bled from the marginal ear vein. Euthanasia was by CO_2 asphyxiation (rabbits) or cervical dislocation (mice). Protocols were reviewed and approved by the institutional animal care and use committee.

Peptide Synthesis and Immunogenic Conjugate Preparation

Peptides STNDNIKDLLDWYSSGC-amide (TT1–16C) and SPAFTKGEKVDLNC-amide (TT53–65C), corresponding to residues 1–16 and 53–65, respectively, of TSST-1 protein, included a cysteine-NH_2 residue added to the carboxyl terminus to allow coupling to carrier proteins. Peptides were synthesized by standard Fmoc solid phase chemistry on Rink amide resin. Cleavage from the resin was carried out in TFA/water/1,2-ethanedithiol/thioanisole/phenol (88/3/2/3/4 by volume). Peptide purification was performed by reverse phase high performance liquid chromatography on a C_{18} semi-preparative column. Peptides were characterized by MALDI-TOF mass spectrometry.

Peptides were coupled with bovine serum albumin (BSA) using heterobifunctional cross-linker *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) (Pierce Biotechnology, Rockford, IL). In brief, BSA (30 mg in 3 ml PBS) was activated by adding a 30-fold molar excess of SPDP in 0.5 ml of absolute ethanol. The mixture was left at room temperature for 1 h and then dialyzed for 6 h against PBS. A solution of 4 mg of peptide in 2 ml of 10% DMF in PBS was added dropwise to 6 ml of PBS containing 10 mg of activated BSA. The mixture was allowed to react at room temperature for 2 h. The change in absorbance at 343 nm due to the release of pyridine-2-thione upon coupling was used to estimate the progress and extent of peptide conjugation reactions with BSA. The peptide-BSA conjugates were then dialyzed against PBS, pH 7.4, and concentrated to 2 mg/ml by ultrafiltration for storage at −20 °C or subsequent modification. Prior to use, the conjugate was modified by mixing with an equal volume of 10 mM glycolaldehyde in PBS and the reaction allowed to proceed for 3 h at 37 °C. The mixture was then dialyzed for 8 h at 4 °C against four changes of PBS. Glycolaldehyde-modified conjugates were analyzed by SDS-PAGE electrophoresis using BSA and unmodified conjugates as controls. SDS-PAGE electrophoresis was repeated after 2 months of storage at −20 °C. No high molecular weight products due to polymerization of the glycolaldehyde-modified conjugates were observed.

Circular Dichroism Spectral Analysis

The propensity of the short synthetic peptides to adopt secondary structure in solution was assessed by measurement of circular dichroism (CD) ellipticity values. CD spectra were recorded on a Jasco J-600 spectropolarimeter. Peptide solutions (0.1 mg/ml) in 100% TFE or 50% TFE/water mixture, or in PBS, were used to collect data over a wavelength range of 190–250 nm at 20 °C in a cuvette of 0.1 cm path length. Each spectrum was corrected for the background spectrum of the solvent alone.

Immunizations and Immunoassay

Glycolaldehyde-modified conjugates were freshly prepared just before use. Peptide-BSA conjugates at 1 mg/ml in PBS were mixed 1 : 1 with a suspension of alum (Pierce Biotechnology, Rockford, IL), vortexed vigorously and allowed to adsorb for 20 min. Each immunogen was used to immunize two male New Zealand white rabbits using 250 μg/dose, injected subcutaneously. All animals were boosted twice in the same manner at 4-week intervals with 200 μg of the conjugate. Sera were collected 2 weeks after each boost.

For determination of antiserum titers 96-well microtiter plates (NUNC Maxisorb) were coated with peptide (1 μg/well), peptide-BSA conjugate (1 μg/well), BSA, or TSST-1 (0.5 μg/well) in PBS, pH 7.6, for 3 h at 23 °C and then blocked for 1 h with 200 μl of 5% NFM in PBS. Rabbit antisera were serially diluted in PBS, or PBS 0.02% tween (PBST), and 100 μl aliquots were dispensed into the wells. Plates were incubated

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for 1 h at room temperature and then washed 3–5 times with the dilution buffer. Goat anti-rabbit–HRP conjugate diluted 1 : 10 000 in PBST was added (100 µl/well), the plates were incubated and washed as above and finally developed with *O*-phenylenediamine–hydrogen peroxide (Pierce Biotechnology). Absorbance was read at 450 nm on a TiterTek microplate reader. The Ab titer was defined as the lowest dilution producing absorbance with three standard deviations greater than the mean absorbance of control wells incubated with control rabbit serum at 1 : 200 dilution.

Detection of TSST-1 by Western Blotting

Mixtures containing BSA (5 µg/lane) and TSST-1 (0.5 or 0.1 µg/lane) were resolved by SDS-PAGE on precast 4–15% acrylamide gels along with prestained MWS and the gels were electroblotted onto PVDF. The membranes were then blocked with 5% NFM in PBS and incubated in rabbit antisera diluted 1 : 500 in PBST. Membranes were washed with PBST (3×20 ml), incubated 1 h with goat anti-rabbit IgG-HRP (1 : 10 000 in PBST) and developed with ECL substrates (Amersham Biosciences, Piscataway, NJ). Prestained MWS were imaged directly from the membrane.

Toxin Neutralization in Lymphocyte Proliferation Assay

Spleens were collected from BALB/c mice at 8 weeks of age. Single cell suspensions of splenocytes in Iscove's medium supplemented with 2% fetal calf serum, 50 µM 2-mercaptoethanol, 100 U penicillin and 100 µg/ml streptomycin (complete media) were dispensed in 96-well flat bottom cell culture plates at 5×10^6 cells/well. An equal volume of complete media containing TSST-1 or Con A (5 µg/ml) and rabbit antisera was added in triplicate wells and the plates were kept at 37 °C in a CO_2 incubator. Complete media contained only rabbit serum, and no mitogen or superantigen was added to the control wells for determining background proliferation. Cells were pulsed with 0.5 µCi/well of ^{3}H-thymidine (Amersham Biosciences) at 72 h and incubation continued for an additional 18 h. Cells were then harvested on 96-well filter plates, washed and the counts were read on a TopCount plate reader (Perkin Elmer Instruments, Shelton, CT). Proliferation was expressed as the stimulation index [(total counts of wells incubated with antigen – background counts from buffer stimulated cells)/background counts of buffer stimulated cells]. Values represent mean of triplicate determinations ± SD.

RESULTS

TSST-1 Peptides and Peptide Conjugates

Two peptides, TT1–16C and TT53–65C, describing an *N*-terminal and an internal sequence of TSST-1, respectively, were selected on the basis of structural considerations and previously reported epitope mapping experiments [22]. These epitopes are thought to comprise determinants for the superantigen binding sites of TSST-1. Peptides prepared by solid phase synthesis included a *C*-terminal cysteine residue in order to facilitate coupling to carrier proteins by disulfide bond formation. The circular dichroism spectra suggested a propensity for α-helical conformation of TT1–16C in 100% and 50% TFE, whereas TT53–65C adopted a random coil structure in all solvent mixtures (Figure 1).

The reaction of each peptide with thiolated BSA provided conjugates TT1–16-BSA and TT53–65C-BSA with a coupling ratio of 7 to 8 peptide molecules per molecule of BSA, as estimated from the release of chromogenic pyridine-2-thione. Amadori reaction with glycolaldehyde was performed according to the reported procedure [5] to obtain aldehyde-modified conjugates gTT1–16-BSA and gTT53–65C-BSA. Modification of native BSA under these conditions introduced six or more aldehyde groups per mol of protein, estimated as previously described [24]. Since lysine residues of native BSA were effectively blocked in the coupling reactions with peptides, glycolaldehyde modification was presumed to occur at lysine and *N*-terminal amine residues of the coupled peptides. Since TT1–16C and TT53–65C provide two and three amino groups per peptide, respectively, it can be estimated that up to 12 and 18 aldehyde groups are introduced in the conjugates. Analysis by SDS-PAGE did not detect any cross-linking or oligomerization of the aldehyde-modified conjugates.

Characterization of Immune Responses in Rabbits

Antisera collected at the end of a standard course of immunization were used for immunoassay on peptide conjugates, free peptides and native TSST-1 adsorbed on 96-well ELISA plates. Reactions could be detected on microtiter plates coated with free peptides. However, enhancement due to glycolaldehyde modification was seen only for the antisera to the TT53–65C-BSA conjugate bearing the internal peptide (Figure 2). The low reactivity in ELISA using the *N*-terminal peptide was likely due to poor absorption of the free peptide on the plates. In the case of both peptide conjugates, the glycolaldehyde-modified antigen elicited a higher titer Ab response to the respective BSA conjugates (Figure 2). For both the peptide conjugates, the difference in antiserum reactivity for the respective peptide-BSA conjugate or BSA alone suggested enhanced peptide-specific responses in the animals immunized with aldehyde-modified conjugates.

Significant reactions against the aldehyde-bearing conjugates were detected at serum dilutions up to 1 : 4000, whereas the binding of antisera elicited against the unmodified conjugates was negligible. Moreover, using TSST-1 as the coating antigen, the antisera induced against aldehyde-modified peptide conjugates showed greater reactivity compared with those produced against the corresponding unmodified conjugates (Figure 2). No reactivity was observed under these conditions using pre-immune sera. With more

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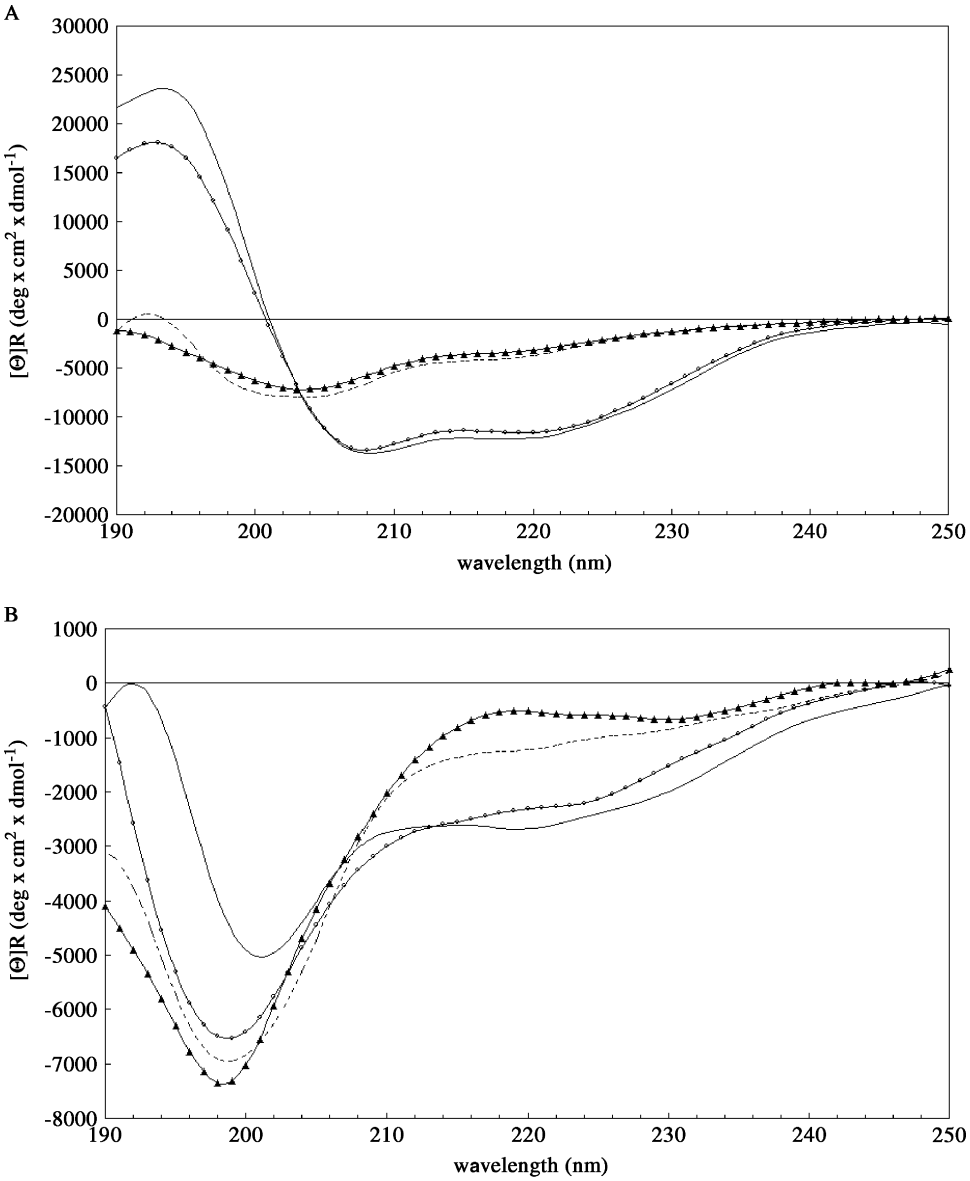

Figure 1 CD spectra of TT1–16C (A) and TT53–65C (B) in 100% TFE (———), 50% TFE (O), 10 mM sodium phosphate buffer, pH 7.5, PB at 0 °C (▲), or PB at 20 °C (- - - -). Data were expressed in terms of $[\theta]_R$, the molar ellipticity per residue.

rigorous washing conditions using PBST, greater ELISA specificity was demonstrated with the antiserum generated to gTT1–16C-BSA, although the titers were reduced significantly (data not shown). The reduced binding of the antisera against peptide TT53–65C under these conditions was attributed to inaccessibility or a substantially different conformation of the residues comprising this epitope in TSST-1.

Reactivity of the anti-peptide antisera with TSST-1 was confirmed by western blot analysis. Samples containing a mixture of TSST-1 and BSA were resolved by SDS-PAGE and blotted with each of the rabbit antisera, as described in experimental methods. In agreement with the ELISA observations, the TSST-1 band was specifically stained with antiserum elicited to gTT1–16-BSA but not with the serum elicited against gTT53–65C-BSA (Figure 3). Both these sera included anti-carrier Abs, which stained BSA in the blots. Interestingly, the sera produced against the unmodified BSA conjugates stained neither TSST-1 nor BSA (not shown).

Neutralization of Cell Proliferation Induced By TSST-1

The ability of Abs to neutralize the biological activity of TSST-1 was assessed by its effect on the superantigen-induced proliferation of mouse splenocytes. Stimulation in the presence of TSST-1 was dose dependent and was inhibited in the presence of increasing concentration of the antiserum generated to the gTT1–16-BSA but not by the antiserum to the unmodified conjugate (Figure 4). Antiserum induced to the glycolaldehyde-modified conjugate suppressed the proliferation of

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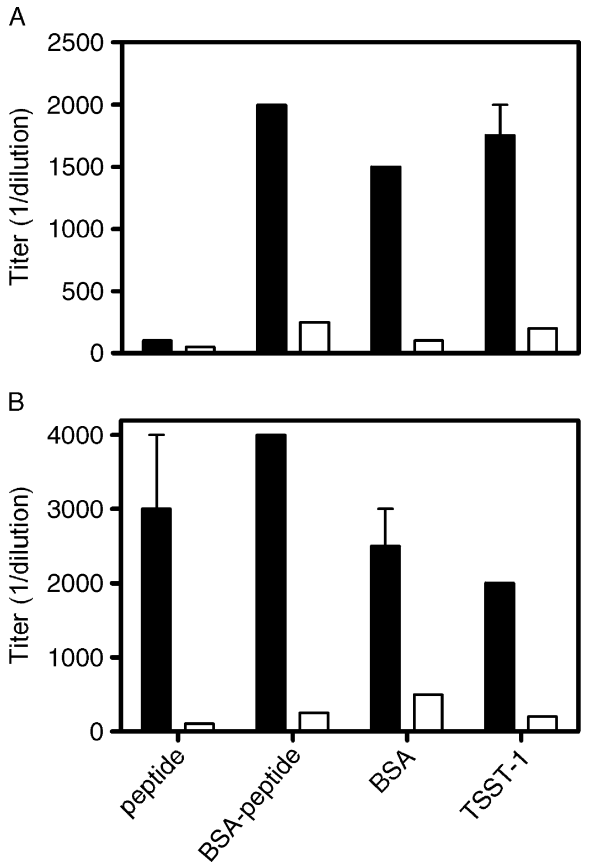

Figure 2 ELISA reactivity of antisera towards free peptides, conjugates and TSST-1. Antisera of rabbits immunized with modified (filled bar) or unmodified (open bar) conjugates gTT1–16C-BSA or TT1–16C-BSA (A) and gTT53–64C-BSA or gTT53–64C-BSA (B) were serially diluted and applied to wells coated with the respective free peptide, peptide-BSA conjugate, BSA, or TSST-1. Data represent mean ± SD for antisera from two immunized rabbits.

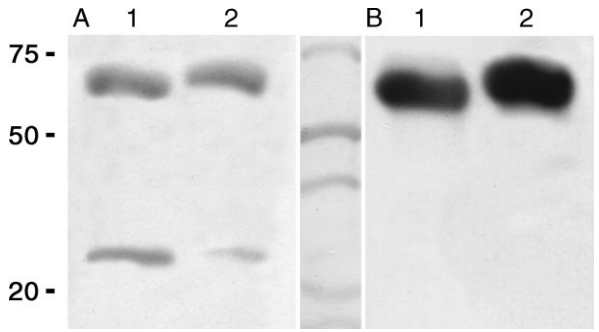

Figure 3 Reaction of TSST-1 with rabbit antisera. TSST-1 (lane 1, 0.5 μg; lane 2, 0.1 μg) diluted in PBS containing BSA (5 μg/lane) was resolved by SDS-PAGE. Proteins were transferred to PVDF membranes and blotted with rabbit antisera (1 : 500 in PBST) elicited against gTT1–16C-BSA (A) or gTT53–64C-BSA (B). Blots were developed with goat anti-rabbit-HRP and visualized by ECL as described in 'Materials and Methods'. Positions of the molecular weight standards (kDa) are indicated in the center lane.

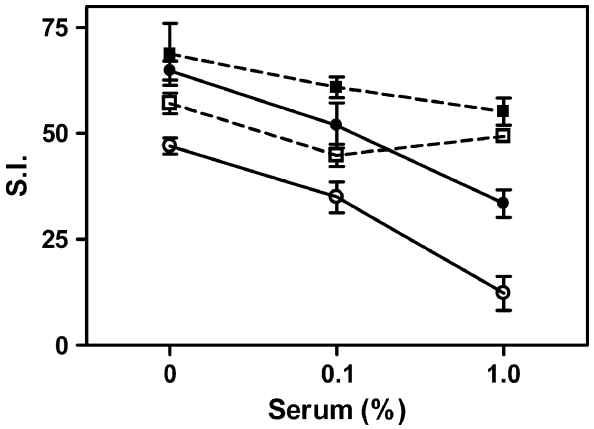

Figure 4 Inhibition of TSST-1 mediated mouse splenocytes proliferation by rabbit antisera. Cells were incubated with TSST-1 at 100 ng/ml (filled square/circle) or 10 ng/ml (open square/circle) in complete media containing 0–1% of rabbit antiserum elicited to TT1–16C-BSA (square) or with gTT1–16C-BSA (circle). Each combination was performed in triplicate. Values (mean ± SD) are expressed as the stimulation index as defined in 'Materials and Methods'.

splenocytes stimulated by 100 and 10 ng/ml of TSST-1 by 43 and 74%, respectively. Under identical conditions, antiserum to the unmodified conjugate reduced proliferation by only 19.5 and 13.6%. Neither pre-immune rabbit serum nor immune sera from rabbits immunized with gTT53–65C -BSA or TT53-65C-BSA diminished proliferation to the toxin at concentrations up to 2%.

DISCUSSION

The specificity of neutralizing Abs for conformational epitopes and the requirement for potent adjuvants for immunogenicity represent constraints in the development of synthetic peptides as vaccines. Recently, appreciation of the role of innate immunity in the initiation of the adaptive immune response suggested new modalities for augmenting the immunogenicity of synthetic antigens. The recognition of molecular patterns or chemical features of bacterial pathogens by specific receptors of accessory cells provides a trigger for the innate response and also for the presentation of antigens for T- and B-cell responses. The efficacy of lipopeptide constructs [1–3] and plasmid DNA vaccines [25] has been attributed to its uptake by receptors of the TLR family expressed on professional antigen presenting cells. Aldehyde modification is believed to direct antigens for uptake via the macrophage scavenger receptor, providing entry to the antigen-presentation pathway [5]. Here, we provide evidence to suggest that aldehyde modification of synthetic peptides conjugated to a carrier protein could also serve to enhance protein-reactive immunity.

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Serum Ab responses induced to peptide conjugates administered in alum were generally of low titer compared to typical responses induced with protein antigens in CFA. However, the enhancement was evident in the response provided by glycolaldehyde-modified antigen relative to the unmodified one. Moreover, the more relevant test of vaccine potential is the cross-reactivity to native TSST-1. Thus, ELISA comparisons suggested that the modified conjugates elicited toxin-reactive Abs with titers similar to the anti-albumin response, whereas Abs induced by the unmodified conjugates had no significant TSST-1 cross-reactivity. Furthermore, antisera generated to the *N*-terminal peptide also reacted with TSST-1 in western blots. The efficacy of these sera may be attributed to the recognition of a common secondary structure of the peptide and the *N*-terminus in the protein presented on the membrane. The CD data suggested that the *N*-terminal peptide has a high propensity to retain α-helical structure similar to that of the corresponding epitope in the native protein. The lack of anti-toxin reactivity in the western blot observed with antisera produced against TT53–65C could be due to inaccessibility or conformational differences at the respective epitope on the protein. Calculations using the program MOLMOL suggested that residues 55–61 in the TSST-1 crystal structure are highly accessible to the solvent [26]. Consequently, this sequence may adopt a conformation not recognized by Abs generated against TT53–65C. Binding of low affinity Abs to the denatured protein absorbed on plastic could account for the detergent-sensitive reaction observed by ELISA.

Design of the synthetic peptide epitopes was assisted by several structural considerations. Determinants of TSST-1 involved in MHC class II or TCR-Vβ2 recognition or sequences that are spatially close to these sites would have greatest potential to neutralize the toxin. Structural analysis by X-ray diffraction has shown that TSST-1 includes two domains: A (residues 1–89) and B (residues 90–194) [27,28]. Residues G31 and S32 and residues 47–56 of the A domain have a major role in interacting with MHC class II molecules [29–31], whereas H135 and certain residues of the α2 helix (residues 125–141) are involved in the binding of the TCR-Vβ2 structure [32]. Since the His135 residue is in close proximity with residues 15 and 16, it was expected that the Abs that bind to the *N*-terminal α1 helix could have neutralizing activity. This is supported by reports on anti-toxin and anti-toxoid antisera. These studies also suggested neutralizing activity directed against the epitope that comprised residues 53–65, with antiserum generated against the toxoid but not the toxin [22]. The potential of the anti-peptide antiserum to neutralize TSST-1 was confirmed by the functional assay based on inhibition of superantigen-induced lymphocyte proliferation. This strategy could therefore be effective for inducing protective immune responses directed against this class of bacterial toxins.

The improved protein reactivity of anti-peptide Abs elicited by our strategy could suggest a selective response to conformational structures that are more relevant to protein epitopes. In this respect, aldehyde modification may generate a more specific B-cell response against structured peptides compared to typical anti-peptide immune responses. Abs elicited to glycolaldehyde-modified proteins were similarly reported to react preferentially with native unmodified protein [5]. In conclusion, these studies demonstrate that a strategy for potentiation of immune responses by aldehyde modification could be applied to peptide conjugates or other molecular vaccines. We have shown that cross-reacting Abs can be directed against neutralizing epitopes of a relevant protein toxin of an infectious agent. Improvement of the responses should be possible by selection of conformationally defined epitopes, construction of appropriate peptide mimics and ultimately by introduction of aldehyde-bearing moieties. It is possible that peptide-specific responses could be further enhanced by selection of appropriate carriers. Aldehyde modification is relatively mild, simple and nontoxic. The chemically modified antigens may be effective even in the absence of any adjuvant. These qualities suggest a number of potential benefits in the development of synthetic vaccines.

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