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In Vivo Efficacy of a Chimeric Peptide Derived from the Conserved Region of the M Protein against Group C and G Streptococci

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The J8 peptide from the conserved region of the M protein protects against group A streptococcus infections. In this study, we demonstrate that vaccination with a J8-containing formulation induces IgG that recognizes and binds group C and G streptococci. Moreover, this formulation has the potential to provide protection against infections caused by these organisms.

Group G and C streptococci (GGS and GCS) have been acknowledged as pathogens causing infections similar to those caused by group A streptococci (GAS) (1, 2, 6). Serious complications from GGS and GCS, such as toxic shock-like syndrome and rheumatic fever, have also been reported (16, 17, 32), a finding which might be due to the fact that GGS and GCS, similarly to GAS, share epitopes with keratin and human cartilage (14).

GGS, GCS, and GAS are genetically closely related and share genes for several virulence determinants, such as the M protein (5, 11, 12, 15, 20, 22, 27, 28, 30, 31). We have defined a peptide sequence within the conserved region of the M protein, referred to as p145, which can induce bactericidal antibodies in mice against multiple GAS serotypes (24, 26). This epitope was recognized by sera from the majority of adults living in areas of high GAS prevalence, and purified human p145-specific antibodies were also bactericidal (4). The minimal non-host-reactive peptide was embedded within a non-M protein peptide sequence (GCN4), designated J8, and conjugated to diphtheria toxoid (DT) (18, 25). J8-DT induced opsonic antibodies that were able to protect outbred mice from virulent challenge when adjuvanted with the human-compatible SBAS2 and alum (3, 23). Taken together, the J8-DT conjugate is a highly interesting vaccine candidate for GAS. If the J8 peptide is to be considered in a vaccine against GAS, it is critical to know whether the induced antibodies also have consequences for other related pathogens.

DNA sequence analysis of the *emm* gene that encodes the M protein from a number of GCS and GGS clinical isolates from different geographical locations, including regions of GAS endemicity and nonendemicity, was performed (Table 1). *emm* typing was performed according to the protocol developed by Bernard Beall, CDC (http://www.cdc.gov/). The 12-amino-acid sequence (SREAKKQVEKAL) from the J8 peptide that was originally derived from the conserved region of the M protein of GAS is 100% identical to the corresponding region found in GCS and GGS analyzed in this current study. A recent epidemiological study, which supports the findings presented here, expanded this approach to determine the sequence identity of the vaccine target epitope in a larger set of group C and G isolates from Fiji (29).

To determine whether the J8 epitope was expressed, surface proteins from six GGS strains and six GCS strains were extracted using lysozyme and mutanolysin. The surface proteins were separated on SDS-PAGE gels analyzed in a Western blot using murine J8 sera, followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse polyclonal antibody (pAb). The J8 epitope was demonstrated in all strains tested, and the detected proteins were between 45,000 and 80,000 Da (Fig. 1). This pattern of binding is similar to that previously observed using monoclonal antibodies generated against the M protein of M6 GAS (21). The M protein of GAS is known to vary significantly in size, as variations from 41,000 to 80,000 Da in molecular mass have been determined for M proteins extracted from 20 different group A streptococcal serotypes (13). While the M protein of GAS usually contains between 1 and 3 C-repeat regions, the M protein of GCS and GGS can have up to 5 C-repeat regions contributing to the size variation in the M proteins from different bacterial strains.

We have previously demonstrated that immunization with the J8 peptide conjugated to diphtheria toxoid (DT) protects inbred and outbred mice from virulent challenge with GAS (3). As the J8 sequence and epitope have been detected in both GGS and GCS strains, our next question was whether J8-DT would be protective against GGS and GCS as well. The synthetic J8 peptide was produced as described elsewhere (19) and was conjugated via a C-terminal cysteine residue to DT (CSL, Australia) using 6′-maleimido-caproyl *n*-hydroxy succinimide (MCS) (7). The sequence for the J8 peptide is QAEDKVKQSR EAKKQVEKALKQLEDKVQ.

All mouse experiments were approved by the QIMR Animal Ethics Committee, in compliance with the National Health and Medical Research Council of Australia (NHMRC). Thirty micrograms of J8-DT or DT alone, emulsified 1:1 in complete Freund's adjuvant (CFA; Difco Laboratories), was administered subcutaneously to BALB/c mice (Animal Resources Centre, Western Australia). A preparation of total surface proteins from the respective bacteria was used as a positive control, whereas a negative-control group received sterile-filtered phosphate-buffered saline (PBS). Mice were given 2 subsequent booster injections at days 21 and 28 after primary immunization. Blood samples were obtained on days 20, 27, and 35 after primary immunization. An enzyme-linked immunosorbent assay (ELISA) was used to measure immunogen-specific murine serum IgG titers. All groups responded

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TABLE 1 Bacterial strains[a]

Strain group and name	Other name	*emm* type	Isolation site	Country of origin
Group A				
M1	2031	*emm1*		
Group C				
GCS4	MD323	STG62647	Blood culture	Australia
GCS5	MD894	STG62647	Urine	Australia
GCS7	MD128	STG93464 /STC1400.1	Throat	Australia
GCS8	MD929	STG62647	Blood culture	Australia
GCS19		STC5345	Blood culture	Fiji
MD605		STG62647	Blood culture	Australia
Group G				
GGS9	MD075	STG166b	Foot	Australia
GGS17		*emm12.8*	Blood culture	Fiji
GGS18		STG480	Blood culture	Fiji
GGS20		STC74A	Blood culture	Fiji
GGS21		STC36	Blood culture	Fiji
GGS22		STC74A	Blood culture	Fiji
NS3396[b]		STG480.3	Throat	Australia

[a] The corresponding J8 amino acid sequence for all strains was SREAKKQVEKAL.
[b] Bacteria isolated from a patient who subsequently developed rheumatic heart disease (8).

to the immunization, and immunogen-specific titers were all above 100,000 (Fig. 2).

A direct bactericidal assay was performed as previously described (3, 4, 24). Pooled immune sera from cohorts of mice immunized with MD605 surface protein or J8-DT were capable of the opsonization of the MD605 GCS, with 78% and 39% reductions in CFU, respectively (P values of <0.05 and <0.05, respectively, in a nonparametric t test compared to the PBS control group). Similarly, pooled immune sera from cohorts of mice immunized with NS3396 surface protein or J8-DT were capable of the *in vitro* opsonization of GGS strain NS3396, with 62% ($P < 0.05$) and 41% ($P < 0.05$) reductions in CFU, respectively. In contrast, pooled sera from cohorts of mice immunized with DT (7%) or PBS (0%) did not significantly kill the NS3396 strain.

Immunized and control mice were challenged with MD605 GCS or NS3396 GGS intraperitoneally 10 days after the final immunization. At time points 8, 24, 32, 48, and 72 h after challenge, spleens from three mice per group were collected and homogenized and aliquots of 50 μl were plated out, and on the following day, the numbers of CFU were determined. In the experiment in which the mice were challenged with MD605, a significant difference in clearance in the groups immunized with J8-DT or total surface proteins compared to the group immunized with PBS was detected after 24 h (Fig. 3a). After 32 h, the bacterial load was completely cleared in the total surface proteins group (not shown) and only a few counts were left in the J8-DT group. In parallel, in the experiment with NS3396, mice immunized with J8-DT cleared the bacteria efficiently (Fig. 3b). A decrease of the bacterial load in spleens was detected after 24 h, and the counts were lower at all time points than those in all the other groups. No difference was seen between the naive mice and the mice immunized with PBS-CFA (not shown). We have previously observed similar partial protection induced by administration of DT formulated with adjuvant in our GAS studies. In summary, immunization with J8-DT induces an efficacious immune response *in vivo* against both GCS and GGS, as MD605 and NS3396 were cleared more quickly.

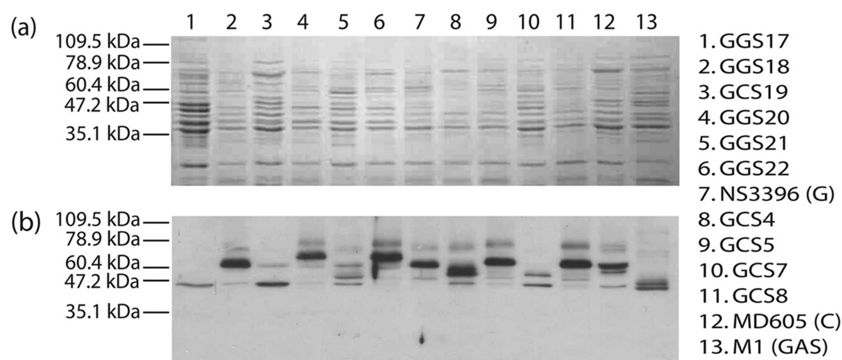

FIG 1 A protein with a molecular mass of 45 to 70 kDa was identified in all strains by using antibodies against the J8 epitope. (a) Coomassie blue-stained SDS-PAGE gel showing surface proteins. (b) Western blot analysis with antibodies against the J8 epitope. The group A streptococcus strain M1 was used as a positive control.

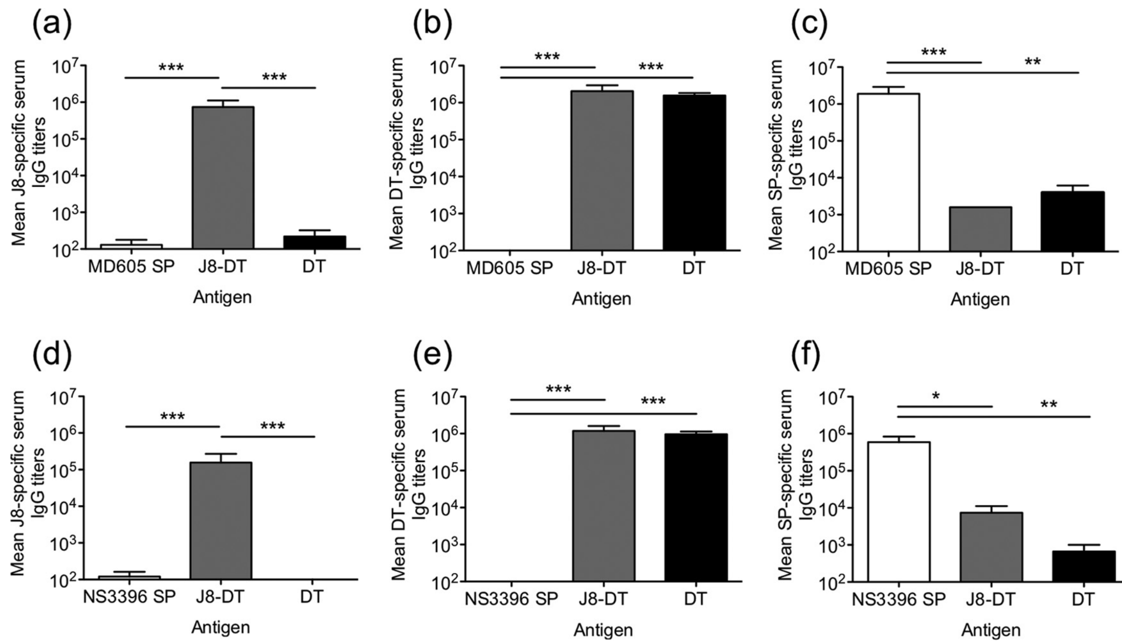

FIG 2 Means and standard deviations of antigen-specific serum IgG antibody titers of BALB/c mice ($n = 10$) immunized with J8-DT, DT, or surface proteins (SP) from MD605 or NS3396. (a to c) J8-specific serum IgG (a), DT-specific serum IgG (b), and total surface protein (MD605)-specific serum IgG (c) titers of cohorts of BALB/c mice. Mice were subsequently challenged with the MD605 bacterial strain. (d to f) J8-specific serum IgG (d), DT-specific serum IgG (e), and total surface protein (NS3396)-specific serum IgG (f) titers of cohorts of BALB/c mice, which were subsequently challenged with the NS3396 bacterial strain. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (as determined by a Mann-Whitney analysis using Prism 5.0c).

Severe streptococcal infections and sequelae are usually due to GAS; however, recent studies suggest that GCS and GGS might also cause acute rheumatic fever (ARF). In a study of an Aboriginal population, cross-reactivity of antibodies to GCS and GGS with human cardiac myosin was demonstrated, suggesting a correlation between GCS and GGS and acute rheumatic fever (16). Another hypothesis of ARF is that formation of a complex of human basement membrane collagen IV and GAS acts as an autoantigen with rheumatogenic potential (10). Recently, it has been suggested that GCS and GGS may have a similar ability to cause ARF through collagen autoimmunity (9).

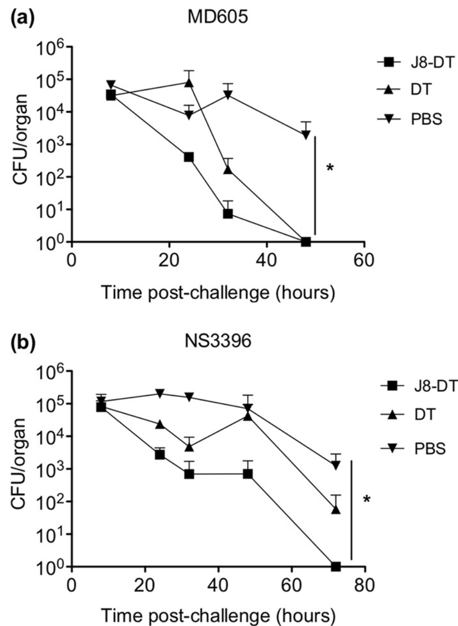

FIG 3 Immunization with J8-DT resulted in faster clearance of bacterial load in spleens after challenge with GCS strain MD605 (a) or GGS strain NS3396 (b). Means and standard deviations of numbers of CFU/organ for each time point are shown. When the data were combined, there was a significant difference ($P < 0.05$) observed between cohorts of animals administered J8-DT, DT, and PBS. *, $P < 0.05$ (determined by polynomial nonlinear regression analysis).

Even though GCS and GGS are increasingly recognized as pathogens causing a variety of infections similar to those caused by GAS, this is the first vaccine study for GCS and GGS. The studies presented above indicate that a J8-based vaccine to prevent acute rheumatic fever may cover not only GAS infections but also GCS and GGS infections.

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