Protective and Nonprotective Epitopes from Amino Termini of M Proteins from Australian Aboriginal Isolates and Reference Strains of Group A Streptococci

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The M protein is the primary vaccine candidate to prevent group A streptococcal (GAS) infection and the subsequent development of rheumatic fever (RF). However, the large number of serotypes have made it difficult to design a vaccine against all strains. We have taken an approach of identifying amino-terminal M protein epitopes from GAS isolates that are highly prevalent in GAS-endemic populations within the Northern Territory (NT) of Australia. Australian Aboriginals in the NT experience the highest incidence of RF world-wide. To develop a vaccine for this population, 39 peptides were synthesized, representing the amino-terminal region of the M protein from endemic GAS. Mice immunized with these peptides covalently linked to tetanus toxoid and emulsified in complete Freund's adjuvant raised high-titer antibodies. Over half of these sera reduced bacterial colony counts by >80% against the homologous isolate of GAS. Seven of the peptide antisera also cross-reacted with at least three other heterologous peptides by enzyme-linked immunosorbent assay. Antiserum to one peptide, BSA10_{1–28}, could recognize six other peptides, and five of these peptides could inhibit opsonization mediated by BSA10_{1–28} antiserum. Cross-opsonization studies showed that six of these sera could opsonize at least one heterologous isolate of GAS. These data reveal vaccine candidates specific to a GAS-endemic area and show the potential of some to cross-opsonize multiple isolates of GAS. This information will be critical when considering which epitopes may be useful in a multiepitope vaccine to prevent GAS infection.

Group A streptococci (GAS) are a major human pathogen responsible for suppurative and nonsuppurative pathology. The former group of conditions range from pharyngitis to the far more serious toxic shock-like syndrome and necrotizing fasciitis, whereas the latter include poststreptococcal glomerulonephritis and rheumatic fever (RF). RF and rheumatic heart disease (RHD) are responsible for 25 to 50% of cardiac conditions in children in developing countries (23). Australia's Aboriginal population experiences the highest rate of RF and RHD in the world. Aboriginal communities in the Northern Territory of Australia have RF incidence rates as high as 650 per 100,000, and the prevalence of RHD approaches 30 per 1,000 in some communities, compared to only 0.14 per 1,000 non-Aboriginals living in the same region (6). The median age for acquiring RF among Aboriginals is 11 years, and the life expectancy of Aboriginals who die of the disease is less than 35 years. Since RF and RHD only follow an infection with GAS, a strategy to prevent these conditions is to prevent GAS infections. The current approach of administering penicillin by injection has had limited success, as compliance with this regimen is low (approximately 50%), resulting in many recurrences in developing countries (6, 23). The best prospects for controlling RF rest with developing a vaccine to prevent streptococcal infection.

Immunity to GAS is mediated by antibodies to the M protein, which exists as a coiled-coil protein on the surface of the bacteria. The amino acid sequence of the amino terminus of

the M protein is responsible for the serotype of the organism, with at least 80 distinct serotypes having been defined (19). Antibodies directed to the M protein opsonize streptococci in the presence of neutrophils; however, these antibodies are serotype specific and generally only opsonize the homologous GAS isolate (1, 2, 10, 13, 14, 21).

Potential problems exist when immunizing with subunits of the M protein, as accumulating evidence suggests that RF is likely to be an autoimmune disease, although the pathogenesis is not clear. It may be that an immune response directed to GAS proteins can also react with host tissues, including the heart (12, 16, 17, 24, 29). Therefore, care must be taken when designing vaccines for GAS. The safest approach is to use sequences which do not evoke host cross-reactive antibody or T-cell responses. Previous studies have focused on defining opsonic epitopes from the amino-terminal region, and most of these epitopes do not evoke cross-reactive antibody to human host tissues (1, 9, 10, 12, 13, 14).

Epidemiological studies of endemic GAS isolates suggest that some serotypes are much more common than others within a population, and these vary between distinct geographic locations (15, 22, 25, 27, 28, 30). Although amino-terminal M protein epitopes from common reference strains of GAS have been identified, protective epitopes from GAS strains prevalent within a GAS-endemic region have yet to be investigated. In the current study, we synthesized peptides to the amino-terminal region of the M protein from GAS isolated from Northern Territory Aboriginal communities. We show that mice immunized with these peptides covalently linked to tetanus toxoid (TT) and emulsified in complete Freund's adjuvant (CFA) raise high-titer antibodies to the immunizing peptide. We define numerous opsonic epitopes from the M

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TABLE 1. List of synthetic peptides, corresponding GAS isolates, M and ST type, OF, and murine serum antibody titer to immunizing peptide^a

Peptide	Sequence	Gas isolate	Origin ^b	M/ST ^c	OF	Titer to immunizing peptide ^d
ML2 family						
2032 ₁₋₁₉	NSKNPVPVKKEAKLSEAELC	2032	T	M2	+	204,800
2032 ₄₆₋₅₉	KVEEEHKKDHEKLEC	2032	T	M2	+	204,800
BSA10 ₁₋₂₈	NSKTPAPAPAVPVKKEATKSKLSEAELHC	BSA10	NTS	STBSA10	+	204,800
BSA10 ₁₋₁₉	NSKTPAPAPAVPVKKEATKC	BSA10	NTS	STBSA10	+	204,800
NS14 ₁₋₁₉	DNPSSVPVKKAAELYDKIKC	NS14	NTS	STNS14	+	6,400
ML60 family						
2080 ₁₋₂₀	ESSTVKAESSTVKAESSTISC	2080	T	M60	+	3,200
2024 ₁₋₂₀	AEIKKPQADSAWNWPKEYNAC	2034	T	M4	+	12,800
2034 ₄₀₋₅₉	DDKEKDPQYRALMGENDLRC	2034	T	M4	+	102,400
2551 ₁₋₂₀	ESSNNAESSNISQESKLINTC	2551	T	M22	+	12,800
Y530S ₁₋₂₀	EAPKSHSISNNEQLINELNDC	Y530S	NTS	M8	+	25,600
M5 family						
2035 ₁₋₂₀	AVTRGTINDPQRAKEALDKYC	2035	T	M5	—	12,800
2036 ₁₋₂₀	RVFPRGTVENPDKARELLNKC	2036	T	M6	—	12,800
2045 ₁₋₂₀	PLTRATADNKDELIKRANDYC	2045	T	M18	—	12,800
2054 ₁₋₂₀	TIITRDIARDPDKLRKIAENC	2054	T	M30	—	12,800
NS1 ₁₋₁₉	RVTTRSQAQDAAGLKEKADC	NS1	NTB	STNS1	—	25,600
TIN family						
Y504S ₁₋₂₀	TEVKAAGQSPKGTNVVSADLC	Y504S	NTS	M11	+	204,800
Y504S ₄₅₋₆₄	EETKNKELDKKNKELDSRVTC	Y504S	NTS	M11	+	204,800
90/85 ₁₋₂₀	DGERVPKNNRLSKYSELSEC	90/85	NTS	ST90/85	—	204,800
88/544 ₁₋₂₀	DGPQKSVSQGGVSLDLYSKLC	89/544	NTS	ST89/544	—	204,800
2041 ₁₋₂₀	ADPQKQNSVSNDSVTINVYNC	2041	T	M13	+	12,800
M52/53/80 family						
41 ₁₋₁₉	EGNARLAQAQEEALRDVLNC	41	T	M41	—	51,200
2072 ₁₋₂₀	DQPVDDHRYTEANDAVLQGRG	2072	T	M52	—	3,200
NS27 ₁₋₁₉	ADDHPGAVAARNVDVLSGFSC	NS27	NTS	STNS27	—	25,600
NS5 ₁₋₁₉	ADHPSYTAAKDEVLSHFVSC	NS5	NTB	STNS5	—	25,600
156 ₁₋₁₉	DNPRYTDHNAVTQGRTPVC	87/156	NZ	ST156	—	25,600
88/25 ₁₋₃₂	DRYTDHNAVTQGRTPVPLRNLLEMDKNSKLC	88/25	NTS	ST88/25	—	12,800
88/25 ₃₃₋₅₃	SENELLOAGLQEKERELEDKLC	88/25	NTS	ST88/25	—	12,800
2317 ₁₋₁₉	HQLADAARREVLKGETVPAC	2317	T	M80	—	2,631,680
M12/M55-related sequence						
2033 ₁₋₂₀	DARSVNGEFPRHVKLKNEIEC	2033	T	M3	—	51,200
2040 ₁₋₁₂	DHSDLVAEKQRLC	2040	T	M12	—	25,600
2040 ₅₀₋₆₉	LKMLNRDLEQAYNELSGEAHC	2040	T	M12	—	102,400
2040 ₂₃₉₋₂₅₈	AALGHQHAHNEYQAKLQDC	2040	T	M12	—	102,400
2075 ₁₋₁₉	NQTEPSQTHNRLYQERQLC	2075	T	M55	—	12,800
Unrelated sequences						
2069 ₁₋₂₀	AEKKVEAKVEVAENNVSVC	2069	T	M49	+	12,800
2077 ₁₋₁₉	NDDITSMTPILSGVPGNAC	2077	T	M57	—	12,800
P73554 ₁₋₂₀	EEERTFTPEYARYKAWKSC	P73554	NZ	M75	+	51,200
88/30 ₁₋₂₀	DNGKAIYERARERALQELGPC	88/30	NTS	ST88/30	—	12,800
STBSB24 ₁₋₂₀	DEGPKDITDLPAPMWRDKAC	BSB24	NTS	STBSB24	+	204,800
PL1 ₁₋₂₀	DNPSSVPVKKAAELYDKIKC	PL1	NTS	M54	—	2,621,450

^a Sequences are grouped into M families based on dendrogram analysis of amino-terminal nucleotide and amino acid sequence relatedness (25).

^b T, reference type strain, NZ, isolate from New Zealand derived from blood culture; NTS, Northern Territory isolate derived from skin swab; NTB, Northern Territory isolate derived from blood culture.

^c M type determined by serotyping; sequence type (ST) determined through sequence analysis of the amino terminus of the M proteins of reference strains and M-nontypeable proteins (25).

^d Antibody titer of pooled murine sera to immunizing peptide following primary immunization and three booster injections of the conjugate peptide.

proteins of endemic GAS isolates and determine the degree to which these induce antibodies that cross-opsonize heterologous GAS strains. This study lays the foundation for rationally designing a polyvalent immunogen which will prevent GAS infection in targeted communities.

MATERIALS AND METHODS

Syntheses of peptides and conjugation to TT. Synthetic peptides were produced as described and were purified by high-pressure liquid chromatography (18). All peptides were conjugated via the C-terminal cysteine residue to TT using 6'-maleimido-caproyl *n*-hydroxy succinimide (MCS) coupling (7). Peptide sequences are given in Table 1.

Immunization of animals. Peptides were administered subcutaneously at the tail base to B10.BR (*H-2^k*) mice (Animal Resources Centre WA). Each of three mice received 30 µg of peptide emulsified in CFA (Difco Laboratories, Detroit, Mich.) in a total volume of 50 µl. Mice were boosted after 21 days with 30 µg of peptide in phosphate-buffered saline (PBS), and two subsequent booster injections were given at 10-day intervals. Mice were bled via the tail artery at 2, 4, 6, and 8 weeks following primary immunization. If, after three booster injections, individual mice had titers of <12,800, mice were boosted with a further 30 µg of peptide in PBS prior to using sera in the bactericidal assay.

Detection of antibodies. An enzyme-linked immunosorbent-assay (ELISA) was used to measure murine serum antibodies to the peptides as described (17, 26). Titer is defined as the lowest dilution that gave an optical density (OD) reading of more than 3 standard deviations above the mean OD of the control wells containing normal mouse serum. Western blot analysis of anti-peptide

cross-reactivity against cardiac myosin (Sigma), tropomyosin (Sigma), keratin (Sigma), and murine whole heart extract (26) was performed as described (26).

GAS reference strains and isolates. GAS isolates and reference strains were obtained from the Menzies School of Health Research, Darwin, Northern Territory, Australia, and Diana Martin, Institute of Environmental Science and Research, Wellington, New Zealand. Details for each strain are given in Table 1. Full designations of reference strains are as follows (reference name in parentheses): 2032 (M2B), 2033 (B930/24 PHLS), 2034 (SS241), 2035 (T5/B/PS PHLS), 2036 (S43/75/8/W14), 2040 (R53/1077 PHLS), 2041 (SS31), 2045 (PHL J17C), 2054 (D24/46 PHLS), 2069 (B737/71/1), 2072 (10870), 2075 (Trinidad A-75), 2077 (3890-V-Ramisson), 2080 (D335 Lfd), 2317 (R75/268), and 2551 (68/3116). Isolates from the Northern Territory were derived from blood culture or skin swabs from Aboriginal patients. All isolates were subjected to serotyping (M, T, and opacity factor [OF]) using standard methods (Table 1) (27, 28).

Bactericidal assay. Murine anti-peptide sera were assayed for their ability to opsonize GAS essentially as described (21). Briefly, the bacteria were grown overnight at 37°C in 5 ml of Todd-Hewitt broth (THB), and then 200 µl of overnight culture was subinoculated into 5 ml of warm (37°C) THB and allowed to grow for 2 h at 37°C. GAS were then serially diluted to 10⁻⁴ in saline. Fifty microliters of the bacteria dilution was mixed with 50 µl of fresh heat-inactivated serum and 400 µl of nonopsonic heparinized donor blood, as a source of neutrophil and complement. All donor blood was screened prior to assay to ensure that it could support the growth of the GAS strain to at least 32 times the inoculum level in a 3-h incubation at 37°C (4, 5). The mixture was incubated end over end at 37°C for 3 h, 50 µl from each tube was plated out, in duplicate, on THB blood agar pour plates and incubated overnight, and the number of colonies on each plate was counted. Opsonic activity of the anti-peptide sera (percent kill) was calculated as [(1 - mean CFU in the presence of anti-peptide sera)/(mean CFU in the presence of normal mouse sera)] × 100. Peptide inhibition opsonization assays were performed as described (4, 5) using log-phase bacteria.

RESULTS

Synthetic peptides from the N terminus of M proteins of endemic GAS. The amino-terminal peptide sequences and sub-amino-terminal peptide sequences from the A, B, and C repeat regions (not containing the amino terminus of the protein) were derived from DNA sequence analysis of streptococcal reference strains and clinical isolates collected from the Northern Territory of Australia and New Zealand (15, 27, 28). Table 1 shows the amino acid sequence of each of the 39 synthetic peptide sequences with their corresponding GAS strain, M type, and OF type. Amino-terminal peptide sequences were derived from sequence typing (ST), a PCR-based sequencing method, of M proteins and M-like (ML) proteins (highly homologous to M protein) of reference strains and Australian and New Zealand GAS isolates (27, 28). Based on dendrogram analysis, sequences have been grouped into five ML protein families according to their nucleotide and protein sequence relatedness; ML2, ML60, M5, TIN, M52/53/80, an M12/55-related sequence group, and an unrelated sequence group (Table 1) (27). Within each ML family, sub-amino-terminal sequences have higher homology between family isolates than amino-terminal sequences (Table 2). Therefore, we also synthesized six sub-amino-terminal peptides to determine whether opsonic epitopes exist within these common domains.

Definition of opsonic amino-terminal epitopes. Mice (three per group) were immunized with the peptide-TT conjugates listed in Table 1. An ELISA was used to detect the level of antibody in the pooled murine sera to the homologous peptide (unconjugated) on the plate. Pooled murine sera had high-titer antibodies (>3,200) against homologous peptides following one primary immunization and three boosts (Materials and Methods) (Table 1). In all cases, immunoglobulin G2b (IgG2b) was the predominant antibody isotype produced. As negative controls, sera from mice immunized with PBS or TT emulsified in CFA were tested by ELISA against each of the peptides. There was no significant recognition (titer of <100) by these sera of any of the amino-terminal peptides listed in Table 1.

Each of the pooled anti-peptide sera with a titer greater than >12,800 to the immunizing peptide was examined by Western blot to determine whether the antibodies cross-reacted with

TABLE 2. Comparison and homology of sub-amino-terminal sequences within ML family isolates

Peptide ^a	Sequence ^b	% Similarity	Location in M protein
ML2 family			
2032 ₄₆₋₅₉	KVEEEHKKDHEKLE		B repeat
BSA10 ₍₅₄₋₆₇₎	-----	100	
NS14 ₍₄₅₋₅₈₎	--K-----K----	93	
ML60 family			
2034 ₄₀₋₅₉	DDKEKDPQYRALMGENQDLR		Nonrepetitive region
2080 ₍₅₆₋₇₅₎	-NIR-----M	85	
Y530S ₍₄₁₋₆₀₎	-NTR-----M	85	
TIN family			
Y504S ₄₅₋₆₄	EETKNKELDKKNKELDSRVT		Follows A repeat
90/85 ₍₃₉₋₅₈₎	LLD-QE--E-E-EK---Q-A	45	
88/544 ₍₄₁₋₆₀₎	LTE--E-----K---Q-D	65	
M52/53/80 family			
88/25 ₃₃₋₅₃	SENEELQAGLQEKERELEDL		A1 repeat
2072 ₍₃₉₋₅₈₎	-----K-D--K--Q--KN-	70	
NS27 ₍₃₇₋₅₆₎	--K---ETE-----Q--KN-	65	
NS5 ₍₃₆₋₅₅₎	----G-K-----Q--KN-	75	
87/156 ₍₃₅₋₅₄₎	-----K-D--K--Q--KN-	70	
2317 ₍₃₄₋₅₃₎	-A---ETT--K--Q--KG-	60	
M41 ₍₃₅₋₅₄₎	RR-N--EKII-----G-	60	
M12/M55 family			
2040 ₅₀₋₆₉	LKMLNRDLQAYNELSGEAH		B1 repeat
2075 ₍₅₈₋₇₇₎	--E--DEF-----DGV	85	
2033 ₍₅₆₋₇₅₎	--G--DWA-RLQ--N--DV	45	
2040 ₂₃₉₋₂₅₈	AALGHQHAHNEYQAKLAEKD		
2033 ₍₂₆₈₋₂₈₇₎	-----	100	

^a Subscripts in parentheses are the amino acid positions of each sequence corresponding to the peptide. Adapted from Relf (27).

^b Identical residues are shown as dashes.

cardiac myosin, tropomyosin, keratin, or whole murine heart extract. None of the sera investigated recognized any of the proteins investigated (data not shown).

Sera with high-titer antibodies raised to each of the peptides were then tested for their ability to opsonize the representative GAS reference strains and endemic isolates (listed in Table 1) in an in vitro bactericidal assay (Fig. 1). For the 33 individual amino-terminal peptides, 19 (58%) peptide-specific antisera demonstrated greater than 80% mean reduction in colony counts against the homologous GAS isolate. An opsonic activity of 60 to 80% was observed with four (12%) peptide antisera; two (6%) had between 40 and 60% opsonic activity; and eight (24%) had less than 40% opsonic activity against the homologous GAS isolate. Antisera to all six sub-N-terminal peptides were also tested for their ability to opsonize the corresponding GAS isolate: four antisera (Y504S₄₅₋₆₄, 88/25₃₃₋₅₃, 2040₅₀₋₆₉, and 2040₂₃₉₋₂₅₈) had an opsonic activity of between 40 and 60%, and two peptide antisera (2032₄₆₋₅₉ and 2034₄₀₋₄₉) had no opsonic activity at all against the homologous GAS isolate (Fig. 1).

Epitope specificity. To determine the specificity of the murine amino-terminal and sub-amino-terminal peptide antisera, pooled anti-peptide sera with a titer of >12,800 to the immunizing peptide were tested in an ELISA for binding to homologous and heterologous peptides. The majority of the antisera bound only the homologous peptide. However, seven antisera

FIG. 2. Antibody and bactericidal cross-reactivity of murine antisera to amino-terminal peptides. Columns represent mean percent reduction in CFU from at least two separate experiments (left vertical axis). Symbols represent mean antibody titer from pooled murine sera to amino-terminal peptides (right vertical axis). (A) GAS isolate BSA10, peptide BSA10₁₋₂₈ (peptide on ELISA plate). (B) 2032 GAS, peptide 2032₁₋₁₉. (C) 2034 GAS, peptides 2034₁₋₁₉ and 2034₄₀₋₆₉. (D) Y530S GAS, peptide Y530S₁₋₂₀. (E) 2040 GAS, peptide 2040₅₀₋₅₉. (F) 88/30 GAS, peptide 88/30₁₋₂₀. (G) Y504S GAS, peptide Y504S₁₋₂₀.

TABLE 3. Inhibition of opsonic activity by cross-reactive peptides

Antiserum	GAS isolate (inoculum size)	Inhibiting peptide	Mean CFU ^a	% Inhibition ^b	ELISA cross-reactivity to peptide ^c
BSA10 ₁₋₂₈	BSA10 (87)	None	530		
		BSA10 ₁₋₂₈	3,100	83	+
		2032 ₁₋₁₉	3,300	84	+
		2034 ₁₋₁₉	970	45	+
		88/30 ₁₋₂₀	2,460	78	+
2032 ₁₋₁₉	2032 (101)	Y504 ₁₋₂₀	2,640	80	+
		None	280		
		BSA10 ₁₋₂₈	5,300	95	+
		2032 ₁₋₁₉	5,000	94	+
		2034 ₁₋₁₉	4,000	93	+
		Y530S ₁₋₂₀	180	NI	-
		88/30 ₁₋₂₀	2,000	86	+
		Y504S ₁₋₂₀	5,600	95	+

^a Mean colony count (CFU) from two plates times the dilution factor.

^b Percent inhibition was calculated as described in the text. NI, no inhibition. In controls containing blood and bacteria (no antiserum, no peptide), the BSA10 titer was 2,800 and the 2032 titer was 3,650.

^c +, ELISA cross-reactive titer to inhibiting peptide >1,600; -, titer 800. Data derived from Fig. 2.

to amino-terminal and sub-amino-terminal peptides, BSA10₁₋₂₈, 2032₁₋₁₉, 2034₁₋₁₉, 2034₄₀₋₆₉, Y504S₄₅₋₆₀, 2040₅₀₋₆₉, and 88/30₁₋₂₀, cross-reacted (defined as a titer of $\geq 1,600$) with at least three other heterologous peptides in an ELISA (Fig. 2). Antisera to BSA10₁₋₂₈ and 2032₁₋₁₉ cross-reacted with six and four heterologous peptides, respectively (Fig. 2). Peptide antisera to 2032₄₆₋₅₉, NS14₁₋₁₉, NS1₁₋₁₉, 90/95₁₋₂₀, 88/544₁₋₂₀, 2040₁₋₁₂, and 2033₁₋₁₉ cross-reacted with at least one other heterologous peptide (data not shown).

The epitope specificity of antisera to BSA10₁₋₂₈ and 2032₁₋₁₉ was further evaluated by comparing the ability of cross-reactive and non-cross-reactive peptides to inhibit opsonization against GAS isolates BSA10 and 2032, respectively. Antisera to BSA10₁₋₂₈ and 2032₁₋₁₉ showed bactericidal activity against their respective GAS isolates. Peptides BSA10₁₋₂₈, 2032₁₋₁₉, 2034₁₋₁₉, 88/30₁₋₂₀, and Y504S₁₋₂₀, which cross-react to BSA10₁₋₂₈ antisera by ELISA, were added to BSA10₁₋₂₈ antiserum prior to addition of nonopsonic donor blood and BSA10 GAS. Bactericidal activity of the BSA10 antiserum was inhibited significantly by peptides BSA10₁₋₂₈, 2032₁₋₁₉, 88/30₁₋₂₀, and Y504S₁₋₂₀ by between and 78 and 84% and by 43% for peptide 2034₁₋₁₉ (Table 3). Antiserum to peptide 2032₁₋₁₉ also shows ELISA cross-reactivity to peptides BSA10₁₋₂₈, 2032₁₋₁₉, 2034₁₋₁₉, 88/30₁₋₁₉, and Y504S₁₋₂₀ but not peptide Y530S₁₋₂₀. Following addition of these peptides to 2032₁₋₁₉ antiserum in a peptide inhibition opsonization assay

against GAS strain 2032, all cross-reactive peptides could inhibit bactericidal activity, whereas in the presence of the non-cross-reactive peptide Y530S₁₋₂₀, the 2032₁₋₁₉ antiserum could still significantly opsonize 2032 GAS (Table 3).

Bactericidal cross-reactivity of the amino-terminal peptide antisera. We have shown that antiserum raised to ML2 family peptide BSA10₁₋₂₈ reacted with itself and six other heterologous peptides, and antisera raised to three of the six peptides that cross-react with BSA10₁₋₂₈ antiserum (2032₁₋₁₉, 2034₁₋₁₉, and 2040₅₀₋₆₉) also recognized peptide BSA10₁₋₂₈. To determine whether these antisera could also cross-opsonize the representative heterologous GAS isolates, opsonization assays were performed using antisera to peptides BSA10₁₋₂₈, 2032₁₋₁₉, 2034₁₋₁₉, 2034₄₀₋₆₉, Y530S₁₋₂₀, 2040₅₀₋₆₉, 88/30₁₋₂₀, and Y504S₁₋₂₀ against the homologous (amino-terminal representative GAS) and heterologous isolates represented by the peptides listed above.

Antisera to both BSA10₁₋₂₈ and 2032₁₋₁₉ could clearly opsonize both corresponding ML2 family GAS isolates (Fig. 2A and B). This result is not surprising, as the peptide sequence of 2032₁₋₁₉ has 90% sequence homology to BSA10₁₋₂₈ (Table 4). The only other isolate that was repeatedly opsonized by BSA10₁₋₂₈ antiserum was Y504S (Fig. 2G). Interestingly, antiserum to peptide Y504S₁₋₂₀ could opsonize isolate BSA10 even though the cross-reactive ELISA titer to peptide BSA10₁₋₂₈ was only 100 (Fig. 2A), suggesting that other cross-reactive epitopes may exist on BSA10. In addition, antiserum to 2040₅₀₋₆₉ also cross-opsonized GAS isolate BSA10 (Fig. 2A).

Although antiserum to 2040₅₀₋₆₉ was only moderately opsonic (40%) against the representative isolate, 2040, it was able to cross-opsonize three other isolates, BSA10 (89.3%), 2034 (96.8%), and 88/30 (70%) (Fig. 2A, C, and F, respectively), which correlated to ELISA cross-reactivity. Antiserum to 88/30₁₋₂₀ could also cross-opsonize two other isolates, 2034 (97.1%), which correlated with ELISA cross-reactivity, and Y530S (97.4%), which did not correlate with ELISA cross-reactivity (Fig. 2C and D, respectively). Similarly, antiserum to peptide Y530S₁₋₂₀ reduced mean colony counts of two nonhomologous isolates of GAS, Y504S (79.2%) and 2034 (80%) (Fig. 2C and G, respectively), even though no ELISA cross-reaction was previously observed. Antisera to peptides 2034₁₋₁₉ and 2034₄₀₋₅₉ only poorly cross-opsonized heterologous isolates of GAS.

DISCUSSION

This study is the first to take the approach of investigating and defining opsonic epitopes from a specific region where GAS are endemic. We have synthesized amino-terminal and

TABLE 4. Amino acid homology between ELISA-cross-reactive amino-terminal peptides

Peptide	% similarity ^a with peptide:							
	BSA10 ₁₋₂₈	2032 ₁₋₁₉	2034 ₁₋₁₉	2034 ₄₀₋₅₉	Y530S ₁₋₂₀	2040 ₅₀₋₆₉	88/30 ₁₋₂₀	Y504S ₁₋₂₀
BSA10 ₁₋₂₈	100							
2032 ₁₋₁₉	90	100						
2034 ₁₋₁₉	36	42.8	100					
2034 ₄₀₋₅₉	35	40	22	100				
Y530S ₁₋₂₀	24	30	21	34	100			
2040 ₅₀₋₆₉	38	38	47	50	42	100		
88/30 ₁₋₂₀	33	45	58	40	32	44	100	
Y504S ₁₋₂₀	18	40	75	41	34	43	43	100

^a Calculated using the GCG Pileup program.

TABLE 5. Secondary-structure characteristics of the ELISA-cross-reactive amino-terminal peptides^a

Peptide	Peptide sequence and predicted secondary structure
BSA10 ₁₋₂₈	NSKTPAPAPVPVKKEATKSKLSEAELH ttccceehhhhhhhhhhhhhhhhhhh
2032 ₁₋₁₉	NSKNPVPVKKEAKLSEAEL ttccceehhhhhhhhhhhhh
2034 ₁₋₁₉	AEIKKQADSAWNWPKEYNA hhhhhhhhcttcctttt
2034 ₄₋₅₉	DDKEKDPQYRALMGENDLR hhhhhhhhheehhhhhhh
Y530S ₁₋₂₀	EAPKSHSISNNEQLINELND hhhhcccccheeehhht
2040 ₅₀₋₆₉	LKMLNRDLEQAYNELSGEAH hhhhhhhhhhhhhhhhhh
88/30 ₁₋₂₀	DNGKAIYERARERALQELGP hhhhhhhhhhhhhhhtct
Y504S ₁₋₂₀	TEVKAAGQSAPKGTNVSADL hhhhhhhhcctteeeeeee

^a Secondary-structure analysis was determined by Garnier secondary-structure prediction. h, helix; t, beta turn; c, random coil; e, extended strand.

sub-amino-terminal peptides from M protein of clinical GAS isolates collected from Australian Aboriginal communities and RF outbreaks in the Northern Territory and reference strains. All 33 amino-terminal peptides and six sub-amino-terminal peptides were highly immunogenic in mice when conjugated to TT, and over half of the amino-terminus-specific antibodies were highly opsonic to the homologous GAS strain. Antisera to the sub-amino-terminal peptides did not significantly opsonize the homologous strains of GAS (less than 50% kill). These findings are in agreement with previous studies that show that antibodies to sub-amino-terminal domains generally do not effectively opsonize GAS (1, 13).

We also identified several anti-peptide sera that could opsonize not only the homologous strain of GAS, but heterologous strains as well. Although other studies have identified anti-peptide sera that cross-react with heterologous serotypes of M protein by ELISA, none of these sera cross-opsonized the related heterologous GAS (1). We were able to show that opsonization mediated by antisera to BSA10₁₋₂₈ and 2032₁₋₁₉ (against BSA10 and 2032 GAS, respectively) could be inhibited by those peptides that were cross-reactive by ELISA. Antisera to BSA10₁₋₂₈ and 2032₁₋₁₉ could each directly opsonize two GAS isolates represented by the ELISA-cross-reactive peptides. In addition, antisera to peptides Y530S₁₋₂₀, 2040₅₀₋₆₉, 88/30₁₋₂₀, and Y504S₁₋₂₀ could opsonize at least one nonhomologous isolate. In some cases where there was cross-opsonization between strains, no ELISA cross-reactivity was observed between amino-terminal peptide antisera and the nonhomologous amino-terminal peptide. It may be that other regions within the M protein, or indeed other surface antigens of GAS, cross-react and are opsonized by antibodies to these sequences.

With the exception of BSA10₁₋₂₈ and 2032₁₋₁₉, which have 90% similarity and are both in the ML2 family, very little sequence homology between the other amino-terminal peptides existed (Table 4). Cross-opsonization by the peptide antisera may in part reflect structural similarities of the peptides. The predicted secondary structures of peptides BSA10₁₋₂₈, 2032₁₋₁₉, 2040₅₀₋₆₉, 88/30₁₋₂₀, and 2034₄₀₋₅₉ are predominantly alpha-helical (Table 5). Investigation of the helical propensity of BSA10₁₋₂₈ using circular dichroism indicated that this peptide has high alpha-helix potential, with 62 to 81% alpha helix

in 100% trifluoroethanol and 40 to 63% alpha helix in 50% trifluoroethanol (data not shown). Therefore, the cross-reactive anti-peptide sera may recognize a common structure produced by the alpha-helical coiled folding of the primary sequence, either as a peptide on an ELISA plate or on the M or ML proteins of the streptococci. Several studies have in fact reported that anti-peptide antibodies from the M protein can cross-react with several alpha-helical proteins, including laminin, myosin, and tropomyosin (8, 9, 31). It is thus conceivable that the cross-reactive anti-peptide antibodies can recognize and cross-opsonize other nonhomologous alpha-helical M or ML proteins.

The advantages of using a vaccine incorporating peptides representing amino-terminal sequences of the M protein is that they are more opsonic than C-terminal antisera (26) and less likely to evoke tissue-cross-reactive antibodies (10, 12, 14). None of the peptide-specific antibodies cross-reacted with host proteins, suggesting that a vaccine using these epitopes may not induce antibody-mediated pathology. In terms of vaccine development, we have now identified numerous opsonic epitopes specifically targeted to GAS-endemic region. In order to protect against the numerous strains of GAS, a vaccine would need to incorporate multiple protective epitopes. Several studies have shown the potential of constructing multivalent streptococcal vaccines capable of opsonizing multiple serotypes of GAS (1, 2, 10, 11, 14). Thus far, up to eight amino-terminal epitopes have been linked in tandem to produce a hybrid protein that can produce bactericidal antibodies in immunized rabbits (10, 11, 14). To develop a successful antistreptococcal vaccine, it is critical to define epitopes that would induce broad protective coverage. The frequency of GAS isolates collected from the Northern Territory of Australia, represented by the amino-terminal M protein peptides used in this study, has recently been investigated (15; unpublished data). Based on these studies, it may be possible to design a vaccine that will incorporate opsonic amino-terminal epitopes from GAS commonly found in this region in combination with cross-opsonic epitopes, including conserved-region epitopes (3, 4, 5, 24, 26), to produce coverage against the significant diversity of GAS strains found within the Aboriginal communities of the Northern Territory.

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