Journal of Microbiology, Immunology and Infection (2016) 49, 352—358

Available online at www.sciencedirect.com

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ORIGINAL ARTICLE

Immune response against M protein-conserved region peptides from prevalent group A *Streptococcus* in a North Indian population

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Received 12 November 2013; received in revised form 5 May 2014; accepted 6 May 2014
Available online 30 July 2014

KEYWORDS
group A streptococci;
immune response;
J14-like sequence;
M protein-conserved C region

Background: Group A streptococci (GAS) cause infections with a high prevalence in most developing countries. A GAS vaccine under trial that is based on the amino-terminus of the M protein provides type-specific immunity, and hence seems ineffective in India because of heterogeneous *emm* types. However, the conserved C-terminal region of the M protein protects against multiple serotypes. In this paper, the immune response generated against the conserved C-repeat region of the M protein was checked in an Indian population to establish their vaccine candidature.

Methods: When screened for GAS, patients with pharyngitis, rheumatic fever/rheumatic heart disease (RF/RHD), and invasive disease showed heterogeneous *emm* types, out of which five prevalent types (1-2, 11, 49, 75 and 112) were selected for the study. The C-terminal region of their M proteins showed conserved C1-, C2-, and C3-repeats. The C1-repeat was more diverse and had two different J14-like sequences. Peptides to these C-terminal regions (J14.1 and J14-R6) were designed. Antibodies against these peptides were analyzed using the sera of 130 GAS-infected volunteers.

Results: Serum antibodies were significantly higher in patients with acute rheumatic fever, RHD, and invasive disease than in patients with pharyngitis or the healthy controls. The serum antibodies to these peptides was higher in teenagers and adults than in children.

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http://dx.doi.org/10.1016/j.jmii.2014.05.008

Conclusion: Results showed an association between streptococcal disease progression and the age-related development of immunity to the conserved regions. Hence, these peptides could be considered protective in impeding streptococcal infections worldwide.

Introduction

Group A streptococus (GAS; *Streptococcus pyogenes*) is responsible for a wide spectrum of human infections ranging from pharyngitis and impetigo to invasive diseases such as streptococcal toxic shock-like syndrome, cellulitis, and necrotizing fasciitis. The primary infections are self limiting and innocuous. Of greater concern are post-infectious sequelae such as aute rheumatic fever (ARF), rheumatic heart disease (RHD), and acute glomerulonephritis,[1] which are a cause of severe morbidity and mortality in developing countries such as India.[2,3] There is no licensed vaccine available against GAS in the market. Thus, a GAS vaccine that could prevent even a fraction of these cases could have a major impact on the socioeconomic status of the Indian population.

Intense research has unveiled a detailed understanding of the molecular pathogenesis of GAS infections. Many discoveries have identified several vaccine candidates, which are in various stages of development.[4–11] A leading candidate antigen is the surface M protein, a major virulence factor of GAS that confers protection against infections in animal models.[5,12–14] The M protein forms a coiled-coil α-helix that consists of a hypervariable amino terminal domain.[15] Antibodies directed to this hypervariable region opsonize bacteria of homologous serotypes. With the advent of serological diversity of the M types that are isolated from patients with endemic disease, the main efforts to develop a GAS vaccine have been hampered. To date, more than 150 different *emm* types have been identified,[16] which makes it impossible to effectively immunize against the heterogeneous GAS strains. To develop a broader strain coverage GAS vaccine, multivalent constructs have been investigated that use fused recombinant amino terminal peptides derived from M protein associated with epidemiologically important prevalent serotypes of GAS.[4,8,17] A 26-valent vaccine is undergoing clinical trials in the United States of America.[8] However, it seems quite unlikely that it will provide protection to the Indian population because different *emm* types are pervasive in India; this indicates the need for the development of an indigenous/or universal vaccine.[18]

Another approach in designing a GAS vaccine has been to investigate the highly conserved C-terminal region of the M protein. In 2000, Cunningham reported that antibodies directed to the C-terminal region protect against multiple serotypes and hinder bacterial colonization at the mucosal surface.[1] Previous studies reveal opsonic antibodies specific against the conserved C-terminal repeats of the M protein in humans, and antibodies raised in mice to a conserved 20-mer peptide (referred to as p145) have been known to opsonize reference strains of GAS and GAS isolates from Australian aboriginal and Thai patients with rheumatic fever.[19,20] However, a few studies also indicate that p145 contain B cell and T cell epitopes and that T cell epitopes share determinants on human cardiac myosin.[20] However, by placing overlapping segments of p145 within another helical peptide derived from a yeast DNA-binding protein, GCN4 (which is essential to sustain the exact helical folding and conformational structure of the peptide), the peptide J14 was mapped within p145 with minimal B cell epitope and free from probable deleterious T cell autoepitopes.[21]

J14 (KQAEDKVKASREAKKQVEKALEQLEDRVK) is a peptide with 14 amino acids from the C region of M proteins and is flanked by yeast-derived GCN4 sequences. J14 seems to elicit protective opsonic antibodies against multiple different GAS isolates. It has been suggested that approximately 60% of GAS strains contain the J14 sequence, whereas the remaining GAS strains contain J14-like sequences.[13,22,23] Two peptide sequences, J8 and J14, from a conserved repeat induce opsonic antibodies against multiple GAS strains.[23] In this study, to understand their potential as vaccine candidates, the prevalent GAS *emm* types showing two peptides, J14.1 and J14-R6 (which represent the J-14 like sequences), were analyzed for their ability to elicit opsonic antibodies in an Indian population with GAS infections.

Methods

Screening of prevalent GAS isolates for J14 and J14-like sequences from the conserved carboxy terminal segment of the M protein

During the community surveillance from year 1997 to 2007, throat samples (n = 14019) and skin samples (n = 3063) were collected from patients who had pharyngitis, skin infections, and ARF and resided in or around Chandigarh, India. The samples were screened for GAS on blood agar plates. The study was approved by Institute Ethics Committee, Post Graduate Institute of Medical Education and Research, Chandigarh-160012, India (Ref. No. 43/IAEC/177). A total of 407 cases of GAS infection were confirmed by latex agglutination kits (Murex Kit, UK) and preserved under proper storage conditions at the Department of Experimental Medicine and Biotechnology at the Postgraduate Institute of Medical Education and Research (PGIMER) (Chandigarh, India). There were 70 types representing 53 known types, which included 33 subtypes, 12 sequence types, and five novel M-nontypable (MNT) strains. The first 10 most prevalent *emm* types—81, 11, 112, 77, 44, 15, 49, 75, NT, 1-2—comprised 51.7% isolates. They were screened for their national as well as worldwide prevalence[18], based on which five types (i.e., *emm* 11, *emm* 112,

emm 49, *emm* 75, and *emm* 1-2) were selected for the present study. The most prevalent type was *emm* 81; however, it was not included in this study since our earlier study showed that *emm* 81 strains of North India are less virulent with respect to adhesion and invasion.[24]

The genomic DNA of the GAS strains was isolated by a modified SDS-phenol method.[25] The *emm* gene was amplified as previously described.[26,27] A pairwise comparison of the nucleotide homology for the first 160 bases of the hypervariable region of the *emm* gene against the bacterial database (which can be accessed at http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm) was conducted to[37] designate *emm* types to a particular strain. The obtained nucleotide sequences of the conserved C-terminal segment of the M protein (*emm* types 1-2, 11, 49, 75 and 112) were subsequently deduced into amino acid sequences and searched for J14 or J14-like sequences. The sequences and number of their repeats were recorded.[23]

Synthesis of J-14 like sequence peptides

J14.1 (KQAEDKVKASREAKKKVEADLAQEEDKVK) and J14-R6P (KQAEDKVKASRAAKKELENHQQEEDKVK),[23] containing 29 amino acid sequences and 28 amino acid sequences, respectively, were commercially synthesized by the fluorenylmethoxycarbonyl (FMOC) method from ISOGEN Life Sciences (De Meern, Netherlands). Peptides were conjugated with Keyhole Limpet Hemocyanin (KLH; Boehringer Mannheim, Germany) in the ratio of 1:10 and purified by high pressure liquid chromatography (HPLC). Peptides were dissolved in water at a concentration of 10 mg/mL and maintained at −20°C until use.

Estimation of human serum antibodies against J14-like peptides

Blood sample collection

Blood samples were obtained after consent from 130 volunteers from in and around Chandigarh, India who had established RHD (n = 40), ARF (n = 40), pharyngitis (n = 40), and invasive disease (n = 10). Blood from an equal number of age- and sex-matched controls (n = 130) was also collected and their sera were segregated. Patients having any other chronic illness or who did not give consent for the sampling were excluded from the study. All patients were assessed by physicians. The ARF patients were diagnosed on the basis of the revised Jones criteria[28] and RHD was diagnosed on the basis of echocardiography. The sera were obtained during the acute stage of pharyngitis, rheumatic fever, and invasive disease. The sera from patients with ARF and invasive disease was collected when they were admitted for treatment in the hospital (i.e., PGIMER in Chandigarh, India). By contrast, the sera were collected from RHD patients when the patients were admitted to the hospital for valve replacement surgery.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was used to measure human serum antibodies to the synthesized peptides[29] with the addition that standard curves of optical density versus concentration of human immunoglobulin G (IgG) were used to calculate the antibody concentration.[30] The peptides were initially diluted to 5 μg/mL in carbonate-bicarbonate buffer (at pH 9.6), and coated onto 96-well ELISA plates (Nunc, Schwerte, Germany) in a volume of 100 μL per well overnight at 4°C. Excess antigen was removed and the wells were blocked with 100 μL of 2% bovine serum albumin in phosphate buffered saline (PBS) for 2 hours at 37°C. Plates were washed five times with PBST (PBS containing 0.05% Tween-20). Serum dilutions were prepared in 1.0% skim milk in PBS, and 100 μL were added and incubated for 1.5 hours at 37°C. The plates were washed five times with PBST and then incubated with rabbit-antihuman IgG conjugated with horse-radish peroxidase [(Bangalore Genei, India) (diluted 1:15000 in PBS-skim milk (1.0%)] for 1.5 hours at 37°C. The plates were washed five times with PBST and 100 μL of o-phenylenediamine substrate was added and incubated for 30 minutes. The reaction was stopped with 50 μL of 2N sulfuric acid (H_2SO_4) and the plates were read at 450 nm in an ELISA plate reader.

Statistical analysis

The cross-reactivity was expressed as the arithmetic mean and standard deviation. All statistical differences between the control and different groups of GAS-infected patients and between different age groups were assessed by the t test for the unpaired mean. A p value of less than 0.05 was considered statistically significant for all analyses.

Results

Identification of prevalent GAS types with J14 and/or J14-like sequences

After sequencing the C-terminal region of prevalent *emm* type encoding M protein and deducing the corresponding amino acid sequences for the distribution of J14 and J14-like sequences, it was observed that the selected *emm* types contained the three C-repeats (i.e., C1 repeat, C2 repeat, and C3 repeat). The C1 repeat was more diverse and contained two different J14-like sequences (i.e., J14.1 (ASREAKKKVEADLA) and J14-R6 (ASRAAKKELEANHQ). The molecule J14-R6 was present in *emm* types 1-2, 11, 75, and 112, but was not present in *emm* 49.The C2- and C3-repeats were identical with both having the J14.1 sequence (Fig. 1). Our study showed that the J14.0 sequence was absent in the C-repeat region for all the selected prevalent GAS strains in the Indian population.

Prevalence of serum antibodies to J14.1 and J14-R6 in an Indian population

The sera of healthy controls elicited a high antibody titer to both peptides (J14.1 and J14-R6), even at 1:4000 dilutions. The sera from patients with pharyngitis, ARF, RHD, and invasive disease also had measurable levels (approximately 5 μg/mL) of serum antibodies to J14.1 and J14-R6. The mean antibody concentration against these peptides was similar in healthy controls and in patients with pharyngitis. However, when compared to patients with pharyngitis, the

emm Type	C1-Repeat	C2-Repeat	C3-Repeat
M 1-2	J14-R6	J14.1	J14.1
M 11	J14-R6	J14.1	J14.1
M 75	J14-R6	J14.1	J14.1
M 112	J14-R6	J14.1	J14.1
M 49	J14.1	J14.1	J14.1

Figure 1. The J14 sequence types in the C-repeat region of the M protein of group A streptococci strains in a North Indian population.

serum antibodies to these peptides were significantly higher (approximately 8 μg/mL, $p < 0.001$) in patients with ARF, RHD, and invasive disease (Fig. 2).

The sera of 130 Indian subjects with GAS infections were categorized as "children" [i.e., patients aged 1–10 years ($n = 28$)], "teenager" [i.e., patients aged 11–20 years ($n = 42$)], and "adult" [i.e., patients aged over 20 years ($n = 60$)]. All three age groups contained detectable quantities of anti-J14.1 antibodies (at 5.68 μg/mL, 7.14 μg/mL and 6.82 μg/mL respectively) and anti-J14-R6 IgG antibodies (at 5.63 μg/mL, 7.02 μg/mL, and 6.67 μg/mL for anti-J14-R6 IgG antibodies, respectively). The teenagers and adults had a significantly higher concentration of serum antibodies to the two peptides, compared to the children ($p < 0.001$) (Fig. 3).

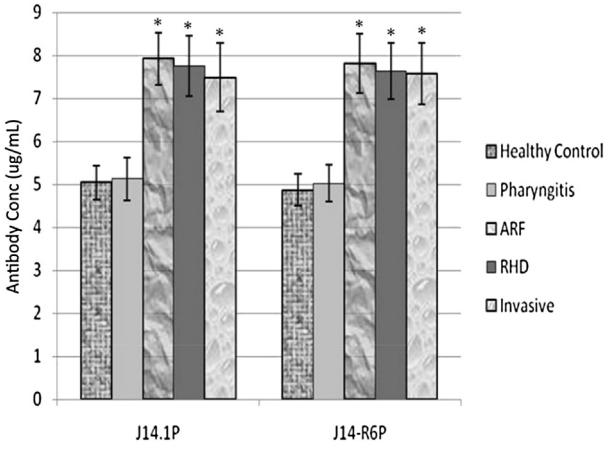

Figure 2. Analysis of the concentration of the serum antibody against J14.1 and J14-R6 in North Indian patients with streptococcal infections and in healthy controls. ARF = acute rheumatic fever; Conc. = concentration; RHD = rheumatic heart disease.

Discussion

Studies from North Indian communities have shown a heterogeneous *emm* type distribution of GAS with seasonal,

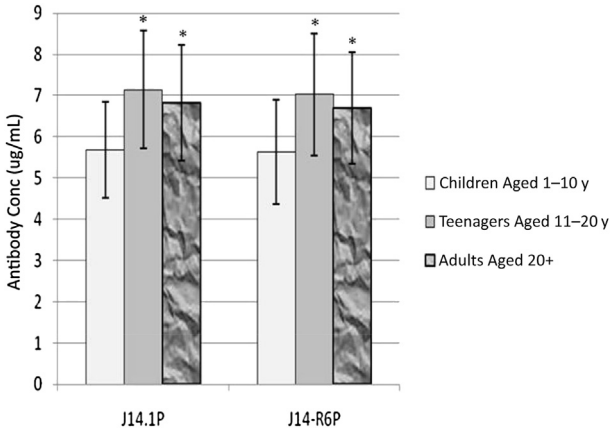

Figure 3. Comparative analysis of the serum antibody concentration against J14.1 and J14-R6 peptides in an age-stratified North Indian population.

yearly, and geographical variations.[27,31] The specific *emm* types were associated with the source of isolation.[18] The *emm* types circulating in North Indian communities differ from those in south Indian communities and the developed nations.[8,16] The most prevalent *emm* types, comprising 51.7% isolates, were screened. Because *emm* 81 has been shown to be less virulent,[24] it was excluded from this study. Selected for the present study were *emm* 11, which is a common serotype that is often prevalent in the United States and South India, along with the *emm* types 112 and 49, *emm* 1-2 (based on the role of *emm* 1 in invasive diseases in developed nations), and *emm* 75 (which is prevalent in our region). Our past observations showed that only 50% of the *emm* types considered in the 26-valent multiple-epitope vaccine were present in the North Indian community. This suggests that the efficacy of current vaccines under trial that are based on the N terminal region of M proteins may not be successful in India.[18] Therefore, we considered the C-repeat conserved region of the M protein in the present study to detect a vaccine candidate effective against heterogeneous GAS strains, thereby making it a universal vaccine.

All selected *emm* types showed the three C-repeats in a conserved region of the M protein with the C1 repeat being more diverse and containing J14.1 and J14-R6 sequences. In another study, only 60% (12/20) of the isolates contained three C-repeats, 35% contained two C-repeats, and one isolate had a single C-repeat.[23] The types *emm* 49 and *emm* 75 from a Northern Thai population.[23] Our study interestingly showed a similar C-repeat pattern, whereas an earlier report from the same Indian region showed a variation in the J14 sequence type.[22] Among the 521 GAS isolates examined in Fiji, most (91.6%) isolates had the three C-repeats, 40 GAS isolates contained two C-repeats, and four GAS contained only one C-repeat.[11]

In spite of the conserved sequence (greater than 98%), variations have been reported towards the N-terminal region of the conserved C-repeat region,[22,32] which was also reflected in our study. Distribution of the J14 sequence in isolates from the same geographical region have been assessed that showed the C1-repeat had the greatest diversity (17 types) of J14 types; the C2 repeats contained three J14 types (J14.1, 14.4, and 14.8) and C3-repeats J14.1 type accounted for 72.7% isolates.[22] Yoonim et al[23] also found that the C1—repeat had the greatest diversity (nine different types) of J14 types and the C2-repeat contained eight different types of J14 types (with the greatest being J14.1).The largest report of J14 sequence type from Fiji identified 25 different J14 sequence types.[11]

Many studies show the potential of J14 to be a vaccine candidate for GAS infection,[22,33] although our study showed that J14 sequence was absent in the C-repeat region for all selected GAS strains. A few relevant studies also show that the J14 epitope is present only within the C3-repeats.[22,23] Four conserved J14 peptides, (i.e., J14, J14.1, J14-R1, and J14.R2) are potential GAS vaccine candidates to prevent streptococcal infections in an endemic area.[23] Across all C-repeat regions, 55 different J14 sequences types (i.e, J14.0 to J14.54) have been described in Indian GAS isolates.[22] Fourteen new J14 sequence types (i.e., J14.55 to J14.68) that have not been described previously have recently been reported from Fiji,[11] and have been numbered in order of their discovery. In our study, and similar to previous results, J14.1 and J14-R6 among the different J14-like sequences are present in the C-repeat region of the M protein.[22] Antibodies raised against the J14.0 peptide in mice opsonize the GAS strains belonging to a variety of *emm* subtypes that contain J14 sequences other than the J14.0 sequence type.[22] Apart from GAS, 12 overall J14 types among 248 GCS/GGS isolates have also been reported,[11] which makes these two J14 peptides promising vaccine candidates against streptococcal infections in the near future.

When serum antibodies to these peptides in patients with ARF, RHD, and invasive disease were compared to patients with pharyngitis, statistically significant results were found. This suggests a greater immune response is generated as the disease progresses towards chronicity. No significant difference in the mean anti-p145 serum antibody level has been recorded between RHD, ARF, and other streptococcal diseases from Australian aborigines from a highly endemic area,[12] which is similar to our observation of the same serum antibody concentration in patients with ARF, RHD, and invasive disease.

Protective immunity from GAS is achieved through antibodies directed against the M protein. Studies have provided evidence that the relative resistance of adults to streptococcal pharyngitis was not because of the presence of type-specific antibodies, but may be because of the presence of antibodies to the conserved determinants.[33] In the present study, all three age groups contained detectable quantities of anti-J14.1 IgG and anti-J14-R6 IgG antibodies, respectively. The teenagers and adults had a significantly higher concentration of serum antibodies, compared to children. The acquisition of antibodies to the conserved epitopes with increasing in age may explain, in part, the reduced incidence rates of GAS infections and their sequelae in adults. These observations are comparable to a previous study in which adult sera showed strong antibody responses to the M6 protein-conserved region.[34] Teenaged and adult aborigines show significantly high serum antibody levels to the conserved region epitope (i.e., p145, J1, J2, J3, J7, and J8), compared to children.[12] In the endemic areas, it has been well established that children up to 10 years old have high

incidence rates of streptococcal skin and respiratory infections, which decreases rapidly with increasing age.[35] A similar phenomenon has been observed in a case of superantigen-mediated disease-toxic shock-like syndrome (TSLS, in which a peptide was created from the homologous region of staphylococcal enterotoxins and streptococcal pyrogenic enterotoxins.[36] The acquisition of antibodies against p145 with age also paralleled the acquisition of GAS immunity. This increases the likelihood of using this epitope as a target in a prophylactic vaccine administered during early childhood.[12,29] Human sera with antibodies to p145 opsonize against heterologus GAS strains. However, p145 contains T cell epitope shared with determinants on human cardiac myosin and keratin in the mouse.[29] Henceforth, J14-like sequences are searched for.

Thus, our data provide an insight into the development of immunity against GAS with high antibody concentration to conserved epitopes in the C-terminal region of M protein as the infection progresses from pharyngitis to ARF, RHD, and to an invasive state with increasing age. It appears that immunization early in childhood with the peptides J14.1 and J14-R6 may induce accelerated immunity to many GAS serotypes. However, considering that ARF/RHD is an autoimmune disease, a potential risk associated with immunization with these peptides J14.1 and J14-R6 derived from M protein may be that it might actually promote disease by reacting with host tissues, including the heart. To develop a successful antistreptococcal vaccine, it is vital to identify epitopes that would provoke broad protection coverage without evoking tissue cross-reactive antibodies. In this study, we have identified the opsonic epitopes that are particularly aimed to GAS-endemic areas that would offer protection against multiple GAS strains and cover the wide array of streptococcal infections in the Indian population.

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