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Original Article

M12 protein expressed in the plasma membrane of Group A Streptococcal identified as a vaccine target in Taiwan

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Group A *Streptococcus* (GAS) grows in the throat or skin and can lead to streptococcal illnesses, such as scarlet fever. Therefore, identifying the specific expressed proteins as antigens is urgent for the development of an efficient vaccine. The aim of this study was to identify and evaluate the GAS antigen that be recognized in adult sera. Using western blotting and epidemic statistical analysis, we identified a 55kDa GAS protein that is immunoreactive with the sera of adults but not the sera of children. The 55kDa GAS protein was further confirmed as M12 protein by MALDI-TOF MS. Moreover, we constructed and expressed the NH_2-terminal and COOH-terminal of M12 recombinant proteins to determine the crucial epitope of M12. Opsonization and indirect bactericidal experiments showed that the recombinant M12 proteins can interfere the adult serum GAS opsonization and be recognized by adult antibody. Therefore, M12 antigen expressed in GAS could possibly serve as a potential target used in production of a vaccine that might prevent diseases by GAS in Taiwan.

Key words: Group A Streptococcus, M12 protein, Scarlet fever

Introduction

Streptococcus pyogenes (Group A *Streptococcus*, GAS)[1,2], a gram positive pathogenic bacteria, grows in the upper respiratory tract or skin. GAS can cause suppurative infections such as bacterial pharyngitis, scarlet fever, impetigo[2-5], and non-suppurative sequelae such as acute rheumatic fever, acute glomerulonephritis, reactive arthritis, and rheumatic heart disease[2-7]. Based on the different immunogenic recognitions of the surface M proteins by antibodies[8] and the polymorphisms of GAS in the emm gene[9,10], more than 100 serotypes of GAS have been classified. Studies of the M protein serotype show that the prevalence of GAS infection differs geographically[9,11-16]. The M12 type of GAS is frequently distributed in Europe, America, Africa and Asia, though it was most dominant in Africa and Asia when it was discovered[13,14,16,17]. In Europe or America, the most common serotypes of GAS are M1 (19%), M28 (12%), and M12 (5 %)[18].

Previous studies have shown that GAS infections and asymptomatic carriers mainly occur in children, and thus GAS is typically considered a childhood infectious disease[11,15]. Recently, results of epidemic studies of severe GAS infections such as scarlet fever have reported a higher scarlet

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fever-infection rate in Asia[19], where it is present in more than 90 percent of child cases[20]. Other studies have also shown that different age groups have various GAS-reacted immunities[9,15,21,22], suggesting that that M protein-derived antibodies might be produced in adults. We hypothesized that M protein can serve as a target for the development of a vaccine to prevent GAS infection.

It is urgent to search for and identify a GAS antigen that can be used to further prevent and diagnose GAS. The GAS secreted proteins SpeB and C5a peptidase can interfere with the host's defense mechanisms and cause an inflammatory response. Therefore, antibodies against SpeB and C5a peptidase can be used to treat patients with GAS infection. In addition, antibodies against SLO (Streptolysin O) and GAS's membrane glycoprotein can be used in the diagnosis of GAS infections[23]. Recent studies have demonstrated that M protein expressed on the surface of GAS can be used as a vaccine to prevent GAS infection[24,25]. Moreover, specific M protein expression in GAS can be used for epidemiological analysis[21,26] and as a marker for prognosing GAS infection[21,27,28]. However, it is not clear what specific M protein expressed in GAS can be used as a target for vaccine design.

In this study, we investigated a diverse array of serum antibodies in adults and children by western blotting analysis to search for a specific GAS protein as a vaccine for preventing the GAS infection. We found that the membrane protein M12 of GAS can be recognized by the antibody from adult sera. We also used opsonization assay to investigate the interference effect of the recombinant NH2- or COOH-terminal of M12 protein to confirm our location of the recognition site of serum antibody. It was found that M12 protein expressed on the membrane of GAS can be targeted for a vaccine to be used in the prevention of GAS infection in Taiwan.

Materials and Methods

Bacteria strains

GAS strains used in this study were isolated from the Tai-An Hospital in Taichung City, Taiwan between1996 and 2003 (Table 1). All cases were documented by subjects, and GASs were isolated from the nasopharynx of scarlet fever patients (n=115). GAS was grown in tryptic soy broth supplemented with 0.5% yeast extract (TSBY) or Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.2% yeast extract (THY) at 37 °C. Tryptose agar with 5% sheep blood was used as a solid medium.

Table 1. Distribution of GAS isolated from the scarlet fever patients

Age	Patient (n)	Expression of 55kDa in isolated GAS
<14	101 (88%)	16.46%
>14	14 (12%)	95.79%

Total 115 patients were enrolled. The individual isolated clone from each patient was analyzed using western Blotting to observe the 55kDa protein expression.

Subcellular Fractionation and Preparation of Membrane Protein Extract

GAS was cultured in sheep blood medium at 37 °C overnight with 2 ml of TSBY medium (3% Tryptic soy broth and 0.5% yeast extract). The GAS was then added with 200 ml of TSBY medium and cultured at 37 °C. After 24 h, the GAS was harvested by centrifugation at 10000 rpm (Hitachi CR-21 rotor 41) for 10 min, and subsequently broken by a sonicator (Fish Sonicator). After removal of unbroken cells by centrifugation at 12000 rpm for 10 min, the membrane fraction of GAS protein was extracted using ultracentrifugation at 56000 rpm (Beckman L80 rotor Ti 90) for 60 min. M12 protein was purified by High-Q anion exchange chromatography as previously described[29]. GAS membrane protein was stepwise eluted with different concentrations of NaCl, 0.05 M, 0.1 M, 0.2 M, 0.3 M, and 0.5 M, respectively. Five fractions (1 ml/tube) were collected. The collected fractions were analyzed by immunoblotting.

Western Blotting

Western blotting was performed to detect M12 protein using sera from patients as primary antibody as previously described[13]. The second antibody against human conjugated with horseradish peroxidase was added and the sera was incubated for one hour. The ECL detection system (Millipore) was used and the images were acquired

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by Imaging System (Gel Doc XR System, Bio-Rad).

Opsonization assays

Opsonic antibodies were detected using *in vitro* opsonization assays as previously described[30]. The mixture consisted of 0.05 ml of a standard suspension of streptococci which was grown to mid-log phase in 0.05 ml of test serum, 0.2 ml of heparinized (10 U/ml), and non-immune human blood. For these assays, the number of streptococcal CFU per leukocyte was set to approximately ten. The tubes were rotated for 45 min at 37°C. Their smears were made on glass slides and stained with Liu's reagent. The efficiency of opsonization was quantitated by counting 100 consecutive neutrophils and calculating the percentage with associated streptococci (percent opsonization).

Indirect bactericidal tests

Bactericidal assays were performed as previously described[24,30] and 0.05 ml of Todd-Hewitt broth containing GAS was added to 0.1 ml of test serum and 0.35 ml of blood and the mixture was rotated at 37 °C for 3 h. Then, 0.1 ml aliquots of this mixture were added to melted sheep blood agar, pour plates were prepared, and viable organisms (CFU) were counted after overnight incubation at 37 °C.

Opsonization interference assays

The serial dilution of test serum were made in 20 µl aliquot increments, adding 20µl, 40µl, 60µl, 80µl of GAS extract, incubating at 37 °C for 30 min, and then mixing with 10 µl of streptococci (grown to mid-log phase) and 200 µl of whole, heparinized (10 U/ml) nonimmune human blood. The tubes were rotated end over end at 37 °C for 45 min. Their smears were made on glass slides and stained with Liu's stain. Opsonization interference was quantitated by counting 100 consecutive neutrophils and calculating the percentage with associated streptococci (percent opsonization).

Protein identification

The protein was identified as previously described[13]. Peptide mass fingerprinting was used for protein identification from tryptic fragment sizes located through the MASCOT search engine (www.matrixscience.com) tapping the entire NCBI nr protein database under the assumption that peptides are monoisotopic, oxidized at methionine residues, and carbamidomethylated at cysteine residues. Up to one missed trypsin cleavage was allowed, although most matches did not contain any missed cleavages. Mass tolerance of 31 ppm was the window of error to be allowed for matching the peptide mass values. A score greater higher than 109 was considered significant and accepted.

Preparation of NH_2- and COOH-terminal of M12 recombinant proteins

In order to define the conserved region of M12 protein, we first retrieved the M protein homologs from the NCBI protein database. We performed multiple sequence alignment of these M12 protein homologs by ClustalW program to identify the highly conserved regions of NH_2-terminal and COOH-terminal. TACG program (http://workbench.sdsc.edu) was used to select restriction enzyme sites of M12 nucleotide sequence. *Bam* HI site was selected as M12 NH_2-terminal and COOH-terminal forward primer restriction enzyme site, *Hind* III site was selected as M12 NH_2-terminal reverse primer restriction enzyme site and *Eco*RV site was selected as M12 COOH-terminal reverse restriction enzyme site. The plasmids pET32a-NT and pET32a-CT could produce the NH_2-terminal and the COOH-terminal M12 protein, respectively. All pET32a-NT, pET32a-CT, and pET32a plasmids encode a 6×His-tag on the COOH-terminal of recombinant protein[13]. Primers for NH_2-terminal M12 protein recombination plasmid pET32a-NT: Forward primer: CACTTGGGATCCATGGCTAAAAATAACACGAATAG; Reverse primer: CACTTGAAGCTTTTACACGTGATGCGTCCAAGTC; Primers for COOH-terminal M12 protein recombination plasmid pET32a-CT: Forward primer: CACTTGGATATCTCAGACGCAAGCCGTCAAG; Reverse primer: CACTTGCTCGAGTTAAGCTGTTGCCATAACAGTAAG.

Metal Chelating Chromatography

Five mg of membrane proteins was gently mixed with 1 ml of Ni^+-NTA resin (Qiagen) at room temperature for 30 min before the resin was packed into a column. The collected flow-through

was then passed through the column three times before the last one (flow-through) was saved. The removal proteins that were nonspecifically bound to the resin were washed using the wash buffer (35 mM imidazole, 1 mM PMSF, 0.3 M NaCl, 20 mM Tris-HCl). Finally, the resin-bound protein was eluted with five-fold the resin volume of the elution buffer (0.5 M imidazole, 1mM PMSF, 0.3 M NaCl, 20 mM Tris-HCl) at 1 ml/fraction. Flow through (FT), first wash (W1), final wash (Wf), and all five elute fractions were individually mixed with equal volumes of 2×SDS-PAGE sample buffer containing 10% β-mercaptoethanol and boiled for 10 min before separation in SDS-polyacrylamide gel.

Statistical analysis

The statistical software package, SPSS, was used for all statistical operations. A p-value of < 0.05 was considered significant (Student's t-test).

Results

GAS 55 kDa protein is a membrane M12 protein

To analyze the major expressed proteins of GAS isolated from the patients with scarlet fever, we performed western blotting to evaluate the specific GAS-recognized antibody in adult sera. One previous study, analyzing (i) the 6688 samples aged from 0 to 14, and (ii) the 141 samples over age 15 has reported that child patients (age<14) made up most those diagnosed in Taiwan[22]. Our results indicated that GAS expresses a specific antigen protein that can be inhibited by the antibody in the adult serum. Fourteen isolated GAS from scarlet fever were analyzed and one specific protein at ~55 kDa was detected in western blotting (Fig 1A). Other bacteria groups such as Streptococcus group B, C, and G, *S. pneumonia*, and *S. aureus* were also examined to exclude non-specific reactions. The ~55 kDa protein was a specific protein in isolated GAS from the the sera of a patient with scarlet fever (Fig 1B). Moreover, in order to determine the subcellular location of GAS ~55 kDa protein, we separated soluble and insoluble fractions of GAS by ultracentrifugation, and found ~55 kDa protein to be clearly located in membrane extraction (Fig 1C), suggesting that the ~55 kDa protein is a membrane protein. Furthermore, the ~55 kDa protein was isolated from the gels of High-Q chromatography, and identified by MALDI-TOF MS. The ~55 kDa protein was found to be the M protein serotype 12 (M12 protein) (Fig 2).

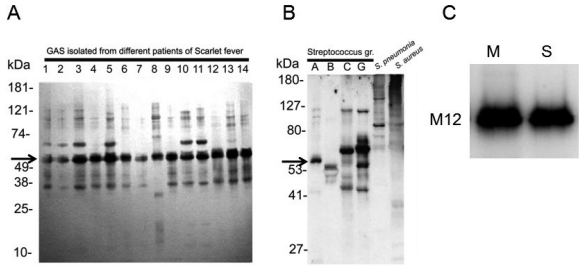


Fig. 1. **M12 (~55kDa) specifically presented in GAS as a membrane protein.** (A) Isolated GAS was analyzed to observe the protein target using adult sera as the primary antibody. The arrow indicates the position of significant GAS protein which was ~55 kDa. (B) The ~55 kDa protein of GAS was specifically recognized by adult sera compared to other pathogens. A, group A streptococcus; B, group B streptococcus; C, group C streptococcus; G, group G streptococcus. (C) GAS ~55 kDa protein existed in the membrane (M) fraction after isolation by ultracentrifugation at 56000 rpm for 60 min. S, soluble.

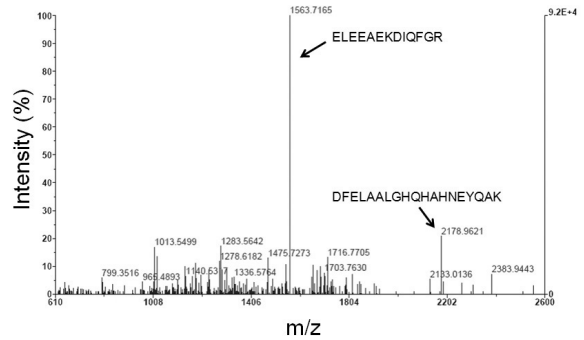


Fig. 2.**Identification of M12 protein by MALDI-TOF MS.** The extracted ~55kDa protein was identified by peptide mass fingerprinting after in-gel trypsin digestion. The result shows that observed masses matched to the theoretical masses of M 12 protein with covered 65% of the M12 protein sequences ($p<0.05$). Two peptides are shown as examples: MW 1563.72, ELEEAEKDIQFGR; MW 2178.96, DFELAALGHQHAHNEYQAK.

M12-responding antibody in the sera of adult patients

To investigate whether there was M12-responding antibody in the adult sera, we analyzed 108 serum samples, including samples from those aged <14 (n=91) and those aged >15 (n=17), using M12-expressed GAS strain 1 and strain 2 as standard to identify the M12 antibody in child or adult sera. Furthermore, a selected M12-expressed strain 1 (Fig 3A) recognized by the adult sera was used as a positive control (100%) in western blotting, whereas background was used as a negative control. Western blot data indicated that the average distribution for each child sera ranged 0 to 55.87% and for adults 4.4 to 162.6%. With a cut off value to compare with the intensity of standard protein, only 16.5% sera of the those aged <14-year-old children had the M12 antibody (Table 1). However, ~90% of sera from those >15-year-old adult had the M12 antibody (Table 1). This result suggests that the lower infection rate of scarlet fever in adults may be due to the fact that most of them had the M12 antibody.

GAS activity was inhibited by M12-responding antibody in adult sera

In order to determine the inhibitory ability of M12-responding antibody in adult sera, in vitro opsonization assay and indirect bactericidal tests were performed. Isolated GAS was mixed with sera obtained from children or adults. The phagocytic percentage of polymorphonuclear cells was calculated in the opsonization assay. Fig 3A shows that M12 protein of GAS can be detected in adult serum. Moreover, GAS could be lysed by neutrophils incubated with adult serum (Fig 3B). In contrast, the opsonization efficiency of children's sera to GAS was reduced to less than 20% (Fig 3C), indicating that the GAS had resistance of opsonization due to an absence of anti-M12 antibody in child sera. As expected, the opsonization percentage of adult sera was 80~90% (Fig 3C). In addition, the indirect bactericidal test revealed that adult sera improved the cytotoxic effect of white blood cells (WBCs) against the isolated GAS (Fig 3D). In contrast, GAS had tolerance to sera from children even in the presence

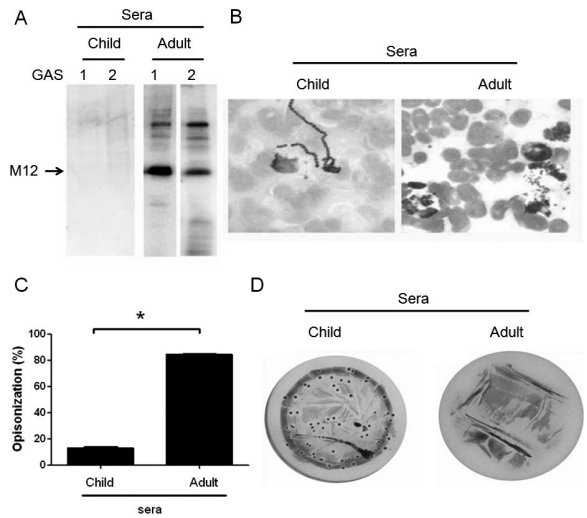


Fig. 3. **Opsonization analysis and indirect bactericidal assay.** (A) Western blotting was performed to detect the M12 protein expressed in the isolated GAS strain 1 and strain 2 and demonstrated that adult sera as primary antibody can recognize M12 protein. (B) Phagocytosis of polymorphonuclear leukocytes (PMN) was observed under a microscope, indicating that the isolated GAS strain 1 was marked for ingestion and destruction (opsonization) in the adult serum; (C) Quantification of opsonizartion for the isolated GAS strain using the sera from child or adult (n=3). (D) Indirect bactericidal assay using the sera from children and adults against the GAS strain demonstrated that adult sera inhibited the growth of GAS. This result suggested that there was specific recognized antibody in the adult sera. GAS, group A streptococci. *p<0.05 (Student's t-test).

of WBCs. These results suggest that M12 antibody presented in adult sera was crucial for preventing the GAS infection and capable of inhibiting GAS activity.

Evaluation of M12 protein as a target of vaccine

The extracted and recombinant M12 protein was used to perform an opsonization interference assay to determine the epitope of M12. First, the membrane extract of GAS was mixed with adult serum in a dose dependent manner, which inhibited the phagocytosis of WBC in the opsonization assay (Fig 4A), compared to those extracts without the addition. This result showed that the

extracted M12 protein competed and reduced M12 antibody ability in adult sera. Moreover, the His-tagged thioredoxin::NH2-terminal (1-433 amino acid) M12 fusion protein and His-tagged thioredoxin::COOH-terminal (315-563 amino acid) M12 fusion protein were constructed and used in opsonization interference assay. We found that both NH2- and COOH-terminal M12 fusion proteins had opsonization interference ability, and were capable of reducing the phagocytosis of WBC to 38-55 % (Fig 4B). Combination of the recombinant M12 proteins synergistically reduced the effect of phagocytosis, killing less than 30% of that of the control (Fig 4B). This result suggests that the interference of both recombinants had an additive effect.

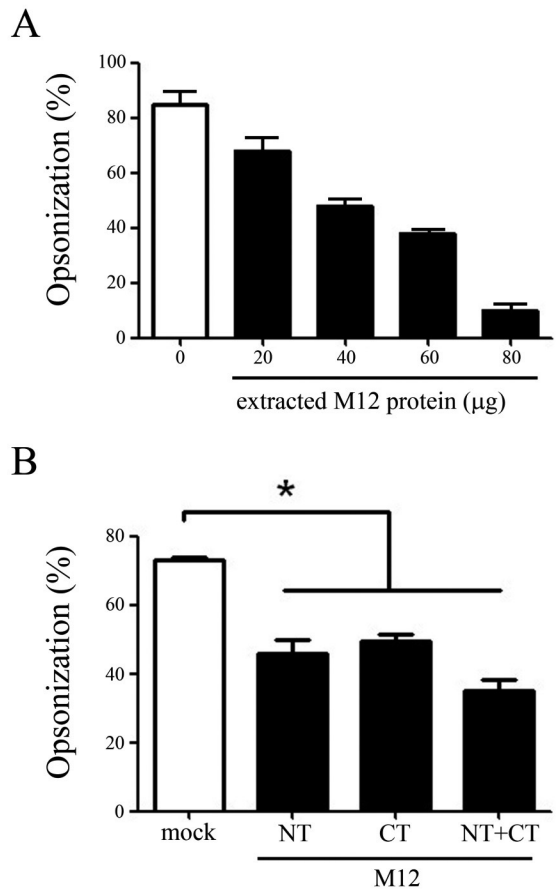


Fig. 4. **N- and C-terminal of M12 were both recognized by the adult sera.** (A) Opsonization interference was quantitated by counting 100 consecutive neutrophils and calculating the percentage with associated streptococci. The extracted membrane M12 protein was added (20~80 μg) to block the opsonization. The result shows that the opsonization gradually decreased with the increased addition of M12 protein extraction. (B) Opsonization interference (%) was measured using the recombinant NH2- or COOH-terminal GAS M12 protein. The result indicated that both NH2- and COOH-terminal M12 protein can be recognized by adult sera and interfere the opsonization effect. NT, M12 protein NH2-terminal; CT, M12 protein COOH-terminal; Mock, cell extract from pET32a host. This figure was created after five individual experiments and calculated by SigmaPlot program. *p<0.05 (Student's t-test).

Discussion

In this study, we found that the high-level expressed protein on the surface of isolated GASs from the scarlet fever patients was M12, identified by MALDI-TOF MS. Totally, 102 isolated GASs were analyzed using western blotting revealing an M12-responding antibody in the sera of normal adults. Moreover, the recombinant M12 protein intefered with the recognition of adult serum. These results indicate that M12 protein can be potenially be used to develop vaccine target for preventing GAS-leading diseases such as scarlet fever.

One previous study has reported that 70 isolated GAS strains obtained from patients with severe infectious disease can be categorized into 51 pulsed-field gel electrophoresis types, and according to *emm* gene typing, M12-serotype GAS is the most commonly found one in Taiwan[14]. Here, we confirmed that M12-serotype GAS is the main pathogen in the central Taiwan area, as has been found previously[26,28], suggesting that M12 protein may be used as the target of GAS prevention vaccine. Opsonization analysis and indirect bactericidal assay confirmed that the adult blood serum had the anti-GAS antibody. Previous studies have found that human serum containing the M-specific type antibodies were capable of preventing mice from being infected by GAS[31,32]. In addition, our survey showed that scarlet fever was more common in children than adults, suggesting that adult sera may have a GAS-

recognized antibody. The results of this study also demonstrated that combination of recombinat N- and C-terminal M12 protein inhibited the recognition of antibodies in adult sera, indicating that M12 needs both N- and C-terminal M12 protein fragmentations to serve as a vaccine. However, in our study, the interference opsonization analysis of N- or C-terminal recombinant M12 had little effect on phagocytic WBCs. It is possible that the adult serum had polyclonal antibodies, but the recombinant M12 protein only inhibited the part of M12-corresponding antibodies in adult serum, or needed additive GAS protein to enhance antigen-antibody binding[33]. Currently, the main focus of our work to develop a vaccine development is to find the most effective antigenic epitope of M protein. On the basis of epidemiological data, M12 protein has also been used in many studies focused on the development of vaccines, including the 26-valent GAS vaccine, which has had a significant impact on streptococcal disease[25,34]. Additionally, 30-valent M protein vaccine has been found to evoke bactericidal antibodies against non-vaccine serotypes of GAS[35].

In conclusion, we identified the M12 protein expressed on the surface of GAS as the protein recognized by antibody in adult sera and found M12 to be the crucial protein in GAS. Therefore, it could potentially be used potential target of a vaccine aimed to prevent GAS-causing diseases such as scarlet fever in Taiwan. It is hoped that this study may help the diagnosis and prevention of GAS infections in clinical practice.

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