Analysis of the coverage capacity of the StreptInCor candidate vaccine against *Streptococcus pyogenes*

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**A B S T R A C T**

*Streptococcus pyogenes* is responsible for infections as pharyngitis, sepsis, necrotizing fasciitis and streptococcal toxic shock syndrome. The M protein is the major bacterial antigen and consists of both polymorphic N-terminal portion and a conserved region. In the present study, we analyzed the in vitro ability of StreptInCor a C-terminal candidate vaccine against *S. pyogenes* to induce antibodies to neutralize/opsonize the most common *S. pyogenes* strains in Sao Paulo by examining the recognition by sera from StreptInCor immunized mice. We also evaluated the presence of cross-reactive antibodies against human heart valve tissue. Anti-StreptInCor antibodies were able to neutralize/opsonize at least 5 strains, showing that immunization with StreptInCor is effective against several *S. pyogenes* strains and can prevent infection and subsequent sequelae without causing autoimmune reactions.

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1. Introduction

*Streptococcus pyogenes* causes diseases as pharyngitis, impetigo, streptococcal toxic shock syndrome and necrotizing fasciitis. Rheumatic fever (RF), acute streptococcal glomerulonephritis and rheumatic heart disease (RHD) are non-suppurative autoimmune post-streptococcal sequelae that arise from a delayed immune response to infection in genetically predisposed individuals [1]. Several markers are described as risk factors for RF/RHD, including HLA-DR7, the allele most commonly associated with RHD in Brazil and other countries [2].

According to the World Health Organization (WHO), *S. pyogenes* is responsible for 15–20% of bacterial pharyngitis cases, which primarily affect 5–to-18-year-old individuals [3]. The incidence of bacterial pharyngitis varies among countries, and even within the same country, there are variations in different regions due to age, socioeconomic and environmental factors and quality of health services [4,5].

M1 is the most common strain worldwide and, due to its high virulence, is involved in invasive and non-invasive infections in several countries [12,13]. There is a large diversity of strains in Brazil. The most prevalent strains found in a sample from Sao Paulo city were the M1, M6, M12, M22, M77 and M87 compatible with those found in the rich districts from Salvador [5,14]. These M-types are also predominant in most of the world western countries [15].

The M protein has been described as the major bacterial antigen [6]. The protein consists of two polypeptide chains in an alpha double helix coiled-coil that forms fibrils extending up to 60 nm away from the bacterial surface. It is approximately 450 amino acids long and is divided into tandem repeat blocks distributed over four regions (A, B, C and D). The N-terminal portion (regions A and B) is polymorphic and differences within the first 150 amino acid residues of the A region allow for the classification of different serotypes [7,8]. The C-terminal portion (regions C and D) is highly conserved, responsible for binding the bacteria to the oropharynx mucosa and has antiphagocytic properties [6,7].

RF/RHD pathogenesis is related to the production of autoantibodies and autoreactive T cells that recognize and cross-react with epitopes from both the M protein and human heart tissue by molecular mimicry [9,10] and it was demonstrated by analyzing the T cell repertoire that infiltrated cardiac tissue and led to damage in RHD [11].
Besides that, there is a much higher diversity of M-types in the poor districts from Salvador and Brasilia typically found in low incomes regions [5,16].

The classification of strains according to their tissue tropism for throat (A–C pattern), skin (D pattern) or both (E pattern) is based on the organization of emm and emm-like genes located in the mga locus within S. pyogenes genome and constitute the base for emm pattern genotyping [17,18]. Strains belong to A–C pattern, as the M1, M6 and M12, has been historically associated with rheumatic fever [10,19] while the M22 and M87 strains belong to the E pattern are considered a group associated with both throat and skin infections [19,20].

The development of a vaccine against S. pyogenes would provide many benefits, preventing streptococcal infections and sequelae. Several vaccine development studies have focused on the M protein due to its high immunogenicity and have been tested since 1923 [21,22]. The first vaccines used whole inactivated bacteria. The use of the entire M protein from specific strains started in 1979, but the results were not satisfactory. In the 1980s, synthetic peptide models were introduced. Later, molecular biology models based on the N-terminal portion were developed, and hexavalent and 21-valent vaccines containing the most prevalent serotypes in United States entered into phase I/II clinical trials [23]. Simultaneously, new approaches for defining protective epitopes were designed based on both N and C-terminal regions. Currently, researchers are studying models that are based on streptococcal antigens other than the M protein [24].

Approximately 15 years ago, our group started to develop an effective vaccine against S. pyogenes. The approach considered how the immune system could be more effective in inducing a protective immune response via T and B lymphocytes without triggering autoimmunity [25].

Briefly, the vaccine is based on amino acid sequences from the M5 protein conserved region (C2 and C3 regions). Reactivity was evaluated by humoral and cellular analyses to define potentially protective epitopes. The B epitope, composed of 22 amino acid residues, is linked by 8 amino acid residues to the T epitope, which consists of 25 amino acid residues, using a segment of the natural M5 protein. We synthesized a peptide with 55 residues called StreptInCor (medical ID), which contained both the B and T epitopes [25].

The analysis of StreptInCor sequence binding to different HLA class II molecules was conducted using theoretical possibilities of processed peptides to fit into the pockets of antigen presenting cells (APC), followed by T cell activation via T cell receptor (TCR) that stimulates B cells to secrete antibodies with protective potential. The StreptInCor sequence contain seven potential binding sites that were recognized by HLA class II (DRB1* DRB3*/DRB4*/DRB5*), making StreptInCor a candidate vaccine with broad capacity of coverage [26].

The vaccine peptide was tested in animal models. Inbred and outbred mice showed strong humoral response against StreptInCor with high IgG production [27]. Challenge with M1 strain in immunized Swiss mice showed a survival rate of 100% for up to 21 days, compared to the control group's lower survival rate (40%) [28]. HLA class II transgenic mice, that have the capacity of present the vaccine epitope to the TCR in the context of human molecules, were immunized with StreptInCor in aluminum hydroxide (Alum) and produced high titers of IgG1 (Th2-dependent IL-4) and IgG2a (Th1-dependent IFN–γ). Specific antibodies were observed after a period of one year without reactivity against human heart proteins. No lesions were observed in several organs [29], indicating that StreptInCor is safe and has protection potential.

In the present study, we analyzed the in vitro ability of anti-StreptInCor antibodies to neutralize/opsonize S. pyogenes strains frequently found in Sao Paulo. We also analyzed the absence of humoral autoimmune reactions against human heart valve tissue.

The results presented here showed that anti-StreptInCor antibodies were able to neutralize/opsonize M1, M5, M12, M22 and M87 S. pyogenes strains, indicating that the vaccine can be effective against the bacteria, preventing infection and subsequent sequelae without causing autoimmune reactions.

2. Methods

2.1. StreptInCor vaccine epitope

The vaccine epitope consists of the following 55 amino acid residues: KGLRRLDASREAAKQLEAEQKLEEQNKISEASKRLR-DLDDASREAAKQVEKA. The peptide was synthesized using a 9-α-fluorenylmethoxy-carbonyl (Fmoc) solid-phase strategy, purified by reverse phase high-pressure liquid chromatography (RP-HPLC, Shimadzu, Japan). Peptide quality was assessed by matrix-assisted desorption ionization mass spectrometry (MALDI-ToF, Ettan Maldi ToF Pro, Amersham-Pharamcia, Sweden) as previously described [25]. Patents PCT-BR07/00184.

2.2. Mice

Inbred BALB/c and outbred Swiss mice with mature immune system (6- to 8-week-old) specific pathogen-free from CEMIB (Unicamp, Campinas, Brazil) were maintained in autoclaved cages (Alesco, Brazil) and handled under sterile conditions in the animal facility at the Tropical Medicine Institute, University of Sao Paulo, Brazil. Procedures were performed in accordance with the Brazilian Committee for animal care and use (COBEA) guidelines approved by the Tropical Medicine Institute Ethics Committee (project number 002/08).

2.3. Immunization

Mice sera previously immunized with 10 μg of StreptInCor adsorbed onto 60 μg of aluminum hydroxide gel (Sigma–Aldrich Corp., USA) in saline via subcutaneous with two doses 14 days apart. Animals that received saline plus 60 μg of adjuvant were used as negative controls. Positive controls were immunized with recombinant streptococcal M1 full protein (clone kindly provided by Prof. Patrick Cleary, University of Minnesota Medical School, MN, USA), produced and purified in our lab. Sera samples were obtained under light anesthesia by retro-orbital puncture on day 28 following immunization. Samples with high specific antibody titers (>1:1,200) detected by Enzyme-Linked Assay Immunoabsorbent (ELISA) [28] were used.

2.4. S. pyogenes strains

The strains were obtained from patients treated at the Clinical Hospital, University of Medicine – Sao Paulo, between 2001 and 2008 and identified by genotyping [30]. The M1, M5, M6, M12, M22 and M87 specimens were cultured on sheep blood agar (Vetec, Brazil), followed by growth in Todd-Hewitt broth (Himedia, India) until OD600 of 0.4 and stored at –80 °C.

2.5. M protein C-terminal region sequence alignment of different S. pyogenes strains

Amino acid sequences from the M protein C-terminal region of M1, M5, M6, M12 and M87 strains were aligned using the StreptInCor amino acid sequence through the online program BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences are available at Pubmed (http://www.ncbi.nlm.nih.gov/pubmed),
Swissprot (http://www.uniprot.org/help/uniprotkb) and CDC (http://www.cdc.gov/ncidod/biotech/strep/streplast.htm). The alignment was colored using the Jalview 2.7 program with Zapo staining to indicate the amino acids’ chemical groups.

2.6. Neutralization assay by flow cytometry

*S. pyogenes* isolates were cultured as described in Section 2.4. The bacteria were incubated with 1:100 BALB/c hyperimmune or control mice sera (*n* = 9) for 30 min. After incubation, the bacteria were incubated with murine IgG phycoerythrin (PE) – (Invitrogen, USA) specific antibody (1:50) for 30 min. After, washed and fixed in 1% paraformaldehyde. Subsequently, 10,000 events were acquired using a flow cytometer FACS Canto II (BD Biosciences, USA), and the results were analyzed using FlowJo software version 3.4.1. Statistical analysis was performed using Mann–Whitney test after analyzing normalization using the Shapiro–Wilk test.

2.7. *S. pyogenes* strains Western Blotting

M1 and M5 strains were cultured as described in Section 2.4. The bacteria were disrupted by sonication (Sonic Dismembrator 60, Termo Fisher Scientific, Sweden). The proteins were precipitated in TCA/Acetone solution at −20 °C and concentrated in filter columns (Millipore, USA). The Bradford assay (Bradford, 1976) was used for quantitation of proteins (Bio-Rad, USA). After SDS–PAGE electrophoresis, the gel was blotted onto nitrocellulose membranes [31,32], subsequently blocked with Tris-buffered saline containing 5% skim milk. The membrane was treated with immunized or control BALB/c mice sera pools (*n* = 6), incubated with anti-mouse IgG alkaline phosphatase and revealed with NBT-BCIP solution (Invitrogen, USA). The molecular weight marker used was Full-range Rainbow (GE Healthcare, Sweden). Membranes and gels images were obtained using an ImageScanner photo-scanner with the scanning software Labscan (GE Healthcare, Sweden). Densitometry was performed by TL ImageQuant software (GE Healthcare, Sweden).

2.8. Opsonophagocytic assay

*S. pyogenes* strains were cultured until they reached an optical density of 0.4–0.5. After, approximately 2.5 × 10^8 colony-forming units (CFU) were incubated with 1:100 anti-StreptInCor or control sera (*n* = 6) from BALB/c mice, previously heat-inactivated by incubation at 56 °C for 30 min, to destroy the activity of serum complement. Pre-immunization sera from 6 BALB/c mice were used as negative control. After incubation, 10% of normal mouse serum (NMS) was added as complement source. To stimulate the recruitment of mice immune cells, 10 μg of Concanaavalin A (Canavalia ensiformis-ConA, Sigma) was injected intraperitoneally. The animals were sacrificed 48 h after injection, and the peritoneal cavity was washed with 5 mL of cold PBS on ice. The concentration of peritoneal cells was adjusted to 4 × 10^7/ml in HBSS (Invitrogen, USA) containing 0.01% gelatin (opsonization buffer). The bacteria treated with hyperimmune or control mice sera were harvested and incubated with 4 × 10^5 peritoneal cells at 37 °C for 45 min with shaking (220 rpm). Ten-fold dilutions of the samples were performed and 10 μL aliquots of each dilution were cultured on blood agar plates. The count live colonies were performed as previously described [33]. After 20 min, slides of the M1 strain opsonophagocytic assay were prepared by cytopsin, stained with Instant-Prov (Newprov, Brazil), subsequently analyzed by light microscopy using an Axion Vision Zeiss Imager A1 and photographed by Axion Vision software (Zeiss, Germany). Statistical analysis was performed using Kruskal–Wallis test.

2.9. Heart tissue valve Western Blotting

Heart tissue was obtained from the lysate of a postmortem normal human mitral valve, separated by SDS–PAGE and blotted onto nitrocellulose membranes [31,32]. The blots were blocked with Tris-buffered saline containing 5% skim milk. The membrane was sequentially treated with a pool (*n* = 6) of BALB/c or Swiss immunized mice sera and anti-mouse IgG alkaline phosphatase and revealed with NBT-BCIP solution (Invitrogen, USA).

3. Results

3.1. Anti-StreptInCor recognized M1 and M5 strains

We observed that anti-StreptInCor antibodies from the BALB/c mice sera pool were able to cross-recognize both the M5 and M1 proteins in total protein extracts from each strain (Fig. 1).

3.2. Several streptococcal strains were neutralized by anti-StreptInCor antibodies

The anti-StreptInCor antibodies from Swiss mice were able to neutralize the M1, M5, M12, M22 and M87 strains by cross-recognizing the M protein on the bacterial surface with a Median Fluorescence Intensity (MFI) 2 or 3 times greater than the MFI of control sera (Fig. 2).

3.3. Anti-StreptInCor antibodies were able to opsonize, and promote the phagocytosis and death of several strains

Anti-StreptInCor antibodies from BALB/c and Swiss mice were able to promote opsonophagocytosis and death of the M1, M5, M12, M22 and M87 strains (Fig. 3a and b, respectively). The amino acid sequences alignment of the M protein C-terminal region of the strains used in this study had, on average, 72% identity with the StreptInCor amino acid sequence (Fig. 3c). The M1, M6 and M12 strains had an additional block of 7 amino acids, while the M87 strain contained two fewer amino acids than the StreptInCor sequence. M1 strain was killed in peritoneal cells by phagocytosis 20 min after the opsonization assay as observed by optical microscopy (Fig. 4a–d).

3.4. StreptInCor did not induce autoimmune reactions

No autoreactive antibodies against human heart mitral valve protein extracts were observed (Fig. 5).

4. Discussion

The development of a vaccine against multiple *S. pyogenes* strains without causing autoimmune will bring numerous benefits to human health. A vaccine would prevent streptococcal infections and sequelae and could be more effective and longer-lasting than the currently used treatment.

In addition to have broad coverage against strains, a vaccine should promote the production of neutralizing and opsonophagocytic antibodies, which are the body’s major defense lines against extracellular microorganisms.

In the 70 and 80s several models of anti *S. pyogenes* vaccines were assayed without satisfactory results, however by using new approaches several models were proposed [24], strain-specific vaccines based on recombinant N-terminal portions of the M protein serotypes most prevalent in the US entered into phase I/II clinical trials [23]. A new approach based on the 30 most prevalent serotypes is being tested and the results indicate that the vaccine
The pool.

\[ (b) \] the

\[ \text{could evoke cross-protective antibodies capable of covering most of the serotypes not included in the vaccine design [34]. Therefore, as the prevalence of strains can vary depending on the region of the world a vaccine based on the conserved region of the M protein probably will present a broad coverage.} \]

The StreptInCor is a vaccine model developed from the M5 protein C-terminal region [25], specifically located on C2 and C3 region that is conserved among the serotypes. It is interesting to note that the sequences KLEEQKNI that link both the T and B epitopes in the StreptInCor peptide, is located after the C0–C1, C1–C2, C2–C3 conserved linkers as showed by McMillan et al. (2013) [19], and this sequence is in accordance with the natural M5 protein segment.

Antibodies induced by the vaccination should be capable of binding to the same cross-conserved region of the M protein from different S. pyogenes strains around the world. This process would neutralize the adhesion function, leading to phagocytosis and killing by APCs.

We observed that immunization with StreptInCor in mice was able to promote the antibody production against C-terminal epitopes capable of cross-recognizing similar regions in both the M5 and M1 proteins. In addition, anti-StreptInCor neutralizing antibodies had the capacity to bind to M1, M5, M12, M22 and M87 proteins on the surface of each bacterial cell, opsonizing and leading to phagocytosis and death as observed in the opsonophagocytic assays. The M1 strain, the most common worldwide, also one of the most virulent strains [12], was rapidly killed on the APCs phagocytosis vacuoles induced by StreptInCor immunization, as compared with controls. These results indicate the capacity of anti-StreptInCor antibodies to neutralize/opsonize the most prevalent strains.

By amino acid sequences alignment in the present study, we observed that the C-terminal region of the M proteins had, on average, 72% identity with StreptInCor. The M1, M6 and M12 have an additional block of 7 amino acid residues in their sequences, while M87 has two fewer amino acids than the StreptInCor sequence. These differences did not interfere with antibody recognition, as observed in the opsonization assays with several strains.

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**Fig. 1.** Reactivity of sera pool from BALB/c mice immunized with StreptInCor and controls against M1 and M5 protein extracts. Western blotting of (a), M1 protein extract (b), M5 protein extract; lanes: (1) molecular weight marker, (2) anti-recombinant M1 sera pool, (3) pre-immune sera pool, (4) control sera pool, (5) anti-StreptInCor sera pool. M1 and M5 molecular weights are available at [http://www.uniprot.org/help/uniprotkb](http://www.uniprot.org/help/uniprotkb). The pool was composed of 6 BALB/c mice sera per group.

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**Fig. 2.** Neutralization of different strains by anti-StreptInCor antibodies. Analysis of neutralization of M1, M5, M12, M22 and M87 strains was performed by flow cytometry; the Median Fluorescence Intensity (MFI) obtained for each serum sample is represented as data point. Anti-StreptInCor BALB/c mice sera (△); control BALB/c mice sera (○); *P value < 0.5; **P value < 0.1; ***P value < 0.01; ****P value < 0.001 (Mann–Whitney). Data are representative of three independent experiments with nine mice per group. The error bar represents the standard deviation (SD).
Fig. 3. Opsonophagocytosis and death of several S. pyogenes strains by mice peritoneal cells. Opsonophagocytosis of M1, M5, M12, M22 and M87 strains by anti-StreptInCor antibodies from: (a) BALB/c mice sera (n = 6), (b) Swiss mice sera (n = 6), immunized with 10 μg of StreptInCor or controls. The CFU units obtained at 10⁶ dilution are represented as a data point. Anti-StreptInCor BALB/c mice sera (▲); control BALB/c mice sera (●); pre-immune mice sera pool (♦). *P value < 0.5; **P value < 0.1; ***P value < 0.01; ****P value < 0.001 (Kruskal–Wallis). Data are representative of three independent experiments with six mice per group. The error bar represents the standard deviation (SD). (c) C-terminal amino acid sequences from M1, M5, M6, M12 and M87 strains aligned with the StreptInCor amino acid sequence; identity percentage is represented in red; (*): identical amino acid residues; (-----): lack of residues; (’’’) different amino acids from the same chemical group. Colors – blue and red: amino acids with electrically charged side chains; green: amino acids with uncharged polar side chains; pink: glycines; light orange: amino acids with hydrophobic side chains. Different colors in the same column indicate an amino acid from a different chemical group; ([___]) natural link between the B and T cell epitopes.

Fig. 4. Opsonization, phagocytosis and death of M1 strain induced by anti-StreptInCor antibodies. Optical microscopy of M1 strain phagocytosis and death within peritoneal cells stained with Instant-Prov. Acquisition at ocular lenses 20 min after the opsonization assay. Magnification 10×, objective ranged from 20× to 40×. Bacteria treated with pre-immune control sera (a) magnification of 200×, (b) magnification of 400×; bacteria treated with hyperimmune sera (c), magnification of 200×; (d) magnification of 400×. Black arrows: S. pyogenes; red arrows: S. pyogenes phagocytosed by APC; blue arrows: S. pyogenes in APC digestive vacuoles.
In addition, M-types amino acid sequences from UniprotKB database were aligned in the Short-Blast program against StreptInCor. The results showed that the StreptInCor sequence is on average 71% conserved amongst the 541M protein sequences available at the public database. This block of results indicates that anti-StreptInCor antibodies can bind directly to multiple parts of the M protein C-terminal sequences due to the repeat blocks of amino acids. Consequently, differences between StreptInCor and the M protein sequences do not affect opsonization of the target strain, indicating that StreptInCor have broad capacity of coverage against the diverse M-types around the world.

Previously we showed that StreptInCor can be recognized by several HLA class II molecules, making it a candidate vaccine with broad capacity of coverage. The binding prediction of the C-terminal amino acid sequences of the M1, M5, M6, M12 and M87 proteins with different HLA class II molecules shows that the possibility of recognition/processing of M proteins and peptides in the pockets (P1, P4, P6 and P9) of different HLA class II molecules agree with previous human studies from our group [26].

Another important data present here is that the anti-StreptInCor opsonizing and neutralizing antibodies did not induce cross-reactivity with human valve protein extracts, indicating the absence of cross-reactive antibodies. These results agree with previous studies with HLA class II transgenic mice, in which no cross reactivity against heart-tissue derived proteins and no tissue lesions were observed in several organs up to one year post-vaccination [29].

5. Conclusions

The present work reinforces the safety of and strong immune response triggered by the StreptInCor mice vaccination. Productions of antibodies that opsonize and neutralize a broad range of S. pyogenes strains indicate the potential of StreptInCor to prevent streptococcal infections without causing deleterious reactions.

Conflict of interest

The authors declare that there is no conflict of interest.

References


Intellectual properties

StreptInCor intellectual properties are in the names of Luiza Guilherme and Jorge Kalil.

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