HLA class II transgenic mice develop a safe and long lasting immune response against StreptInCor, an anti-group A streptococcus vaccine candidate


Abstract

Streptococcus pyogenes infections remain a health problem in several countries because of post-streptococcal sequelae, such as rheumatic fever and rheumatic heart disease. We developed a vaccine epitope (StreptInCor) composed of 55 amino acid residues of the C-terminal portion of the M protein that encompasses both T and B cell protective epitopes. Recently, by using human blood samples, we showed that the StreptInCor epitope is able to bind to different HLA class II molecules and that it could be considered a universal vaccine epitope. In the present work, we evaluated the immune response of HLA class II transgenic mice against aluminum hydroxide-absorbed StreptInCor. After a period of one year, several organs were analyzed histologically to verify the safety of the candidate vaccine epitope. Our results showed that StreptInCor is able to induce robust and safe and long lasting immune response without deleterious reactions in several organs. In conclusion, the results presented here indicate that StreptInCor could be considered a safe vaccine against severe streptococcus-induced diseases.

1. Introduction

Group A streptococci (GAS) are responsible for several human diseases, such as pharyngitis. These diseases may lead to post-streptococcal sequelae, including autoimmune disorders glomerulonephritis and rheumatic fever (RF). Non-autoimmune post-streptococcal sequelae that are caused by the cutaneous infections include necrotizing fasciitis and toxic shock syndrome.

The global incidence of diseases caused by GAS is not clearly resolved. In developing countries, many cases go untreated, and consequently, the health system is not notified. Although the estimates are misinformed, it is estimated that there are more than 15 million people around the world with rheumatic heart disease (RHD), the most severe sequel of RF. An estimated 300,000 new cases of RHD occur each year, and over 200,000 deaths caused by RHD each year [1]. The Brazilian public health system spent over 90 million U.S. dollars for treatment of RF and RHD patients. Furthermore, 31% of all cardiac surgeries in children are related to RF, which is also responsible for 7.5% mortality per year. Finally, it is estimated that Brazil has over 10 million cases of throat infections caused by Streptococci that lead to 30,000 new cases of RF each year [2].

The M protein is the major virulence factor of GAS. The M protein involves bacterial adhesion, evasion, and promotes immune responses to GAS because of its immunogenicity [3]. It is composed of N and C-terminal portions; the N-terminal region is hypervariable and highly immunogenic whereas the C-terminal region is highly conserved among the most GAS strains. The mechanisms leading to RF and RHD involve a cross-reaction between the N-terminal region of the alpha-helical coiled-coil M protein and self-proteins, mainly cardiac proteins. Accordingly, the homology between the M protein and human proteins myosin, tropomyosin, keratin [4] and fibrillar collagen, the major component of heart valves [5], could be involved with the autoimmune response by the molecular mimicry mechanism [6–11]. In other words, the production of cross-reactive antibodies raised against GAS could be specifically within cardiac tissue, which would lead to an increased expression of the adhesion molecule VCAM-1 [12] that facilitate the lymphocytic infiltration through the valve surface endothelium. This mechanism appears to be the initiating step for tissue damage and disease pathogenesis [12]. Both streptococcal primed CD4+ and CD8+ T lymphocytes are recruited probably under specific chemokine. This scenario might promote enhanced infiltration
of mononuclear cells to the lesion and the production of inflammatory cytokines, such as IFN-γ and TNF-α, resulting in further tissue destruction and necrosis [12–14].

The triggering of an autoimmune response involves antigenic presentation by macrophages via human leukocyte antigen-II (HLA-II) molecules to the T cell receptor. These molecules are genetically controlled and some alleles have already been described as being associated with the development of RF/RHD. Briefly, DR2 and DR4 were found in association with individuals in America; DR4, in Saudi Arabia; DR1 and DR6, in South Africa; DR7 and DR11, in Turkey; and DR7 and DR53, in Brazil. It is interesting to note that a DR7 defined molecular approach was also found in Latvians and Egyptians, and this was associated with the worsening of the valve damage [15].

The epidemiological growth of streptococcal diseases and the high treatment costs generated by complications caused by RF have encouraged many groups to study vaccine candidates to prevent GAS infections. Because of the importance and immunogenicity of the M protein in GAS infections, some vaccine models against GAS are being developed that involve different regions of this protein. A vaccine currently under clinical trials is based on the N-terminal region of the M protein and contains sequences from 26 of the most prevalent serotypes of GAS in the USA [16–19]. Additionally, an Australian group has developed a vaccine based on a C-terminal B epitope in the M protein that is conjugated to a universal T epitope and Toll-like receptor target lipopolysaccharides [20].

We have been studying a sequence of amino acids present in the C-terminus of the M protein to develop a subunit vaccine that is able to induce protection against different GAS strains. To define the vaccine epitope, we tested a large panel of approximately 900 sera and peripheral blood mononuclear cell (PBMC) samples that enabled us to identify both B and T immunodominant epitopes and then to construct a candidate vaccine composed of 55 of these amino acid residues [21]. Recently, we showed that this vaccine epitope, identified as StreptInCor (medical identity), has three-dimensional structural features that make it recognizable to any HLA class II resulting in T cell activation and differentiation into effectors and memory cells [22]. Specific antibodies raised against StreptInCor were able to recognize heterologous M1 protein in immunized isotopic mice, which suggests that our candidate vaccine has broad coverage.

MHC-II transgenic mouse models have a complete deletion of murine H2 molecules [23]. These models are an important approach to study the relationship of HLA-II molecules and autoimmunity [24–27] and therefore could be an important model to study the immune response to vaccines. In the present work, MHC class II transgenic mice carrying human HLA class II alleles were evaluated. HLA DRB1.1502 (DR2), DRB1.0401 (DR4), DQB1.0601 (DQ6) and DQB1.0302 (DQ8) transgenic mice were used to study humoral immune responses after immunization with StreptInCor. These animals were followed for 12 months to monitor the humoral immune responses and safety control. The results presented here showed high titers of specific antibodies, and no signs of tissue damage or autoimmune disorders were observed, indicating that the StreptInCor could be an immunogenic and safe vaccine.

2. Methods

2.1. StreptInCor vaccine epitope

The vaccine epitope consists of 55 amino acid residues as follows: KGLRRLDASREAAKQLEAEQQQLEENKKSEASKGLRRLDLASREAAKQVEK, as previously described [21] (patents INPI 0501290/0604997–4, PCT-BR07/000184).

2.2. Mice

Specific pathogen-free, 6- to 8-week-old HLA-class II DRB1*1502 (DR2), DRB1*0401 (DR4), DQB1*0601(DQ6) and DQB1*0302 (DQ8) transgenic mice were used in this study [24,25,28]. All transgenic mice were kindly provided by Dr. Chella S. David (Department of Immunology, Mayo Clinic, Rochester, USA) and were maintained and manipulated in the animal facility of the Tropical Medicine Institute, Medical School, University of São Paulo, Brazil. The mice were housed in autoclaved micro isolator cages (Alesco, Brazil) and manipulated under aseptic conditions. All procedures were performed in accordance with the Brazilian Committee for Animal Care and Use (COBEA) guidelines.

The presence of the HLA-class II transgene in all mice studied was verified by molecular biology techniques using skin biopsies. All mice that did not have the HLA class II transgene were discarded and were not used in this study. We also evaluated the presence of the HLA class II molecules on the surface of antigen presenting cells from the peripheral blood to control for the expression of the specific transgene (data not shown).

2.3. Immunization

HLA-class II transgenic mice received two subcutaneous doses (100 µL) on days 0 and 14 of a suspension containing 50 µg of StreptInCor absorbed onto 300 µg of Al(OH)₃ (aluminum hydroxide). Animals receiving saline plus adjuvant were used as experimental controls for immunization. Sera samples were obtained from mice on day 28 following immunization while under light anesthesia by retro-orbital puncture.

2.4. Sera antibody measurement

Sera antibody titers were determined by ELISA. Briefly, 1 µg of StreptInCor vaccine epitope and overlapping peptides, porcine cardiac myosin (Sigma, USA), or M1 recombinant protein (clone kindly provided by Prof Patrick Cleary, University of Minnesota Medical School, MN, USA) produced and purified in our lab, were diluted in coating buffer (0.05 M carbonate–bicarbonate, pH 9.6, 50 µL/w) and was added to a 96-well MaxiSorp assay plate (Nunc, Denmark). After overnight incubation, the plates were blocked with 0.25% gelatin (Sigma) diluted in 0.05% Tween-20 (Sigma, USA) in PBS (dilution buffer) for 1 h at room temperature. Starting at 1/100 in dilution buffer, serial 2-fold dilutions were added to the plates (50 µL/w). After a 2 h incubation at 37 °C and three washes (200 µL/w) with 0.05% Tween 20 in PBS (rinse buffer), the plates were incubated for another hour at 37 °C with peroxidase–conjugated anti-mouse IgG (Pharmingen, USA) at 1:2000 in dilution buffer (50 µL/w). The plates were then washed three times (200 µL/w) with rinse buffer, and the reaction was revealed with 50 µL/w of 0.4 mg/mL orthophenylenediamine (OPD, Sigma, USA) in 100 mM sodium citrate (Merck, Germany) containing 0.03% H₂O₂ (Merck). After 10 min at room temperature, the reactions were stopped using 4 N H₂SO₄, and the optical density was evaluated using a 490 nm ELISA filter in an MR4000 ELISA plate reader (Dynatech, USA). To study IgG isotypes, the biotinylated conjugates anti-mouse IgG1, IgG2a, IgG2b and IgG3 (Pharmingen, USA) were used at 2 µg/mL (50 µL/w) and incubated for 1 h at 37 °C. After three washes with rinse buffer, the plates were incubated with streptavidin peroxidase (Pharmingen, USA) at 1/1000 (50 µL/w) for 1 h at 37 °C. Finally, the reaction was finished as described above.

2.5. Western blot

Lysate of heart tissue was obtained from post mortem normal human myocardium, separated by 10% SDS–PAGE and blotted
onto nitrocellulose membranes as described [29,30]. The blots were divided into strips and blocked with Tris buffered saline containing 5% of skim milk. The strips were sequentially treated with a pool of immunized and non-immunized (controls) transgenic mice sera, followed by a treatment with anti-mouse IgG alkaline phosphatase and revealed in the presence of NBT-BCIP solution (Invitrogen, USA). Positive control: mouse anti-porcine myosin serum. Negative control: pre-immune mouse serum.

### 2.6. Histopathological analysis

After 12 months, immunized mice and controls were sacrificed and the heart, liver, spleen, brain, kidney and articulations were collected. The tissues were immediately fixed in PBS containing 10% formaldehyde, paraffin-processed, and histological sections were evaluated after staining with hematoxylin and eosin (H&E).

### 3. Results

#### 3.1. StreptInCor induces high IgG antibody titers

StreptInCor was able to induce a robust immune response in all HLA class II transgenic mice studied 28 days after immunization. DQ6 and DQ8 transgenic mice presented the highest titers of total IgG (>1:12,800) (Fig. 1). We observed variable IgG production among the DR4 transgenic mice (>1:800 and 1:12,800) (Fig. 1). Among the IgG isotypes, IgG1 and IgG2b were induced in all the transgenic mice and IgG3 was only produced in the DQ8 transgenic mice (Fig. 1). Control animals receiving only aluminum hydroxide did not present any reactivity to StreptInCor (data not shown). To verify whether the immune response against StreptInCor was specific, we analyzed the reactivity of the immunized transgenic mice recognize the immunogenic vaccine epitope in the heterologous M1 recombinant (rM1) protein. Our results showed that all DR2, DR4, and DQ8 mice and 3 out of 6 DQ6 mice were reactive against rM1 protein (Fig. 2). It is interesting to note that the levels of anti-IgG antibodies against rM1 protein were lower (1:100 to 1:3200) (Fig. 2). Additionally, none of the transgenic mice developed antibodies against either porcine cardiac myosin (Fig. 2) or human myocardium-derived proteins (Fig. 3) indicating the absence of cross-reactivity with cardiac proteins.

All the mice were followed for one year before they were sacrificed. The amount of IgG was evaluated at 1, 4, 8, and 12 months. Our results showed a decreased amount of IgG present in immunized mice after 4 months (Fig. 4), and most of the mice maintained low reactivity IgG titers until 1 year post-immunization (Fig. 4).

#### 3.2. Immunized HLA class II transgenic mice recognize StreptInCor overlapping peptides

We analyzed the humoral immune response of HLA class II Tg-mice against 8 StreptInCor-derived overlapping peptides that cover the entire vaccine epitope sequence and encompassed the possibilities of processing and presentation by antigen-presenting cells.
Table 1

<table>
<thead>
<tr>
<th>Overlapping peptides sequences (20 aa residues)</th>
<th>DR2</th>
<th>DR4</th>
<th>DQ6</th>
<th>DQ8</th>
</tr>
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<tbody>
<tr>
<td>KGLRRLDAASREAAQKLEAE</td>
<td>6'/6</td>
<td>5'/6</td>
<td>2'/6</td>
<td>5'/6</td>
</tr>
<tr>
<td>KGLRRLDAASREAKQVEKAL</td>
<td>5'/6</td>
<td>5'/6</td>
<td>3'/6</td>
<td>4'/6</td>
</tr>
<tr>
<td>LDASREAKQLEAEQKLEE</td>
<td>4'/6</td>
<td>5'/6</td>
<td>3'/6</td>
<td>6'/6</td>
</tr>
<tr>
<td>KLEQNKISEASRGLRDL</td>
<td>5'/6</td>
<td>4'/6</td>
<td>3'/6</td>
<td>5'/6</td>
</tr>
<tr>
<td>KISEASRGLRDLDAASREA</td>
<td>5'/6</td>
<td>2'/6</td>
<td>2'/6</td>
<td>3'/6</td>
</tr>
<tr>
<td>SEASRGLRRLDAASREK</td>
<td>5'/6</td>
<td>4'/6</td>
<td>3'/6</td>
<td>5'/6</td>
</tr>
<tr>
<td>ASRGLRRLDAASREAKQV</td>
<td>4'/6</td>
<td>4'/6</td>
<td>1'/6</td>
<td>2'/6</td>
</tr>
</tbody>
</table>

Humoral immune response of six transgenic mice for each lineage after 28 days post-immunization was tested by ELISA against overlapping peptides sequences, as previously described by us [21,22]. Positive immune response was considered when antibodies titers were ≥1:100 and ≤1:3200.

4. Discussion

We developed a vaccine epitope (StreptInCor) composed of 55 amino acid residues of the C-terminal portion of the M protein that encompasses both T and B cell-protective epitopes [21]. The structural, chemical, and biological properties of this peptide were evaluated, and we show that StreptInCor is a very stable molecule, which is an important property for a vaccine candidate. Additionally, our previous results show that humans, bearing different HLA class II molecules recognize StreptInCor, which demonstrates the universal character of this vaccine [22]. It is interesting to note that both healthy individuals and rheumatic fever and rheumatic heart disease patients were able to respond to StreptInCor peptide. No cross reactivity against human myocardium and valve proteins was observed, indicating that StreptInCor is immunogenic and safe [21].

The role of HLA class II molecules in the antigen presentation and that this vaccine should avoid autoimmune reactions, were considered in the present work; therefore, we evaluated the capacity of HLA class II transgenic mice to recognize the vaccine epitope combined with aluminum hydroxide adjuvant while not inducing autoimmune reactions. This adjuvant has been used in
Fig. 5. Histological analysis of tissues from immunized HLA class II transgenic mice. (A) Histological sections of the heart chambers, (10×); (B) tricuspid valve (25×); (C) mitral valve (50×); (D) aortic valve (25×); (E) joint (25×); (F) kidney (200×); (G) spleen (400×); (H) brain, and (I) liver. RA, right atrium; RV, right ventricle; LA, left atrium; LV, left ventricle; S, septum; A, aortic valve; T, tricuspid valve; M, mitral valve; JC, joint cavity; ST, synovial tissue; BM, bone marrow; EH, extramedullary hematopoiesis; moderate **intense. H&E, hematoxylin and eosin stain.

veterinarian and human vaccines since 1930 and causes very little systemic toxicity [31].

The presence of the HLA class II transgene will affect the immune response in the whole mouse since thymic selection will interfere with the interactions between T lymphocytes and antigen presenting cells and with the activation of B lymphocytes in the periphery. The biological properties of HLA class II molecules, together with testing their role in a transgenic mice model, are useful for new vaccine studies. Recently, our group showed that the HLA class II transgenic mice are able to respond to multi-epitopic vaccines against HIV by inducing proliferation of both CD4+ and CD8+ T lymphocytes and the production of IFNγ [32].
The data presented here show that all HLA class II transgenic mice (DR2, DR4, DQ6 and DQ8) immunized with StreptInCor plus aluminum hydroxide were able to produce specific IgG antibodies that also recognize the vaccine epitope in the context of a heterologous M protein. According to our previous data on human sera and peripheral blood cells [22], the results presented here also indicated that antigen presenting cells of HLA class II transgenic mice bearing DR2, DR4, DQ6 and DQ8 molecules were able to present several peptides encompassed by StreptInCor vaccine epitope, probably via the TCR with activation of both T and B lymphocytes. In addition, it is interesting to note that transgenic mice bearing the HLA-DR molecules were more responsive than those bearing the second HLA class II molecules (DQ6 or DQ8). In agreement with these data the IgG specific responses in DR2 and DR4 transgenic mice were slightly better than in mice bearing DQ6 and DQ8 molecules. Although some mice became nonresponsive a year after the immunization, the immune responses to StreptInCor were maintained for up to a year. These results also indicated that the vaccine epitope is able to induce a long period of specific immune responses, with IgG1 predominance due to the effects of the adjuvant. The balance between humoral and cellular immune responses induced by adjuvant formulations can be addressed through the isotype profile of the vaccine-specific IgG1 and IgG2a antibodies produced. The IgG1 isotype switch is dependent of IL-4 production in opposite to isotype IgG2a, which is IFN-γ dependent. We observed a huge predominance of specific IgG1 when compared to IgG2a and also to IgG3, another IFN-γ dependent isotype. It is interesting to note that some IgG2b, a TGF-β-dependent switch, was seen in some animals from all groups studied (DR2, DR4, DQ6 and DQ8). Finally, aluminum adjuvants are responsible for Th2 polarization, resulting in increased humoral immunity, mediated by production of IgG1 isotype.

Considering pharyngitis is among the most common S. pyogenes infections, the induction of mucosal immune responses, mainly by IgA secretions, is attractive. Accordingly, other adjuvants are being assayed to obtain both systemic and mucosal immune responses. One of the major challenges of producing a vaccine against S. pyogenes is to not induce autoimmune responses and diseases such as RF and RHD. Although we know the mechanisms that lead the disease in humans [13], there have been no ideal in vivo models of the disease, except for in the Lewis rat [33], until our current study.

As myosin is a putative auto-antigen involved in RHD development [33–39], we used both human myocardium-derived proteins and porcine cardiac myosin to evaluate the presence of cross-reactive antibodies that could be triggered by the immunizations. No specific cross reactivity against heart proteins was observed indicating that StreptInCor did not induce autoimmune reactions.

Myosin heavy chains have been categorized into several classes based on comparisons and phylogenetic analysis of the conserved regions [40–42]. It is interesting to note that the coiled coil region differs among the species studied however global results suggested a common coevolution of myosin head, neck and tail domains [40–42]. So, the fact that we used both porcine myosin and human cardiac protein extract, in which cardiac myosin is the major protein, strongly indicated that StreptInCor vaccine epitope is unable of inducing autoimmune reactions.

Although the histopathology of mice assessed a year after the last immunization showed some alterations, such as extramedullary hematopoiesis, liver steatosis, and infiltration of mononuclear cells in the kidney, these observations were also observed in the control animals. This finding suggests that these features are not due to the immunization with the vaccine epitope and are most likely due to aging of the mice. In support of this finding, the analysis of the heart tissue, with a special focus on the valves, and the other organs after 1 year did not display any specific RF lesions.

Despite these promising results, humans are the only hosts for GAS. Although several studies have been conducted to find a suitable animal model, there is no suitable animal model that can desiccate the autoimmune process of RF and RHD.

5. Conclusions

All the results presented here indicate that the StreptInCor vaccine epitope induces a robust and long lasting immune response in transgenic mice and not induces autoimmune reactions and can be considered a promising vaccine candidate to prevent RF.

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References


