Effects of immunization with *Streptococcus mutans* surface antigens in auto-reactivity of BALB/c mice

Efeitos da imunização com antígenos de superfície de Streptococcus mutans *na auto-reatividade de camundongos BALB/c*

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Abstract

BALB/c mice were hiperimmunized with surface antigens of *Streptococcus mutans*, GS5 strain. The immunization increased significantly anti-heart and anti-myosin IgG, free and present in immunecomplexes. In Western-blotting, the autoantibodies displayed a notable reactivity with cardiac myosin and cardiac tissue proteins, mainly with a 35 kDa peptide. The histological analysis of hearts demonstrated absence of significant damage in valves and myocardium, and immunohistochemistry showed absence of antibodies linked in cardiac tissue. The treatment with cyclophosphamide was able to reduce autoantibody levels but did not alter de histological aspect of heart. These findings demonstrated that surface antigens *S. mutans*, GS5 strain, were able to induce autoantibody synthesis without potential to initiate cardiac damage.

Uniterms

Streptococcus mutans; molecular mimicry; heart; myosin.

Introduction

Rheumatic fever is an inflammatory disease that represents the main cause of cardiovascular morbidity and mortality among individuals from five to 24 years of age in developing countries. In general, this disease develops in genetically susceptible individuals, after oropharyngeal infections caused mainly by *Streptococcus pyogenes* that shares antigenic determinants with cardiac valves[1].

Probably the bacterium antigen involved is the M protein, an important component of the streptococcal cellular surface with anti-phagocytic properties[3,4]. The cardiac myosin has been identified as the target-antigen[4], however antibodies to myosin are not exclusive of rheumatic fever, appearing in other conditions such as in the myocarditis induced by the virus Coxsackie B3 (CB3)[14].

The antigenic mimicry between the myocardium and cariogenic streptococci species, such as *Streptococcus mutans*, has been also studied. Van de Rijn et al.[18] (1976) related cross-reactivity between the sera of rabbits hiperimmunized with *S. mutans* and human cardiac tissue. Since then, it has been a great obstacle to the development of an anti-caries vaccine.

The bacterium antigen involved in the induction of these autoantibodies is still unknown. Studies with the antigen I/II (B, P1, SR), the main cellular surface antigen of the microorganism, did not confirm the initial suspicions of cross-reactivity[19]. It was also unknown if these antibodies would be sufficient to induce a myocarditis in laboratory animals[11].

Although there is a strong tie between the cardiac pathology and the bacteria of *Streptococcus* genus,

little attention has been given to the most prevalent species in the human dental caries.

In this study, we intended to verify if the immunization with *S. mutans* surface antigen would be able to produce cardiac damage in BALB/c mice, if the antibodies would be present in these lesions, and if concomitant use of an immunossupressor, such as the cyclophosphamide, would alter the development of the inflammatory condition.

Materials and Methods

Preparation of antigens:

a) Streptococcus mutans: The methodology used here was described by Leão et al, 2000[10]. Strain GS-5 was cultured in triptic soy broth (TSB, Difco) and incubated at 37°C, for 24 h, in atmosphere with CO_2 5%. After this period, growth was interrupted with formaldehyde 0.075% and the culture maintained for 18 h at 4°C. The cells were harvested by centrifugation and washed for three times in Tris-HCl 0.125 M; EDTA 0.01 M, pH 7.5. Then, the cells were ressuspended in 50mL of the same buffer solution and phenylmethylsulphonylfluoride (PMSF-Sigma) was added in a final concentration of 0.005 M. Glass beads were added to this suspensions and strongly mixed in Kline agitator overnight at 4°C. Then, the lisate was centrifuged for 30 minutes at 4°C, and the supernatant was discharged. Five milliliters of the extraction buffer (Tris-HCl 0.150 M, urea 6 Mm /Tween-20 1%), was added to the precipitate and boiled at 100°C for five minutes. Then, this preparation was conserved at 4°C for 24 hours. After this period, the extract was centrifuged and the supernatant was dialyzed against PBS, concentrated by ultra-filtration, lyophilized and stored at -20°C. This preparation constituted the surface antigen;

b) Heart extract: Twenty mice of BALB/c race of both genders were sacrificed and their hearts were removed. After, the material was washed with PBS for removing the blood. Hearts were macerated and filtered in sterilized cotton gauze. The filtrate obtained was stored at -20°C. Five milliliters of a buffer solution containing Tris-HCl 0.15 M, urea 6 M, β-mercaptoethanol and 1% Tween 20 were added. The mixture was boiled for five minutes at 100°C and stored at 4°C, for 24 hours. After this period, the material was centrifuged and the supernatant was dialyzed against PBS overnight. Then, the filtrate and the dialyzed product were mixed and concentrated by ultra-centrifugation. The concentration of the protein was estimated in 30µg/µl, by the methodology described by Bradford[2] (1976);

c) Myosin from cardiac muscle was obtained from Sigma.

Immunization:

BALB/c mice, with six weeks of age, were divided in three groups. Mice from group I (n = 5) were immunized weekly, for six weeks, with 50µL of S. mutans surface antigen, ressuspended in 100µL of sterile isotonic physiologic solution, emulsified in 100µL of the adjuvant. The first injection was administered intradermically and the others by intraperiotoneal via. Two injections in Freundís incomplete adjuvant and more four in 1% aluminum sulfate and potassium were performed. One week after the last injection, a booster injection with 300µL of the surface antigen in sterile saline solution was given. Group II (n=5) was immunized following the same protocol used for group I, however, one day before each one of the last 3 injections, including the booster one, 100mg/kg of cyclophosphamide was administered for each animal. The control group received injections with 100µL of sterile physiologic solution and 100µL of the same adjuvants, in the same days in relation to the other groups. One week after the booster, animals were anesthetized, bled through retroocular via and the serum was obtained for antibodies and immunocomplexesí analysis. After bled, the animals were sacrificed by cervical vertebra dislocation and hearts were removed to the histological analysis.

Antibodies analysis:

a) ELISA: Polystyrene plates, with ninety-six-well, were coated with heart extract (100µg/mL), S. mutans surface antigen (100µg/mL) and myosin (5µg/mL), in 0.1 M carbonate buffer (pH 9.6). The plates were incubated for 2 hours at 37°C, washed with PBS and the free sites of the polystyrene were blocked with 0.5% gelatin (G) in phosphate buffered saline (PBS) for 30 minutes, at 37°C. After this period, plates were washed with 0.1% Tween 20 PBS (T- PBS). Mice sera were, then, diluted at 1/25 in PBS-T with 0.5% gelatin (PBS-T-G) and added to the wells. In the other wells, sequential dilutions

in the ratio of two were performed. Plates were incubated at 37°C, for 2 hours, washed with PBS-T, and then, 50µL of anti-IgG of mice peroxidase-labelled (Sigma), in the concentration of 1 µg/mL were added to the wells (one hour, at 35°C). After washing with PBS-T-G, peroxidase activity was revealed using the substrate o-phenylenediamine (OPD-Sigma): 6mg in 12mL of 0.1M citrate buffer (pH 5.5) and 10µL of 30% H_2O_2, 100µL per well. The reaction was developed for 15 minutes at room temperature and immediately blocked with 2.5 N H_2SO_4. Optical densities (OD) were measured at 490 nm;

b) Polyacrylamide gel electrophoresis and Western-blotting: The technique of Western-blotting was developed according to Towbin et al.[17] (1979). After the polyacrylamide gel electrophoresis9, proteins were transferred to nitrocellulose paper (Amersham). 50µg of the heart antigen, 25µg of the S. mutans surface antigen and 5µg of myosin were applied in the gel, in each site. Then, the free sites of the nitrocellulose paper were blocked with PBS-T containing 5% protein milk, for two-hours, under shaking. After this, the incubation with the serum obtained from the mice and diluted to 1/50 or 1/100 in the same buffer was accomplished overnight. In the day after, the nitrocellulose was washed with PBS-T, and after, incubated with the conjugate anti-IgG of mice peroxidase-labelled (two-hours at room temperature, under shaking). After this period, the nitrocellulose papers were washed again with PBS-T and then, incubated with the substrate diaminobenzidine (DAB-Sigma) at 0.01% in 0.1 M Tris-HCl (pH 7.5) and 0.003% H_2O_2. The reaction was developed for 20 minutes, and immediately blocked with distilled water.

Immunocomplexes analysis:

The precipitation technique by PEG (MW 6000), described by Ohlson and Zetterstrand[15] (1985), was used. I was added 50µL of PBS (pH 7.4) to 50µL of the serum. Then, to the 100µL of the diluted serum, 100µL of 5% PEG 6000 in 0.02 M phosphate buffer (pH 7.4) containing 0.15 M NaCl. The solution was shaken and incubated overnight at 4°C. After the incubation, the solution was centrifuged at 1500 g for 20 min at 4°C. The supernatant was discharged and the precipitate was washed twice with 2.5% PEG prepared as previously described. Then, the precipitate was dissolved in 100µL of 0.15 M NaCl, 0.02 M phosphate buffer, 0.01 M EDTA and 0.05% Tween 20 and incubated at 37°C for 30 minutes. After this, the suspension was diluted five times and tested by ELISA, in a maximum period of 30 minutes.

Histological analysis:

Hearts were removed and put in a fixing solution (10% formaldehyde) for 24 hours. After fixation, pieces were hemi-sectioned and imbedded in paraffin. Five-micrometer sections were cut in and stained with hematoxylin and eosin. Histological analysis was performed, in light-microscopy, where inflammation focus and lesion in the myocardium and cardiac valves were researched.

Immunohistochemical analysis:

From the pieces obtained for the histological analysis, 4 to 5µm slices were cut. The slides were submitted to pressure in citrate buffer (pH 6.0) for 30 min. After they were cold and washed in water for 5 minutes, the laminas received 4 baths of 5 minutes in a solution of 20V hydrogen peroxide. They were washed and immersed in PBS and incubated with PBS with 1% BSA, in a humid chamber at 3°C overnight. In the day after, they were washed and incubated with the conjugate avidin-biotin-peroxidase anti-immunoglobulin, for 30 minutes each step, at room temperature. Then, they were well washed with PBS, and the reaction was revealed with 0.06g of DAB, 100mL of PBS and 1mL 20v hydrogen peroxide, for 5 minutes. Slides were counterstained by Harrisí hematoxylin for 2 minutes and washed. They were submitted to light microscopy were the positive sites exhibited brownish color.

Results

Detection of antibodies by ELISA:

The immunization of mice with *S. mutans* surface antigen induced a strong response of specific antibodies to this antigen. The increase in levels of anti- *S. mutans* IgG was accompanied with an increase in the level of antibodies capable of recognizing cardiac antigens and myosin (Figures 1 and 2). The immunized animals and treated with cyclophosphamide presented a great reduction in the levels of IgG reactive with these antigens, whose values were similar to the values observed for control animals.

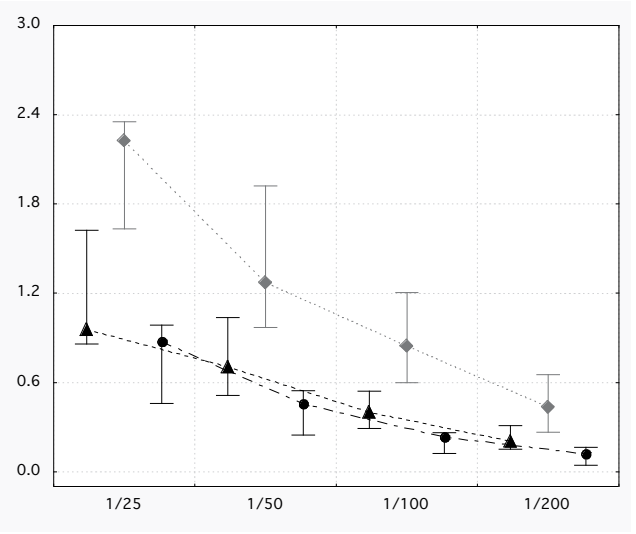

Figure 1 – ELISA- Median values of the IgG levels reactive with cardiac antigens in control animals (red-triangle), immunized with the antigen – group I (green-losangle) and immunized and treated with cyclophosphamide- group II (blue-circle).

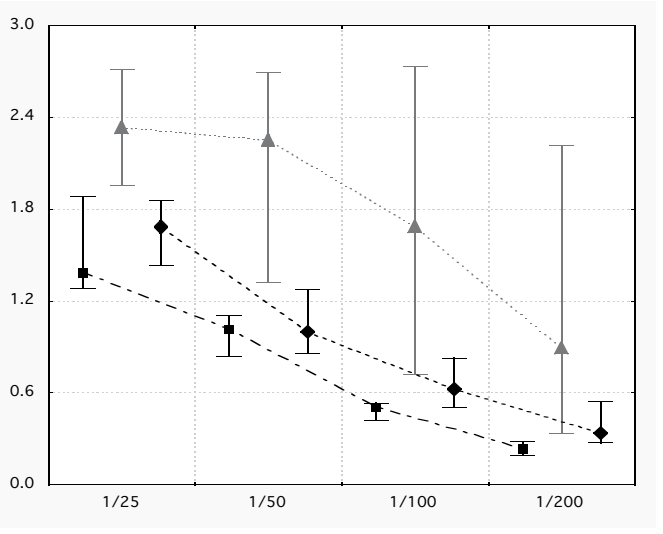

Figure 2 – ELISA – Median values of the IgG levels reactive with cardiac muscle myosin in control animals (square-red), immunized with the antigen – group I (triangle-green) and immunized and treated with cyclophosphamide – group II (losangle-blue).

Detection of antibodies by Western-blotting:

IgG of the immunized animals from groups I and II recognized several fractions of different molecular weights of the streptococcal antigenic extract. Control animals did not recognize any band of this antigen. Antibodies of these animals were not able to recognize fractions of the cardiac extract, while the sera of the animals included in group I (3 and 4) and group II (5 and 6) mainly recognized a band of 35 kDa (Figure 3). The reactivity of the IgG among the animals from groups I and II for cardiac myosin could be weakly visualized.

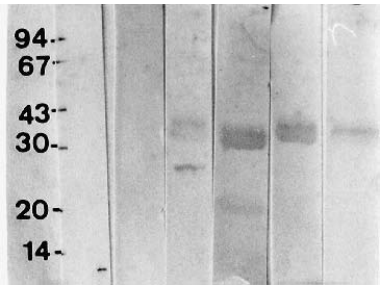

Figure 3 – Western-blotting – Reactivity for the cardiac antigens of the serum IgG of the control (1 and 2), animals immunized with S. mutans surface antigens – group I (3 and 4) and immunized and treated with cyclophosphamide – group II (5 and 6).

Detection of antibodies fractioned from immunocomplexes:

These results were similar to free antibodies levels. The animals from group I presented higher level of fractioned IgG from immunocomplexes reactive with streptococcal antigens when compared with the animals from groups II and control. The levels of IgG for cardiac antigens and cardiac myosin could be observed. Higher levels among the animals from group I and lower levels for animals from groups II and control group.

Histological analysis:

Very discreet alterations could be observed in all the histological slices studied. Ischemic necrosis areas of some myocytes, observed as cells with a more eosynophilic staining with picnotic nuclei and some retracted fibers and cells with increased nuclei. The cardiac valves, endocardium and pericardium, showed normal aspect in all the studied groups (Figure 4). Just in two immunized animals inflammatory reaction signs in the epicardial site were observed. The infiltrates were mainly mononuclear (Figure 5).

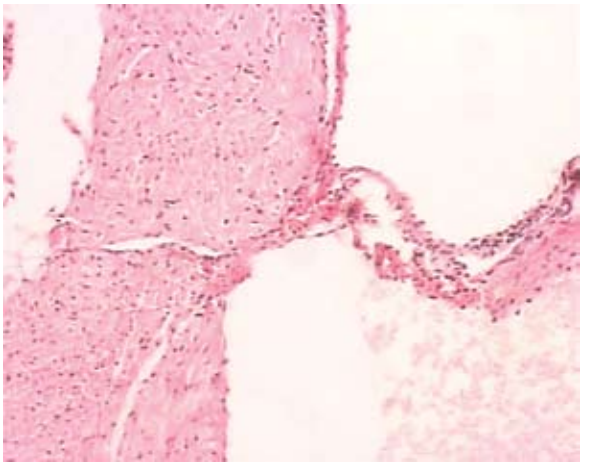

Figure 4 – Histological slice of BALB/c mouse. Normal aspect of control mice, x 40, stained by HE

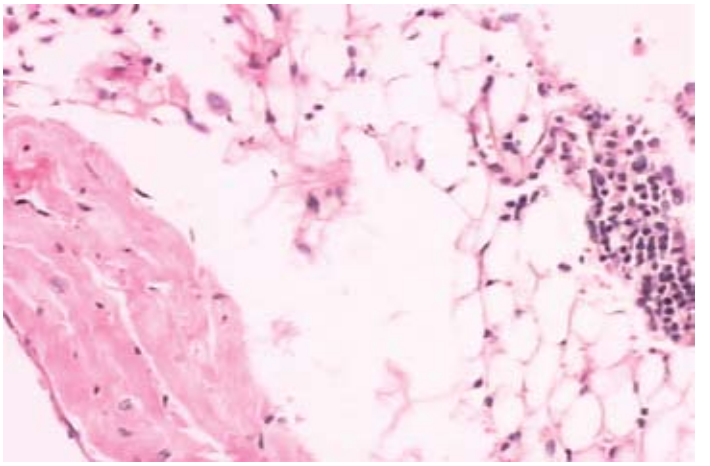

Figure 5 – Histological slice of BALB/c mouse. Mononuclear inflammatory infiltrates in the epicardium of mice from group I, x200, stained by HE.

Immunohistochemical analysis:

The results observed in immunohistochemical analysis showed no cellular staining in the myocardium, pericardium, endocardium and cardiac valves, indicating absence of antibodies in these sites. Some reaction was observed inside vessels and hemorrhagic regions, where the presence of antibodies is natural. Curiously, the most marked site, in all the studied animals, was the epicardial, mainly around adipose cells.

Discussion

In the present study, we verified that the hiperimmunization of BALB/c mice with *Streptococcus mutans* surface antigen increased the serum levels of antibodies reactive with cardiac antigens and myosin. Similar results were observed by Van de Rijn et al. [18] (1976) and Ferretti et al.[5] (1980) who observed the presence of specific antibodies for the cardiac tissue and muscle antigens after hiperimmunization of rabbits with whole cells of the microorganism.

Transient increasing of natural autoantibodies was observed in the sera of patients infected by different microorganisms[12,16]. Then, the immunization with *S. mutans* surface antigen could be induced by a normal transient increase of natural autoantibodies.

For Neu et al.[13] (1990), the antibodies reactive with myosin do not participate of the induction of the autoimmune myocarditis, once these immunoglobulins do not bind to intact myocytes. Liao et al.[11] (1995) demonstrated that anti-myosin antibodies could cause myocarditis in susceptible animals. DBA/2 mice developed the pathology after the administration of monoclonal anti-myosin antibodies, while BALB/c mice did not. In this study, we included BALB/c mice that are resistant to cardiac lesions by autoantibodies according to the last authors. In fact, however the animals increased the production of antibodies reactive with the cardiac tissue, the cardiac lesion observed was not significant.

In a previous study[10], we observed that animals immunized with whole cells of *S. mutans* and restimulated with surface antigen of the microorganism presented significant cardiac lesions, although the levels of anti-heart antibodies were not so elevated. Considering this, we suspect that the cardiac lesions could be more related with cellular response or most of the antibodies could be connected to tissues forming complexes with antigens.

The role of the surface antigen in the induction of cellular response for the cardiac tissue could not be confirmed in this study. We found high levels of antibodies reactive with the cardiac tissue in the animals immunized with the antigenic extract and, in the histological analysis, we verified that these animals did not develop significant cardiac lesions. Our results demonstrated that the autoantibodies synthesized by the animals were not sufficient to cause extensive lesions in the cardiac tissue.

When we fractionated the immunocomplexes in the sera of the animals, we could observe reactive levels similar to that of free antibodies. In the immunohistochemical analysis, we could not prove the participation of the immunoglobulins in the process of tissueís lesion. The evidences in the cardiac tissues were rare, weak and heterogeneous, and suggested few or no binding of antibodies to the myocytes. Curiously, the adipose membranes of the epicardium were the most marked areas, in all the studied groups, including the control one. These antibodies can represent a natural response of auto-antibodies to the oxided molecules of low-density lypoprotein (LDL-cholesterol). Findings of this nature have been described in man and associated to atherosclerotic diseases of the coronaries[7].

Hughes et al.[6] (1980) showed that the antigen B, very similar to the Ag I/II, presented homology with the cardiac tissue. On the other hand, Wu and Russell[19] (1990) showed that the Ag I/II did not induce the production of anti-heart antibodies. The antigenic preparation in our experiments did not contain high quantities of Ag I/II since the isolate GS 5 do not produce the 190kDa protein but other with a lower molecular weight[8]. The antigen was mainly composed by these other proteins of the cellular membrane of the microorganism, in this case, were able to induce the production of anti-heart antibodies, but not cardiac lesion. In our previous study *S. mutans* surface antigen used to the restimulation of the second group of animals contained higher quantities of the AgI/II because the isolate CCT 1910 was employed[10].

Western-blotting technique permitted us to observe that the antigen of the cardiac tissue recognized by the sera of the hiperimmunized animals was mainly a protein of 35kDa. This finding is in accordance to our previous study[10] and with Latif et al.[9](1993) that demonstrated in patients with dilated cardiomiopathy, anti-heart antibodies that recognized, among other proteic fractions, a polypeptide of 35kDa, identified as tropomyosin.

Different authors consider the autoantibodies an epyphenom in the autoimmune myocarditis[13], and where the role of the T lymphocytes in the processí induction was widely proved. On the other hand, a participation of these immunoglobulins in the diseaseís progression by the antibody dependent cytotoxicity has been suggested. New studies might be performed aiming to clarify the immunopathogenic potential of different streptococci isolates in BALB/c mice, and define the true pathogenic mechanism of the autoimmune cardiac lesion.

Conclusion

The hiperimmunization of BALB/c mice with *Streptococcus mutans* surface antigen, isolate GS5, increased the serum levels of immunocomplexes and reactive free antibodies with extracts of heart and cardiac myosin.

Cyclophosphamide reduced the levels of free antibodies and of antibodies present in the immunocomplexes, especially in those reactive with the auto-antigens.

The immunization did not induce significant inflammation. Histological analysis of miceí hearts showed only few tissue alterations. Moreover, no antibodies bound to the cardiac tissue of the immunized animals were found.

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Resumo

Camundongos BALB/c foram hiperimunizados com antígenos de superfície de *Streptococcus mutans*, cepa GS5. Observamos que a imunização elevou significativamente os níveis de IgGs anticoração e antimiosina livres e presentes em imunocomplexos. No *Western-blotting*, estes auto-anticorpos reagiam com miosina cardíaca e principamente uma banda de 35 kDa do extrato cardíaco. A análise histológica dos corações demonstrou ausência de alterações significativas nas válvulas ou miocárdio. Também não foram encontrados anticorpos ligados ao tecido cardíaco. O tratamento com ciclofosfamida foi capaz de reduzir os níveis de auto-anticorpos, não alterando porém o aspecto histológico do coração. Os resultados deste trabalho mostraram que os antígenos de superfície desta cepa de *S. mutans* não foram capazes de iniciar uma lesão cardíaca em camundongos BALB/c, embora elevassem consideravelmente o nível de anticorpos livres e ligados em imunocomplexos para coração e miosina.

Unitermos

Streptococcus mutans; coração; reatividade cruzada; miosina.

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