Antibodies Targeting Hsa and PadA Prevent Platelet Aggregation and Protect Rats against Experimental Endocarditis Induced by Streptococcus gordonii

Stefano Mancini, Carmen Menzi, Frank Oechslin, Philippe Moreillon, José Manuel Entenza
Department of Fundamental Microbiology, University of Lausanne, Lausanne, Switzerland

Streptococcus gordonii and related species of oral viridans group streptococci (VGS) are common etiological agents of infective endocarditis (IE). We explored vaccination as a strategy to prevent VGS-IE, using a novel antigen-presenting system based on non-genetically modified Lactococcus lactis displaying vaccinogens on its surface. Hsa and PadA are surface-located S. gordonii proteins implicated in platelet adhesion and aggregation, which are key steps in the pathogenesis of IE. This function makes them ideal targets for vaccination against VGS-IE. In the present study, we report the use of nonliving L. lactis displaying at its surface the N-terminal region of Hsa or PadA by means of the cell wall binding domain of Lactobacillus casei A2 phage lysine LysA2 (Hsa-LysA2 and PadA-LysA2, respectively) and investigation of their ability to elicit antibodies in rats and to protect them from S. gordonii experimental IE. Immunized and control animals with catheter-induced sterile aortic valve vegetations were inoculated with 10⁶ CFU of S. gordonii. The presence of IE was evaluated 24 h later. Immunization of rats with L. lactis Hsa-LysA2, L. lactis PadA-LysA2, or both protected 6/11 (55%), 6/11 (55%), and 11/12 (91%) animals, respectively, from S. gordonii IE (P < 0.05 versus controls). Protection correlated with the induction of high levels of functional antibodies against both Hsa and PadA that delayed or totally inhibited platelet aggregation by S. gordonii. These results support the value of L. lactis as a system for antigen delivery and of Hsa and PadA as promising candidates for a vaccine against VGS-IE.

The viridans group streptococci (VGS) are commensal bacteria of the human oral cavity but can cause infective endocarditis (IE) when they enter the bloodstream (1). VGS-IE accounts for ca. 20% of IE cases (1) and generally results from cumulative exposure to recurrent bouts of transient low-grade bacteremia, occurring during normal day-to-day activities, including tooth brushing, flossing, and chewing (2–4). Under these circumstances, antibiotic prophylaxis regimens cannot be recommended to prevent VGS-IE. Based upon this assumption, the American Heart Association (AHA) and the European Society of Cardiology (ESC) drastically restricted the use of antibiotic prophylaxis for IE in at-risk patients undergoing dental procedures (5, 6). The British National Institute for Health and Clinical Excellence (NICE) went even further and suggested the total abolition of antibiotic-based prophylaxis (7). However, since the AHA guidelines’ revision in 2007, a significant increase in the incidence of VGS-IE has been reported in the United States (8). This suggests that the development of an effective prophylactic strategy against VGS-IE is an unmet medical need.

A number of immunization strategies for the prevention of VGS-IE have been explored in the past and have been shown to protect animal models from IE (9–13). However, no further step has been made toward the development of vaccines against oral streptococci, and no vaccine currently exists against VGS-IE in the market.

The oral VGS bacterium Streptococcus gordonii is a major etiological agent of IE (14). S. gordonii is well known for its ability to interact with human platelets, a step that is considered crucial for the initiation and progression of IE (15, 16). S. gordonii adheres to platelets via the surface-anchored proteins Hsa (hemagglutinin salivary antigen) and PadA (platelet adherence protein A). Hsa mediates the initial interactions with platelets by binding the membrane glycoprotein GPIIb/IIIa. The high on-off rate of GPIIb/IIIa allows rapid loss and formation of new interactions between platelets and the immobilized bacteria, leading to platelet rolling over the microorganisms. This process, which slows down platelets from the high shear stress experienced in the bloodstream, is then followed by the interaction of PadA with the platelet receptor GPIIb/IIIa, which promotes firm bacterium-platelet adhesion and ultimately leads to platelet aggregation (21, 22).

Due to their role in platelet aggregation, Hsa and PadA (18, 22) represent intuitively logical candidates for vaccine development against IE induced by VGS. In the present study, we employed a recently developed antigen display system (23) to immunize rats with both adhesins. This system is based on nonliving, non-genetically modified Lactococcus lactis cells displaying on the cell wall the functional N-terminal region (directly involved in platelet activation) of Hsa or PadA fused to the C-terminal domain of Lactobacillus casei A2 phage lysine (LysA2), which was previously shown to bind to the cell wall of a wide spectrum of lactic acid bacteria (24). The immunizations with L. lactis displaying Hsa-LysA2 (L. lactis Hsa-LysA2) and L. lactis displaying PadA-LysA2 (L. lactis PadA-LysA2), individually or after coimmunization,
were evaluated for their ability to induce specific antibodies in rats and to protect against S. gordonii experimental IE.

Our results indicate that immunization of rats with L. lactis Hsa-LysA2 and/or L. lactis PadA-LysA2, individually or together, was effective in inducing functional Hsa- and PadA-specific antibodies that inhibited platelet aggregation and protected against S. gordonii experimental IE. Taken together, these results support the suitability of Hsa and PadA as potential candidates for the development of an anti-VGS-IE vaccine.

MATERIALS AND METHODS

Bacterial strains and growth conditions. L. lactis (strain MG1363) (25) was grown at 30°C in M17 broth medium (Difco-Becton Dickinson, Sparks, MD) containing 1% glucose (GM17). S. gordonii Challis (strain DL1) (19) was grown at 37°C in brain infusion broth (Difco-Becton Dickinson) in the presence of 5% CO₂. Escherichia coli DH5α (Invitrogen, Carlsbad, CA) and E. coli BL21(DE3)pLysS (24) were grown in Luria-Bertani (LB) broth (Difco-Becton Dickinson).

Construction of the plasmids carrying Hsa-LysA2 and PadA-LysA2 fusion cassettes. Genomic DNA was extracted from S. gordonii using a genomic DNA purification kit (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer’s instructions. The N-terminal regions coding for amino acids (aa) 39 to 449 of hsa and 35 to 1327 of padA were PCR amplified from S. gordonii genomic DNA using forward primers hsa-fw (ATATGGATCCTGATCCTCTGAATATTGAGACG) and padA-fw (ATATGGATCCTTTAAGATTAATGAAGGGTGCTG) containing BamHI recognition sites (underlined) and reverse primers hsa-rv (TAAGCATATTACAAAAATGCC) and padA-rv (TATACGGCGCCTTTCAAGGTATCAGAAAAATGACA) containing Eagl recognition sites (underlined). The resulting products of 1,258 bp and 3,901 bp were digested with BamHI and Eagl (Promega, Madison, WI) and ligated into the pLysA2 vector, a plasmid containing the gene coding for bacteriophage A2 of L. casei (24), previously digested with the same enzymes. The resulting constructs, namely, pHsa-LysA2 and pPadA-LysA2, encoded the N-terminal regions of Hsa and PadA with an N-terminal 6-His tag and Xpress epitope and fused to the cell-binding domain of L. casei LysA2 at the C terminus (Fig. 1). The correct insertion of the genes for 1H/cm² (26). UV-induced killing was confirmed by the absence of viable organisms. The cells (100 µl) were then mixed with increasing amounts of Hsa-LysA2 or PadA-LysA2 in order to determine the amounts of proteins necessary for the complete saturation of the L. lactis surface and incubated for 1 h at 30°C to promote binding of the proteins. L. lactis
cells were also incubated with BSA (control). Cell protein suspensions were then centrifuged, washed with PBS, and resuspended into 100 μl of the same buffer.

**Assessment of Hsa-LysA2 and PadA-LysA2 levels on L. lactis surface by enzymelinked immunosorbent assay (ELISA).** One hundred microliters of UV-treated L. lactis cells (final concentration, 10⁶ CFU/ml) pre-incubated with different amounts of Hsa-LysA2 and PadA-LysA2 was adsorbed on an 96-well MaxiSorp microplate (Thermo Fisher Scientific) for 18 h at 4°C. The presence of Hsa-LysA2 and PadA-LysA2 on the surface of immobilized L. lactis cells was detected using a mouse anti-Xpress antibody (Invitrogen) and a peroxidase-conjugated goat anti-mouse secondary antibody (Dako, Baar, Switzerland). Fifteen minutes after the addition of the tetramethylbenzidine (TMB) solution (Thermo Fisher Scientific), the reactions were stopped with 2 M H₂SO₄. The absorbance at 450 nm was then measured with the Infinite 200 Pro microplate reader (Tecan, Maennedorf, Switzerland). The assays were carried out in triplicate.

**Western blot analysis for the detection of surface-associated Hsa-LysA2 and PadA-LysA2.** Twenty microliters of UV-inactivated L. lactis used for the immunization studies (see below) was mixed with 10 μl NuPAGE lithium dodecyl sulfate (LDS) sample buffer (4×; Invitrogen) and boiled for 10 min at 99°C. The resulting protein samples together with 1 ng of each purified protein were resolved by 10% SDS-polyacrylamide gel electrophoresis. Western blotting (WB) was performed using a standard procedure (27). Blots were incubated with a 1:1,000 dilution of a mouse anti-Xpress antibody (Invitrogen) and successively with a 1:3,000 dilution of a peroxidase-conjugated goat anti-mouse secondary antibody (Dako). The bands were detected by chemiluminescence with the enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ).

**Animal studies.** All animal protocols were reviewed and approved by the Cantonal Committee on Animal Experiments of the State of Vaud, Switzerland (permit number 879.9). A mixture of ketamine (75 mg/kg of body weight) and midazolam (5 mg/kg) anesthetics was administered to the animals before any surgical procedure.

**Immunization studies.** UV-killed L. lactis (10⁶ CFU) mixed with 2 μg of Hsa-LysA2 (L. lactis Hsa-LysA2) or 5 μg of PadA-LysA2 (L. lactis PadA-LysA2), the smallest amounts of proteins sufficient to saturate the L. lactis surface (see Results), was used for immunization.

Rats (6-week-old female Wistar rats) were injected subcutaneously with an initial dose of L. lactis Hsa-LysA2, L. lactis PadA-LysA2, or the combination of the two emulsified with complete Freund’s adjuvant (Sigma-Aldrich, Buchs, Switzerland), followed by two booster doses in incomplete Freund’s adjuvant (Sigma-Aldrich) at 14-day intervals. Control groups of rats were sham immunized with L. lactis plus the adjuvant. Blood samples were collected at days 0 (before immunization) and 45 (at the time of catheter insertion, 14 days after the third immunization dose; see below). Following centrifugation (3,000 rpm for 15 min at 4°C), sera were stored in aliquots at −80°C until use.

**Animal model of endocarditis.** The production of catheter-induced aortic vegetations and the implantation of an intravascular (i.v.) line to deliver the bacterial inocula were performed in rats 2 weeks after the last immunization dose, i.e., on day 45, as described previously (28).

Twenty-four hours later, immunized and control rats were inoculated i.v. by continuous infusion (0.0017 ml/min over 10 h) of 10⁹ CFU of S. gordonii, to simulate cumulative exposure to low-grade bacteremia (28). By virtue of this property, LysA2 CBD was here used to efficiently immobilize Hsa and PadA on the surface of L. lactis, which served as an antigen-presenting system. Since Hsa and PadA are large surface-associated proteins composed of 2,178 and 3,646 amino acid residues, respectively, only the functional surface-exposed N-terminal regions were used to generate the fusion proteins. Hsa contains two serine-rich regions (SRs), which are associated with the binding to the sialylated N-terminal region of GP Ibα on platelets. SR1 is relatively short (81 amino acid residues) and is located in the N-terminal region between two non-petitive regions (NR1 and NR2). In contrast, SR2 is a long serine-rich region containing 113 dodecapeptide repeats, which are comprised between the N-terminal region and the C-terminal cell wall anchoring domain (Fig. 1). Since SR1, NR1, and NR2, but not SR2, were previously shown to be indispensable for the binding to sialoglycoconjugates (18), the N-terminal region spanning SR1, NR1, and NR2 was used to generate Hsa-LysA2. PadA consists of a leader peptide, an N-terminal region comprising a von Willebrand factor-like domain, a long C-terminal domain with 14 repetitive regions (NR1 and NR2). In contrast, SR2 is a long serine-rich region containing 113 dodecapeptide repeats, which are comprised between the N-terminal region and the C-terminal cell wall anchoring domain (Fig. 1). Analogously to Hsa, the N-terminal re-
region was used for the generation of PadA-LysA2, as it was shown to be directly involved in the binding to platelets (21).

**Binding of surface-saturating levels of Hsa-LysA2 and PadA-LysA2 on *L. lactis***. The ability of the Hsa-LysA2 and PadA-LysA2 fusion proteins to bind to the *L. lactis* cell wall and the optimal protein/cell ratio for antigen presentation were assessed by ELISA and Western blotting (WB). As shown in Fig. 2A, the absorbance increased with the amounts of proteins added to *L. lactis* cells, with maximal signal intensities obtained upon addition of 2 μg of Hsa-LysA2 and 5 μg of PadA-LysA2. Interestingly, further addition of the fusion proteins did not result in increased chemiluminescent signals, suggesting that the binding capacity of the *L. lactis* cells was saturated at those concentrations. Consistently, bands corresponding to Hsa-LysA2 and PadA-LysA2 appeared in *L. lactis* preincubated with 2 μg of Hsa-LysA2 and 5 μg of PadA-LysA2 (Fig. 2B), further corroborating the idea that the chimeric proteins were firmly attached on the cell wall.

**Vaccination with *L. lactis***. Animals were immunized with three subcutaneous doses of *L. lactis* Hsa-LysA2, *L. lactis* PadA-LysA2, or the combination of the two bacteria given at intervals of 15 days. Antibody titers of Hsa and PadA in sera were assessed after the third dose. As shown in Fig. 3, sera obtained from rats immunized with *L. lactis* Hsa-LysA2 or *L. lactis* PadA-LysA2 showed an approximately 10- to 15-fold increase in specific antibodies compared to those of control rats and animals before immunization. Coimmunization with *L. lactis* Hsa-LysA2 and *L. lactis* PadA-LysA2 induced antibody levels comparable to those of single-antigen-immunized animals. Anti-Hsa and anti-PadA antibodies inhibited *S. gordonii*-induced platelet aggregation. As shown in Fig. 4, sera from *L. lactis* Hsa-LysA2- or *L. lactis* PadA-LysA2-immunized animals significantly delayed *S. gordonii*-induced platelet aggregation (lag time of 14.0 ± 1.6 min and 15.0 ± 1.2 min, respectively), compared to sera from sham-immunized rats (lag time of 8.0 ± 0.8 min; *P* < 0.0005). Moreover, upon preincubation with anti-Hsa or anti-PadA, *S. gordonii* exhibited comparable maximum platelet aggregation percentages (64% and 56%, respectively), which were
significantly lower than that induced after the addition of control sera (73%; $P < 0.005$). Remarkably, sera taken from animals co-immunized with \( L. \text{ lactis} \) Hsa-LysA2 and \( L. \text{ lactis} \) PadA-LysA2 completely prevented \( S. \text{ gordonii} \)-induced platelet aggregation (lag time, $> 20 \text{ min}; P < 0.0001$) and reduced maximum aggregation to 13% ($P < 0.0001$).

**Vaccination with \( L. \text{ lactis} \) Hsa-LysA2/PadA-LysA2 successfully protected rats against \( S. \text{ gordonii} \)-induced experimental IE.** As shown in Fig. 5, while 14/15 (93%) sham-immunized rats developed \( S. \text{ gordonii} \) IE, only 5/11 (45%) animals immunized with \( L. \text{ lactis} \) Hsa-LysA2, 5/11 (45%) animals immunized with \( L. \text{ lactis} \) PadA-LysA2, and 1/12 (9%) animals coimmunized with \( L. \text{ lactis} \) Hsa-LysA2 and \( L. \text{ lactis} \) PadA-LysA2 showed infected vegetations ($P < 0.05$). Interestingly, significantly higher levels of protection against \( S. \text{ gordonii} \) IE were obtained with coimmunization than in single-immunized groups ($P < 0.05$).

Bacterial counts in vegetations that remained infected were not significantly different between immunized and control rats (6.38 to 7.09 log\(_{10}\) CFU/g).

**DISCUSSION**

In the present study, we investigated the potential of \( L. \text{ lactis} \) displaying \( S. \text{ gordonii} \) Hsa or PadA as a vaccine candidate against \( S. \text{ gordonii} \) infection in a rat model of IE.

We used for the immunization a recently developed novel display system that allows highly efficient immobilization of antigens on the surface of lactic acid bacteria, such as \( L. \text{ lactis} \) (23). This system is based on non-genetically modified \( L. \text{ lactis} \) used as a vehicle for antigens bound on the surface by means of a high-affinity cell-binding domain (CBD) from \( L. \text{ casei} \) A2 phage lysine lysA2 (24). Such a system offers several advantages over a previously used immunization method based on recombinant \( L. \text{ lactis} \) expressing bacterial antigens, such as the \( Staphylococcus aureus \) clumping factor A (ClfA) or the fibronectin binding protein A (FnbpA) (26). First, it allows use of a non-genetically modified antigen-presenting system. Second, it permits the clustering of antigens on the \( L. \text{ lactis} \) cell wall, a feature that increases antigen stimulation of the immune system. Consistent with this notion, while in a pilot study anti-Hsa antibody levels were barely detectable in rat plasma after immunization with recombinant \( L. \text{ lactis} \) expressing Hsa (data not shown), vaccination with \( L. \text{ lactis} \) with cell wall-saturating amounts of Hsa-LysA2 or PadA-LysA2 fusion proteins triggered production of antibodies against either immunogen with levels approximately 10 to 15 times higher than those found in the sham-immunized rats. Also, whether this strategy is more effective than the vaccination with proteins alone remains to be investigated.

Why then Hsa and PadA? Hsa and PadA appear to be major factors in triggering \( S. \text{ gordonii} \) platelet adhesion and aggregation, which are crucial steps in the pathogenesis of IE (15).

Consistent with this notion, we here showed that \( S. \text{ gordonii} \)-
induced platelet aggregation was delayed by anti-Hsa and anti-PadA and, most importantly, completely abolished by the combination of the two. These observations, however, are in contrast with previous results, which showed that concomitant genomic inactivation of hsa and padA did not have any effect on S. gordonii-induced platelet aggregation (21). These contradictory results might be attributed to the cross-reactivity of the anti-Hsa and anti-PadA antibodies with other S. gordonii (DL1) adhesins that interact with platelets, such as SspA and SpdB (19). In support of this assumption, SspA and SpdB sequences exhibit 20% identity to Hsa (see Fig. S1 in the supplemental material). Clearly, this hypothesis requires experimental validation.

The results of the vaccination studies also identified Hsa and PadA as factors primarily responsible for the early steps of IE induced by S. gordonii. In fact, rats immunized with Hsa or PadA were significantly protected from S. gordonii IE. Most importantly, communication with the two proteins conferred full protection, suggesting that Hsa and PadA act synergistically to promote IE.

Thus, Hsa and PadA represent potential targets for vaccination against S. gordonii IE. To be ideal vaccinogens, however, S. gordonii Hsa and PadA should afford cross-protection against other VGS. PadA appears to be conserved among viridans streptococci, including Streptococcus sanguinis (99% identity), Streptococcus mitis (98% identity), Streptococcus parasanguinis (68% identity), and Streptococcus oralis (66% identity) (see Fig. S2 in the supplemental material). Although less well conserved, S. gordonii Hsa homologues can be found in S. mitis (83% identity), Streptococcus sanguis (named SrpA; 63% identity) (31), S. gordonii M99 (named GspB; 40% identity) (17, 32), and S. oralis (26% identity) (see Fig. S3). Whether the anti-Hsa and anti-PadA antibodies also cross-react with and block the function of the Hsa- and PadA-like proteins of other VGS is under investigation in our laboratory.

ACKNOWLEDGMENTS

We thank Juan Evaristo Suarez (University of Oviedo, Spain) for providing the pLysS2 vector and Christiane Gerschheimer (Service and Central Laboratory of Hematology, Lausanne University Hospital) for help with aggregometry.

None of the authors has any conflict of interests relevant to this study.

This work was funded by the Swiss National Science Foundation (310030-143799).

FUNDING INFORMATION

This work, including the efforts of Jose M. Entenza, was funded by Swiss National Science Foundation (310030-143799).

REFERENCES


