Title: VACCINE TO PREVENT STREPTOCOCCAL ENDOCARDITIS

Abstract

FimA proteins from streptococci and enterococci, which are responsible for bacterial adherence to damaged heart tissue, are potent vaccines against a patient developing endocarditis. Recombinantly produced FimA proteins from S. parasanguis provide protection against S. parasanguis derived endocarditis, as well as endocarditis derived from several other streptococci. Conserved DNA regions in viridans streptococci and enterococci suggest that FimA from these strains will be broadly applicable as a vaccine.
VACCINE TO PREVENT STREPTOCOCCAL ENDOCARDITIS

DESCRIPTION

BACKGROUND OF THE INVENTION

Field of the Invention

The invention is directed to vaccines and, more particularly, to a vaccine for the prevention of endocarditis.

Background Description

Infective endocarditis is a serious endovascular infection causing substantial morbidity and mortality despite medical and surgical advances over the last several decades (11,18,38). In the United States, there are 30-40 cases per million a year (1,6,11). In Europe, the annual incidence of disease ranges from 14-24 cases per million (19,25,41). Epidemiologic surveys reveal that the incidence of endocarditis increases significantly with age and that in developed countries with a growing population of elderly people, endocarditis is a disease of increasing medical importance (18,19,25,41). Native valve endocarditis occurs predominantly in patients with predisposing heart lesions. High and moderate risk patients are those with a history of infective endocarditis, prosthetic heart valves, surgical systemic-pulmonary shunts, congenital cardiac malfunctions, rheumatic valvular disease, mitral valve prolapse, and hyperthropic cardiomyopathy (10). In patients with valve disease, daily low-grade bacteremia which occurs during eating and tooth brushing affords the opportunity for circulating bacteria to attach to the abnormal endocardium (21). Other high-risk patient populations include those without preexisting valve lesions.
who have a history of recent exposure to invasive dental, upper respiratory, gastrointestinal, and genitourinary diagnostic and surgical procedures (22,31,37).

Prevention of endocarditis is vital because the disease is always fatal if untreated. Current practice in the United States, Britain, and Europe favors the use of antibiotic prophylaxis for patient at high risk of endocarditis undergoing health care techniques that can cause bacteremia (8,11,36). The most common pathogens associated with native valve endocarditis are the viridans streptococci which account for over 60% of cases (9). Antibiotic prophylaxis is targeted at these organisms. However, because only about half of the patients with endocarditis have recognizable predisposing cardiac conditions and since endocarditis associated with health care procedures constitutes a minority of cases, only a small fraction of endocarditis cases may be preventable with prophylaxis (11,19,21).

Other preventive strategies are warranted and are being explored. There have been several reports on the effect of vaccination on susceptibility to experimental endocarditis. Immunization with killed whole cells of Streptococcus sanguis, Streptococcus mutans, Streptococcus pneumoniae, Pseudomonas aeruginosa, nutritionally variant streptococci, and Candida albicans was protective against the development of endocarditis or early septicemia in rabbits (2,4,12,30,32,40). In contrast, anti-whole cell antibody did not protect rabbits from Staphylococcus aureus endocarditis (17). Immunization with staphylococcal capsular polysaccharide/adhesin (PS/A) prevented Staphylococcus epidermidis endocarditis in rabbits (39) and immunization with fibronectin binding protein from Staphylococcus aureus was protective in rats (34).

Vaccination studies in endocarditis models have provided insights about how immunoprophylaxis confers protection. Specific antibody conferred immunity by increasing bacterial clearance and by inhibiting bacterial attachment is a crucial early step in the pathogenesis of this disease (2,30,33,34,39).
FimA is an important virulence determinant in *S. parasanguis* endocarditis and is implicated in promoting bacterial adherence to fibrin in vegetations (7).

**SUMMARY OF THE INVENTION**

It is an object of this invention to provide a means to prevent infections of heart valves (endocarditis) by the most common bacterial cause, viridans streptococci (e.g., oral streptococcal bacteria). This invention contemplates a composition of matter which takes the form of a protein found on the surface of many streptococcal species present in the human mouth. This protein, in purified form, can be administered as a vaccine and confers protection against endocarditis. Although modeled around one species of streptococci, *Streptococcus parasanguis*, the material, called FimA, is found on many streptococci and enterococci bacteria. Protection in animals has been demonstrated using a rodent model system, which reliably mimics human endocarditis.

The *S. parasanguis* FimA protein was over produced and purified using the Qiagen pQE30 plasmid expression system. Purified FimA was used to investigate its usefulness as a vaccine in a rat model of endocarditis. The vaccination regimen was as follows. Nine-week old male Sprague-Dawley rats were given an initial dose of 100 µg of purified FimA emulsified in Freund's Complete Adjuvant. The antigen preparation was given in an area of the animal's flank in six intradermal injections. The same site was used for a booster dose of 100 µg of protein in Incomplete Freund's Adjuvant three weeks later. Two weeks after vaccination, trauma to the heart valves was induced by catheterization in vaccinated and control animals. Twenty-four hours after catheterization, the animals were challenged with *S. parasanguis* FW213. A $10^7$ inoculum of organisms grown to an OD$_{660} = 0.6$ in BHI broth was injected intravenously via tail vein. Forty-eight hours following inoculation, animals were euthanized and
endocarditis was determined by the presence of bacteria in vegetations following necropsy. Catheterized rats immunized with FimA were protected against challenge with *S. parasanguis* FW213 when compared to unimmunized control animals (p<0.001). These results demonstrate that FimA can serve as a vaccinogen to protect against endocarditis.

Experiments were also conducted which demonstrate that FimA from *S. parasanguis* is protective against heterologous infectious challenge. Rats were vaccinated with FimA of *S. parasanguis* origin as described above, and then challenged with fimA-expressing streptococci including *S. mitis*, *S. salivarius*, and *S. mutans*. A significant decline in FimA-vaccinated rats was observed.

The principal advantages of this invention are that it would be reasonably inexpensive, safe, reliable, and effective protection against endocarditis. In short, this invention includes a primary protective vaccine against endocarditis, a method for preventing endocarditis, and formulations useful in protecting against endocarditis and methods for producing the vaccine formulations.

The vaccine of this invention may also be used to prevent streptococcal bacteremia, a clinical condition seen increasingly in immuno-compromised patients. In this use, the FimA would be provided to an immuno-compromised patient (e.g., a bone marrow transplant patient) by intramuscular injection or other route prior to high dose chemotherapy or radiation therapy, and would elicit opsonic antibodies to invading streptococci in the patient’s bloodstream, thus enhancing clearance of these infectants.

**BRIEF DESCRIPTION OF THE DRAWINGS**

The foregoing and other objects, aspects and advantages will be better understood from the following detailed description of the preferred embodiments of the invention with reference to the drawings, in which:
Figure 1 is a schematic diagram showing the cloning of *fimA* into the pQE30 expression vector. *fimA* DNA from pVT781 was amplified by PCR. Primers were designed to modify the ends of the *fimA* DNA for subcloning as an *SphI-HindIII* fragment at the multicloning site of the pQE30 vector. This expression vector contained a phage T5 promoter and two *lac* operator sequences. The *E. coli* host cell has multiple copies of the plasmid pREP4 which carries the *lacI* gene ensuring tight regulation of protein expression. The construct pVA2341 has the six histidine residue affinity tag 5' to *fimA*.

Figure 2 is a photograph of a protein analysis gel of purified recombinant FimA. Specifically, the photograph shows a Coomassie blue-stained SDS-polyacrylamide gel. Lane 1 has a broad molecular weight marker (Bio-Rad); Lane 2 has native FimA after metal chelate chromatography (MCAC); Lane 3 has MCAC purified FimA after gel filtration with FPLC.

Figure 3 is a graph showing the comparison of serum anti-FimA titers in immunized and non-immunized rats. Rats were immunized and boosted once with FimA. Antibody levels were measured by EIA. Mean serum anti-FimA titers standard deviation (SD) were plotted for each antibody dilution tested. •: Immunized rats (n=6). ▲: Non-immunized rats (n=7).

Figures 4a-c are bar graphs showing the adherence properties of *S. parasanguis* strains. Bacterial adherence to platelet-fibrin matrix and adherence of *S. parasanguis* FW213 incubated with adsorbed sera to platelet-fibrin matrix were examined. Bars show mean percent adherence to platelet-fibrin matrices with standard deviations. Figure 4A shows the results when *S. parasanguis* strains were incubated on platelet-fibrin coated disposable Petri dishes (60 by 15 mm) for 30 min at 37°C. The adherence of wild type FW213 was significantly different from the mean percent adherence obtained with *fimA* mutant VT930 at a level of P<0.05. Figure 4B shows the results when *S. parasanguis* FW213 incubated in rabbit serum adsorbed with *S. parasanguis* FW213 was exposed to a platelet-fibrin matrix. The adherence of *S. parasanguis* FW213 preincubated with immune sera was
not significantly different from that found in preimmune sera (P=0.34).

Figure 4C shows the results when *S. parasanguis* FW213 incubated in rabbit serum adsorbed with VT930 was exposed to a platelet-fibrin matrix. Incubation of *S. parasanguis* FW213 with anti-FimA sera adsorbed with VT930 blocked adherence of *S. parasanguis* FW213 to the platelet-fibrin matrix (0.34%) but no such blocking effect was observed by incubation with adsorbed preimmune sera (5.04%) (p<0.001).

Figure 5 are nucleotide sequences (SEQ. ID. Nos. 1-10) showing the alignment of portions of the nucleotide sequences of *fimA* (SEQ ID No. 1 and SEQ ID No. 6) and its homologs derived from the GCG program Pileup. The primer pair in **bold** letters and identified with brackets 10 and 12 corresponds to nucleotides 151-173 and 868-893 of *fimA* and represents conserved regions in the lipoprotein receptor antigen (LraI) family. The average size of the genes in this family is 930 bp in length. The primers are 5' GCTGGGGATAAGATCGAGCTCCACAG 3' (SEQ ID No. 11), and 5' TTCATCATGCTGTAGTAGCTATCGCC 3' (SEQ ID NO. 12).

Figures 6a and 6b are photographs of gels showing the detection of *fimA* homologs. Figure 6a shows a Southern blot of EcoR1-digested genomic DNA from streptococcal strains using *fimA* DNA as a probe. Lanes: 1, *fimA* DNA; 2, *S. mutans* ATCC 25175; 3, *S. bovis* ATCC 43144; 4, *S. oralis* ATCC 10557; 5, *S. salivarius* ATCC 7073; 6, *S. mitis* ATCC 6249; 7, *S. anginosus* ATCC 27823; 8, *E. faecium* ATCC 19434. Figure 6b shows 0.8% gel electrophoresis of PCR amplified genomic DNA from various streptococcal strains. The primer pair corresponds to nucleotides of *fimA* described in Figure 5. Lanes: 1, molecular size markers; 2, *S. parasanguis* FW213; 3, *S. mutans* ATCC 25175; 4, *S. bovis* ATCC 43144; 5, *S. oralis* ATCC 10557; 6, *S. salivarius* ATCC 7073; 7, *S. mitis* ATCC 6249; 8, *S. anginosus* ATCC 27823; 9, *E. faecium* ATCC 19434. Sizes in base pairs are given on the left of Figure 6b.

Figure 7 is a photograph of a gel showing the expression of FimA-like proteins in clinical isolates. Clinical isolates from bacteremic
patients were grown anaerobically in 50 ml of BHI broth for 48 hours at 37°C. Bacterial cells were disrupted using a MiniBead™ beater. Protein samples were separated by 10% TrisGlycine SDS PAGE and electrotransferred to a nitrocellulose membrane and probed with polyclonal anti-FimA. The bound antibodies were visualized by addition of anti-rabbit IgG horseradish peroxidase conjugate, H2O2, and 4-chloronaphthol. Lanes: 1, broad molecular weight markers (BioRad); 2, *S. mutans* ATCC 25175 (this laboratory strain did not express FimA. Subsequent experiments showed FimA to be expressed in multiple clinical isolates of *S. mutans*); 3, *S. parasanguis* FW213; 4, *S. sanguis*, V2426; 5, *E. faecium* V2424; 6, *S. salivarius* V2471; 7, *S. anginosus* V2470; 8, *E. faecalis* V2437. The sizes of protein markers are indicated on the left of the photograph in Figure 7. The arrow corresponding to the 36 kDa size range indicates the reactive proteins.

**DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION**

Present technology deals with endocarditis in one of two ways:

1. Patients at risk for endocarditis are given prophylactic antibiotics prior to scheduled procedures that might result in invasion of the bloodstream with oral streptococci (e.g., dental procedures).

2. Patients who contract endocarditis usually has sustained heart valve damage. Depending on the nature of the damage, their natural valves are surgically replaced with porcine or prosthetic valves.

This invention provides a different approach and describes the first and only primary protective vaccine against endocarditis.

Infected bacterial endocarditis cases in the United States alone number between 10 and 40 thousand per year. The majority of these
infections are caused by viridans streptococci, including such species as *S. sanguis*, *S. parasanguis*, *S. mutans* and others. Bacterial endocarditis is life threatening and its treatment is expensive; 4 to 6 weeks of hospitalization are required in order to complete effective intravenous antibiotic therapy protocols. Predisposing factors to streptococcal endocarditis include valve damage, congenital heart defects, and rheumatic heart disease. Ninety percent of all patients who contract infective endocarditis have one or more predisposing factors. Based on identification of risk factors there would likely be an annual steady state population of 75 to 100 thousand patients who would require vaccination. This base would be significantly expanded if the vaccine were administered to the elderly population as well. There is increasing concern among clinicians that infective endocarditis is appearing more frequently in this patient population. Reasons for this include calcification of heart valves and possible diminished effectiveness of the immune system with age. There are approximately 31 million individuals over the age of 65 years in the United States population. Coverage of this population with proven vaccines is actively increasing each year; e.g., flu vaccination and pneumococcal vaccinations are widely recommended and administered to this group. The segment of our population representing the elderly is growing dramatically. The group comprising individuals over the age of 65 years grew by 21% from 1980 to 1989. The rest of the population grew by only 8% during the same period. Effective vaccines are likely to enjoy broad usage in the elderly, a group which is growing strikingly as medical advances lengthen life span.

Experiments have been conducted which demonstrate that FimA and related proteins or protein fragments are useful vaccines against endocarditis. In the experiments, the efficacy of FimA immunization was evaluated in the rat model of endocarditis, the effect of anti-FimA on the adherence of *S. parasanguis* FW213 to platelet-fibrin matrix *in vitro* was investigated, the presence of fimA homologs among viridans streptococci was determined, and the occurrence of FimA-like proteins among various streptococci and
enterococci was assessed. The results showed that FimA immunization conferred antibody-mediated protection against \textit{S. parasanguis} endocarditis in rats. FimA from \textit{S. parasanguis} was also demonstrated to confer protection from endocarditis derived from other species including \textit{S. mitis, S. salivarius,} and \textit{S. mutans}. Southern hybridization, PCR amplification and Western analyses indicated the occurrence of \textit{fimA} homologs and the expression of FimA-like proteins among viridans streptococci and enterococci, and it is expected that these \textit{fimA} proteins, and fragments thereof, can also provide protection against endocarditis.

In the practice of this invention, a patient would be provided with a vaccine comprised of FimA, or fragments thereof, which is derived from viridans streptococci and enterococci, to protect the patient from endocarditis or bacteremia. The protein sequence for FimA, and its corresponding DNA sequence are described in Fenno et al. \textit{Infect. Immun.} 57:3527-3553 (1989). The vaccine could be provided by parenteral (e.g., intravenous, intramuscular, intradermal, subcutaneous), oral, sublingual, transdermal and other routes of administration well known in the art. The preferred mode of delivery is parenteral. The FimA could be provided in combination with carrier fluids (e.g., water based (saline, etc.) or oil based or emulsions), stabilizing agents, preservatives (e.g., parabens, benzalkonium chloride (BAK), etc.), and the like as appropriate to the delivery route. For example, in an oral vaccine, the carrier may be a solid lactose based material.

The FimA protein of the vaccine should be provided in quantities sufficient to confer protection by the patient's body raising antibodies to FimA, and could be provided as a bolus dose with follow up boosters, or a single bolus dose, or according to other dosing regimens depending on the patient and formulation of the vaccine.

The FimA protein or fragment thereof in the vaccine can be isolated from a variety of streptococci or enterococci, or, as discussed in detail below, be recombinantly produced in a bacterial, mammalian or plant cell host, or be manufactured by other means. In the methods below, it is shown
that the gene for FimA can be isolated and transferred to a plasmid for subsequent production in an E. coli host; however, it will be apparent to those of skill in the art that the gene might be transferred and expressed in and retrieved from a wide variety of different cell systems or from a living animal or plant.

Recombinant production of FimA also might be accomplished so as to render FimA or its subsequence peptides as fusion proteins. Fusion to other proteins to increase the immunogenicity of FimA and/or to increase its stability would be desired outcomes of such fusion protein construction.

Based on the results of the heterologous infectious challenge study, the source of the FimA protein should not limit the protein's effectiveness as a vaccine against endocarditis or bacteremia derived from either viridans streptococci and enterococci. However, a vaccine could take the form of a mixture of FimA proteins derived from a mixture of viridans streptococci and enterococci. In the case of fragments of the FimA protein being used as the vaccine, enough of the protein should be present such that immunization causes antibody-mediated protection.

MATERIALS AND METHODS

Bacterial strains, plasmids and media. Wild type S. parasanguis FW213, its isogenic fimA insertion mutant, VT930 (does not express FimA protein), and E. coli, VT786, a recombinant FimA producing strain, have been described previously (13,14). The M15 E. coli host strain, pQE30 expression vector, and pREP4 repressor plasmid were from the Qiaexpress system (Qiagen Inc. Chatsworth, CA). Streptococcal strains were grown anaerobically (10% CO₂, 10% H₂, 80% N₂) at 37°C in brain heart infusion (BHI) broth with 0.35% glucose. E. coli strains were grown in Luria Bertani (LB) broth (Life Technologies, Inc., Gaithersburg, MD). Agar was added to a final concentration of 1.5% to prepare solid medium. BHI, glucose, and agar were obtained from Difco (Detroit, MI). Ampicillin (100 μg/ml) and
kanamycin (25 µg/ml to maintain pREP4 containing strains or 100 µg/ml to maintain VT930) (Sigma Chemical Co., St. Louis, MO) were added for bacterial and plasmid selection. Bacterial cultures were stored at 70°C in BHI with 30% glycerol.

**DNA methods.** Plasmid DNA was isolated using the Qiagen® plasmid purification protocol. Agarose gel electrophoresis protocols were those of Sambrook et al. (28). Restriction endonucleases were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, MD) and enzymatic digestions were performed according to the manufacturer's directions.

Preparation of *Streptococcus* chromosomal DNA was as described previously (35).

**Protein production and purification.** Expression and purification of recombinant FimA was performed using the Qiaexpress expression and the Nickel-nitrilotriacetic acid (Ni-NTA) protein purification system. The cloning strategy used to construct the overexpression plasmid, pVA2341, is shown in Fig.1. Plasmid pVT781 was isolated from *E. coli* VT786 (26). pVT781 was constructed by subcloning *fimA* as an *NdeI-BclI* fragment into a pET3a expression vector. *fimA* DNA was amplified by polymerase chain reaction (PCR). Oligonucleotides used to PCR amplify *fimA* were synthesized by Bio-Synthesis, Incorporated™ (Lewisville, TX). The oligonucleotide sequences corresponding to *fimA* nucleotides 1-17 and 916-930 were: 5' ACATGCATGCAAAAAATCGCTTC 3' (SEQ ID No. 13) and 5' CCCAAGCTTACTGACTCAATCC 3' (SEQ ID No. 14). The primers were designed so that the ends of the *fimA* DNA could be subcloned as an *SphI-HindIII* fragment into a pQE expression vector. The restriction sites imbedded in these oligonucleotides are shown in **bold** font (GCATGC=*SphI*, AAGCTT=HindIII). The multicloning region of pQE30 contains restriction endonuclease sites for *BamHI*, *SphI*, *SacI*, *KpnI*, *SmaI/XmaI*, *SaiI*, *PstI* and *HindIII*. Integration into pQE30 using *SphI* and
HindIII directional cloning resulted in a six histidine residue extension at the amino terminus of fimA. The expression construct was transformed into the M15 host strain carrying the plasmid pREP4 which carries the lacI gene. Transformants were selected on LB agar plates containing ampicillin and kanamycin and screened for correct insertion of the fimA gene by DNA restriction endonuclease cleavage analysis.

Expression of recombinant FimA was verified by preparation and analysis of the protein from small scale cultures. A single colony of the transformant was inoculated in 1.5 ml of LB medium containing 100 µg/ml ampicillin and 25 µg/ml kanamycin and grown overnight. 1.25 ml of the saturated culture was added to 8.75 ml of prewarmed LB medium with the appropriate antibiotics. A 1 ml sample was taken prior to induction to serve as the uninduced control. Expression was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 2 mM. A time course of expression was determined by taking 1 ml samples at hourly intervals. Cells were grown for up to 5 h. Cells were harvested by centrifugation and cell pellets were stored at -20°C until all samples were ready for processing. Cells were purified under denaturing conditions according to the manufacturer's instructions. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) and proteins were visualized by Coomasie Blue stain.

For large scale expression of FimA, a 20 ml starter culture was first grown overnight in LB broth containing 100 µg/ml ampicillin and 25 µg/ml kanamycin with 100 µl of stock culture. One liter of LB broth with antibiotics was inoculated 1:50 with the uninduced overnight culture and these cells were grown at 37°C with vigorous shaking to an OD$_{600}$ of 0.7. The cells were induced with 2 mM IPTG and incubated for an additional 5 h. Cells were harvested by centrifugation, suspended in 10 mM Tris-HCl (pH 8.0), and disrupted with the French® Pressure Press (SLM Instruments Inc., Urbana, IL). The lysate was centrifuged at 25,000 rpm in a Beckman SW28 rotor (Beckman Instruments, Inc., Palo Alto, CA) and the supernatant was
loaded onto Ni-NTA column. The column was washed with 10 mM Tris-HCl (pH 8.0) containing 10 mM imidazole until the A280 had returned to the baseline value, and the protein was eluted with 100 mM ethylenediaminetetraacetic acid (EDTA) in Tris-HCl (pH 8.0). The protein was further fractionated by gel filtration with Sepharyl®S-100 using fast protein liquid chromatography (FPLC) (Pharmacia LKB, Piscataway, NJ).

The nucleotide sequence of the subcloned DNA in the construct which expressed FimA was confirmed by DNA sequencing. Automated sequencing reactions were performed by the Sanger-based dideoxy chain termination method (PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit, Applied Biosystems Incorporated, Foster City, CA) according to the manufacturer's directions.

**Protein analysis.** 15% SDS-PAGE (Bio-Rad Laboratories, Hercules, CA) and Western immunoblots (Promega Corp., Madison, WI) were performed according to manufacturers' instructions. The gels were stained with Coomassie Brilliant Blue (Sigma) and immersed in destaining solution (40% methanol, 10% acetic acid, and 50% distilled water) until the background was clear. Molecular weight standards (size range: 7,200-208,000) from Bio-Rad were used. Protein concentrations were determined by the Lowry method (23) using bovine serum albumin (BSA) as a standard.

**Production of polyclonal antisera.** Antisera directed against FimA were prepared by subdermal injection of female New Zealand White rabbits at the back of the neck with 0.5 mg FimA suspended in 0.5 ml phosphate buffered saline (PBS) (pH 7.4) and emulsified in an equal volume of complete Freund's adjuvant (CFA). A booster injection of 0.5 mg FimA in incomplete Freund's adjuvant (IFA) was given three weeks later. Antisera were collected and tested for antibody titer by enzyme immunoassay. All preimmune sera were negative by this method and immune sera had anti-FimA titers of ≥ 100,000. All sera were stored at 70°C until needed.
Rabbit injections were carried out under the Virginia Commonwealth University Institutional Animal Care and Use Committee (VCU IACUC) authorization no. 9504-2137.

**Enzyme immunoassay (EIA).** The procedure for immunodetection of FimA was adapted from Sigma (Biochemicals and Organic Compounds for Research and Diagnostic Reagents. 1995). EIA plates (Costar, Cambridge, MA) were coated with 10 μg/ml FimA in carbonate-bicarbonate buffer (pH 9.5) and blocked with washing buffer. Serum from each animal was serially diluted in PBS. The optimal dilutions for the secondary antibodies were determined in titration assays. The peroxidase-conjugated goat anti-rabbit antibody (Sigma) or peroxidase-conjugated mouse anti-rat antibody (Jackson Immunoresearch laboratories, Inc., West Grove, PA) was detected by TMBBlue substrate (TSI Center for Diagnostics Products, Milford, MA) and color development was stopped with IN H₂SO₄. Plates were read with a 700 MR microplate reader (Dynatech Laboratories, Inc., Chantilly, VA). Antibody titers were expressed as the reciprocal of the highest serum dilution with A₄₅₀ of ≤ 0.10 10 min after addition of substrate.

**Immunization protocol.** The immunization dose per Sprague-Dawley rat contained 100 μg of FimA in CFA. The dose was given by intradermal injection at 6 different sites in a shaved area of the rat's right flank. The same area was used for a booster dose of 100 μg of protein in IFA three weeks later. In the first study, catheterization and bacterial challenge as described below were performed six weeks after the initial immunization. In the second study, nonimmunized and immunized rats were exsanguinated by cardiac puncture two weeks after the booster dose to determine serum antibody titers.

**Rat model of endocarditis.** The rat model of endocarditis employed in this study was as described by Munro and Macrina (24). Approval for animal use
was obtained from the VCU IACUC (protocol no. 9410-2082) prior to initiation of experiments. Male Sprague-Dawley rats (Harlan, Indianapolis, Ind.) were challenged with 1 $\times 10^7$ bacteria 1-5 days after cardiac catheterization. The significance of differences between the numbers of *Streptococcus* infected vegetations obtained from immunized and non-immunized rats was calculated by Fisher's exact test.

**Platelet-fibrin adherence assay.** Methods adapted from Scheld, *et al* (32) and Munro and Macrina (24) were used. To prepare bacteria, an overnight culture of streptococci in BHI was diluted 1:10 in fresh BHI and was grown anaerobically to an optical density at 660 nm of $\approx 0.6$. Bacteria were washed in PBS, sonicated, and diluted to yield $1 \times 10^8$ cells per ml. In some experiments, these bacteria then were incubated with either preimmune or immune sera for 30 min at 37°C. Every ten minutes during the incubation period, the cells were blended in a Vortex mixer for 30 sec to ensure that the streptococci were single cells (not in chains). The sample was centrifuged and washed with PBS. In other experiments, immune and preimmune sera were adsorbed with 1 ml of an overnight culture of *S. parasanguis* FW213 or VT930 ($1 \times 10^8$ CFU) at 4°C for 24 h. To prepare the fibrin-platelet matrices, 1 ml of human platelet-poor plasma (platelet count, $\leq 50,000$/mm$^3$) purchased from the Medical College of Virginia (MCV) Hospitals Blood Bank was mixed with 0.4 ml of 0.2 M CaCl$_2$ and 0.4 ml of bovine thrombin (Baxter Diagnostics Inc., Deerfield, IL) at 100 NIH U/ml. To determine adherence, 1 ml of bacteria was placed on the platelet-fibrin matrix and incubated at 37°C for 30 min with gentle agitation. Non-adherent cells were removed from the platelet-fibrin matrix and the surface was washed four times with PBS. The matrix was dissolved by the addition of 0.5 ml of 2.5% trypsin solution. The fluid was sonicated, serially diluted, and inoculated onto BHI agar plates. The plates were incubated anaerobically at 37°C for 48 h. The percent adherence was calculated as: (number of colony forming units recovered/number of cells introduced onto the platelet-fibrin plate) X
Statistical analysis was calculated by Student's t test.

**Southern hybridization.** Three μg of chromosomal DNA digested with *EcoRI* were electrophoresed in a 0.8% Tris-borate (TBE) agarose gel on a model H5 horizontal gel apparatus (Life Technologies, Inc.) at 15V for 24h. The gel was depurinated in 0.25 M HCl for 15 minutes, denatured in a solution containing 0.05 M NaOH and 1.5 M NaCl for 30 min, and neutralized in a solution containing 2.5 M NaOH and 1.0 M CH₃COONH₄ for 1 h. DNA fragments were transferred to 0.45 μm pore size nitrocellulose membrane (Micron Separations Incorporated, Westboro, MA) by capillary action (35). The DNA was immobilized on the membrane by ultraviolet irradiation in a model 2400 UV Stratalinker™ (Stratagene, La Jolla, CA). Random-primed radioactive labeling of full length *fimA* probe was generated by using Prime-a-Gene® (Promega Corp.). The nitrocellulose membrane was incubated for 1 h at 42°C in a prehybridization buffer consisting of 5X SSPE (0.75M NaCl, 5 mM EDTA, 0.05 MM NaH₂PO₄), 5X Denhardt's reagent, 100 μg/ml salmon sperm DNA and 25% formamide. Hybridization with the randomly labeled probe was carried out at 42°C for 18 h in a solution of 5X SSPE, IX Denhardt's reagent, 100 μg/ml salmon sperm DNA, and 25% formamide. After hybridization, the membrane was washed twice (15 min each) in 2X SSPE with 0.1% SDS and then washed twice (15 min each) in 0.1X SSPE with 0.1% SDS at room temperature to remove unbound probe. Prehybridization, hybridization and washing steps were performed in a Savant Gene Roller™ hybridization oven (Savant Instruments, Inc., Holbrook, NY). The membrane was exposed to Reflection™ autoradiography film (Du Pont-NENG® Research Products, Wilmington, DE).

**PCR amplification to demonstrate *fimA* homologs.** Oligonucleotides were designed to amplify *fimA* homologs from *Streptococcus* spp. The synthetic oligonucleotides 5' GCTGGGGATAAGATCGAGCTCCACAG 3'
(SEQ ID No. 11) (nucleotides 151 to 173 in Figure 5) and
5'TTCACTCATGCTGATAGCTATCGCC 3' (SEQ ID No. 12)
(complementary to nucleotides 868 to 893) derived from fimA related
sequences found in well-conserved regions of the lipoprotein receptor
antigen I (LraI) family of genes were used as primers to amplify by PCR
DNA fragments from genomic DNA of streptococcal strains used in the
Southern blot. Nucleotide coordinates corresponded to the 930 bp native
fimA gene. GenAmp® PCR core reagents (Perkin Elmer Corp., Norwalk,
CT) were used and reactions were carried out for 28 cycles (94°C for 30
sec, 55°C for 20 sec, and 72°C for 45 sec) with an automated thermal
cycler, GeneAmp PCR System 9600 (Perkin Elmer Corp.). The reaction
products were analyzed by 0.8% agarose gel electrophoresis.

Preparation of cell Lysates for protein analysis. Clinical strains of
streptococci from patients who had positive blood cultures were obtained
from the diagnostic microbiology laboratory, MCV Hospitals (Virginia
Commonwealth University). Bacteria were grown anaerobically for 48 hrs at
37°C in 50 ml of BHI broth. The cells were harvested by centrifugation at
4000 X g for 10 min at 4°C. The cell pellets were suspended in BHI to a
final volume of 1 ml, and transferred to microcentrifuge tubes containing
one-half volume of 0.1 mm zirconium beads (Biospec Products, Bartlesville,
OK). The cells were disrupted in a Mini-Bead Beater homogenizer (Biospec
Products) for 2 minutes. Beads and cellular debris were removed by
centrifugation at 12,000 X g for 5 minutes to obtain a clear lysate. The
lysates were kept at 4°C until protein analyses were performed.

RESULTS

Overexpression and purification of FimA. It has been demonstrated that a
fimA insertion mutant, VT930, had significantly reduced virulence in the rat
endocarditis model compared to wild-type S. parasanguis FW213. It was
deduced from *in vitro* experimental data that virulence was associated with adherence of FimA to fibrin. Recombinant FimA was made using the Qiaexpress™ System for further *in vivo* and *in vitro* studies. The cloning of *fimA* into a pQE expression vector was done as described in Materials and Methods (Fig. 1). Oligonucleotide primers were synthesized to amplify *fimA* by PCR. The DNA product was subcloned into a pQE30 vector. The expression vector contained a phage T5 promoter and two *lac* operator sequences thereby increasing the probability of *lac* repressor binding and ensuring effective repression of the T5 promoter. This plasmid had a synthetic ribosomal binding site for more efficient translation and two transcriptional terminators, t₅ from phage lambda and t₁ from the *rrnB* operon of *E. coli*, which prevented read-through transcription thus stabilizing the expression construct. The six consecutive histidine residue tag and the start codon (ATG) were upstream of the polylinker sequence. The *E. coli* M15 host expression strain carried the pREP4 plasmid. The nucleotide sequence of the subcloned DNA in the construct was analyzed and confirmed. The 6X histidine residue served as a convenient affinity tag for purification of FimA from crude *E. coli* lysates under native conditions. The Ni-NTA resin metal chelate adsorbent allowed for separation of most contaminating proteins. Other contaminants were subsequently removed by gel filtration. The recombinant strain, VA2341, expressed 0.5 mg/liter of FimA.

As illustrated in Figure 2, FimA with the 6X histidine tag migrated more slowly and appeared larger than its expected size of 36kDa on SDS-PAGE gel. Presumed lower molecular weight degradation products were apparent. Based on molecular size analysis, native FimA appeared in monomeric and dimeric forms (see arrows to right of Fig. 2).

**Susceptibility of nonimmunized and immunized rats to endocarditis.**

The effect of FimA immunization in rats' susceptibility to endocarditis was investigated. Vaccinated and non-vaccinated rats were inoculated with *S. parasanguis* FW213 twenty-four hours after catheterization. Forty-eight
hours post-challenge, the animals were sacrificed and their hearts resected. Correct catheter placement and the presence and absence of vegetations were assessed visually. Only those animals with proper catheter placement were included in our analyses. Development of endocarditis was determined by recovery of streptococci from cultured vegetations. Twenty-one out of 33 nonimmunized rats (61%) developed *S. parasanguis* endocarditis compared with 2 of 34 rats (6.1%) immunized with FimA (*p*<0.001) (Table 1). Thus, vaccination with FimA conferred protective immunity against endocarditis in this model.

Table 1. Protective Effect of Immunization with FimA in Rats Challenged with *S. parasanguis* FW123.

<table>
<thead>
<tr>
<th>Rats</th>
<th>No. infected/total</th>
<th>% infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonimmunized</td>
<td>21/33</td>
<td>61.8</td>
</tr>
<tr>
<td>Immunized</td>
<td>2/34</td>
<td>6.1</td>
</tr>
</tbody>
</table>

The rat model is considered predictive of human endocarditis infection because rat cardiovascular anatomy is very similar to that of the human, and the course and outcome of infection are clinically similar. Infected vegetations from rats and humans are visually indistinguishable microscopically.

**Heterologous challenge.** FimA from *S. parasanguis* was tested to determine protection against heterologous infectious challenge. Rats were vaccinated as described above with fimA of *S. parasanguis* origin, (*S. parasanguis* FW213) and then challenged with different fimA expressing streptococci five days post catheterization. Three species of streptococci were tried in these experiments and the results are shown in Table 2.
Table 2. Protective effect of immunization with FimA from one organism against other FimA expressing organisms

<table>
<thead>
<tr>
<th>Organism</th>
<th># rats infected/total (%) unvaccinated</th>
<th># rats infected/total (%) FimA-vaccinated</th>
<th>p value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. mitis</td>
<td>5/7 (71%)</td>
<td>4/21 (26%)</td>
<td>0.0196</td>
</tr>
<tr>
<td>S. salivarius</td>
<td>5/8 (63%)</td>
<td>2/24 (8%)</td>
<td>0.048</td>
</tr>
<tr>
<td>S. mutans</td>
<td>10/15 (66%)</td>
<td>7/23 (21%)</td>
<td>0.0077</td>
</tr>
</tbody>
</table>

<sup>a</sup>p values were calculated using the Fisher’s Exact Probability test.

All isolates tested were of clinical origin from blood cultures. The S. mutans data represents pooled data from infections using three clinical isolates of this species, all with identical genotypic HaeIII-DNA restriction fragment patterns. All of the p values were highly significant indicating protection conferred by the FimA vaccine. Thus, these data indicate FimA is a virulence factor in these other strains as has been demonstrated in S. parasanguis.

While the data do not suggest the requirement, the vaccine may include several different FimA proteins from several different sources.

**Evaluation of anti-FimA titers in rats.** Humoral immunity is an important defense mechanism in many diseases. To establish that the induction of antibodies correlated with the protection observed with immunization with FimA, anti-FimA levels were compared with immunized and non-immunized animals. As illustrated in Figure 3, immunized rats developed anti-FimA titers ranging from 1:10,000 to 1:100,000 whereas no anti-FimA antibodies were detected in control rats. 6 out of 7 immunized animals had high antibody levels. One immunized animal, and 7 out of 7 non-immunized animal had no demonstrable levels of anti-FimA.

Thus, the FimA used in the vaccine should be of a sufficient quantity, and of a sufficient size in the case of a FimA fragment, to allow a patient’s
body to raise antibodies against the FimA in an immune response. Figure 3 shows that the antibody titers raised in an effective vaccine could be 1:10,000 to 1:100,000.

**Bacterial adherence in vitro.** A crucial step in the development of endocarditis is the initial colonization by bacteria of endothelial lesions with sterile vegetations. Previous experiments showed that wild type *S. parasanguis* FW213 bound significantly better (2.1% of added cells) to fibrin monolayers than a fimA insertion mutant (0.12% of added cells) (7). To more closely simulate vegetations *in vivo*, we performed adherence experiments on platelet-fibrin matrices.

Platelet-fibrin matrices were prepared and the percent adherence of streptococci was determined. The results of adherence and immune blockade assays from three replicate experiments, each of which were performed in triplicate, are illustrated in Figure 4a-c.

The ability of wild type *S. parasanguis* FW213 to adhere to platelet-fibrin matrices *in vitro* was tested and compared with that of its isogenic fimA insertion mutant, VT930 (Fig. 4A). VT930 adhered less readily (0.74% of added cells) than did the wild type strain (7.44% of added cells) (p<0.05).

Since FimA is a fibrin-binding adhesin, it was tested whether anti-FimA antibodies would interfere with colonization of vegetations. As shown in Figure 4B, prior adsorption of immune sera with *S. parasanguis* FW213 did not affect the ability of *S. parasanguis* FW213 to adhere to platelet-fibrin matrix (4.9% of added cells) (Fig. 4B). In contrast, incubation of *S. parasanguis* FW213 with anti-FimA sera adsorbed with VT930 blocked adherence of *S. parasanguis* FW213 to the platelet-fibrin matrix (0.34% of added cells) but no such blocking effect was observed by incubation with adsorbed preimmune sera (5.04%) (p<0.001) (Fig. 4C).

These results suggest that a protective mechanism of FimA immunization is inhibition of viridans streptococci and enterococci
attachment to platelet-fibrin thrombi.

*fimA* homologs among viridans streptococci and enterococci. *fimA* is one of five known genes which encode proteins belonging to the LraI family of adhesins (20). The presence of *fimA* homologs among viridans streptococci and enterococci which commonly cause native valve endocarditis were determined to explore the feasibility of utilizing FimA as a broadly protective vaccine against streptococcal endocarditis. Southern blot analysis of streptococcal genomic DNA digested with EcoRI and probed with full length *fimA* DNA showed the presence of reactive fragments in six of seven streptococci tested (Fig. 6A). Hybridizing fragments which co-migrated with *fimA* were found with *S. mutans* ATCC 25175, *S. oralis* ATCC 10557, and *S. salivarius* ATCC 7073. Less well-hybridizing fragments of differing molecular weights were observed with *S. salivarius*, and *S. anginosus* ATCC 27823. The probe did not react with *S. bovis* ATCC 43144 nor *E. faecium* ATCC 19434.

As shown in Figure 6B, PCR reactions amplified 800 bp DNA fragments from *S. mutans*, *S. oralis*, *S. salivarius* and *S. anginosus* which co-migrated with the amplified fragment from *S. parasanguis* FW213. Larger DNA fragments were amplified from *S. salivarius* and *E. faecium*.

Taken together, results from the Southern blot and PCR analysis show that *fimA* homologs are present in a variety of viridans streptococci and enterococci studied.

The results above indicate the presence of closely related genes in streptococci and enterococci. This can be seen from the finding that identically sized DNA fragments were amplified in *S. mutans* ATCC 25175, *S. oralis* ATCC 10557, and *S. salivarius* ATCC 7073 using oligonucleotide primers from the *fimA* sequence of *S. parasanguis*. The fainter components observed with *E. faecium* ATCC 19434, *S. salivarius* ATCC 7073, and *S. anginosus* ATCC 27823 suggest their *fimA* like genes exhibit reduced sequence homology to *fimA*. 
As explained above, in vivo testing with FimA from *S. parasanguis* demonstrated protection was conferred to different species of streptococci.

Expression of *fimA* homologs among viridans streptococci and enterococci. FimA is a protein found on the cell surface of streptococci and enterococci and belongs to the lipoprotein receptor adhesin family (5,16,29). Several species of viridans streptococci and enterococci that cause endocarditis are known to have genes that encode for these proteins. According to Mandell et al. (Principles and Practice of Infectious Diseases 4th edition, Churchill Livingstone, NY, 1995, P.753) streptococci account for 60-80% of the cases of endocarditis. The viridans streptococci alone account for 30-40% of all cases.

Polyclonal antisera raised against FimA from *S. parasanguis* were used to evaluate whether a related antigen is present in other streptococci and enterococci. Blood isolates from clinical patients were screened by immunoblot (Western blot) using polyclonal antisera. Table 3 shows that FimA is broadly expressed among bacteria which most frequently cause endocarditis.

**TABLE 3**
Reactivity of Streptococcal and Enterococcal Blood Isolates to Polyclonal Anti-FimA.

<table>
<thead>
<tr>
<th>Organism</th>
<th>FimA-Positive</th>
<th>FimA-Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. faecalis</em></td>
<td>10</td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. anginosus</em></td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. salivarius</em></td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td><em>S. sanguis</em></td>
<td>9</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td><em>S. mutans</em></td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td><em>S. mitis</em></td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>
Nutritionally deficient streptococci (*S. defectivus*)

In the case of *S. mitis* and *S. defectivus* collectively, multiple isolates from the blood of patients with endocarditis were evaluated and found uniformly to express a FimA-like antigen. The *S. mutans* data seen in Table 3 were obtained from endocarditis isolates of ten species. In contrast, only 1 of 4 dental plaque isolates of *S. mutans* were found to express FimA protein.

In addition to the data shown in Table 3 which demonstrate FimA-like proteins are commonly expressed by clinical strains of streptococci and enterococci from patients with bacteremia, Figure 7 shows that FimA antiserum detected proteins which co-migrated with FimA, further indicating their similarity.

Taken together the results shown in Table 3 and Figures 6A, 6B, and 7 provide molecular and immunological proof of concepts that are seminal to the use of FimA as a vaccinogen. First, the *fimA* gene and its encoded protein are evolutionarily conserved in gram positive bacteria, especially in organisms frequently associated with endocarditis etiology. Second, these proteins are naturally expressed and by direct evidence and by inference play a role in the organisms ability to incite endocarditis. Third, a paradigm FimA from *S. parasanguis* can be used to provide immunity against endocarditis in animals challenged with fimA producing organism other than *S. parasanguis* (e.g., *S. mitis, S. mutans, S. salivarus*). Thus, for purposes of this invention, FimA shall mean FimA and FimA like proteins, and fragments or fusion proteins thereof, which are derived from any species known to express these proteins.

The propensity of viridans streptococci to cause endocarditis is likely due to their adherence abilities. Several FimA like cell-surface proteins of oral streptococci and enterococci belonging to the LraI family of adhesins have been identified (5,16,29). In addition to FimA from *S. parasanguis*,

| Nutritionally deficient streptococci (*S. defectivus*) | 4 | 0 | 4 |
these include EfaA from *Enterococcus faecalis* (3,5), PsaA from *Streptococcus pneumoniae* (29), ScaA from *Streptococcus gordonii* (3,5,29), and SsaB from *Streptococcus sanguis* (15). Of these, EfaA and FimA have been implicated in endocarditis pathogenesis (3,5,16,29). As discussed above, the Qiaexpress System can be used for expression and purification of recombinant FimA in *E. coli*. As illustrated in Figure 2, a relatively pure preparation of native FimA was eluted from the Ni-NTA column and other nonspecific contaminants were effectively removed by gel filtration. FimA monomers and dimers were evident in the Coomassie-stained SDS PAGE gel. Polymeric forms of FimA may also be produced other overexpression and renaturation protocol (26).

The rationale for the use of the FimA adhesin as a vaccine is that antibody formed against it may interfere with bacterial adherence and thereby reduce virulence. Once the vegetation is colonized and bacteria are overlaid with fibrin and platelets, the pathogens are less likely to be opsonized and phagocytosed. The data presented above shows that immunized animals were less susceptible to subsequent challenge with *S. parasanguis* FW213 than the nonimmunized group. Six out of 7 (86%) immunized rats developed high anti-FimA titers; in contrast, all control animals showed no demonstrable antibody to FimA (Fig. In addition, the FimA from *S. parasanguis* was protective against heterologous infectious challenge.

The vaccine of this invention may also be used to prevent streptococcal bacteremia, a clinical condition seen increasingly in immuno-compromised patients. In this use, the FimA would be provided to an immuno-compromised patient (e.g., a bone marrow transplant patient) by intramuscular injection or other route prior to high dose chemotherapy or radiation therapy, and would elicit opsonic antibodies to invading streptococci in the patient's blood stream, thus enhancing clearance of these infectants.

While the invention has been described in terms of its preferred embodiments, those skilled in the art will recognize that the invention can
be practiced with modification within the spirit and scope of the appended claims.

LITERATURE CITED


Streptococcus pneumoniae gene encoding a 37-kilodalton protein homologous to previously reported Streptococcus sp adhesins. Infection and Immunity 62:319-324.


We claim:

1. A method of vaccinating a patient against endocarditis, comprising the step of administering to said patient an effective dose of FimA protein.

2. The method of claim 1 wherein said FimA protein is derived from streptococci.

3. The method of claim 2 wherein said FimA protein is derived from *S. parasanguis*.

4. The method of claim 1 wherein said FimA protein is derived from enterococci.

5. The method of claim 1 wherein said step of administering is performed parenterally.

6. A vaccine for endocarditis or bacteremia comprising an effective dose of FimA protein distributed in a carrier.

7. The vaccine of claim 6 wherein said FimA protein is derived from streptococci.

8. The vaccine of claim 7 wherein said FimA protein is derived from *S. parasanguis*.

9. The vaccine of claim 6 wherein said FimA protein is derived from enterococci.
10. The vaccine of claim 6 wherein said carrier is an injectable fluid.

11. A method of producing a vaccine for endocarditis, comprising the steps of:
   obtaining FimA protein from a cell; and
   combining said FimA protein in a carrier at an effective dose.

12. The method of claim 11 wherein said cell in said obtaining step is a recombinantly transformed cell.

13. A method of vaccinating a patient against bacteremia, comprising the step of administering to said patient an effective dose of FimA protein.

14. The method of claim 13 wherein said FimA protein is derived from streptococci.

15. The method of claim 14 wherein said FimA protein is derived from S. parasanguis.

16. The method of claim 13 wherein said FimA protein is derived from enterococci.

17. The method of claim 13 wherein said step of administering is performed parenterally.
FORWARD PRIMER WITH SphI SITE
REVERSE PRIMER WITH HindIII

PCR AMPLIFY fimA

SphI fimA HindIII FRAGMENT

PROMOTER OPERATOR

RBS

ATG

6X His

MCS WITH Sph & HindIII

SphI-HindIII DIGEST

LIGATE

FIG. 1
FIG. 2

<table>
<thead>
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<th>kDa</th>
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<tr>
<td>33.6</td>
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</table>
ANTI-FimA TITERS IN RATS

FIG. 3

ANTIBODY DILUTION (LOG)
MEAN % ADHERENCE OF S. PARASANGUIS STRAINS TO PLATLET-FIBRIN MATRIX

FIG. 4A

MEAN % ADHERENCE OF S. PARASANGUIS FW213 TO PLATLET-FIBRIN MATRIX

FIG. 4B

MEAN % ADHERENCE OF S. PARASANGUIS FW213 TO PLATLET-FIBRIN MATRIX

FIG. 4C
FIG. 5
<table>
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<th>kDa</th>
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<th>6</th>
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</table>

\[\text{FIG. 7}\]
A. CLASSIFICATION OF SUBJECT MATTER
IPC(6): A61K 39/00, 39/02, 39/38; C07K 1/00
US CL: 424/184.1, 200.1; 530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S.: 424/184.1, 200.1; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, MEDLINE, BIOSIS, CA, EMBASE, WPIDS
terms: FimA protein, S. parasanguis, enterococcus, Streptococcus, endocarditis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U.S. 5,422,427 A (RUSSEL ET AL) 06 June 1995 (06/06/95), see entire document.</td>
<td>3-17</td>
</tr>
<tr>
<td></td>
<td>WO 94/25598 A2 (UNIVERSITY OF VICTORIA INNOVATION AND DEVELOPMENT CORPORATION) 10 November 1994 (10.11.94), see entire document.</td>
<td>3-17</td>
</tr>
</tbody>
</table>

Further documents are listed in the continuation of Box C. See patent family annex.