Vaccination of Mice with Gonococcal TbpB Expressed In Vivo from Venezuelan Equine Encephalitis Viral Replicon Particles

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We investigated the immunogenicity of gonococcal transferrin binding protein B (TbpB) expressed with and without a eukaryotic secretion signal from a nonpropagating Venezuelan equine encephalitis virus replicon particle (VRP) delivery system. TbpB was successfully expressed in baby hamster kidney (BHK) cells, and the presence of the eukaryotic secretion signal not only apparently increased the protein's expression but also allowed for extra-cellular localization and glycosylation. Mice immunized with VRPs produced significant amounts of serum antibody although less than the amounts produced by mice immunized with recombinant protein. The response of mice immunized with VRPs encoding TbpB was consistently more Th1 biased than the response of mice immunized with recombinant protein alone. Boosting with recombinant protein following immunization with TbpB VRPs resulted in higher specific-antibody levels without altering the Th1/Th2 bias. Most of the immunization groups produced significant specific antibody binding to the intact surface of the homologous *Neisseria gonorrhoeae* strain. Immunization with TbpB VRPs without a eukaryotic secretion signal generated no measurable specific antibodies on the genital mucosal surface, but inclusion of a eukaryotic secretion signal or boosting with recombinant protein resulted in specific immunoglobulin G (IgG) and IgA in mucosal secretions after TbpB VRP immunization. The TbpB VRP system has potential for an *N. gonorrhoeae* vaccine.

Neisseria gonorrhoeae is the causative agent of gonorrhea and causes 62 million new infections worldwide each year (44). Despite being easily treatable in most cases, it represents a major drain on public health resources and is a cofactor for the transmission and acquisition of human immunodeficiency virus (13). Ten percent of untreated gonococcal infections in women can progress to pelvic inflammatory disease, increasing the risk of ectopic pregnancy and infertility (29, 43). Antibiotic resistance of *N. gonorrhoeae* is increasing, including resistance to ciprofloxacin, a widely used oral treatment (34, 45). Gonococcal disease is thus an underemphasized cause of morbidity and mortality, and treatment is becoming more difficult. The single most cost-effective strategy for control of infections generally is a vaccine. Even a partially effective vaccine could be useful in reducing the prevalence of this disease. Because of the sexual-network mode of transmission of gonorrhea, it might even be possible to reduce the overall incidence of gonorrhea by vaccinating a limited core population.

Based on a survey of the key surface antigens and their roles in pathogenesis (38), we chose TbpB as a potential vaccine target. TbpB is the lipoprotein member of a two-component bacterial receptor for human transferrin. It is expressed under iron limitation on the outer surface of the outer membrane (36) and reasonably well conserved (6). The ortholog of this protein in a closely related pathogen, *Neisseria meningitidis*, has been the subject of considerable vaccine research (8, 35, 42). The transferrin

receptor is required for gonococcal infection in the human urethral challenge model of infection in the absence of a functional lactoferrin receptor (7). Since only half of gonococci express a lactoferrin receptor (27), the transferrin receptor is crucial to infection and is a promising vaccine target.

One of the key issues in vaccine development is the mode of antigen delivery. Recent success with vaccines based on Venezuelan equine encephalitis virus (VEE) (1, 2, 9, 14, 16, 21–23, 30, 32, 33) prompted us to examine VEE as a potential platform for a gonococcal vaccine. VEE is an *Alphavirus* in the family *Togaviridae*. It contains an 11.4-kb, positive-sense, single-stranded RNA genome encoding three structural proteins and four nonstructural proteins (19). VEE was adapted to serve as a nonpropagating vaccine delivery system by dividing the viral genome into three separate RNAs (33). Two of the RNAs (helper RNAs) contain the structural genes for the viral coat, the capsid protein and the glycoproteins, respectively. The third RNA encodes the nonstructural proteins responsible for viral replication and was modified to express heterologous antigens (replicon RNA). When all three RNAs are cotransfected into permissive cells, they are all amplified and expressed; however, only the replicon RNA is packaged into viral coats because it is the only RNA that has the signal necessary for packaging. In this way, nonreplicating viral replicon particles (VRPs) are formed with the native viral coat and retain the tropism of the intact virus for dendritic cells (26). These VRPs are capable of delivering the replicon RNA to the first target cell but do not have the genetic information to produce progeny particles. Because the replicon is designed to express the heterologous gene from the subgenomic RNA promoter normally driving expression of the structural coat proteins, mRNA encoding the inserted gene is amplified up to 10 times

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TABLE 1. Plasmids and strains

Plasmid or strain	Relevant feature	Source or reference(s)
Plasmids		
pCR2.1	TA cloning vector	Invitrogen
pET30a	T7 expression vector	EMD Biosciences
V1Jns-tPA	Vector with tPA signal sequence	25
pVR21	VEE replicon vector	33
pCA50	VEE capsid helper	33
pK8.1	VEE glycoprotein helper	33
pUNCH677c	<i>tbpB</i> in pCR2.1	This study
pUNCH681	<i>tbpB</i> in pET30a	This study
pUNCH689	V1Jns-tPA with BglII-AccI adapter	This study
pUNCH692	<i>tbpB</i> with tPA signal sequence	This study
pUNCH696	tPA- <i>tbpB</i> replicon	This study
pUNCH698	<i>tbpB</i> replicon	This study
Strains		
<i>N. gonorrhoeae</i>		
FA1090 A23	Prototype gonococcal strain (PIB-3)	5, 28
FA6916	FA1090 A23 Δ <i>tbpB-tbpA</i>	7
<i>E. coli</i>		
DH5 α MCR	Cloning strain	Invitrogen
Novablue(λ DE3)	T7 expression strain	EMD Biosciences

the level of the genomic RNA and is capable of high-level heterologous-antigen expression (39, 40).

The VEE VRP system has been used to express a growing list of heterologous antigens, including antigens from Lassa fever (33), influenza (33), Marburg (16), Ebola (32), simian immunodeficiency (9, 10), human papilloma (4, 41), equine arteritis (1, 2), and Norwalk (15) viruses. More recently, a number of bacterial proteins have been expressed in this system, including botulinum neurotoxin (23), *Borrelia burgdorferi* OspA (14), staphylococcal enterotoxin (21), and the protective antigen from *Bacillus anthracis* (22). In this study, we tested VEE VRPs as a potential platform for a gonococcal vaccine. We constructed TbpB VRPs with and without the eukaryotic secretion signal from tissue plasminogen activator (tPA). Mice immunized with VRPs generated a Th1-biased immune response, and anti-TbpB immunoglobulin A (IgA) antibodies were detected in vaginal washes. These findings suggest that TbpB VRPs are a potential vaccine for gonorrhea.

MATERIALS AND METHODS

General bacteriology. *N. gonorrhoeae* strain FA1090 (28) variant A23a was used throughout these experiments and was obtained from Marcia Hobbs (5). Passage of FA1090 and its derivatives was kept to a minimum to avoid phase or antigenic variation. Gonococcal strains were routinely maintained on GC base medium (Difco) supplemented with Kellogg's supplement I (18). All relevant strains and plasmids are shown in Table 1.

Cloning. Gonococcal *tbpB* was amplified by PCR from FA1090 by use of primers GCV-1 and GCV-2 (all oligonucleotide primers are shown in Table 2). This PCR product was then cloned using the TA cloning system (Invitrogen) to create pUNCH677c (*tbpB*). Where necessary, multiple clones were combined to create a complete clone containing the authentic FA1090 sequence. This clone was used as the starting point for several subsequent subclonings and amplifications. Comparison of the published sequence of FA1090 *tbpB* (6) (GenBank accession no. U65219) with the sequence determined as part of the FA1090 genome (GenBank accession no. NC_002946) revealed two single base differences at bases 114 and 743 of the *tbpB* open reading frame. The sequence that we determined for our clone agreed with the published FA1090 *tbpB* sequence at base 114, a difference that does not affect the encoded amino acid, and with the genome sequence at base 734, which would change alanine 248 of the published sequence to a glycine. These sequence differences could represent authentic sequence variations or sequencing errors. We used the sequence we determined for isolate A23 of FA1090 as our reference sequence.

The insert from pUNCH677c (*tbpB*) was isolated as a Klenow blunted ClaI fragment and ligated into Klenow blunted NotI-digested pET30a, a bacterial expression vector. Transformants of this ligation were screened by size and hybridization. Insert-positive clones were pooled and transformed into *Escherichia coli* expression strain Novablue(λ DE3) (EMD Biosciences) and screened by Western blotting for antigen expression. This clone was designated pUNCH681 (T7-His-*tbpB*).

To place the tPA secretion signal upstream of the mature coding sequences of *tbpB*, the insert from pUNCH677c (*tbpB*) was cloned downstream of the tPA signal sequence in pUNCH689 (tPA). The plasmid pUNCH689 is a derivative of VJns-tPA (25) with the BglII-AccI adapter (Table 2) inserted into the BglII site, creating a new unique AccI site which is compatible with the ClaI sites bounding the insert of pUNCH677c (*tbpB*). The tPA-containing clone was designated pUNCH692 (tPA-*tbpB*). This clone was used as the template for the corresponding replicon constructs discussed below.

The replicon constructs were assembled essentially as previously described (2) by a process of overlapping PCR amplification to create an authentic fusion between the 26S VEE viral structural gene promoter and the synthetic start codon of the bacterial gene. All amplifications used Vent DNA polymerase (New England Biolabs) in an effort to reduce replication errors. The insert from pUNCH677c (*tbpB*) was amplified by PCR using primers GCV-5/GCV-6 to incorporate an upstream overlap with the 26S VEE promoter and a downstream AseI site. In a similar fashion, the insert from pUNCH692 (tPA-*tbpB*) was amplified with GCV-12/GCV-6. The 26S promoter was amplified with primers GCV-9/GCV-10 and combined with each of the previous PCR products in an overlapping PCR fusion reaction using GCV-9 as the upstream primer and GCV-6 as the downstream primer. These two PCR fusion products were digested with ApaI (cuts the 26S promoter) and AseI (cuts the downstream end of each of the PCR products) and ligated into similarly cut replicon vector,

TABLE 2. Oligonucleotides used

Primer designation	Length (nt) ^b	Relevant feature	Sequence (5' to 3') ^a
GCV-1	24	5' <i>tbpB</i> (adds ClaI site)	<u>ATCGATG</u> gtgtctggcgaggcg
GCV-2	22	3' <i>tbpB</i> (adds ClaI site)	<u>ATCGat</u> tgttcggcagccgtgc
BglII-AccI adapter	14	Self-annealing with BglII cohesive ends (adds AccI site)	5' GATCGCGTCGACGC 3' 3' CGCAGCTGCGCTAG 5'
GCV-5	41	5' <i>tbpB</i> (adds overlap with 26S VEE promoter)	<u>AGTCTAGTCCGCCAAGATG</u> gtgtctggcgaggcgagtc
GCV-6	44	3' <i>tbpB</i> (adds AseI site)	ATTATCAGGCGCGCcttatttcacaagctgttggcggttcgca
GCV-9	30	5' 26S VEE promoter	GGTGATCCAGGCTGCCGATCCGCTAGCAAC
GCV-10	45	3' 26S VEE promoter	<u>CATCTTGGCGGACTAGACT</u> ATGTCGTAGTCCATTAGGTTAGCCG
GCV-12	36	5' tPA (adds overlap with 26S VEE promoter)	<u>AGTCTAGTCCGCCAAGatg</u> gatgcaatgaagagagg

^a Added restriction sites are underlined, start codons are in boldface, bacterial and tPA sequences are in lowercase, and viral and synthetic sequences are in uppercase. Overlapping sequences for PCR fusion are double underlined.

^b nt, nucleotides.

TABLE 3. Mouse inoculation schedule

Immunization group	Inoculations 1, 2, and 3		Inoculation 4	
	Immunogen	Route	Immunogen	Route
Mock	PBS, Rib	Footpad	PBS, Rib	Footpad
TbpB	rrTbpB, Rib	Dorsal	rrTbpB, Rib	Dorsal
Late TbpB ^a	ND ^b	ND	rrTbpB, Rib	Dorsal
TbpB VRP	TbpB VRP	Footpad	TbpB VRP	Footpad
TbpB VRP plus rrTbpB	TbpB VRP	Footpad	rrTbpB, Rib	Dorsal
tPA-TbpB VRP	tPA-TbpB VRP	Footpad	tPA-TbpB VRP	Footpad
tPA-TbpB VRP plus rrTbpB	tPA-TbpB VRP	Footpad	rrTbpB, Rib	Dorsal

^a Late TbpB, single injection at the time of the fourth inoculation for the other groups.

^b ND, not done.

pVR21 (2), to give rise to pUNCH696 (tPA-*tbpB*) and pUNCH698 (*tbpB*). The inserts of all final constructs were sequenced to confirm fidelity, and any errors were corrected by screening and/or subcloning.

Production of recombinant antigens and rabbit antiserum. Novablu (λDE3) containing pUNCH681(T7-*His-tbpB*) was expanded to produce recombinant protein by inducing a culture of an optical density of 0.4 to 0.5 with 2 mM isopropyl-β-D-thiogalactopyranoside for 30 min and arresting normal cellular RNA synthesis with rifampin for an additional 2 h. Since pUNCH681(T7-*His-tbpB*) expresses recombinant TbpB as an N-terminally six-His-tagged fusion (rHis-TbpB), we purified this fusion on Ni-nitrilotriacetic acid agarose (QIAGEN) under denaturing conditions by using a modification of protocol no. 9 (QIAexpressionist; QIAGEN). Briefly, the cell pellet was lysed for 10 min in 6 M guanidinium hydrochloride buffered with 100 mM NaH₂PO₄ and 10 mM Tris to pH 8.0, DNA was sheared by 10 passages through an 18- to 22-gauge needle, cellular debris was removed by pelleting at 10,000 × *g* for 30 min, His-tagged protein was bound to Ni-nitrilotriacetic acid agarose in batch format for 30 min, resin was washed with 8 M urea buffered as described above to pH 8 in column format, resin was washed again in 8 M urea buffered as described above to pH 6.7, recombinant protein was eluted in 8 M urea buffered as described above to pH 5.0, and quantitation was measured by the Bio-Rad protein assay.

An attempt to renature the recombinant protein was made by slow dilution of the denaturing urea in the presence of low concentrations of detergents. The rHis-TbpB was diluted to 380 μg/ml in 1.6 M urea, 0.04% Triton X-100, 30 mM NaCl, 130 mM Tris (pH 7.5) and slowly diluted to 18 μg/ml with 150 mM NaCl and 50 mM Tris (pH 7.5). At this stage, the protein was designated recombinant "renatured" His-TbpB(rrHis-TbpB). The antigen had assumed some native conformation since the rrHis-TbpB could bind human transferrin by a dot blot assay (data not shown).

The rrHis-TbpB was used as an immunogen to immunize Elite rabbits at Covance Research Products. Rabbits were immunized with 250 μg of recombinant protein in Freund's complete adjuvant and boosted with 125 μg of recombinant protein in Freund's incomplete adjuvant three times at 3-week intervals. Rabbits were bled at 7, 10, and 13 weeks.

The rrHis-TbpB was cleaved by recombinant enterokinase (rEK) (EMD Biosciences) at 20°C for 3 h. Removal of the S tag epitope (encoded along with the His tag) was monitored by Western blotting. The protein was deemed cleaved when no detectable S tag reactivity comigrated with the TbpB band by Western blotting. After the cleavage reaction, rEK was removed with EKapture agarose (supplied in the rEK cleavage/capture kit; EMD Biosciences), and the protein was concentrated and purified by ultrafiltration using a Centricon YM-50 filter (Millipore) and designated rrTbpB.

Replicon-based expression. Vaccine antigen expression from replicon constructs was confirmed in vitro essentially by the methods described by Pushko et al. (33). Briefly, RNase-free, NotI-linearized replicon plasmids were transcribed using an mMESSAGE mMACHINE T7 kit (Ambion), and the capped runoff transcripts were electroporated into baby hamster kidney (BHK) cells by use of the conditions of Liljestrom and Garoff (24) (0.4-cm-gap cuvette; three pulses, 0.85 kV, 25 uF). Efficiency of transfection (percentage of cells expressing antigen) was measured by indirect immunofluorescence. An aliquot of each electroporation was plated onto a chamber slide and incubated along with the rest of the electroporation for 18 to 24 h. The chamber slides were rinsed with phosphate-buffered saline (PBS), fixed for 10 min in ice-cold acetone, dried, and stored at -20°C. Slides were later rehydrated in PBS with 0.2% normal goat serum (NGS) and then blocked in 5% NGS. Antigen was detected by sequential addition of rabbit anti-rrHis-TbpB (1:200, see above), biotin-SP AffiniPure goat anti-rabbit IgG (H+L) (1:100; Jackson ImmunoResearch Laboratories), and cyanine Cy2

streptavidin (1:100; Jackson ImmunoResearch Laboratories), each diluted in PBS containing 5% NGS.

The quantity of expression was measured by subjecting a crude lysate of the transfected BHK cells to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (3, 20). The crude lysate consisted of rinsed BHK cells solubilized in 1% SDS for 5 min at 37°C and diluted to equal total protein concentration determined by a bicinchoninic acid protein assay (BCA; Pierce). SDS-PAGE samples were routinely boiled for 2 min under reducing conditions (5% β-mercaptoethanol) prior to loading. Antigens were detected in standard immunoblot format by using 1:10,000 rabbit anti-rrHis-TbpB, 1:5,000 alkaline phosphatase-conjugated sheep anti-rabbit IgG F(ab')₂ fragment (Sigma), and LumiPhos WB (Pierce).

Replicons were assembled into VRPs as previously described (33) by coelectroporating three RNAs into BHK cells. The three RNAs consisted of runoff transcripts from capsid helper (pCA50), glycoprotein helper (pK8.1), and one of the replicon constructs. The glycoprotein helper plasmid pK8.1 encodes wild-type glycoproteins E1 and E2. We chose the wild-type glycoproteins to take advantage of their efficient targeting to Langerhans cells (26). Supernates from the electroporated BHK cells were harvested after 20 to 27 h, and VRPs were concentrated by centrifugation through a 20% sucrose cushion at 100,000 × *g*. Particle pellets were resuspended in PBS and stored in aliquots at -70°C. Particle preparations were quantitated by infecting subconfluent BHK monolayers on chamber slides with dilutions of particles, followed by detection of expressed antigen by indirect immunofluorescence after 18 to 24 h.

Glycosylation of expressed antigens was investigated by evaluating the effect of deglycosylation on electrophoretic mobility of antigens from transfected or infected BHK cell lysates. Crude BHK lysates were exposed to peptide N-glycosidase F (PNGase F) (New England Biolabs) at a concentration of 4 U per 10 μg protein lysate for 1 h under the manufacturer's suggested conditions. Lysates were boiled, subjected to SDS-PAGE and Western blotting, and detected as described above.

Secretion of expressed TbpB with and without tPA was evaluated by immunoprecipitation of culture supernatants, using rabbit anti-rrHis-TbpB. Antibodies were diluted in IP buffer (1% Triton X-100, 150 mM NaCl, 1 mM EDTA, and 10 mM Tris, pH 7.4) and allowed to bind to protein A Sepharose high performance (Pharmacia) for 1 h at 4°C. Sepharose was rinsed three times in IP buffer and exposed to culture supernatant diluted in IP buffer for 1 h at 4°C. Beads were rinsed three more times in IP buffer and boiled in SDS-PAGE loading buffer. Beads were pelleted, and supernates were subjected to SDS-PAGE and Western blotting and detected as described above.

Mouse inoculation and sample collection. Four- to 5-week-old female BALB/c mice were purchased from Harlan Laboratory Animals and housed in the University of North Carolina animal facility according to federal and institutional animal care and use committee guidelines. Mice in groups of six were immunized as shown in Table 3. The mock-immunized group was inoculated in each hind footpad with 10 μl PBS. The TbpB VRP-immunized groups were inoculated in a similar fashion with PBS containing 1 × 10⁶ TbpB VRPs in a 20-μl total volume. Immunization with rrTbpB was done by subcutaneous injection of 5 μl rrTbpB in 50 μl of Rib R-700 adjuvant system (Corixa) at each of two dorsal sites. Mice were boosted with immunizations identical to their prime immunization at 4 and 7 weeks postprime. Mock controls for the group vaccinated with rrTbpB were given 50 μl Rib R-700 adjuvant in each of the dorsal sites. Five of the groups were given a final boost at 10 weeks that was identical to their earlier immunizations. The remaining three groups were given a final boost at 10 weeks of rrTbpB in Rib adjuvant administered as described above. A group that received only a single dose of rrTbpB in Rib adjuvant at week 10 was included

to determine how much of the response to the rrTbpB boost was a primary response to the rrTbpB and how much was a boost of the response generated by the TbpB VRPs.

Venous tail blood was collected preprime and at 4, 7, 10, and 13 weeks postprime, always prior to immunization. Blood was collected in Microtainer serum separator tubes (Becton Dickinson), allowed to clot for 30 min at room temperature (RT), and stored at 4°C overnight. The following morning, tubes were centrifuged according to the manufacturer's specifications. Serum was filtered through a sterile, 0.45- μ m cellulose acetate centrifuge tube filter (Costar). Equal amounts of serum from each animal were used to make pooled samples for each bleed and group. Vaginal mucosal samples were collected at 2 weeks after the fourth immunization by washing mouse vaginal cavities several times with 50 μ l PBS, followed by addition of 10 μ l 5 $\times$ stabilization buffer (protease inhibitor cocktail no. P8340 [Sigma, St. Louis, MO], according to the manufacturer's recommendations, in PBS with 0.1% sodium azide). Twenty microliters of individual vaginal samples was taken to make a pool. Serum and vaginal fluids were stored at 4°C until needed.

Serum and vaginal mucosal antibody analysis. The quantity of antibodies in serum and vaginal fluids was measured by indirect enzyme-linked immunosorbent assay (ELISA) as previously described by Zhu et al. (47). In brief, microtiter wells (F96 certified MaxiSorp Nunc-Immuno plates) were coated with 250 ng rrTbpB in 50 μ l 50 mM sodium bicarbonate coating buffer (pH 9.6) at 4°C overnight. They were then blocked with 2% bovine serum albumin in ELISA buffer (PBS containing 0.05% Tween 20) at 37°C for 2 h. The samples and standards were serially diluted in ELISA buffer with 1% bovine serum albumin, added to the coated/blocked wells, and incubated at 4°C overnight. The quantity of TbpB-specific antibody bound to the plates was determined by comparing the optical density of the TbpB-coated wells to that obtained by coating the wells with goat anti-mouse IgG-Fc affinity-purified coating antibody at 1:100 (or IgA-affinity-purified coating antibody at 1:100 for IgA ELISA) obtained from Bethyl Laboratories, comparing them to a standard curve of mouse reference serum with known quantities of immunoglobulins (IgG, IgA, IgG1, or IgG2a; also purchased from Bethyl Laboratories) in wells developed in parallel with the TbpB-specific wells. Antibodies were detected with goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies and Ultra-TMB substrate (Pierce, Rockford, IL). Secondary antibodies were purchased from Bethyl Laboratories and were used at the following concentrations: HRP-conjugated goat anti-mouse IgG at 1:5,000; HRP-conjugated goat anti-mouse IgA at 1:2,500; HRP-conjugated goat anti-mouse IgG1 at 1:10,000; and HRP-conjugated goat anti-mouse IgG2a at 1:5,000. Reactions were developed by adding 50 μ l Ultra-TMB substrate (Pierce) and incubating for 30 min at RT. Results were read with a Victor2 1420 multilabel counter (EG&G Wallac) by using a 450-nm filter.

Whole-cell binding assay. The relative amounts of antibodies that recognized the surface of the bacteria were measured by indirect whole-cell radioimmunoassay. This assay consisted of exposing limiting dilutions of serum to whole bacteria that differed only in antigen of interest and detecting bound antibody by using 125 I-labeled goat anti-mouse immunoglobulin (12, 17). Immune sera were diluted 1:100 in 50 μ l Bacto GC medium base broth (GCB) and then mixed with 150 μ l GCB containing 2×10^8 CFU gonococci/ml (final dilution of serum, 1:400) for 15 min at RT. The gonococci were collected by filtration in a multi-screen plate (Millipore, Bedford, MA). Filters were washed five times with PBS-azide (0.1%) buffer before adding 1×10^6 cpm of 125 I-labeled secondary antibody (goat anti-mouse IgG, IgA, and IgM; ICN Biomedicals, Inc., Aurora, OH) in 100 μ l to each well. After incubation at RT for 15 min, the filters were washed five times with PBS-azide (0.1%) and air-dried, and the filter bottom was punched out into borosilicate glass tubes. Antibody binding was assessed by counting gonococcal-associated radioactivity with a 1271 RIA Gamma counter (LKB Wallac). The antibodies recognizing surface-exposed epitopes of TbpB were detected by subtracting the amount of antibody bound to iron-stressed FA6916 (FA1090 Δ tbpB-tbpA) (7) from the amount bound to iron-stressed FA1090 A23.

Bactericidal assay. Bactericidal activity was measured as previously described by Elkins et al. (11). Mouse sera were added to about 200 CFU gonococci in 50 μ l GCB (final dilution, 1:50) and incubated at 37°C for 15 min. Ten percent pooled normal human serum (NHS) was added as a source of active complement, and incubation was continued at 37°C for 45 additional minutes. Samples were plated and viable counts were determined. Significant killing was defined as greater than 50% decrease in viable count compared to that of controls without mouse sera added. Nonspecific killing by the NHS was monitored by incubating gonococci with heat-inactivated NHS and held to less than 20%. Assays were performed using pooled serum from each group in triplicate on at least 2 days.

Statistics. Quantitative assays were performed a minimum of three times on separate days (see the specifics of each assay listed in the figure legends). The

FIG. 1. Western blot showing expression of TbpB from TbpB VEE replicons electroporated into BHK cells. Lane 1 contains 20 μ g of total membranes from iron-stressed FA1090 as a size control for full-length native TbpB, lane 2 contains 50 μ g of crude cell lysate from BHK cells electroporated with green fluorescent protein-expressing VEE replicon (negative control), lane 3 contains 50 μ g of crude cell lysate from BHK cells electroporated with TbpB VEE replicon pUNCH698 (*tbpB*), and lane 4 contains 50 μ g of crude cell lysate from BHK cells electroporated with tPA-TbpB VRP replicon pUNCH696 (tPA-*tbpB*). Numbers to the right of the blot mark the relative mobilities of molecular size markers in kDa. The blot was probed with rabbit anti-rrHis-TbpB.

deviations shown by error bars are the standard deviations between animals within a group. The variability of determinations in the assays was lower than the variability between individuals within a group. The ability to discriminate between the samples from different groups was determined by the use of Student's *t* test and considered significant if *P* was ≤ 0.05 .

RESULTS

Expression of gonococcal antigens in BHK cells. We transfected replicon RNA by electroporation into BHK cells to test the ability of the replicon constructs to express TbpB despite the nonnative codon preference of this bacterial gene in a eukaryotic system. These transfections were consistently efficient, with >80% TbpB-expressing cells as judged by indirect immunofluorescence (data not shown), and were used as the antigen source for the Western blot shown in Fig. 1. Figure 1 demonstrates that the TbpB expressed by these replicon constructs is abundant and full-length. While we acknowledge that Western blotting is not usually considered very quantitative, we consistently saw higher levels of TbpB expressed from the tPA-containing constructs (Fig. 1). The alteration of TbpB's electrophoretic mobility in the tPA-containing constructs was the first indication that the TbpB fused to tPA might be modified by the eukaryotic expression system. One explanation for the slower-than-expected mobility of the primary expression product in the tPA-TbpB replicon transfected lysate (Fig. 1, lane 4) was the addition of carbohydrate groups to TbpB fused to its eukaryotic partner. The immunoreactive bands migrating faster than the full-length TbpB product (Fig. 1, lanes 3 and 4) could represent fragments of the full-length product after proteolytic attack or alternative translation products that remained immunoreactive.

By use of the prediction algorithm of NetNGlyc (version 1.0; Center for Biological Sequence Analysis, Technical University

FIG. 2. Western blot showing the effect of deglycosylation on TbpB expressed from BHK cells infected with TbpB VRPs. Lane 1 contains 10 µg of total membranes from iron-stressed FA1090 as a size control for full-length native TbpB. Each of the remaining lanes contains 10 µg crude BHK cell lysate treated identically, except that PNGase F was added to samples shown in lanes 3 and 5. Lysates in lanes 2 and 3 were derived from BHK cells infected with TbpB VRPs lacking the tPA signal sequence. Lysates in lanes 4 and 5 were derived from BHK cells infected with tPA-TbpB VRPs (containing the tPA signal sequence). Numbers to the right of the blot mark the relative mobilities of molecular size markers in kDa. The blot was probed with rabbit anti-His TbpB.

of Denmark [http://www.cbs.dtu.dk/services/NetNGlyc/], TbpB was predicted to have three asparagines with a glycosylation potential of >0.5 (Asn-364, -542, and -611 of mature TbpB). To test whether the slower-migrating primary expression product in the tPA-TbpB replicon lysate could be explained by N-linked glycosylation (the most widely distributed form of glycosylation in eukaryotes) of the primary expression product,

FIG. 3. Western blot showing TbpB present in the supernatant of BHK cells electroporated with tPA-TbpB VEE replicon RNAs. Lane 1 contains 10 µg of total membranes from iron-stressed FA1090 as a size control for full-length native TbpB. Lanes 2 and 3 contain 10 µl of culture supernatant, while lanes 4 and 5 contain the equivalent of 600 µl of supernatant that had been immunoprecipitated. The samples in lanes 2 and 4 came from cultures electroporated with TbpB VEE replicon RNAs lacking the tPA signal sequence, pUNCH698 (*tbpB*). The samples in lanes 3 and 5 came from cultures electroporated with tPA-TbpB VEE replicon RNAs containing the tPA signal sequence, pUNCH696 (*tPA-tbpB*). Numbers to the right of the blot mark the relative mobilities of molecular size markers in kDa. The blot was probed with rabbit anti-His-TbpB.

FIG. 4. Serum IgG response against gonococcal antigens, as measured by quantitative ELISA. Open bars indicate the average amounts of TbpB-specific IgG at 2 weeks after the third immunization (week 10), and filled bars indicate the average amounts of TbpB-specific IgG at 2 weeks after the fourth immunization (week 13). Late rrTbpB indicates the group receiving a single injection of rrTbpB at week 13. The error bars indicate the standard deviations for individual mice from each group. Specific IgG levels were significantly higher than those of the age-matched, mock-immunized group controls for all experimental groups with the exception of the late rrTbpB group. Symbols: †, samples that significantly increased after final recombinant protein boost (week 10 versus week 13); ‡, samples that significantly increased compared with their age-matched, non-tPA-containing partner (TbpB VRPs with and without tPA signal) (applies to samples from both week 10 and week 13). The significance threshold was $P < 0.05$ by Student's t test.

lysates from BHK cells infected with VRPs expressing TbpB either with the tPA signal sequence (tPA-TbpB VRPs) or without the tPA signal sequence were exposed to PNGase F. These lysates along with their unexposed counterparts were subjected to Western blotting (Fig. 2). PNGase F exposure increased the mobility of the primary tPA-TbpB VEP expressed antigen to near the mobility of its non-tPA signal-containing counterpart, indicating that the reduced mobility was due to N-linked glycosylation. Since none of the other primary expression products changed in mobility, it is unlikely that they were glycosylated.

Glycosylation in eukaryotes normally occurs during the process of export through the endoplasmic reticulum. To determine if any of the expressed gonococcal antigens were being exported by the BHK cells, culture supernatants from replicon transfected BHK cells were subjected to immunoprecipitation and Western blotting (Fig. 3). Only the supernatant from the tPA-TbpB replicon transfected BHK cells had detectable antigen in either the supernatant or the immunoprecipitated supernatant, indicating that the tPA signal was directing the export of TbpB at detectable levels.

Immune responses. (i) Serum IgG. The quantity of anti-TbpB IgG present in the serum from each individual mouse was measured, and the average amount of TbpB-specific IgG for each immunization group is shown in Fig. 4. The background of antigen-specific antibodies observed with the mock-immunized group remained below 0.1 µg/ml throughout the experiments. All of the mice immunized with TbpB VRPs developed specific IgG after one inoculation. The specific IgG

FIG. 5. Serum IgG1 and IgG2a antibody responses to TbpB. The number at the top of each bar is the ratio of IgG1/IgG2a for each sample. In each bar, the top open portion indicates the amount of IgG1 recognizing TbpB, while the lower filled portion indicates the amount of IgG2a recognizing TbpB in the same sample. The error bars indicate the standard deviations for individual mice from each group. Symbols: *, ratios significantly different from age-matched rrTbpB samples (recombinant protein versus TbpB VRPs); †, ratios in TbpB VRP groups that were significantly different from age-matched, tPA-containing partner (TbpB VRPs with and without tPA signal). The significance threshold was P of <0.05 by Student's t test comparing individual-mouse ratios of IgG1 and IgG2a.

responses reached their peak after the second inoculation and did not increase after further immunization (data not shown). At 14 days after the third immunization, TbpB VRP-immunized mice produced specific IgG antibodies ranging from 2 µg/ml to 5 µg/ml. Presence of the tPA signal significantly improved the amount of specific IgG produced by TbpB VRP-immunized mice. Comparing tPA-TbpB VRP-immunized mice to TbpB VRP-immunized mice showed that tPA-TbpB VRP-immunized mice produced at least 54 times more anti-TbpB IgG in serum. A final boost with rrTbpB resulted in an increase of about 2.5-fold in the average amount of specific IgG for each TbpB VRP-immunized group. Immunization with recombinant TbpB in Ribi R-700 resulted in specific IgG levels that were significantly higher than those for all other groups, reaching a peak after three immunizations of 1 mg/ml to 2 mg/ml.

(ii) IgG1 and IgG2a responses. The IgG1 and IgG2a isotype responses are shown in Fig. 5. The antibody subclass distribution revealed predominant expression of IgG2a for all TbpB VRP regimens, indicating an apparent bias toward a cellular response characterized by Th1. As expected, the IgG subclass distribution among the rrTbpB-immunized groups revealed predominant expression of IgG1, indicating a bias toward a humoral response characterized by Th2. It should be noted that the scales of the three graphs in Fig. 5 are vastly different. Although both VRP immunization regimens clearly induced a Th1-biased immune response, based on the predominance of IgG2a, the tPA VRPs unexpectedly gave a slightly more Th1 polarized response. Consistent with the concept of “immunological sin,” the IgG1/IgG2a ratios for TbpB VRP-immunized mice were little changed at week 13 after a boost with rrTbpB at week 10.

(iii) Mucosal antibody responses. Ability to induce IgA responses at mucosal surfaces will probably be a necessary feature of an effective gonococcal vaccine. In order to understand whether subcutaneous inoculation with VRPs resulted in the production of antigen-specific mucosal antibody, we examined the vaginal wash samples from individual mice to determine their total IgG, IgA, and TbpB-specific IgG and IgA antibody levels.

In general, the mucosal TbpB-specific IgG responses showed the same trend as serum IgG (Fig. 6). The groups immunized with rrTbpB gave the strongest response. TbpB VRP-immunized mice generated a mild mucosal IgG response, whereas the tPA-TbpB VRP-immunized animals showed a significantly improved specific IgG response. Boosting with rrTbpB resulted in an increase in the specific mucosal IgG response. Since these results mirrored the serum responses, they may reflect transudation of serum IgG onto the vaginal surface.

Specific mucosal IgA antibody responses were related to the vector (Fig. 7). The TbpB VRP-immunized group generated

FIG. 6. TbpB-specific IgG generated in mouse vaginal secretions. Vaginal wash samples were collected 2 weeks after the fourth immunization. Each bar presents the average amount of TbpB-specific IgG generated by each immunization group. Late rrTbpB indicates the group that received a single injection of rrTbpB in Ribi adjuvant at week 10. The error bars indicate the standard deviations for individual mice from each group. *, samples significantly different from age-matched, mock-immunized samples. The significance threshold was P of <0.05 by Student's t test.

FIG. 7. TbpB-specific IgA generated in mouse vaginal secretions. Vaginal wash samples were collected 2 weeks after the fourth immunization. Each bar presents the average amount of TbpB-specific IgA generated by each immunization group. Late rrTbpB indicates the group that received a single injection of rrTbpB in Ribi adjuvant at week 10. The error bars indicate the standard deviations for individual mice from each group. Specific IgA levels were significantly higher than those of mock-immunized group controls for all experimental groups with the exception of the TbpB VRP group. Symbols: †, samples that significantly increased after final recombinant protein boost; ‡, samples that significantly increased compared with their age-matched, non-tPA-containing partner. The significance threshold was P of <0.05 by Student's t test.

little more specific IgA than the mock-immunized group, whereas the other immunized animals generated significantly higher specific mucosal IgA responses. Boosting with rrTbpB also generated increased specific mucosal IgA. Since we measured neither serum IgA responses nor secretory IgA specifically, we were not able to differentiate how much of the mucosal response was true genital secretory IgA production and how much was transudation of serum IgA such as is assumed for the mucosal IgG.

Surface binding. One of the assumptions in the design of a gonococcal vaccine is that antibodies which recognize the bacterial surface will play a major role in a protective response. The relative amount of surface binding antibody produced by each immunization group was measured by a whole-cell binding assay (Fig. 8). TbpB-specific surface binding antibodies were defined by the amount of antibody bound after the background of antibody bound to an isogenic strain lacking TbpB was subtracted. The amounts of nonspecific surface binding antibodies in the isogenic strain lacking TbpB varied from 2,000 to 5,000 cpm depending on the group. All of the VRP-immunized groups had significantly more surface binding IgG antibodies than the mock-immunized group, ranging from 7,000 to 30,000 net cpm bound. The highest levels of surface-bound IgG were observed for the group immunized with rrTbpB, where the TbpB-expressing strain bound 84,000 cpm more than the isogenic strain lacking TbpB. The TbpB VRP-immunized groups generated more binding after a final boost with rrTbpB. A single inoculation of rrTbpB given to previously unimmunized animals at 10 weeks resulted in no surface-bound IgG. Interestingly, the level of surface binding antibod-

FIG. 8. Whole-cell binding activity of antibodies recognizing surface-exposed epitopes of TbpB. Antibody bound to the surface of iron-stressed FA1090 after subtraction of antibody bound to FA6916 ($\Delta tpbB$ - $tbpA$) is shown. Late rrTbpB indicates the group that received a single injection of rrTbpB in Ribi adjuvant at week 10. The error bars indicate the standard deviations of between three and eight determinations on between 2 and 4 days. Symbols: *, surface binding significantly different from age-matched, mock-immunized samples (immunized versus mock); †, surface binding that increased significantly after final recombinant protein boost (week 10 versus 13); ‡, TbpB VRP-immunized groups that bound significantly more antibodies than their age-matched, tPA-TbpB VRP-immunized partner. The significance threshold was P of <0.05 by Student's t test.

ies was significantly lower for the group immunized with tPA-TbpB VRPs than for age-matched samples of the TbpB VRP group. This was in direct contrast to the serum antibody levels as measured by ELISA, which were higher for the tPA-TbpB VRP-immunized group (Fig. 4).

DISCUSSION

The long-term goal of the work presented here is to devise a vaccine against *N. gonorrhoeae*. While a VEE-based gonococcal vaccine may not be the ultimate solution, it has many features that make it an attractive system for studies of potentially protective immune responses. The VEE system can be thought of as an in vivo expression system with both specific targeting (due to viral coat) and message amplification systems incorporated. These properties give rise to substantial expression and accompanying immunological responses.

We successfully expressed TbpB in BHK cells with and without a eukaryotic secretion signal (tPA), despite the risk of codon preference differences between bacterial and eukaryotic coding sequences. Inclusion of the tPA signal had obvious effects on the expression and fate of the gonococcal antigens expressed in BHK cells, as shown in Fig. 1 to 3. The tPA-fused antigens were consistently more highly expressed, and tPA-TbpB was apparently carbohydrate modified, indicating that it had been targeted to the endoplasmic reticulum. Secretion of tPA-TbpB into the culture supernatant provided further evi-

dence for the targeting of tPA-TbpB to the endoplasmic reticulum pathway. Since a substantial amount of tPA-TbpB was exported to the supernatant, the amount of antigen shown for the tPA-TbpB replicon (Fig. 1, lane 4) underestimated true expression levels, as this experiment assayed only the portion of TbpB that remained cell associated.

Quantitative measurement of antigen-specific IgG by ELISA was used to judge the overall strength of the humoral immune response. The group immunized with rrTbpB in Ribi R-700 adjuvant was consistently the strongest-responding group by a significant margin (minimum of fivefold over other groups). The modest effect of a boost with rrTbpB on the TbpB VRP-immunized groups could be explained if the recombinant protein stimulated a new set of immune cells, acting as a new primary immunization instead of a classical boost of a memory response. The amount of TbpB-specific IgG in serum was significantly increased for the group immunized with tPA-TbpB VRPs compared to that for its non-tPA-containing counterpart, possibly due to increased expression. There could also be significant differences in the ways that tPA-TbpB was presented to the immune system, since it was glycosylated and exported.

Measurement of IgG subclass distribution was used as a surrogate for the immunological bias of the response, Th1 versus Th2. As expected, the rrTbpB-immunized group tended toward a Th2-type response since recombinant proteins tend to be processed and presented through a class II major histocompatibility complex-dependent pathway. In general, the VRP-vaccinated groups tended toward a Th1-type response, also as expected, since the cytoplasmic expression from the VRPs most closely resembles viral expression which is processed and presented via a class I major histocompatibility complex-dependent pathway. It was surprising that the tPA-TbpB VRPs did not elicit a more Th2-biased response, since other experiments showed tPA-TbpB to be exported substantially to the supernatant, which presumably would cause it to be presented in a fashion similar to that for a recombinant protein vaccine. This unexpected result might be explained by the antigenic characteristics inherent in TbpB or by the relatively greater immunogenicity of the nonsecreted fraction of tPA-TbpB. However, this bias is consistent with the observations of Gipson et al. for the OspA protein expressed under similar circumstances (14).

We presume that protective antibodies will need to recognize the surface of whole gonococci, as a minimum requirement. Surface-bound antibodies might act in several ways, including direct bactericidal activity through complement deposition, enhancement of opsonophagocytic attack, or blocking of the normal function of gonococcal surface structures (i.e., transferrin utilization, attachment, or invasion). We observed significant specific binding to whole cells with most of the immunization protocols. Curiously, the TbpB VRP-immunized group had significantly more surface binding antibody than its tPA-fused counterpart (Fig. 6). This is in contrast to the observation that the tPA-TbpB VRP-immunized group had significantly more antibody overall (Fig. 4 and 5). One possible explanation for this incongruity is that the glycosylation of tPA-TbpB might have blocked one or more surface-exposed immunodominant epitopes. Another explanation might be that altered processing of tPA-TbpB resulted in expression of different immunodominant epitopes that did not include as many that were surface

exposed. These two possibilities might be differentiated by mutagenizing the glycosylation sites within the TbpB coding sequence, but these experiments were beyond the scope of the present studies.

We were unable to show consistent bactericidal activity by using any of the mouse sera (data not shown). The bactericidal assays are inherently less quantitative and less sensitive than the other assays used here. They are also constrained by the limited amount of available mouse antiserum. In addition, FA1090 was originally isolated from a patient with disseminated gonococcal infection and is resistant to serum-mediated killing. Very recently, Price et al. showed that a combination of TbpA and TbpB conjugated to the cholera toxin B subunit could elicit bactericidal antibodies against FA1090 when delivered intranasally (31). Use of greater volumes of sera or different target gonococci might affect the results of these experiments.

We have successfully expressed and immunized mice with TbpB of gonococcal antigen by using the VEE VRP vaccine delivery system. We showed that this immune response was substantially different, in its Th1 bias, from that of immunization with rrTbpB, which elicited a Th2 bias. We also showed that inclusion of a tPA secretion signal markedly affected expression and results of immunization for the nonintegral membrane protein TbpB. While the strength of the VEE VRP-immunized group's responses was less than that of the more traditional, recombinant protein-immunized group, the true measure of the efficacy of these immunization strategies will be known only after protection experiments with mice or eventually humans. Since gonococci are now known to grow within epithelial cells (37, 46) as well as on mucosal surfaces, the ability to generate substantial Th1 responses with a VRP-based vaccine might be advantageous.

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