

ISOLATION OF A *TREPONEMA PALLIDUM* GENE ENCODING  
IMMUNODOMINANT OUTER ENVELOPE PROTEIN P6,  
WHICH REACTS WITH SERA FROM PATIENTS AT  
DIFFERENT STAGES OF SYPHILIS

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Our understanding of the role of *Treponema pallidum* surface components in syphilis pathogenesis is limited by the inability to cultivate the spirochete in vitro. Despite this difficulty, three treponemal outer envelope, fibronectin-binding proteins have been identified as putative adhesins responsible for host cell parasitism (1, 2). In addition, eight other (nonadhesin) surface-exposed, immunogenic *T. pallidum* proteins have been detected (3). One of these outer envelope proteins, designated P6 (3), is consistently reactive with sera from experimentally infected rabbits and syphilitic humans; this is shown by immunoprecipitation and immunoblot assays (4–10). Antibodies to this immunodominant surface protein immobilize the parasite in vitro (11) and may play a role in the development of chancre immunity in experimentally infected rabbits (10). An understanding of the biological function of protein P6 on intact spirochetes has not been elucidated because of inadequate quantities of purified P6 for structure-function analysis. Recombinant DNA technology offers a means to overcome the difficulties of *T. pallidum* cultivation for generating treponemal gene products (12–16). In this study, we describe the molecular cloning of the structural gene for the P6 immunogen. The recombinant protein reacts strongly with Ig found in sera of all patients at various stages of syphilis, establishing the potential use of this molecule as a vaccine and immunodiagnostic test reagent.

### Materials and Methods

*Bacteria.* The virulent Nichols strain of *T. pallidum* was maintained by serial passage in the testes of New Zealand white rabbits (1–4). Treponemes were harvested from minced testicular tissue as previously described (1–4), and were stored at  $-70^{\circ}\text{C}$  until needed for DNA extraction (17) and preparation of total proteins (1–4).

*Escherichia coli* strains LE 392 (18) ( $F^{-}$ , *hsdR514*, *supE44*, *supF58*, *lacY*, *galK2*, *galT22*,

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*metB*, *trpR55*,  $\lambda^-$ ), NM539 (19) (*supF*, *hsdR* [P2 *cox*]) and TB1 (20) ( $\Delta$  *lac-pro*, *strA*, *ara*, *thi*, 080 $\Delta$ lacZ,  $\Delta$ M15 *hsdR*) were grown in Luria broth (18).

**Antisera.** We obtained rabbit syphilitic sera (RSS)<sup>1</sup> from rabbits  $\geq 40$  d after infection (4). Before use in the screening of recombinant phage plaques, antibodies were incubated with an equal volume of French Press cell lysate obtained from  $10^{12}$ /ml *E. coli* cells processed at 20,000 lb/in<sup>2</sup>. Absorbed RSS were then clarified by centrifugation at 40,000 g for 30 min at 4°C. An mAb characterized in an earlier study (8, 9) and designated 13F3, that recognizes protein P6 (45 kD) was also used. Adsorption of the mAb with *E. coli* was also performed as just described for RSS. Human syphilitic sera (HSS) were from patients at various stages of syphilis and were kindly provided by Sandra Larsen of The Center for Disease Control, Atlanta, Georgia. These HSS were recently analyzed for their reactivity to defined treponemal proteins (21).

**Construction of *T. pallidum* Genomic Library.** 60 mg of *T. pallidum* DNA were partially digested with Eco RI enzyme (Boehringer Mannheim Diagnostics, Inc., Houston, TX) to maximize the production of 10–20 kb fragments (18). The digested DNA was then fractionated by centrifugation on a 10–40% linear gradient of sucrose (18). Fractions containing DNA fragments between 10 and 20 kb were pooled and dialyzed against a Tris-EDTA (TE; 100 mM Tris, 10 mM EDTA) buffer, pH 8.0, followed by phenol extraction (18) and ethanol precipitation (18). Bacteriophage EMBL-4 DNA (Promega Biotec, Madison, WI) was digested with Bam HI and Eco RI, phenol extracted, and ethanol precipitated. DNA was finally resuspended in TE buffer, pH 8.0, at a concentration of 1 mg/ml.

We added the endonuclease-digested EMBL-4 vector DNA to the sized *T. pallidum* fragments at a vector/insert ratio of 3:1 in ligation buffer (30 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 0.4 mM ATP (pH 7.8), and 1 U T4 DNA ligase [Boehringer Mannheim Diagnostics, Inc.]). Ligation was carried out at 12°C for 16 h. Recombinant DNA was packaged to produce viable phage using a  $\lambda$  in vitro packaging system (Promega Biotec) according to specifications. The library was amplified on *E. coli* NM539 to eliminate nonrecombinants (19).

**Immunological Screening of Clone Bank.** Recombinant phage were plated on *E. coli* LE 392 to produce ~500 plaques per plate. The plates were then incubated at 4°C and overlaid with nitrocellulose overnight. Disks were then removed and placed in a Tris-buffered saline solution (TBS; 500 mM NaCl and 20 mM Tris-HCl), pH 7.4, with 1% BSA for 2 h at room temperature. Disks were then incubated at 4°C for 16 h with adsorbed 13F3 mAb diluted 1:500 in TBS/5% nonfat dry milk (22). Disks were washed three times for 15 min with TBS, followed by addition of 5 ml of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Bio-Rad Laboratories, Richmond, CA) diluted at 1:2000 in TBS/5% milk. Incubation was continued for 3 h followed by washing three additional times with TBS before substrate development (23). Positive clones were spotted in duplicate on *E. coli* transferred onto nitrocellulose and processed as above. These clones were also examined for their reactivity to adsorbed HSS diluted 1:50 in 1 ml of TBS/5% milk with 100  $\mu$ l of the French Press *E. coli* cell extract added to reduce background reactivity further. The blots were then processed as above using HRP-conjugated goat anti-human IgG (Cappel Laboratories, Cochranville, PA) diluted 1:500 in TBS/5% milk.

**SDS-PAGE and Immunoblotting.** SDS-PAGE and immunoblotting of *T. pallidum* protein preparations were as described previously (2). Electrophoresis was also performed on protein preparations from phage-infected *E. coli* harvested from 1-cm diameter plaques. In this case, the soft agarose overlays were scraped from the plates and placed in 150  $\mu$ l of SDS-PAGE dissolving buffer (1–4) before electrophoresis.

**Construction of a Plasmid Containing the P6 Structural Gene.** Recombinant phage were purified from a 2 liter culture of *E. coli* LE392 after lysis (18). The phage DNA was then isolated (18) and treated with Eco RI restriction endonuclease. Insert fragments of 8.5 and 4.5 kb were ligated into the Eco RI site of the plasmid vector pUC19 (20), which was then used to transform *E. coli* TB1 (18). Transformants displaying a white phenotype (20)

<sup>1</sup> Abbreviations used in this paper: HRP, horseradish peroxidase; HSS, human syphilitic sera; RSS, rabbit syphilitic sera; TBS, Tris-buffered saline; TE, Tris-EDTA.

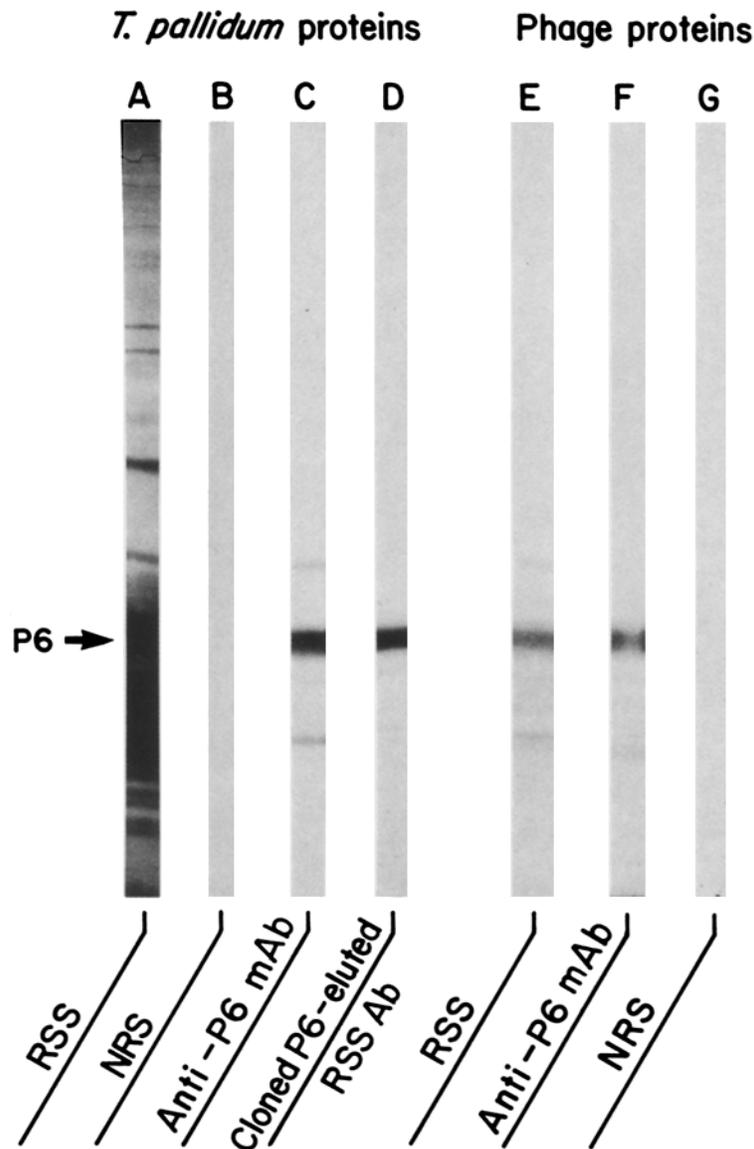
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FIGURE 1. Immunoblot identification of recombinant P6 protein from total *T. pallidum* (A-D) and phage lysate (E-G) protein preparations. Treponemal and recombinant proteins transferred to nitrocellulose after SDS-PAGE were probed with RSS, (A and E), normal rabbit serum (NRS), (B and G), mAb to the 45-kD protein (anti-P6; C and F), and antibody eluted from nitrocellulose blots of phage lysate containing the recombinant P6 protein (D). The generation of the respective protein preparations used for SDS-PAGE and immunoblotting is as described in Materials and Methods.

were analyzed by SDS-PAGE and immunoblotted for expression of a 45-kD protein reactive with 13F3 mAb.

*Fractionation of E. Coli Cells Containing the Structural Gene for T. pallidum P6 Protein.* The subcellular location of recombinant P6 was determined by French Press cell treatment followed by differential centrifugation (24). Briefly, 1 liter of plasmid pTPP6-

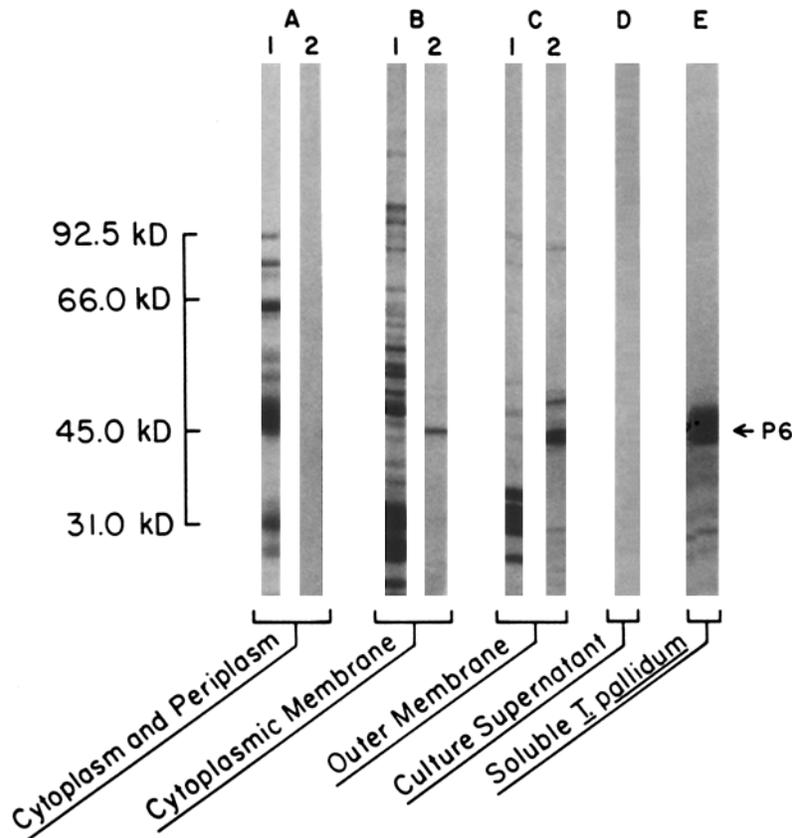


FIGURE 2. Immunoblot localization of P6 in pTPP6 transformed *E. coli*. Recombinant *E. coli* cells were French Press cell treated and separated into cytoplasm/periplasm, cytoplasmic membrane, and outer membrane fractions. After SDS-PAGE, the proteins were either stained with Coomassie Brilliant Blue (A 1, B 1, and C 1) or blotted onto nitrocellulose and incubated with mAb 13F3 (A 2, B 2, and C 2). (D) An immunoblot of culture supernatant; (E) the reactivity of mAb 13F3 to total *T. pallidum* proteins.

transformed cells in mid logarithmic phase growth were harvested by centrifugation and washed once with 10 mM Hepes buffer, pH 7.4. Washed cells were resuspended in 30 ml of Hepes buffer followed by French Press cell treatment at 20,000 lb/in<sup>2</sup>. The cell extract was then separated from whole cells by centrifugation at 6,000 g for 10 min. Crude envelopes were then pelleted at 200,000 g for 1 h in a 60 Ti preparative rotor (Beckman Instruments, Fullerton, CA). The pellet was resuspended in 20 ml of Hepes buffer containing 20 mg/ml Triton X-100. The outer membranes were then separated from cytoplasmic membranes by centrifugation at 30,000 g for 1 h. The final cytoplasmic membrane fraction was precipitated by addition of 2 volumes of ice-cold ethanol. After overnight incubation, the precipitate was pelleted by centrifugation at 10,000 g for 10 min and was resuspended in Hepes buffer.

## Results

**Phage Clones Expressing *T. pallidum* Protein P6.** An EMBL-4 phage library of *T. pallidum* DNA was constructed by in vitro packaging of recombinant DNA into phage particles followed by infection of *E. coli*. We chose EMBL-4 bacteri-

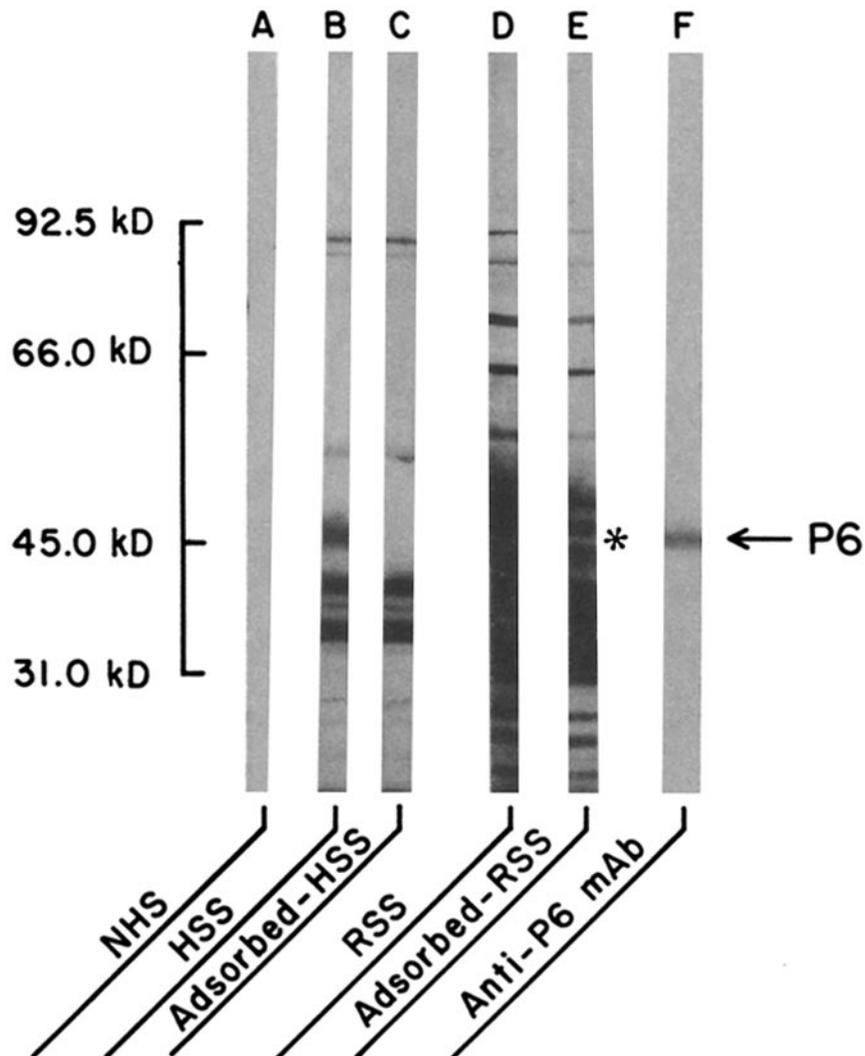
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FIGURE 3. Demonstration of the immunodominant nature of P6 outer envelope *T. pallidum* protein after SDS-PAGE and immunoblotting of total treponemal proteins. (A) Normal human serum as control, (B) HSS, and (D) RSS. Asterisk shows the absence of the P6 band from immunoblots probed with HSS (C) and RSS (E) adsorbed with recombinant P6, indicating that P6 is the major immunogen at this molecular mass. The P6 present in the treponemal protein preparations was identified using mAb 13F3 (F).

ophage  $\lambda$  as a cloning vehicle for *T. pallidum* DNA because of its high cloning efficiency, its large insert size, and its ability to minimize nonrecombinant background phage. The library, which was constructed using 10–20 kb Eco RI partially digested *T. pallidum* DNA, contained  $4 \times 10^4$  individual recombinant phage. We eliminated nonrecombinant phage by amplifying the library in *E. coli* NM 539. The titer of the amplified libraries was  $8 \times 10^9$  PFU/ml. Immunological screening of 10,000 recombinant phage plaques resulted in the detection of

TABLE I  
*Immunoblot Detection of Recombinant P6 in Phage Lysates by Sera  
 from Patients with Different Stages of Syphilis*

Serum reagent used*	Number positive per number tested <sup>†</sup>
Uninfected normal human sera	0/10
Primary syphilitic sera	6/6
Secondary syphilitic sera	10/10
Latent syphilitic sera	12/12

1-cm phage plaques of *E. coli* infected with clone R-12 phage were transferred to nitrocellulose and processed as for immunoblotting by using HSS and HRP-conjugated goat anti-human antibody to produce signal, as described in Materials and Methods.

\* HSS reagents (21) were kindly provided by Sandra Larson, Center for Disease Control, Atlanta, GA.

<sup>†</sup> Positive reactions were scored by comparison with second antibody alone and with nonrecombinant phage lysates.

positive signals using 13F3 mAb directed against a 45-kD *T. pallidum* surface immunogen previously designated P6 (3).

*Analysis of the 45 kD Recombinant P6.* We then performed immunoblot analysis of *T. pallidum* and recombinant *E. coli* initially positive with mAb. As can be seen in Fig. 1, numerous protein bands were detected using absorbed RSS incubated with a nitrocellulose blot of total treponemal proteins (lane A). Only one 45-kD protein, however, was detected using the mAb (lane C) or RSS antibody eluted from the *E. coli* lysate blot containing recombinant (P6) protein (lane D). A protein with identical molecular mass was detected in the EMBL-4 phage clone lysate using RSS (lane E) and 13F3 mAb (lane F). This protein was not detected with normal rabbit serum or irrelevant hybridoma supernatants either in total *T. pallidum* proteins (lane B) or in control phage lysates (lane G). Control phage lysates also did not possess a P6-crossreactive molecule. These initial data indicate that the structural gene for this immunogenic protein was cloned into bacteriophage  $\lambda$  and expressed as a complete protein in *E. coli*.

*Plasmid Expression and Cellular Location of P6 in E. Coli.* To facilitate further analysis of the 45 kD recombinant molecule, we attempted to subclone the structural gene into the multicopy expression plasmid pUC19 (20). The *T. pallidum* gene coding for P6 was removed from the phage DNA by Eco RI treatment producing two fragments. After ligation into the plasmid vector pUC19 and transformation of *E. coli*, we could detect with mAb 13F3 the 45-kD protein in *E. coli* transformed with a plasmid (pTPP6) containing a 4.5 kb piece of DNA.

We also determined the cellular location of P6 in *E. coli* transformed with pTPP6. *E. coli* containing the recombinant plasmid were subjected to French Press cell disruption, and the membranes were differentially separated by centrifugation of Triton X-100 treated membranes (24). Protein P6 was detected in the cytoplasmic and outer membrane fractions (Fig. 2; lanes B2 and C2). In addition, mAb 13F3 detected a higher mol mass protein which may represent a precursor product. No recombinant P6 was detected in the periplasmic or cytoplasmic compartments (lane A2) or in culture supernatant (lane D).

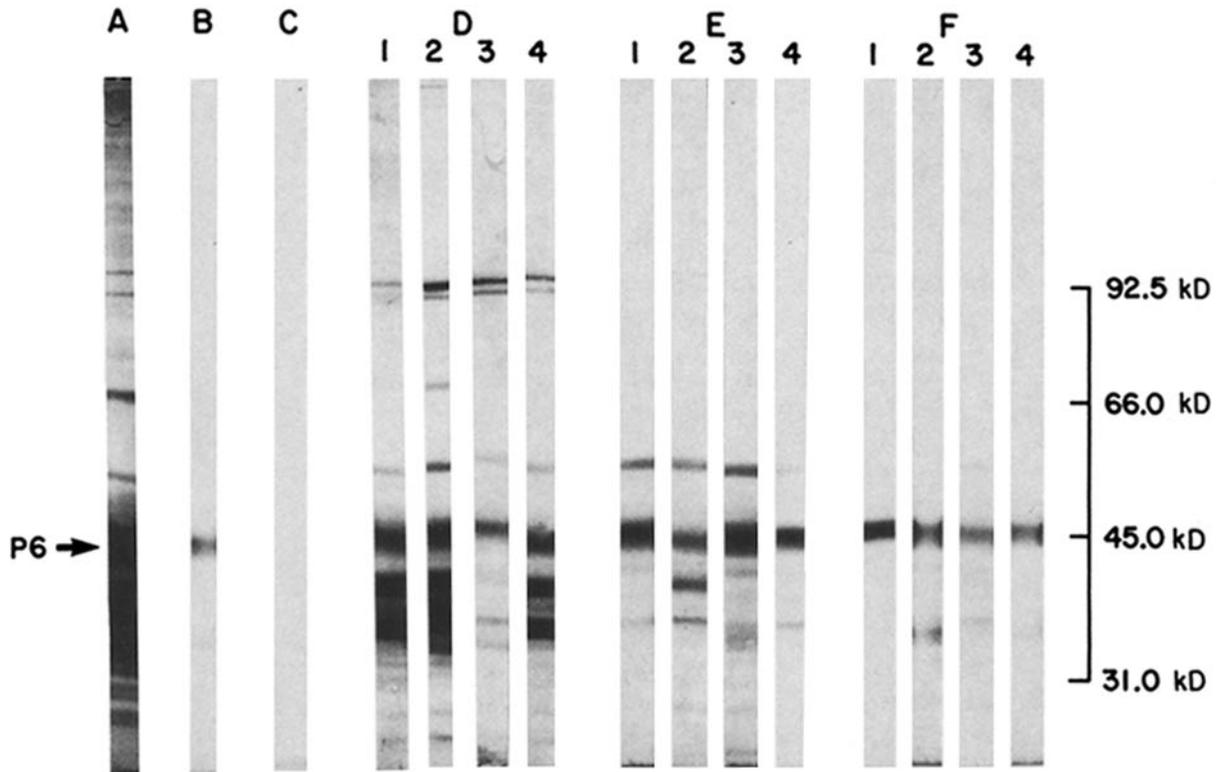
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FIGURE 4. Immunoblot detection of *T. pallidum* proteins by representative sera from patients at different stages of syphilis. Total treponemal proteins were subjected to SDS-PAGE and blotted onto nitrocellulose, then incubated with the following antibody reagents: (A) RSS; (B) mAb 13F3; (C) NHS; (D 1-D 4, primary HSS; (E 1-E 4) secondary HSS; and (F 1-F 4) latent HSS.

**Immunogenicity of P6.** We then tested the immunogenic nature of the recombinant P6 protein. Primary HSS and RSS were adsorbed with a French Press cell extract of *E. coli* containing recombinant P6, and as can be seen in Fig. 3 (lanes C and E, respectively), adsorbed HSS and RSS failed to react with the P6 protein band from a preparation of total *T. pallidum* proteins. In contrast, nonadsorbed HSS and RSS readily detected P6 in duplicates of the same preparations (lanes B and D, respectively). These initial data indicate an antibody response to P6 in humans with primary syphilis and in infected animals.

**Reactivity of HSS with P6.** It was important to determine the reactivity of HSS samples from patients at different stages of infection (20) with recombinant P6. All 28 sera from patients with syphilis were positive by immunodot analysis with phage lysates containing recombinant P6, as compared with control uninfected human serum or nonrecombinant phage lysates (Table I).

These same sera were then examined by immunoblot using total *T. pallidum* proteins separated by SDS-PAGE (Fig. 4). Numerous treponemal antigens were immunologically reactive with the different HSS. Although the number of *T. pallidum* protein antigens detected by these sera appeared to decrease with

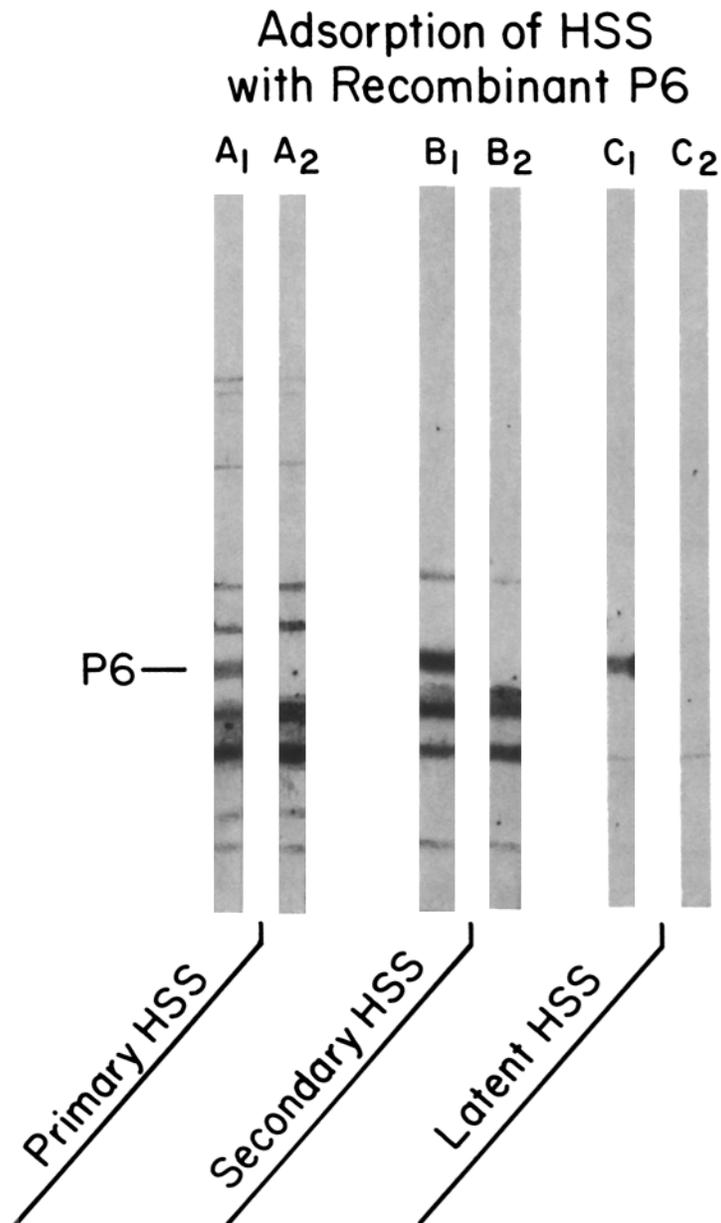


FIGURE 5. Immunoblot reactivity of pooled HSS from patients at different stages of syphilis with *T. pallidum* proteins (A 1, B 1, and C 1). Duplicate samples of pooled sera were also adsorbed with recombinant P6 before use in immunoblot (A 2, B 2, and C 2).

disease progression, high levels of antibody to a 45-kD protein were present throughout, including latent HSS.

To determine the specificity of the antibodies directed toward the 45-kD protein, we adsorbed pooled primary, secondary, and latent HSS with membrane fractions of *E. coli* transformed with pTPP6. As can be seen in Fig. 5, the

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reactivity of these sera to the 45-kD protein was lost after adsorption. These data indicate that a predominant antigen recognized by sera from human syphilitics is treponemal protein, P6.

### Discussion

Our generation of an mAb to the 45-kD treponemal surface protein, previously designated P6, prompted us to construct a *T. pallidum* DNA library in bacteriophage  $\lambda$  with the hope of isolating this gene product from *E. coli*. The mAb was used to select recombinant phage directing the synthesis of this 45-kD protein (Fig. 1). The treponemal origin for the structural gene of this recombinant antigen was confirmed by the ability of plaque-purified antibodies to react with an antigen of the same molecular mass from a total *T. pallidum* protein preparation (Fig. 1). The gene encoding P6 was subcloned into the expression plasmid pUC19. Consistent with the outer envelope location of P6 in *T. pallidum*, the plasmid-encoded protein was efficiently translocated to *E. coli* outer membranes (Fig. 2). The translocation of P6 to the *E. coli* outer membrane is noteworthy and may now allow us to characterize *T. pallidum* peptide signals that are involved in the disposition of proteins within the syphilis spirochete using *E. coli* as a model system (15).

Western blot analysis using recombinant P6, rabbit syphilitic sera, and sera from patients with different stages of syphilis clearly showed the immunodominant nature of native P6 (Figs. 2 and 5). These experiments also showed the presence of long-lasting circulating antibodies to P6 in syphilitic humans (Figs. 4 and 5). These data suggest that this cloned gene product may serve as a specific serodiagnostic test reagent for all stages of syphilitic infection.

Evidence from several laboratories suggest a role for treponemal P6 protein in syphilis pathogenesis. It is interesting to note for example, the generation of hybridoma antibodies directed against this immunodominant surface peptide that possess *T. pallidum*-neutralizing activity (11). Another relevant observation concerns the correlation between chancre immunity in intradermally infected rabbits and the appearance of Ig directed against the 45-kD treponemal protein (10). Our finding that humans with syphilis mount a prolonged response to this antigen reinforces the importance of structure-function analysis of recombinant P6. Such studies may explain the molecular basis of antibody-mediated neutralization of the syphilis spirochete and the biological function of P6 on intact organisms.

Our ability to isolate specific treponemal gene products will allow us to assess both humoral and immune effector cell reactivity to defined treponemal antigens. Such experiments may clarify cellular immune mechanisms in syphilis pathogenesis and may define at a molecular level the role of humoral immunity in syphilis.

### Summary

A phage directing the synthesis of an abundant 45-kD *Treponema pallidum* surface protein was isolated from an EMBL-4 bacteriophage  $\lambda$  library of *T. pallidum* DNA. The recombinant phage was identified using an mAb that was directed toward an immunodominant, outer envelope *T. pallidum* protein des-

ignated P6. The recombinant P6 protein possessed the same mol mass as the native treponemal antigen detected from total *T. pallidum* protein preparations, confirming the cloning of the structural gene for this molecule. Furthermore, *E. coli* was transformed by a 4.5-kb Eco RI  $\lambda$  insert fragment subcloned into the plasmid vector pUC19. These transformed cells expressed and translocated the 45-kD protein to their outer membranes. Finally, all sera from patients with different stages of syphilis (primary, secondary, and latent) contained antibody reactive to this protein.

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