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Identification and Preliminary Characterization of *Treponema pallidum* Protein Antigens Expressed in *Escherichia coli*

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We have previously described the construction in *Escherichia coli* K-12 of a hybrid plasmid colony bank of *Treponema pallidum* (Nichols strain) genomic DNA. By screening a portion of this bank with an in situ immunoassay, we identified six *E. coli* clones that express *T. pallidum* antigens. In this study, the recombinant plasmids from each of these clones have been analyzed in *E. coli* maxicells and have been found to encode a number of proteins that are not of vector pBR322 origin and are, therefore, of treponemal origin. In each case, several of these proteins can be specifically precipitated from solubilized maxicell extracts by high-titer experimental rabbit syphilitic serum. Certain of these proteins are also precipitated by high-titer latent human syphilitic sera (HSS). The *T. pallidum* DNA inserts in these plasmids range in size from 6.2 to 14 kilobase pairs, and from the restriction patterns of the inserts and the protein profiles generated by each plasmid in maxicells, it is apparent that we have recovered a total of four unique clones from our colony bank. Recombinant plasmids pLVS3 and pLVS5 were of particular interest. Plasmid pLVS3 encodes three major protein antigens with molecular weights of 39,000, 35,000, and 25,000. These three proteins, which were not recognized by pooled normal human sera, were efficiently precipitated by most secondary HSS, latent HSS, and late HSS tested. These proteins were also precipitated, although somewhat inefficiently, by most primary HSS tested. Plasmid pLVS5 encodes a major protein antigen with a molecular weight of 32,000 and several minor protein antigens that, although efficiently precipitated by experimental rabbit syphilitic serum, were generally not recognized by the various HSS tested. Evidence is presented indicating that the protein antigens encoded by plasmids pLVS3 and pLVS5 are specific for pathogenic treponemal species. We have also demonstrated that immunoglobulin G antibodies directed against these protein antigens can be detected in rabbits experimentally infected with *T. pallidum* Nichols as early as 11 days postinfection.

Syphilis is an infectious, sexually transmitted disease caused by the spirochete *Treponema pallidum*. Studies in several laboratories suggest that various envelope proteins of *T. pallidum* may have a major role in the pathogenesis of syphilis by mediating motility, the binding of certain host serum proteins, and the attachment of treponemes to host tissues (1, 2, 4, 14, 34). Many of these envelope proteins have been shown to be strongly immunogenic in both infected humans and rabbits, and antibodies to these proteins may be an important component of the host immune response (3, 4, 13, 32). Critical studies that might help to define the role of various *T. pallidum* proteins in the pathogenesis of syphilis are hindered by a major experimental problem, i.e., the inability to culture this bacterium in artificial medium (a new tissue culture system shows some promise; see reference 11). Although *T. pallidum* can be cultivated in rabbits and subsequently purified in small quantities, these procedures usually result in loss of virulence (5, 13, 37). Thus, it is difficult and expensive to obtain sufficient quantities of treponemes for meaningful analyses.

In an attempt to circumvent some of the problems associated with cultivation of *T. pallidum*, several laboratories have sought to apply recombinant DNA techniques to the production of treponemal proteins for experimental studies (recently reviewed in reference 30). We have previously described the construction in *Escherichia coli* K-12 of a hybrid plasmid colony bank of *T. pallidum* (Nichols strain) genomic DNA

(31). We employed an in situ immunoassay, utilizing either human syphilitic sera (HSS) or experimental rabbit syphilitic sera (ERSS), that enabled us to identify *E. coli* clones that express *T. pallidum* antigens. In this study, we describe the identification of the proteins encoded by the *T. pallidum* DNA inserts in the recombinant plasmids obtained from these clones. We found that a subset of these proteins could be specifically precipitated by various HSS and ERSS. Furthermore, we present evidence suggesting that the *T. pallidum* protein antigens we have cloned are specific for pathogenic treponemal species and demonstrate how such protein antigens can be used to investigate the immunobiology of *T. pallidum* infection.

MATERIALS AND METHODS

Bacterial strains. *T. pallidum* Nichols and *Treponema pertenue* Gauthier, obtained from the laboratory of Joel Baseman, University of Texas Health Sciences Center, San Antonio, were cultivated by testicular infection of adult male New Zealand white rabbits (approximately 5×10^7 treponemes per testicle) as previously described (14). Each animal was tested for the absence of *Treponema paraluis cuniculi* infection by the Venereal Disease Research Laboratory test (Fisher Scientific Co., Pittsburgh, Pa.) and the microhemagglutination assay for *T. pallidum* antibodies (MHA-TP) (Ames Co., Elkhart, Ind.). Only animals with nonreactive serological tests were used in this study. Infected rabbits were injected intramuscularly with 15 mg of cortisone acetate daily starting at 3 days postinfection and continuing until the day of peak orchitis (usually day 10 to 12 for *T. pallidum* and day 18 to 21 for *T. pertenue*).

A *T. pallidum* Nichols strain that had never been passaged in cortisone-treated rabbits was obtained from the laboratory of James N. Miller, University of California, Los Angeles. *T. pallidum* street strain 14, isolated by John Clark in 1977 from an infected human, was provided by the Sexually Transmitted Disease Laboratory Program of the Centers for Disease Control (CDC), Atlanta, Ga. Both of these treponemal strains were cultivated in rabbit testes as described above, except cortisone was not administered to the infected animals.

T. phagedenis biotype Reiter (Reiter), also obtained from the laboratory of Joel Baseman, was cultivated at 37°C in Spirolate broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 10% heat-inactivated normal rabbit serum (M. A. Bioproducts, Walkersville, Md.). Organisms were transferred to fresh medium every 4 days.

The *E. coli* K-12 colony bank of *T. pallidum* Nichols genomic DNA inserts has been previously described (31). *E. coli* K-12 strain SE5000, obtained from Ron Taylor, Frederick Cancer Research Center, Frederick, Md., has the following genotype: F^- *araD139 lacU169 rpsL relA thi recA56*.

Plasmid DNA. The recombinant plasmids present in *E. coli* clones expressing *T. pallidum* protein antigens were amplified by a modification of the procedure of Norgard (26). A 10-ml culture of each clone was grown at 37°C in M9 medium (24) containing uridine (1 mg/ml) to an optical density at 650 nm of 0.6 to 0.7. Freshly prepared chloramphenicol (20 mg/ml in 70% ethanol; Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 200 μg/ml, and incubation was continued overnight. Cells were pelleted and washed once in sterile distilled water, and plasmid was isolated by the procedure of Holmes and Quigley (16). Strain SE5000 was rendered competent for plasmid DNA uptake by the method of Dagert and Ehrlich (9), and ampicillin-resistant transformants were selected. Purified plasmid DNA for restriction endonuclease analysis was isolated by the procedure of Norgard (26).

Identification of plasmid-encoded polypeptides (maxicell procedure). Plasmid-encoded polypeptides were identified in vivo by a maxicell procedure similar to that of Taylor et al. (36). Strain SE5000, rather than the originally described strain CSR603, was employed for these experiments (28). In our hands, strain CSR603 exhibited a fairly high level of labeling of chromosome-encoded proteins. Strain SE5000 and derivatives harboring either plasmid pBR322 or one of our recombinant plasmids were grown at 37°C in M9 medium supplemented with 0.5% Casamino Acids (Difco Laboratories, Detroit, Mich.) and 0.4% glucose to an optical density at 600 nm of 0.5. Samples of 5 ml were spread evenly over the bottom of sterile, 100-mm disposable petri dishes and irradiated with 57.6 J of UV light per mm^2. The irradiated cells were incubated with shaking at 37°C for 1 h in the dark. Samples of 0.1 ml from each culture were plated on L agar (24) for the determination of survival rate after UV irradiation, and then D-cycloserine (final concentration, 200 μg/ml; Sigma) was added to kill any growing cells. Incubation was continued overnight in the dark. After determining the number of survivors after UV irradiation (usually none were present), the cells were pelleted, washed twice in 1× M9 salts, resuspended in M9 medium without Casamino Acids, and incubated at 37°C for 1 h. Plasmid-encoded proteins were radiolabeled with 15 μCi of $[^{35}S]$methionine (Amersham Corp., Arlington Heights, Ill.) per ml for 1 h. After labeling, the cells were harvested by centrifugation, washed twice in M9 salts, resuspended in 200 μl of 10 mM Tris–1 mM EDTA–1% sodium dodecyl sulfate (SDS) (pH 7.5), transferred to 1.5-ml Eppendorf tubes, and heated in a boiling water bath for 3 min. Samples were pelleted in an Eppendorf microfuge (10 min at room temperature). The supernatants, which contained the radiolabeled maxicell extracts, were collected and stored at −70°C.

Radiolabeling of *T. phagedenis* biotype Reiter. Reiter treponemes were grown at 37°C in Spirolate broth to a density of approximately 2×10^8 cells per ml, centrifuged at 13,000 × *g* for 15 min, and gently resuspended in 10 ml of labeling medium. The labeling medium was prepared by mixing the following sterile components: 66 ml of distilled water; 5 ml of 10× M9 salts; 3 ml of 20% glucose; 2 ml of 0.1% thiamine; 1 ml of 0.01 M $CaCl_2$; 2 ml of 0.05 M leucine; 1 ml of a stock solution containing threonine (5 mg/ml), proline (5 mg/ml), and arginine (10 mg/ml); 7 ml of heat-inactivated normal rabbit serum; 10 ml of a freshly prepared, autoclaved solution of cysteine (10 mg/ml) and thioglycolate (5 mg/ml); and 4 ml of a stock amino acid solution. This solution contained the following amino acids, each at a concentration of 1.25 mg/ml: aspartate, glycine, glutamate, histidine, isoleucine, lysine, phenylalanine, proline, tryptophan, tyrosine, and valine. Treponemes were radiolabeled with 60 μCi of $[^{35}S]$methionine per

ml for 20 h at 37°C in 15-ml screw-capped tubes. Darkfield examination of the organisms at the end of the labeling period indicated the retention of active motility by virtually all of the oganisms observed. Treponemes were centrifuged, washed once with 10 mM Tris (pH 8.0) and solubilized in 200 μl of 10 mM Tris-1 mM EDTA-1% SDS-2 mM phenylmethylsulfonyl fluoride (pH 7.5), heated for 3 min in a boiling water bath, and centrifuged at room temperature for 10 min in an Eppendorf microfuge. The supernatant containing radiolabeled treponemal proteins was stored at −20°C.

Rabbit sera. All rabbits used for the production of antisera were bled before infection to obtain control sera. Animals experimentally infected with *T. pallidum* Nichols, *T. pallidum* street strain 14, or *T. pertenue* were bled for serum at various intervals during the course of infection.

Rabbit anti-Reiter serum was prepared by injecting an adult male rabbit intravenously with 3.6×10^8 washed organisms (resuspended in phosphate-buffered saline) three times a week for 3 weeks. At the end of week 4, a single intravenous booster of 1.4×10^9 organisms was administered. Serum was obtained 9 days later and stored at −20°C. One additional rabbit anti-Reiter serum was kindly provided by Elizabeth Hunter, CDC.

Human sera. Sera from patients with known primary, secondary, latent, and late syphilis and human yaws sera (HYS), obtained from human patients with yaws, were provided by Sandra Larsen, CDC (Table 1). Pooled normal human sera were provided by Major Wilbur Milhous, U.S. Military Hospital, Fort Bragg, N.C.

Immunoprecipitation of radiolabeled proteins. *T. pallidum* Nichols protein antigens that were radiolabeled in *E. coli* maxicells were identified by an immunoprecipitation procedure with syphilitic sera. Maxicell extracts (25 μl) were mixed with 200 μl of Triton buffer containing 2% Triton X-100, 50 mM Tris, 0.15 M NaCl, and 0.1 mM EDTA (pH 8.0). A 25-μl volume of control or test serum was added and the mixture was incubated at 4°C overnight. The following day, 100 μl of a 10% suspension of formalinized *Staphylococcus aureus* cells (IgGSorb; The Enzyme Center, Inc., Boston, Mass.) in 10 mM Tris (pH 8.0) was added to the precipitation mixture and subsequently incubated with gentle mixing for 1 h at 4°C. After centrifugation in an Eppendorf microfuge for 2 min, the pellet was washed twice in Triton buffer and twice in 10 mM Tris (pH 8.0). The immunoprecipitate was extracted from the pellet by adding 100 μl of SDS sample buffer (6), heating in a boiling water bath for 2.5 min, and centrifuging in an Eppendorf microfuge at room temperature for 10 min. The supernatant containing the precipitate was collected and stored at −20°C.

As described in the Results section, we often combined two radiolabeled maxicell extracts in a single immunoprecipitation procedure. In these instances, 25 μl of each maxicell extract was mixed with 400 μl of Triton buffer and 50 μl of control or test serum. After an overnight incubation at 4°C, the reaction mixtures were processed in a manner identical to that described above.

Reiter protein antigens were also identified by a similar immunoprecipitation procedure. Five microliters of a 1:2 dilution of the radiolabeled treponemal extract was mixed with 500 μl of Triton buffer and 30 μl of control or test serum. After an overnight incuba-

TABLE 1. HSS and HYS utilized in this study

Serum no.	CDC designation	Stage of syphilis	Titer[a]		
			RPR	FTA-ABS	MHA-TP
1	3-142	Primary	1:2	3+	NR[b]
2	D86-81	Primary	1:1	4+	1:80
3	D5-81	Primary	1:4	3+	1:160
4	4-15	Primary	1:64	4+	1:160
5	D87-81	Primary	1:8	4+	1:1,280
6	D14-81	Primary	1:32	4+	1:2,560
7	4-21	Primary	1:64	4+	1:2,560
8	4-32	Primary	1:1	3+	1:5,120
9	4-33	Primary	1:128	4+	1:5,120
10	4-34	Primary	1:2	4+	1:5,120
11	6-10	Secondary	1:32	3+	1:320
12	5-47	Secondary	1:4	4+	1:640
13	5-43	Secondary	1:16	3+	1:640
14	6-16	Secondary	1:16	4+	1:1,280
15	5-39	Secondary	1:64	4+	1:1,280
16	D3-81	Secondary	1:1	2+	1:1,280
17	5-44	Secondary	1:32	4+	1:2,560
18	6-8	Secondary	1:64	4+	1:5,120
19	D34-81	Secondary	1:32	4+	1:10,240
20	5-42	Secondary	1:64	4+	1:20,480
21	6-17	Secondary	1:64	4+	1:40,960
22	D24-81	Secondary	1:512	4+	1:81,920
23	D39-81	Latent	1:2	1+	1:160
24	D2-81	Latent	1:2	3+	1:160
25	8-31	Latent	1:4	4+	1:320
26	8-30	Latent	1:16	4+	1:640
27	8-32	Latent	1:8	4+	1:640
28	7-58	Latent	1:128	4+	1:10,240
29	7-59	Latent	1:2	4+	1:20,480
30	7-56	Latent	1:512	4+	1:20,480
31	D43-81	Latent	1:64	4+	1:20,480
32	8-27	Latent	1:256	4+	1:40,960
33	7-57	Latent	1:128	4+	1:40,960
34	8-29	Latent	1:256	4+	1:81,920
35	10-31	Late[c]	NR	3+	1:1,280
36	10-27	Late	NR	2+	1:1,280
37	10-38	Late	NR	3+	1:1,280
38	10-2	Late	1:4	4+	1:5,120
39	10-36	Late	1:2	4+	1:10,240
Y1	74	Yaws	1:1	ND[d]	1:1,280
Y2	7	Yaws	NR	4+	1:5,120
Y3	188	Yaws	1:4	ND	1:10,240
Y4	203	Yaws	1:16	ND	1:20,480
Y5		Yaws	ND	ND	1:20,480
Y6	151	Yaws	1:128	ND	1:163,840

[a] All HSS and HYS were provided by Sandra Larsen, CDC. Information regarding the stage of disease, rapid plasmin reagin (RPR), and fluorecent treponemal antibody absorption test (FTA-ABS) titers were also provided by the CDC. We determined the MHA-TP titer for each serum according to the recommendation of the manufacturer. The number given represents the lowest serum dilution which gave a reaction of 1+ or better.

[b] NR, Nonreactive.

[c] All late HSS were obtained from patients diagnosed as having cardiovascular syphilis.

[d] ND, Not done.

tion at 4°C, 25 μl of a slurry of protein A–Sepharose CL-4B (Sigma) was added. This mixture was incubated at 4°C for 1 h with gentle agitation and then pelleted and washed, and the precipitate was extracted exactly as described above.

Finally, for precipitation reactions in which our rabbit sera were employed, the corresponding control serum used was always obtained from the same rabbit preinfection or preimmunization. Pooled normal rabbit sera were used as the control for experiments in which the anti-Reiter serum obtained from the CDC was used.

SDS-PAGE. The SDS-polyacrylamide gel electrophoresis (PAGE) system employed has been described previously (6). Radiolabeled cell extracts and immunoprecipitates were electrophoresed on 16-cm 15% acrylamide slab gels. The gels were stained with Coomassie blue, destained, soaked in 1% glycerol–10% glacial acetic acid solution for at least 1 h, soaked in the rapid autoradiography enhancer Enlightning (New England Nuclear Corp., Boston, Mass.), for 30 min, and dried for fluorography. The molecular weights given in the text were determined from the positions of unlabeled known protein molecular weight standards (Bio-Rad Laboratories, Richmond, Calif.) run on the same gel.

RESULTS

Identification of proteins encoded by recombinant plasmids harboring *T. pallidum* DNA inserts. We have described four *E. coli* clones that express *T. pallidum* antigens (31). By further screening of our colony bank with the same in situ immunoassay previously employed, we have recently detected two additional clones that also express treponemal antigens (data not shown). To identify the proteins encoded by the recombinant plasmids present in these clones, we utilized the so-called maxicell technique (28). Plasmid DNA was prepared from each clone and transformed into *E. coli* SE5000, which harbors a *recA* mutation. UV irradiation of this strain results in the preferential degradation of the bacterial chromosome while leaving small plasmids such as pBR322 intact. After a suitable time interval during which plasmid amplification also occurs, the cells are incubated with [^{35}S]methionine, and only those proteins encoded by the plasmid are radiolabled. These proteins are subsequently identified by SDS-PAGE and fluorography (see Materials and Methods).

A fluorograph of maxicell extracts of strain SE5000 and various derivatives is shown in Fig. 1. As expected, no proteins were radiolabeled in strain SE5000 without a plasmid. However, four protein bands are clearly labeled when the same strain harbors the cloning vector pBR322 (8, 28). The protein doublet running midway on the gel represents two forms of the β-lactamase enzyme (molecular weights, 28,000 [28K] and 30K) and are encoded by the single *bla* gene. The higher-molecular-weight protein (34K) is encoded by the plasmid *tet* gene, and the very fast-migrating protein (approximately 10K) is encoded by another gene near the plasmid origin (8). Extracts of six strains harboring recombinant plasmids are also shown. It is interesting to note the following. (i) The 34K *tet*-encoded protein is absent in each of these plasmids. This is an expected result, since *T. pallidum* DNA was cloned into the single *Bam*HI site of pBR322, resulting in insertional inactivation of the *tet* gene. (ii) A number of new protein bands can be discerned for each of the six extracts. Presumably, these proteins are encoded in some manner by the *T. pallidum* DNA inserts present in the recombinant plasmids. However, each of these protein bands may not necessarily represent a unique *T. pallidum* gene product (see Discus-

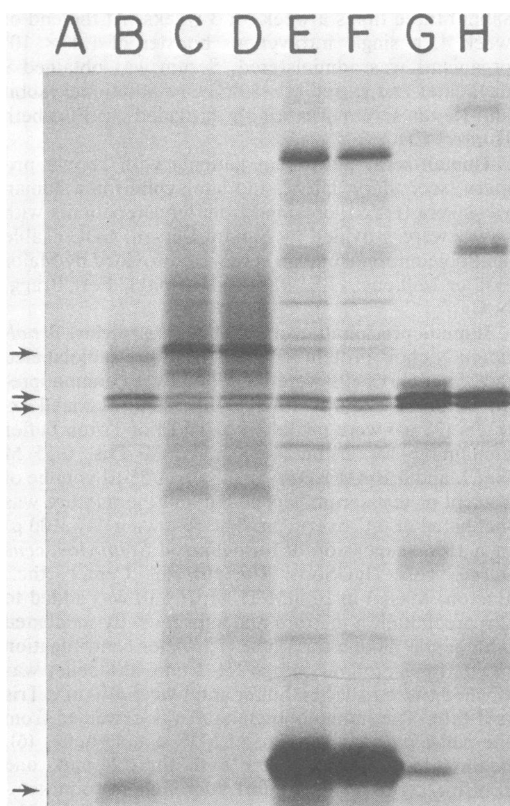

FIG. 1. Maxicell analysis of recombinant plasmids. Plasmid pBR322 and the six recombinant plasmids were transformed into *E. coli* SE5000. [^{35}S]methionine-labeled maxicell extracts were prepared and analyzed by SDS-PAGE and fluorography as described in the text. Lane A, no plasmid; lane B, pBR322; lane C, pLVS1; lane D, pLVS2; lane E, pLVS3; lane F, pLVS4; lane G, pLVS5; lane H, pLVS6. The proteins encoded by the vector pBR322 are indicated by the arrows at left (see text). Plasmids pLVS1, pLVS2, pLVS3, and pLVS4 were derived from clones I28, I39, I86, and H2, respectively (31).

sion). (iii) The protein patterns generated by the recombinant plasmids pLVS1 and pLVS2, like those generated by plasmids pLVS3 and pLVS4, appear to be identical. On the basis of the reactivity of the original *E. coli* clones with different syphilitic sera in our in situ immunoassay and of a preliminary estimate of the size of the *T. pallidum* DNA inserts in the respective plasmids, we had previously suggested that these plasmids represented two sets of related recombinants (31; see below). (iv) Finally, there is a major, rapidly migrating protein species (approximately 12K) encoded by plasmids pLVS3 and pLVS4. This is the only protein synthesized in these maxicells that can be clearly detected by Coomassie blue staining of the gel (data not shown). The remaining proteins can be detected only by fluorography.

Physical characterization of *T. pallidum* DNA inserts in recombinant plasmids. To construct the original colony bank from which each of the recombinant plasmids discussed above was derived, *T. pallidum* DNA had been partially cleaved with restriction endonuclease *Bam*HI and ligated into the single *Bam*HI site of the vector pBR322 (31). The size of the *T. pallidum* DNA insert in each plasmid was determined by examining *Bam*HI digests of purified plasmid DNA (Fig. 2). We found that the treponemal DNA inserts ranged in size from 6.2 to approximately 14 kilobase pairs (kbp). The DNA inserts in four of the six plasmids consist of two nonidentical *Bam*HI fragments and, as expected, the inserts in plasmids pLVS1 and pLVS2, as well as those in plasmids pLVS3 and pLVS4, appear to be identical. This result has been confirmed by preparing preliminary restriction maps for each of the plasmids. It is evident from such maps that these two sets of plasmids not only harbor identical inserts, but also that the orientation of these inserts with respect to the vector DNA is also the same (data not shown). Thus, we conclude that, at this juncture, we have a total of four unique *E. coli* clones that express *T. pallidum* antigens.

Identification of *T. pallidum* antigens expressed in *E. coli*. As previously stated, the original *E. coli* clones expressing treponemal antigens were identified on the basis of an in situ immunoassay. Although our experiments yielded no clue as to the nature of the antigens detected in this assay, we considered it likely that the *E. coli* clones were expressing *T. pallidum* protein antigens. Thus, we next determined whether any of the proteins identified in maxicells as being encoded by the recombinant plasmids could be specifically precipitated by immunoglobulin G (IgG) antibodies present in ERSS or HSS or both. Radiolabeled maxicell extracts were prepared and immunoprecipitations were performed as described in Materials and Methods. The results obtained with four different maxicell extracts, representing proteins encoded by plasmids pLVS1, pLVS3, pLVS5, and pLVS6, are

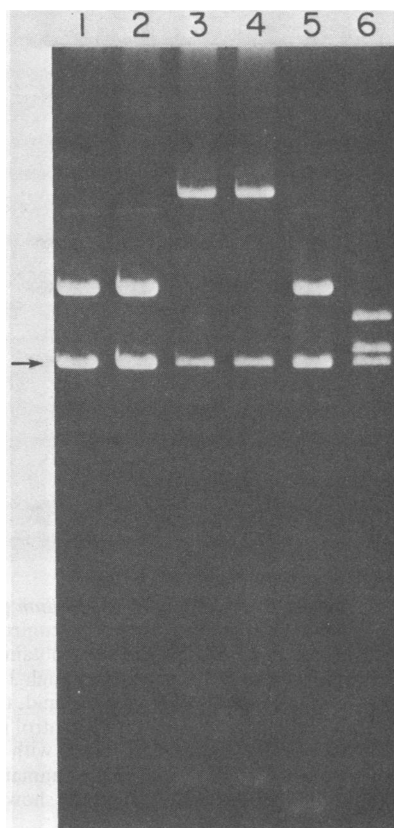

FIG. 2. Agarose gel electrophoresis of *Bam*HI-restricted plasmid DNA. Each of the recombinant plasmids were purified and digested to completion at 37°C with *Bam*HI (Bethesda Research Laboratories, Inc., Bethesda, Md.) in 20 mM Tris-100 mM NaCl-7 mM $MgCl_2$-2 mM 2-mercaptoethanol (pH 7.0). The restricted plasmid DNA was analyzed on a 1% agarose gel with 40 mM Tris-acetate-2 mM EDTA (pH 8.0) buffer. Ethidium bromide was incorporated into the gel at a final concentration of 0.5 μg/ml. The restriction digests of plasmids pLVS1 through pLVS6 are identified by the corresponding plasmid number at the top of each lane. The arrow at left identifies the bands of linearized pBR322 DNA (4.36 kbp). *T. pallidum* DNA insert sizes were determined with λ and ΦX174 replicative form DNA digested with *Hin*dIII and *Hae*III, respectively (not shown). Plasmids pLVS1 and pLVS2 harbor identical 6.2-kbp inserts. Plasmids pLVS3 and pLVS4 harbor an identical insert of approximately 14 kbp. This treponemal DNA insert contains a single internal *Bam*HI site and can be resolved into fragments of 13 and 0.94 kbp. *Bam*HI restriction of plasmids pLVS5 and pLVS6 yields inserts of 6.3 and 0.83 kbp (7.1 kbp total) and 5.2 and 4.6 kbp (9.8 kbp total), respectively.

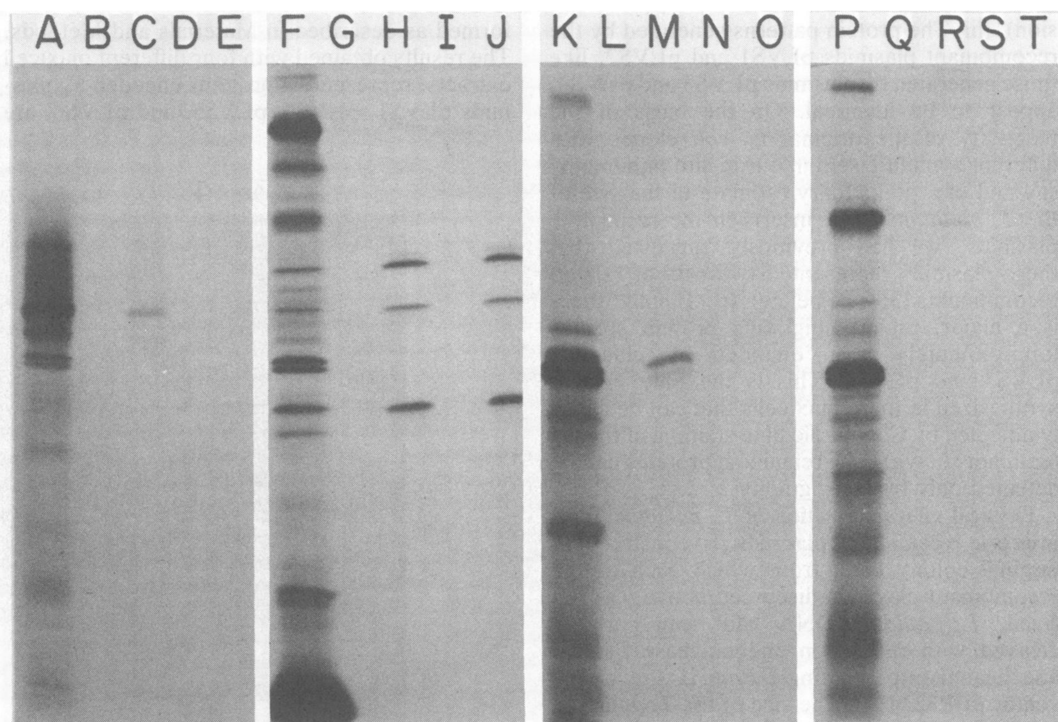

FIG. 3. Precipitation of cloned *T. pallidum* protein antigens by high-titer ERSS and HSS. Immunoprecipitations with radiolabeled maxicell extracts, control or syphilitic serum, and *S. aureus* protein A were performed as described in the text. The precipitates obtained were analyzed by SDS-PAGE and fluorography. Lanes A through E, plasmid pLVS1; lanes F through J, plasmid pLVS3; lanes K through O, plasmid pLVS5; lanes P through T, plasmid pLVS6. For each plasmid, the order of lanes is as follows: first lane, whole maxicell extract; second lane, precipitate obtained with control rabbit serum; third lane, precipitate obtained with serum from a rabbit 79 days after testicular infection with *T. pallidum* Nichols (MHA-TP titer, 1:163,840); fourth lane, precipitate obtained with pooled normal human serum; fifth lane, precipitate obtained with latent HSS (serum number 31, see Table 1). The precipitates shown were obtained from an amount of antigen extract equal to that shown in the figure.

shown in Fig. 3. We found that several proteins were precipitated from each of the extracts by the high-titer ERSS employed. Certain of these proteins were also precipitated by the particular high-titer latent HSS employed in this experiment. Specifically, we found the following. (i) A 35K doublet protein and a minor protein species migrating at 32K, both encoded by plasmid pLVS1, were somewhat inefficiently precipitated by ERSS and even less efficiently precipitated by HSS. (ii) Three of the major proteins encoded by plasmid pLVS3 (molecular weights 39K, 35K, and 25K) were efficiently precipitated by both ERSS and HSS. In addition, a minor protein species migrating just above the 39K protein was also efficiently precipitated by both sera. (iii) A major 32K protein and two minor proteins migrating just below it were efficiently precipitated by ERSS from the maxicell extract of plasmid pLVS5. However, precipitation of these same proteins by HSS was not detectable in this experiment. Likewise, several minor proteins in the molecular weight range of 43K to 66K, encoded by plasmid pLVS6, were precipitated by ERSS but not by HSS.

In considering these results, it is important to note the specificity of the radioimmunoprecipitation reactions. Neither normal rabbit serum (in this instance taken preinfection from the same rabbit used to generate ERSS) nor pooled normal human sera were capable of precipitating any radiolabeled protein species from these various maxicell antigen extracts under identical conditions. Also, proteins specifically precipitated by syphilitic sera were clearly a subset of the proteins encoded by the treponemal DNA inserts. In particular, note that, in most instances, the most intensely radiolabeled protein species were not the ones precipitated. The data we have obtained in similar experiments with various ERSS, HSS, and control sera (see below) have convinced us that the radioimmuno-

precipitation procedure we employed yields results that are both reproducible and reliable. However, it should also be pointed out here that, owing to the method utilized for preparing our antigen extracts, we may not be precipitating all the proteins for which IgG antibodies may be present in syphilitic serum (see Discussion).

Antibodies in HSS and HYS to cloned *T. pallidum* protein antigens. Having identified several protein species encoded by our recombinant plasmids that were specifically and efficiently precipitated by high-titer syphilitic serum, it was of further interest to investigate the ability of various well-characterized HSS to precipitate some of these same protein antigens. We combined the maxicell antigen extracts derived from plasmids pLVS3 and pLVS5 for use in a single radioimmunoprecipitation reaction with each of 39 different HSS (see Materials and Methods). These sera included those from patients with known primary, secondary, latent, and late cardiovascular syphilis. In addition, six HYS were also studied. (Although not previously mentioned, the causative agent of yaws, *T. pertenue*, is very closely related both genetically and antigenically to *T. pallidum* [5, 23].) The information we have on these various HSS and HYS is summarized in Table 1, and the results we obtained with these sera are shown in Fig. 4.

We found that the great majority of the HSS and all of the HYS tested were capable of precipitating one or more of the treponemal protein antigens encoded by plasmid pLVS3. Not surprisingly, the primary HSS, as a group, were the least efficient at precipitating these protein antigens; still, 8 of 10 primary HSS were clearly capable of precipitating, albeit inefficiently, the 39K protein antigen. Each of the secondary and late HSS, each of the HYS, and 11 of the 12 latent HSS precipitated the 39K protein antigen. Most of these sera precipitated the 35K and 25K protein antigens as well. Notwithstanding several prominent exceptions, the ability of these sera to precipitate these protein antigens generally correlated with the serum MHA-TP titer, i.e., the higher the titer, the greater the amount of radiolabeled antigen precipitated.

We found that the protein antigens encoded by plasmid pLVS5 were generally not precipitated by the various HSS and HYS. In some instances, we discerned somewhat inefficient precipitation of one or more of these protein antigens by several of the latent HSS (most notably in lanes 27, 28, 29, 32, and 33). Thus, despite the fact that these proteins were efficiently precipitated by ERSS (Fig. 3; see below), these results would seem to indicate that IgG antibodies to these particular protein antigens are not usually present in HSS or HYS.

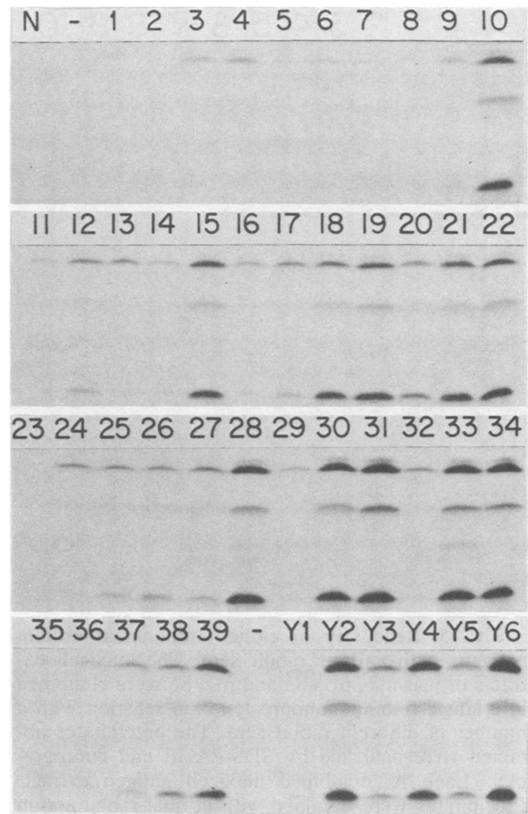

FIG. 4. Reactivity of cloned treponemal protein antigens with HSS and HYS. The maxicell extracts of pLVS3 and pLVS5 were combined in a single radioimmunoprecipitation reaction with a number of different HSS and HYS (see text). The precipitates obtained were analyzed by SDS-PAGE and fluorography. The number above each lane refers to the particular human serum used (listed in Table 1). Lane N, precipitate obtained with pooled normal human sera. The various sera are grouped as follows: lanes 1 through 10, primary HSS; lanes 11 through 22, secondary HSS; lanes 23 through 34, latent HSS; lanes 35 through 39, late HSS; lanes Y1 through Y6, HYS. The precipitates are arranged in such a way that, for each of the above groups, the MHA-TP titer of the corresponding serum increases from left to right on each gel. All reaction volumes were identical, and an equal amount of precipitate was loaded in each lane.

Cloned protein antigens may be specific for pathogenic treponemal species. Using the same combined maxicell antigen extract employed above, we screened a number of different rabbit sera for their abilities to specifically precipitate cloned *T. pallidum* protein antigens (Fig. 5). We found that sera from each of four rabbits experimentally infected with *T. pallidum* Nichols efficiently precipitated each of the protein antigens previously identified. The ERSS were obtained at times ranging from 33 to 139 days postinfec-

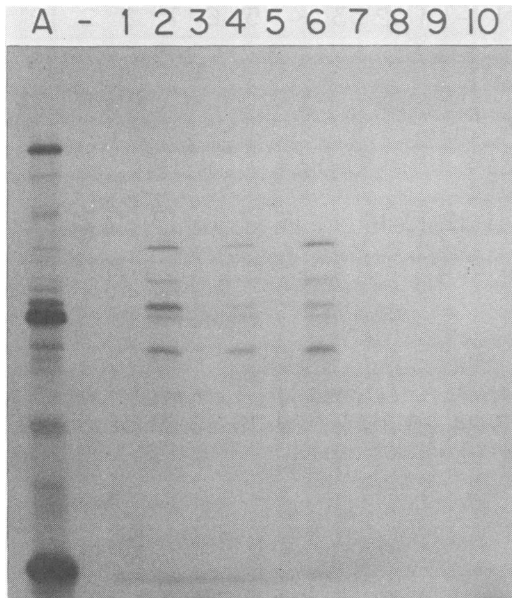

FIG. 5. Reactivity of cloned treponemal protein antigens with various rabbit sera. The maxicell extracts of plasmids pLVS3 and pLVS5 were combined in a single radioimmunoprecipitation reaction with a number of different rabbit sera. The precipitates obtained were analyzed by SDS-PAGE and fluorography. Lane A, combined maxicell antigen extract. Precipitates were obtained with normal rabbit serum (lanes 1, 3, 5, 7, and 9), serum from rabbits experimentally infected with *T. pallidum* Nichols (lane 2), *T. pallidum* street strain 14 (lane 4), and *T. pertenue* (lane 6), our anti-Reiter serum (see text) (lane 8), and CDC anti-Reiter serum (lane 10).

tion. (A total of 12 sera were tested, 3 per animal; the result obtained with just one serum is shown.) In addition, we tested sera from two rabbits experimentally infected with a recently isolated street strain of *T. pallidum* (designated street strain 14; see Materials and Methods). We could discern no qualitative difference in the abilities of these sera to precipitate the same set of protein antigens. The particular ERSS utilized in the experiment shown in Fig. 5 was obtained at 42 days postinfection, and somewhat less efficient precipitation of the protein antigens was observed in this instance. We also experimentally infected one rabbit with *T. pertenue*. Serum taken from this animal at 79 days postinfection efficiently precipitated the cloned protein antigens. Again, specificity of the precipitation reactions was noted. These same proteins were not precipitated by sera taken from the same rabbits preinfection.

It has been demonstrated that some *T. pallidum* protein antigens exhibit immunological cross-reactivity with protein antigens of the nonpathogenic, cultivable treponemal strain, *T. phagedenis* biotype Reiter (20, 25, 29). It was of interest to determine whether any of the treponemal protein antigens encoded by plasmids pLVS3 and pLVS5 would be precipitated by anti-Reiter serum. Rabbit anti-Reiter serum was generated as described in Materials and Methods. A second anti-Reiter rabbit serum was obtained from the CDC. Since anti-Reiter serum cannot be titered by conventional serological tests for syphilis, we wanted to test these sera for their abilities to precipitate Reiter protein antigens before we investigated their reactivities with cloned *T. pallidum* protein antigens. An antigen extract of [^{35}S]methionine-labeled Reiter treponemes was prepared, and immunoprecipitations with rabbit sera were performed as described in Materials and Methods. The precipitates obtained were analyzed by SDS-PAGE and fluorography (Fig. 6). Although both anti-Reiter

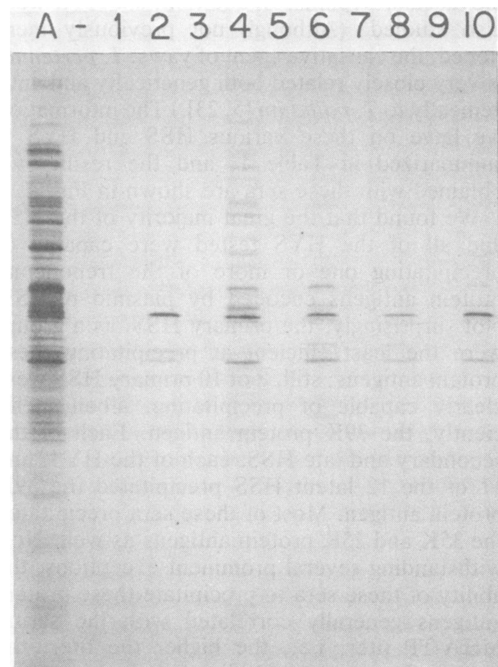

FIG. 6. Reactivity of whole Reiter proteins with various rabbit sera. Reiter treponemes were labeled with [^{35}S]methionine, the antigen extract was prepared, and radioimmunoprecipitations were performed as described in the text. The precipitates obtained were analyzed by SDS-PAGE and fluorography. Lane A, whole cell antigen extract. Precipitates were obtained with normal rabbit serum (lanes 1, 3, 5, 7, and 9), CDC anti-Reiter serum (lane 2), our anti-Reiter serum (lane 4), and serum from rabbits experimentally infected with *T. pallidum* Nichols (lane 6), *T. pallidum* street strain 14 (lane 8), and *T. pertenue* (lane 10). These are the same sera that were used in the experiment shown in Fig. 5.

sera precipitated a number of Reiter proteins, primarily in the molecular weight range of 30K to 90K, we found that the serum we generated recognized a considerably larger subset of total Reiter proteins than did the serum obtained from the CDC. In this experiment, we also investigated the ability of sera obtained from rabbits experimentally infected with *T. pallidum* Nichols, *T. pallidum* street strain 14, and *T. pertenue* to precipitate Reiter proteins. We found that these three sera precipitated a somewhat surprisingly large number of Reiter proteins, indicating that the degree of immunological cross-reactivity among protein antigens of the pathogenic and the nonpathogenic treponemal species may be more extensive than earlier reports indicate (20, 33).

Having demonstrated the ability of the rabbit anti-Reiter sera to precipitate Reiter proteins, we subsequently tested the ability of these sera to precipitate cloned protein antigens from our combined maxicell antigen extract (Fig. 5). We found that one of the anti-Reiter sera did not precipitate these protein antigens at all, and precipitation of only a faint amount of the 39K protein antigen by the other serum was detected. Since each of these protein antigens is efficiently precipitated by sera from rabbits experimentally infected with pathogenic *T. pallidum* or *T. pertenue* strains, it appears that the *T. pallidum* protein antigens encoded by plasmids pLVS3 and pLVS5 may be specific for the pathogenic treponemal species.

Development of IgG antibody response to cloned protein antigens in experimental syphilis. Since we had available radiolabeled treponemal protein antigens and a sensitive radioimmunoprecipitation procedure, we next investigated the development of an IgG antibody response directed against these particular antigens in rabbits experimentally infected with *T. pallidum* Nichols. Three rabbits were intratesticularly infected with *T. pallidum* on day 0. Each rabbit was monitored for development of an orchitis, and blood was drawn for serum at 3, 7, 11, 14, 17, and 21 days postinfection and weekly thereafter. Rabbits were not immunosuppressed with cortisone for any period postinfection. (The particular *T. pallidum* Nichols strain employed in this experiment had never been passaged in cortisone-treated animals.) The sera obtained from these rabbits preinfection and at various times postinfection were tested for their abilities to precipitate treponemal protein antigens from the same combined maxicell antigen extract described above. As before, the preciptitates were analyzed by SDS-PAGE and fluorography. The patterns of reactivity observed for sera from the three infected rabbits were quite similar; the results for just one rabbit are shown in Fig. 7. We found that IgG antibodies capable of precipitating our cloned protein antigens were weakly detectable in serum obtained 11 days postinfection, coinciding with the development of peak orchitis in each animal. The serum obtained from each animal just 3 days later efficiently precipitated several of the cloned antigens. By this assay, the level of IgG antibodies directed against these protein antigens remained fairly high through day 42 postinfection, with a noticeable decrease clearly discernible in the last serum sample tested in this experiment, taken 84 days postinfection.

DISCUSSION

We initially identified six *E. coli* clones from our colony bank of *T. pallidum* genomic DNA that reacted with high-titer ERSS in an in situ

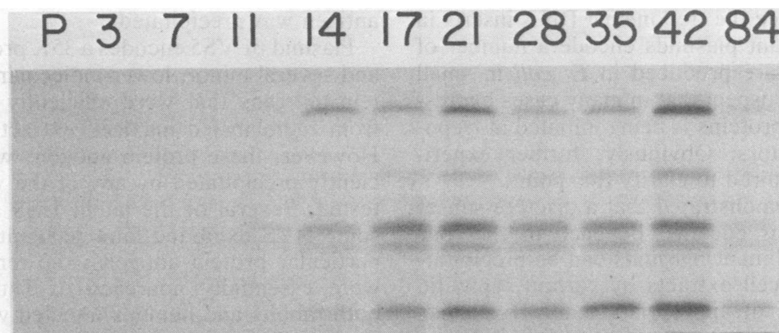

FIG. 7. Development of IgG antibody response to cloned protein antigens in a rabbit experimentally infected with *T. pallidum* Nichols. The maxicell extracts of plasmids pLVS3 and pLVS5 were combined in a single radioimmunoprecipitation reaction with serum samples obtained from a single rabbit at various times postinfection with *T. pallidum*. The precipitates obtained were analyzed by SDS-PAGE and fluorography. The number above each lane refers to the number of days postinfection that the serum sample was taken. Lane P, precipitate obtained with serum from the same rabbit before infection. See text for additional details.

immunoassay (31). In this study, we analyzed the recombinant plasmids derived from these six clones in *E. coli* maxicells. We found that each of the plasmids encoded a number of proteins that were not of vector pBR322 origin. From the protein profile generated by each plasmid, as determined by SDS-PAGE and fluorography, it was apparent that we had recovered a total of four unique clones from the colony bank. This finding was confirmed by comparing the sizes and restriction patterns of the treponemal DNA inserts in each plasmid. Although there is some correlation between the size of the treponemal DNA inserts and the number of new protein bands seen in the corresponding maxicells, it is important to keep in mind that each of these proteins may not necessarily represent a unique *T. pallidum* gene product. Certain of these proteins may represent fusions to the *tet* gene product; some proteins may be degradation products of unstable, higher-molecular-weight proteins; others may be precursor proteins inefficiently processed to the mature form.

With regard to the treponemal DNA inserts in the recombinant plasmids, several additional points should be noted. First, of the six clones originally identified, four of these represented two sets of plasmids harboring identical inserts. The manner in which our original colony bank was generated did not ensure that all the transformants that we obtained harbored plasmids formed from independent ligation events. Since in the two instances cited here the orientation of the insert DNA with respect to the vector DNA was identical, there is little reason to believe that the clones in each pair were independently derived. Second, there is only one transcript originating within pBR322 vector DNA that could account for expression of gene sequences inserted at the *Bam*HI site: the one initiated at the P2 promotor responsible for transcription of the *tet* gene (35). Since the treponemal DNA inserts in our recombinant plasmids encode a number of proteins that are produced in *E. coli* in small amounts, we suspect that in many cases expression of these proteins is being initiated at treponemal promotors. Obviously, further experiments are required to clarify this point.

We have demonstrated that a discrete subset of the proteins encoded by each of the four unique recombinant plasmids can be precipitated from maxicell extracts by certain syphilitic sera. The use of appropriate control sera has served to illustrate the specificity of the radioimmunoprecipitation procedure employed. We noted that certain of the cloned proteins appear to be inefficiently precipitated by high-titer syphilitic sera; i.e., significantly less protein appears in the final precipitate as compared with the amount discerned in the maxicell extract. We solubilized our antigen extracts by resuspending radiolabeled maxicells in a buffer containing 1% SDS, followed by heating in a boiling water bath. Obviously, such treatment can be detrimental, and alternative, less harsh solubilization procedures might be more appropriate to preserve protein antigenicity. Unfortunately, we have found that disruption of radiolabeled maxicells under nondenaturing conditions results in significant loss of our radiolabeled protein species, presumably from proteolytic degradation. This result is expected, since these proteins are foreign to their *E. coli* host and may be particularly susceptible to degradation by endogenous proteases. When it is possible for cloned treponemal proteins to be stabilized under nondenaturing conditions, we may find additional proteins that are efficiently precipitated by syphilitic sera.

In this study, we have focused our attention on the protein antigens encoded by plasmids pLVS3 and pLVS5. *E. coli* maxicells programed with plasmid pLVS3 produced three protein antigens of 39K, 35K, and 25K that were clearly precipitated by most ERSS, HSS, and HYS tested. Since we precipitated immune complexes with *S. aureus* protein A, it must be kept in mind that we were testing specifically for the presence of IgG antibodies directed against these antigens (18, 19). In cases in which little or no reactivity was observed with HSS, the serum titer, as indicated by the quantitative MHA-TP test, was quite low. However, there were several sera, most notably primary HSS, that exhibited some reactivity with one or more of these protein antigens despite very low MHA-TP titers. We did encounter two latent HSS with fairly high MHA-TP titers (sera 29 and 32; see Table 1 and Fig. 4) that exhibited relatively weak reactivity against these protein antigens. Still, there was no doubt that some 39K protein antigen was precipitated.

Plasmid pLVS5 encodes a 35K protein antigen and several minor, lower-molecular-weight protein antigens that were efficiently precipitated from radiolabeled maxicell extracts by ERSS. However, these protein antigens were not efficiently precipitated by any of the various HSS tested. Several of the latent HSS and perhaps some HYS exhibited faint recognition of these particular protein antigens; the remaining sera were essentially nonreactive. Thus, although both rabbits and humans infected with *T. pallidum* or *T. pertenue* appear to mount a strong IgG antibody response to the protein antigens encoded by plasmid pLVS3, it would appear that only infected rabbits respond in like manner to the protein antigens encoded by plasmid pLVS5. At this point, it is difficult to estimate the significance of these findings. The few rele-

vant reports to date have emphasized the similarity of the humoral response of infected rabbits and humans to the protein antigens of *T. pallidum* (3, 4, 13, 20). However, since the course of the disease in infected rabbits and humans is considerably different (12), it would not be surprising if differences in the immune response such as those reported here are important. Further studies in which cloned treponemal antigens are used may be able to provide insight into this possibility.

We have also demonstrated that cloned, radiolabeled treponemal protein antigens can be used to monitor the development of an IgG response in rabbits experimentally infected with *T. pallidum*. In each of three infected rabbits, we detected IgG antibody to our cloned antigens at 11 days postinfection. These results are in agreement with those of Zeltzer et al. (40) and Pepose et al. (27), who demonstrated IgG antibodies to whole *T. pallidum* to be present 9 days after infection of rabbits with this organism. Also, Alderete and Baseman (3) detected IgG antibodies to *T. pallidum* protein antigens as early as 10 days postinfection. It is interesting that the early appearance of IgG antibody coincides with the development of peak orchitis in infected rabbits. During the reactive phase of experimental rabbit syphilis which immediately follows peak orchitis, there is a rapid clearance of treponemes from the infected sites (21, 39), and acquisition of partial immunity to reinfection has been demonstrated (7, 39). It was during this period that we observed a definite rise in IgG antibodies to our cloned treponemal antigens. The precise role of humoral and cellular immune mechanisms in experimental rabbit and human disease remains to be defined (see reference 12 for recent review). Again, we anticipate that the availability of cloned treponemal protein antigens will provide new tools for investigating the immunobiology of syphilis.

We have presented evidence that the treponemal protein antigens encoded by plasmids pLVS3 and pLVS5 may be specific for pathogenic species. Sera from rabbits experimentally infected with either of two strains of *T. pallidum* or one strain of *T. pertenue* efficiently precipitated these antigens. However, these antigens were not recognized by sera from rabbits immunized with the cultivable, nonpathogenic Reiter treponeme. We have not yet tested sera prepared against other nonpathogenic species; such experiments are in progress. However, since the protein antigens encoded by plasmid pLVS3 are recognized by IgG antibodies present in the great majority of HSS and HYS tested, our results to date suggest that these or similar protein antigens could form the basis for a new confirmatory serodiagnostic test for syphilis and other human treponematoses. The use of pathogen-specific antigens would eliminate the requirement for absorbing human sera with an extract prepared from Reiter treponemes, as is now done for most commonly used confirmatory tests (17, 38). For many human sera, the absorption step is necessary to remove antibodies against cross-reacting antigens that presumably arise in response to antigens of the normal bacterial flora (10, 15). Also, we noted that there were a number of Reiter protein antigens that were precipitated by sera from rabbits infected with pathogenic treponemal species. This was a greater number than that indicated in previous studies (20, 33), and it is a result that is somewhat surprising considering the lack of demonstrable genetic homology between Reiter and the pathogenic species (22). This finding is being investigated further.

Finally, we would like to make several additional comments concerning the cloned treponemal protein antigens. When the *E. coli* clones expressing these antigens were initially identified (31), we suggested that we were detecting treponemal antigens expressed on the *E. coli* cell surface. However, we still have no firm evidence that this is the case and, furthermore, we do not know which, if any, of the protein antigens described here are the antigens originally detected by our in situ immunoassay. Experiments are currently under way in an attempt to learn more concerning our cloned protein antigens. For example, preliminary pulse-chase experiments in which maxicells are used have shown us that these protein antigens are fairly stable in intact *E. coli* cells after their biosynthesis, indicating that the 25K and 35K protein antigens encoded by plasmid pLVS3 are not degradation products of the 39K protein antigen. In addition, it appears that the minor protein antigen that migrates just above the 39K protein antigen on SDS-PAGE (see Fig. 3 and 7) is, in fact, the precursor to the 39K protein. Since one of our primary goals has been to clone genes encoding cell surface proteins of *T. pallidum*, it is tempting to speculate that the 39K protein results from the proteolytic processing of an amino-terminal signal sequence on the precursor protein. Such a signal sequence could be responsible for initiating the export of this protein to the cell surface of *T. pallidum* and, when expressed in *E. coli*, to the surface of this organism. We are presently attempting to determine the cellular location of the treponemal proteins expressed in *E. coli* and their relationship to native *T. pallidum* proteins.

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