MOLECULAR CHARACTERIZATION OF
RECEPTOR BINDING PROTEINS AND IMMUNOGENS OF
VIRULENT *TREPONEMA PALLIDUM**

By JOEL B. BASEMAN‡ AND EDWARD C. HAYES

From the Department of Bacteriology and Immunology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514; and the Department of Microbiology and Immunology, Duke University Medical Center, Durham, North Carolina 27710

During the clinical course of syphilis, a complex interrelationship exists between virulent *Treponema pallidum* and the parasitized host. Infection can persist in the presence of a significant immune response, and manifestations of actively developing disease are well documented (1, 2). Although controversies still remain concerning the relative contributions of cellular and humoral immunity to eradication of the disease, it is known that reinfection is unlikely once the disease has progressed through its early stages. Data suggest that humoral mechanisms may in part correlate with increased host resistance to subsequent challenge. For example, passive immunization of rabbits with hyperimmune rabbit serum obtained during experimental infection lengthens the onset and decreases the severity of lesion development, thus providing partial protection (3–5). In other cases extensive immunization regimens in rabbits with gamma-irradiated *T. pallidum* prevent infection with virulent treponemes (6). Unfortunately no specific protective immunogens have been identified by these experiments. There are also indications that cell-mediated immunity is altered during early *T. pallidum* infection. Peripheral blood lymphocytes from syphilitic patients (7) and experimentally infected rabbits (8, 9) respond poorly to in vitro stimulation with mitogenic plant lectins and certain treponemal antigens. Plasma and serum factors have been implicated in the impaired reactivity of lymphocytes during the initial stages of disease by decreasing lymphocyte responsiveness to mitogenic stimuli (10–13). Thus, the simultaneous host expression of seemingly beneficial and detrimental immune mechanisms during treponemal infection is apparent.

We have attempted to understand the pathogenesis of syphilis and outline approaches to interrupt or prevent disease development by examining biological-biochemical properties of *T. pallidum* under a variety of experimental conditions. We felt that identification of treponemal macromolecules that possess functional roles in virulence would be essential. Consistent with this approach, we monitored surface parasitism by *T. pallidum* of host cells in monolayer culture because adherence of certain microorganisms to mucosal surfaces is a prerequisite for the establishment of

disease (14–17). Data implicated the putative role of receptor-like molecules on both *T. pallidum* and host cells (14, 18).

In this paper, methodology is described for detecting proteinaceous components of virulent *T. pallidum* that selectively and avidly bind to host cell membranes and at the same time are immunogenic as determined by radioimmunoprecipitation and gel electrophoresis-fluorography techniques. Furthermore, these experimental techniques permit analysis of the humoral response to parasite components in the infected host and provide a mechanism for differentiating this response during parasitic attack by related microorganisms.

Materials and Methods

Bacteria. Virulent *T. pallidum* (Nichols) were inoculated intratesticularly in New Zealand white rabbits at 2–5 × 10^7 treponemes per testis. Treponemes were extracted from tissue at the approximate time of peak orchitis 8–11 d postinfection, and host cell contaminants were removed as previously outlined with Methocel (Dow Chemical Co., Midland, Mich.) -Hypaque (Winthrop Laboratories, New York) gradient centrifugation in the final clarification step (19). The supernate contained ~1–3 × 10^8 treponemes/ml of extracting medium. The latter was comprised of a salts-pyruvate-glucose-serum mixture previously shown by us to provide a satisfactory environment for radiolabeling *T. pallidum* (20). Treponemes were incubated for 20 h at 34°C under an air atmosphere in 1-oz sterile bottles that contained 10 ml of medium and 150 μCi of [^{35}S]methionine (sp act 978 Ci/mmol, New England Nuclear, Boston, Mass.). The Reiter treponeme (*T. phagedenis* biotype Reiter) was grown in serum-containing Spirolate broth as previously described (19).

Preparation of Treponemal Extracts for Binding Site and Radioimmunoprecipitation (RIP)[1] Assays. Frozen or fresh pellets that contained 5 × 10^8 [^{35}S]methionine-labeled or unlabeled *T. pallidum* or unlabeled avirulent Reiter treponeme were suspended in 200 μl of NET (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.2) buffer that contained 2 mM phenylmethylsulfonyl-fluoride (PMSF). The sodium dodecylsulfate (SDS)-Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.) extracts were prepared as follows. 100 μl of 1% SDS in NET buffer was added to the treponeme suspension that was then incubated at 22°C for 5 min. Next, 100 μl of ovalbumin (10 mg/ml in NET buffer) and 100 μl of 10% Triton X-100 were introduced and the mixture maintained at 37°C for 15 min. This solution was then centrifuged over a 250-μl cushion of 5% sucrose in NET buffer for 1.5 h at 150,000 g with a Beckman SW 50.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) to remove insoluble material. The supernate was then diluted to 1 ml with NET buffer. Triton X-100 extracts were included for comparative purposes. In the latter case, 100 μl of 10% Triton X-100 was added to 200 μl of treponemes suspended in NET-PMSF. This suspension was incubated at 22°C for 15 min followed by the addition of 100 μl of ovalbumin (10 mg/ml) and 100 μl of NET buffer. This mixture was then treated as described above for the SDS-Triton samples.

Test Sera. Serum samples from syphilitic and yaws patients were kindly supplied by S. A. Larsen of the Center for Disease Control, Atlanta, Ga. Additional syphilitic sera and normal human serum controls were provided through the courtesy of J. D. Folds of the University of North Carolina School of Medicine (Chapel Hill, N. C.).

Analysis of T. pallidum Binding Sites. HEp-2 cells grown as monolayers were washed with phosphate-buffered saline (PBS) (pH 7.6) plus 0.02% EDTA to facilitate their release from the vessel surface. Cells were pelleted, resuspended in PBS at a density of 3 × 10^6 cells/ml, and transferred to a 25-ml siliconized glass flask. Formaldehyde was added to a final concentration of 1.0%, and cells were gently stirred for 1 h at 22°C before extensive washing in PBS and resuspension to the original cell density in NET that contained 2 mM PMSF, 0.1% SDS, and

---

[1] *Abbreviations used in this paper:* NET, 150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.2; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonylfluoride; RIP, radioimmunoprecipitation; SDS, sodium dodecylsulfate; Staph A, protein A-bearing *Staphylococcus aureus*.

1.0% Triton X-100. Under these conditions, formaldehyde-treated HEp-2 cells remained intact for the duration of the experiment.

[^{35}S]Methionine-labeled *T. pallidum* solubilized in an SDS-Triton solution as described above were introduced in 0.5 ml vol (equivalent to 5×10^8 treponemes and $1-2 \times 10^5$ cpm) directly to microfuge tubes. Then 300-μl amounts of either NET-SDS-PMSF-Triton buffer or a similar buffer that contained various concentrations of nonradioactive, solubilized treponemes were introduced followed by 100-μl aliquots of formaldehyde-fixed HEp-2 cells. After incubation for 1 h at 34°C with mixing at 15-min intervals, HEp-2 cells were pelleted and washed four times in NET that contained 0.05% Triton to remove nonspecifically bound material. Then 50 μl of 1% SDS in water or NET was added and the HEp-2 cell pellet vortexed vigorously and centrifuged. The supernate that contained avidly bound radioactive treponemal components was processed for gel electrophoresis.

RIP Assay. The SDS-Triton-soluble ^{35}S-labeled *T. pallidum* extracts (1 ml) were preadsorbed with 250 μl of a 10% formaldehyde-treated suspension of Cowan I strain of protein A-bearing *Staphylococcus aureus* (Staph A) (21). After 15 min at 22°C, Staph A were removed by centrifugation at 10,000 g for 1 min. This step eliminated Staph A-associated, nonserum-mediated radioactivity. The adsorbed radiolabeled supernate was then divided into 100-μl aliquots to which 20 μl of various dilutions of immune or nonimmune sera was introduced. After 2 h at 22°C, 50 μl of Staph A suspension was added and incubation continued for 30 min. Staph A were then centrifuged and washed three times in NET buffer that contained 0.05% Triton X-100. The adsorbed radioactive antigens were finally recovered by resuspending the Staph A pellet in 70 μl of solubilizing buffer (100 mM Tris-HCl, pH 6.8; 2% SDS, 2% β-mercaptoethanol, 20% glycerol, and 0.02% bromophenol blue) and boiling the suspension for 3 min. Staph A were then pelleted and the supernate applied to SDS-polyacrylamide gels.

For competitive RIP, the dilution of human syphilitic serum that precipitated ~80% of each of the ^{35}S-labeled antigens in *T. pallidum* SDS-Triton extracts was determined by the RIP assay. Portions (20 μl) of this serum dilution were incubated for 2 h at 22°C with 100 μl of increasing concentrations of unlabeled homologous or heterologous cell extracts (SDS-Triton) or with NET buffer. Then 100-μl aliquots of solubilized [^{35}S]methionine-labeled *T. pallidum* were added and incubation continued for 1 h at 22°C. Immune complexes were precipitated with Staph A as described above and the labeled antigens analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

Gel Electrophoresis and Fluorography. Discontinuous SDS-slab gel electrophoresis was performed using stacking and separating gels of 3 and 10% acrylamide, respectively (15). Radioactively labeled samples that contained bromophenol blue as the tracking dye were added in 50-μl amounts to individual slots in the stacking gel of the acrylamide slab. Electrophoresis was initially performed with a constant current of 8 mA that was increased to 25 mA when the tracking dye entered the separating gel. Slab gels were stained with Coomassie brilliant blue, destained, and processed for fluorography as previously described (22, 23). Protein samples were also electrophoresed in 10% discontinuous slab gels (24) for 3.5 h at 30 mA. Gels were equilibrated for 1 h in 7% acetic acid and fluorograms prepared. Selected fluorograms were scanned (Quickscan, Helena Laboratories, Beaumont, Tex.) and the areas under the peaks integrated with a PDP-11 computer (Digital Equipment Corp., Maynard, Mass.) to quantitate the intensity of individual protein bands (24).

Results

Affinity of T. pallidum for HEp-2 Cells. The unique polarity of attachment of *T. pallidum* to host cell surfaces, along with other biological data, implicated a receptor-ligand interaction (14, 18, 25). To reinforce this concept, we attempted to establish an in vitro model that would permit analysis of treponemal components for their binding properties to eucaryotic cell surfaces. *T. pallidum* radiolabeled with [^{35}S]methionine were exposed to a variety of detergents to facilitate solubilization. In Fig. 1 the efficiency of SDS followed by Triton X-100 vs. Triton X-100 alone is compared. Note that SDS-Triton solubilizes both a wider spectrum of proteins and increased amounts
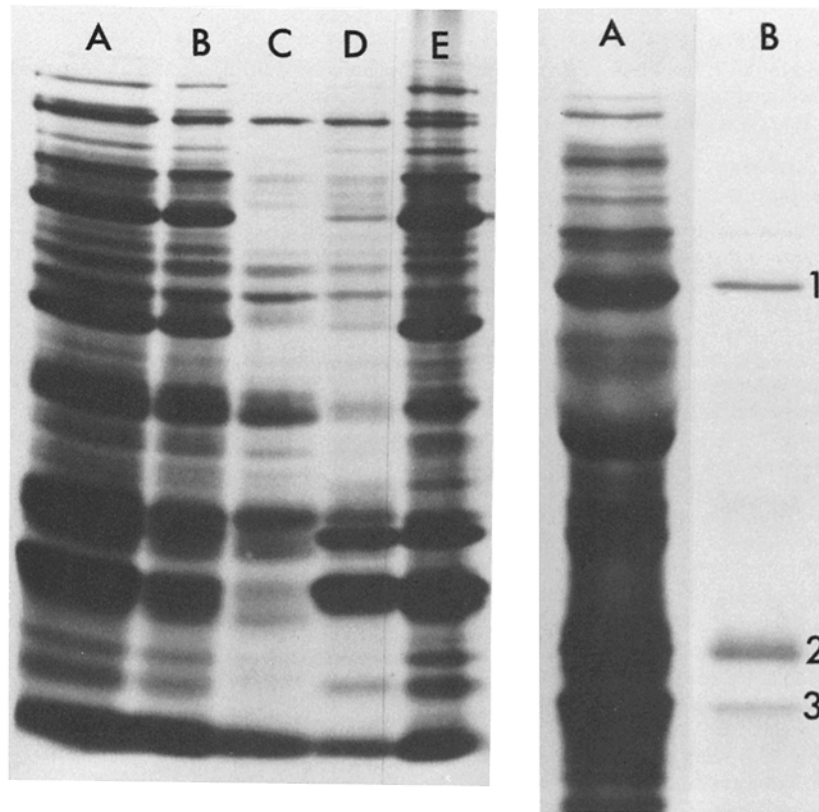
Fig. 1 (Left). Efficiency of detergent solubilization of [³⁵S]methionine-labeled *T. pallidum*. Radiolabeled treponemes were pelleted and resuspended in either (A) final solubilizing buffer before boiling and electrophoresis (Materials and Methods); (B and C) 0.1% SDS-1.0% Triton; or (D and E) 1% Triton. B and D are soluble fractions resulting from the respective detergent treatment. C and E represent the remaining insoluble material that was pelleted and dissolved in final solubilizing buffer. In each case test samples were boiled for 3 min before PAGE and fluorography.

Fig. 2 (Right). Specificity of binding of detergent-solubilized *T. pallidum* proteins to formaldehyde-fixed HEp-2 cells. (A) Total *T. pallidum* ³⁵S-labeled protein profile; (B) Selective binding of treponemal protein bands 1–3. Molecular weight estimations of these proteins were 72,000 (band 1), 28,000 (band 2), and 26,000 (band 3) based upon electrophoretic mobility of known molecular weight standards. In this figure as well as subsequent figures, gel fluorography of the total *T. pallidum* protein profile represents the specific preparation employed in that series of experiments and is included for comparative purposes.

of specific macromolecules as reflected in the intensity of the fluorograph. Other detergents such as NP-40, deoxycholate, and combinations of these agents with and without SDS were less satisfactory than the SDS-Triton mixture.

HEp-2 cells were selected as the indicator system because virulent treponemes readily bind to these cells, monolayer cultures possess cellular homogeneity, and we had already observed receptor-like cooperativity between HEp-2 cells and *T. pallidum* (14, 18). Formaldehyde treatment of HEp-2 cells in suspension permitted their incubation in 0.1% SDS-1.0% Triton without noticeable surface damage or loss of cellular integrity as indicated by light microscopy or reduction in [³⁵S]methionine-associated radioactivity when prelabeled cells were subsequently fixed in formaldehyde and exposed to the detergents. Also, these fixed cells were as competent as
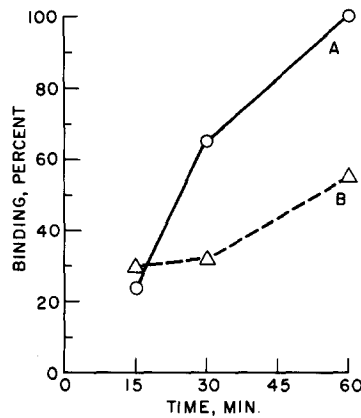
Fig. 3. Binding kinetics of *T. pallidum* protein band 1 to EDTA-treated vs. trypsin-EDTA-treated HEp-2 cells. Formaldehyde-fixed HEp-2 cells were exposed to solubilized [^{35}S]methionine-labeled *T. pallidum* at 34°C for specific time intervals before processing, as described in Materials and Methods. Total binding of band 1 to EDTA-treated HEp-2 cells after 1 h of incubation is defined as 100%. (A) EDTA-exposed cells; (B) Trypsin-EDTA-exposed cells. Quantitative binding of protein band 1 was determined by spectrophotometric scanning of the fluorograms.

untreated cells in permitting attachment of virulent treponemes via their tip-like organelle (14). When solubilized preparations of radiolabeled *T. pallidum* were added to HEp-2 cells, only three major proteins (bands 1–3) remained bound after extensive washing and processing of HEp-2 cells, as described in Materials and Methods (Fig. 2). The kinetics of binding of treponemal proteins was monitored to establish appropriate conditions for biologic assay. At selected time intervals test samples were analyzed by gel electrophoresis fluorography and the binding intensity of protein band 1 recorded by scanning spectrophotometry (Fig. 3). Binding was linear as a function of time during the 1-h incubation. Also presented in Fig. 3 is the effect of brief trypsin treatment (0.125% trypsin for 10 min) of HEp-2 cells followed by formaldehyde fixation, on the capacity of these cells to promote binding of treponemal protein band 1. A decrease of ~50% was detected when trypsin plus 0.02% EDTA-treated cells were compared with cells exposed to EDTA alone. Other data indicated that band 1 was both the predominant ligand and the one whose binding was most sensitive to the effects of trypsin pretreatment of HEp-2 cells. In addition, these proteins were shown to be surface molecules based upon their preferential release during trypsin treatment of *T. pallidum* (26) and the selective ^{125}I-radiolabeling of these proteins after lactoperoxidase-catalyzed iodination of intact virulent treponemes (J. F. Alderete and J. B. Baseman. Unpublished data.).

Competitive Binding of T. pallidum Proteins. Further clarification as to the nature of the receptor-like binding was obtained by performing competition assays with unlabeled, solubilized treponemes. HEp-2 cells were simultaneously exposed to ^{35}S-labeled treponemal preparations plus equivalent or reduced amounts of unlabeled *T. pallidum*. Decreased association of radiolabeled protein bands 1–3 with HEp-2 cells occurred in the presence of increasing amounts of unlabeled treponemal sample (Fig. 4). To quantitatively resolve this relationship, spectrophotometric gel scans of these bands were compared. As depicted in Fig. 5, competition between labeled and unlabeled treponemal material for the putative HEp-2 receptors was readily detected with protein bands 1–3, although the extent of competition differed. In contrast, unlabeled
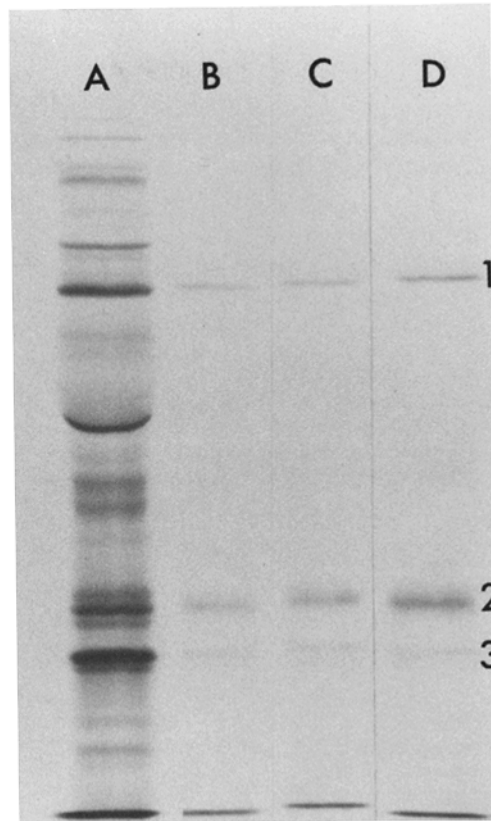
FIG. 4.   Competitive binding analysis of *T. pallidum* proteins. Soluble ^{35}S-labeled *T. pallidum* were exposed to HEp-2 cells for 1 h in the presence of varying amounts of unlabeled soluble *T. pallidum*. (A) Total treponemal protein pattern before incubation with HEp-2 cells; (B) 1.2 parts unlabeled: 1 part radiolabeled; (C) 0.8 part unlabeled: 1 part radiolabeled; (D) 0.4 part unlabeled: 1 part radiolabeled. Gels B-D represent proteins avidly associated with formaldehyde-fixed HEp-2 cells. Unlabeled soluble preparations of the avirulent Reiter treponeme at similar concentrations did not compete.

T. phagedenis biotype Reiter, an avirulent and genetically unrelated spirochete that does not attach to eucaryotic cells (14, 25), was without effect in these competitive binding studies.

Identification of T. pallidum Immunogens.   Because we wanted to define the role of treponemal components both as virulence determinants and as potential protective immunogens, we used the highly sensitive techniques of RIP coupled with staphylococcal protein A adsorption, gel electrophoresis, and fluorography. Human sera from patients in the secondary stages of disease that were serologically positive by both reagin and fluorescent treponemal antibody absorption tests were screened for reactivity against [^{35}S]methionine-labeled *T. pallidum* proteins. Solubilization of the treponeme preparation was identical to the procedure used for analyzing receptor-like interactions. A considerable immunologic response to *T. pallidum* proteins is demonstrated with two representative syphilitic sera (Fig. 6). Detection of antibody against treponemal protein bands 1-3 previously shown to exhibit ligand-like activity was observed.
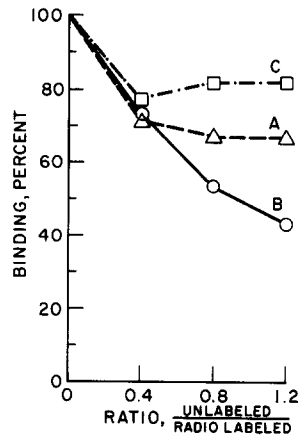
FIG. 5. Quantitative assessment of competitive binding data from Fig. 4. Various concentrations of unlabeled soluble *T. pallidum* preparations, as outlined in the legend to Fig. 4, were combined with a constant amount of radiolabeled treponemal sample before addition of HEp-2 cells. Values were calculated by integration of each peak area after spectrophotometric scanning of the fluorograms. Maximal binding (100%) was defined as total binding of each treponemal protein (bands 1–3) to HEp-2 cells for 1 h in the absence of unlabeled *T. pallidum*. (A) Band 1; (B) band 2; (C) band 3.
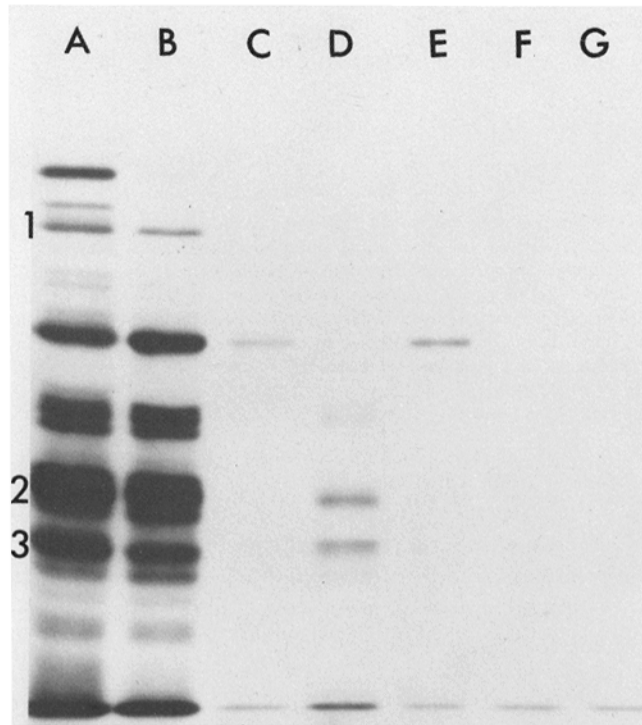

FIG. 6. RIP of [^{35}S]methionine-labeled *T. pallidum* proteins with human syphilitic or control sera. After antigen-antibody formation, complexes were adsorbed to Staph A and processed by PAGE and fluorography, as described in Materials and Methods. Final dilution of serum used was 1/50. (A and B) individual human syphilitic sera obtained during the secondary stage of infection; (C–F) individual control human sera; (G) nonspecific adsorption of ^{35}S-labeled treponemal preparation to staphylococci in the absence of serum.
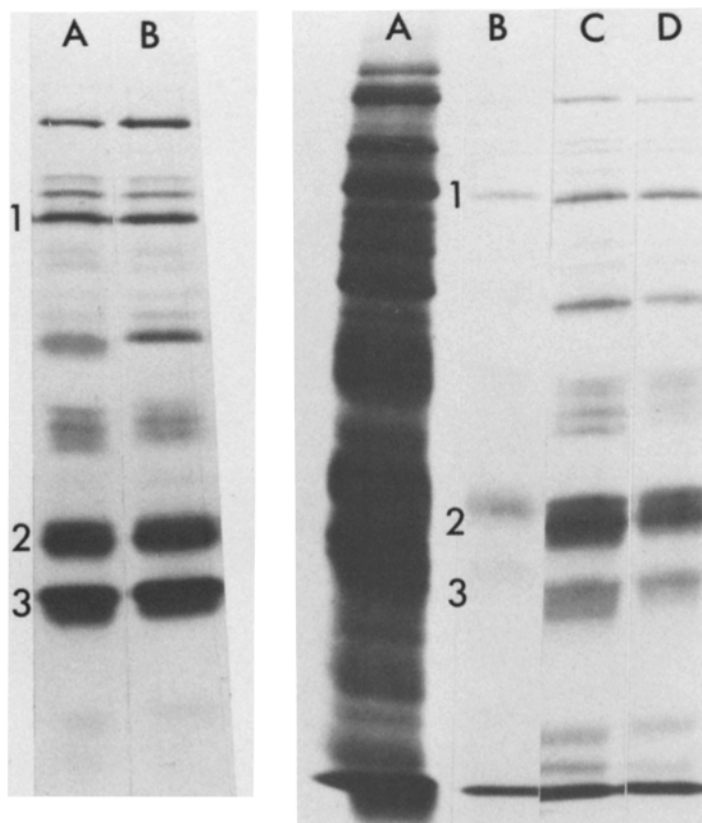
FIG. 7 (Left).   Comparative gel profiles of *T. pallidum* immune complex formation with syphilitic human and rabbit sera. (A) Human; (B) Rabbit. Final serum dilution was 1/50.

FIG. 8 (Right).   Competitive RIP with ^{35}S-labeled *T. pallidum* and human syphilitic serum in the presence of unlabeled *T. pallidum* or *T. phagedenis* preparations. Each test system contained an equal amount of ^{35}S-labeled *T. pallidum*. Concentrations of unlabeled treponeme preparations were made equivalent based upon dark field microscopic counts of the spirochetes before solubilization. (A) Total soluble *T. pallidum* profile; (B) 2 parts unlabeled *T. pallidum*: 1 part radiolabeled *T. pallidum*; (C) radiolabeled *T. pallidum* alone; (D) 2 parts unlabeled *T. phagedenis*: 1 part radiolabeled *T. pallidum*.

The extensive nature of this humoral response is apparent when gel comparisons are made between these patterns and the total *T. pallidum* protein profiles indicated in Fig. 1. Slots C–F on the slab gel (Fig. 6) are profiles obtained when four normal, nonreagin-reactive human sera are incubated with the radiolabeled *T. pallidum* preparation. Only limited reactivity is detected in three of the four sera. If the autoradiogram is exposed for 4 rather than 12 d as shown in Fig. 6, reactivity of treponemal proteins with syphilitic test sera is readily visualized, whereas serologic reactivity in control sera is absent. Slot G of Fig. 6 represents the association of solubilized [^{35}S]*T. pallidum* sample with Staph A in the absence of sera, and nonspecific binding did not occur. A gel profile comparing *T. pallidum* proteins complexed by the human serum used in slot A of Fig. 6 and by serum from a rabbit 4 mo postinfection appeared nearly identical, which suggests a similar humoral response to infection (Fig. 7).
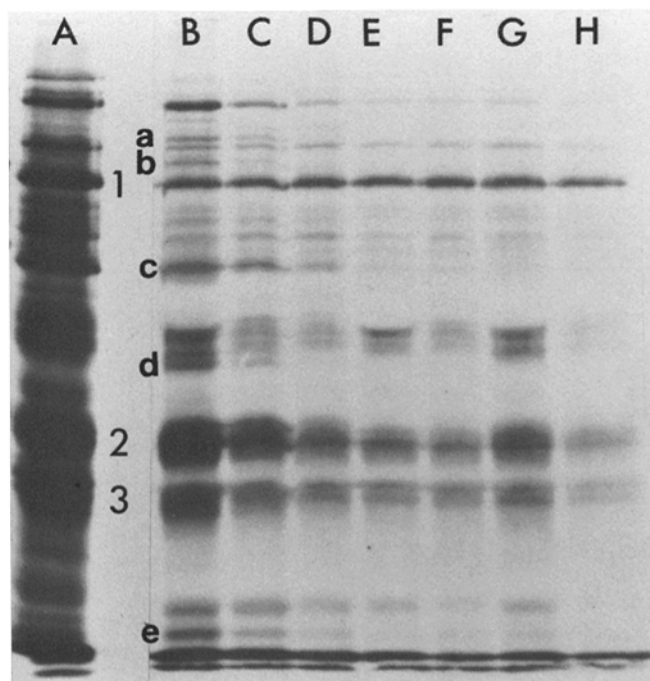
Fig. 9. Comparative RIP fluorograms of ^{35}S-labeled *T. pallidum* proteins exposed to human syphilitic or yaws sera. (A) Total radiolabeled *T. pallidum* proteins; (B–D) Human syphilitic serum at final dilutions of 1/50, 1/200, and 1/800, respectively; (E and F) human yaws serum at dilutions of 1/50 and 1/200; (G and H) different human yaws serum at dilutions of 1/50 and 1/200.

To further support the selectivity of these data, we incubated a constant amount of radiolabeled *T. pallidum* preparation along with unlabeled, solubilized *T. pallidum* or *T. phagedenis* biotype Reiter. With human syphilitic serum (Fig. 6, slot A) to contrast serologic reactivity, we observed extensive reduction in the intensity of the fluorogram at a ratio of 2 parts unlabeled *T. pallidum* to 1 part radiolabeled sample (Fig. 8). Little or no effect is demonstrated with an equivalent amount of the Reiter treponeme (Fig. 8). Note that the *T. pallidum* proteins previously shown to be selectively and avidly bound to the HEp-2 cell surface remain as the major immunogens.

RIP of T. pallidum Proteins with Yaws Sera. Because the biologic relationship between *T. pallidum* and *T. pertenue,* the causative agent of yaws, is unclear, we examined gel profiles with sera from syphilitic and yaws patients. Numerous similarities in the gel fluorograms can be demonstrated that indicate cross-reactivity between these virulent treponemes as well as analogous humoral responses of the host to the infecting agents (Fig. 9). Of interest is the presence of antibody against *T. pallidum* protein bands 1–3 in the yaws sera samples. Equally important is the observation that protein band 1 shows no reduction in intensity over a 16-fold dilution of the test sera in contrast to all other *T. pallidum*-reactive proteins. This suggests the formation of antigen-antibody complexes in the presence of excess specific antibody. Serological differences were also visualized between syphilitic and yaws sera as indicated by the absence or reduction in bands *a–e* in the yaws samples (Fig. 9). The latter is reinforced by gel profile comparisons between the 1/200 dilution of syphilitic serum and 1/50 dilution of yaws sera.

Discussion

The pathophysiology of the disease, syphilis, remains a complicated host response to stress conditions of *T. pallidum* infection characterized by classical stage development and latency. Both humoral and cellular mechanisms of host defense contribute to protection although in certain individuals treponemes are not eliminated and disease progresses. Because *T. pallidum* cannot be cultivated in vitro, complex experimental approaches have been required to clarify the host-parasite interaction. This paper presents our strategy of using molecular technology to dissect the biologic and immunologic properties of virulent *T. pallidum*.

Previous evidence from this laboratory suggested an important role for treponemal parasitism of host cell surfaces (14, 18). It seemed reasonable that identification of ligand-like molecules associated with virulent treponemes would not only reinforce and extend our understanding of virulence determinants but also would offer relevant information for conception and development of potential vaccine candidates. If antibody against such functional molecules could prevent initial attachment and/or colonization of host cells, infection could be interrupted at an early stage.

Highly sensitive probes to identify treponemal macromolecules with selective binding affinity for eucaryotic cells were developed. Incubation of SDS-Triton solubilized [^{35}S]methionine-labeled *T. pallidum* with formaldehyde-treated HEp-2 cells followed by gel electrophoresis and fluorography permitted the resolution of three avidly bound treponemal macromolecules. Competitive binding assays with unlabeled *T. pallidum* over the concentration ranges tested indicated a finite number of membrane sites on HEp-2 cells to accommodate these *T. pallidum* proteins. Differences in the degree of competition of the three proteins (Fig. 5) may suggest modulation of HEp-2 membrane sites as a result of ligand binding. In addition, the inability of solubilized preparations of the Reiter treponème to influence binding of *T. pallidum* proteins further supported the selective nature of the observations and the potential relationship of these treponemal components to virulence. Trypsin treatment of HEp-2 cells before formaldehyde fixation reduced binding of *T. pallidum* protein band 1 by 50%, thus establishing the involvement of proteinaceous membrane components on HEp-2 cells for the recognition event. This observation is consistent with the function of membrane proteins on eucaryotic cells as mediators of ligand interactions (27).

The combination of RIP, gel electrophoresis, and fluorography proved to be a powerful tool for identifying immunogenic *T. pallidum* proteins. It was clear that an extensive and specific humoral response occurs in syphilitic humans based upon the detection of immune precipitates against numerous treponemal components (Fig. 6). The selectivity of this approach was demonstrated by the lack of strong immunologic reactivity with normal human sera and the absence of nonspecific binding of ^{35}S-labeled *T. pallidum* proteins to Staph A. In the case of normal human serum controls, evidence for weak and varied reactivity against *T. pallidum* proteins may signal the existence of cross-reacting antibodies that could arise as a result of antigenic stimulation by treponemes or other microorganisms comprising the resident bacterial flora. The inability of *T. phagedenis* biotype Reiter proteins to significantly alter the *T. pallidum* immunoprecipitation gel profile (Fig. 8) verifies the specificity of the reaction and the fact that little antigenic homology exists between these treponemes, as previously suggested by DNA hybridization studies (28). However, further serologic analysis with an increased antigenic load is required to complete the analysis of antigenic cross-reactivity.

Comparisons of the humoral response of humans infected with *T. pallidum* and *T. pertenue,* the agents of syphilis and yaws, respectively, provided dramatic evidence of the similarities and differences in the serologic response to these infections. The data presented here indicate for the first time the molecular basis for serologic cross-reactivity and would seem to provide a method for distinguishing the humoral response of syphilis and yaws. The latter observations suggest antigenic homology and heterology among these virulent treponemes consistent with cross-protection studies during superinfection (6, 29). It should be pointed out that some variation among different syphilitic sera can also be detected (Fig. 6), which implies possible strain differences among *T. pallidum* organisms (29) and/or variations in individual immunologic responsiveness of susceptible hosts. It was satisfying to detect uniformity in human and rabbit humoral responses to *T. pallidum* infection (Fig. 7), further reinforcing the relevance of the experimental rabbit model for syphilis research (30).

Of special interest was the commonality of antibody against *T. pallidum* bands 1-3 in the reaction mixtures of both syphilitic and yaws sera. This implies that *T. pertenue* possesses similar antigenic molecules, possibly correlating with the ability of *T. pertenue* to attach to tissue culture cells (31). Also pertinent is the observation that band 1 retained equivalent fluorographic intensity over a many fold dilution of serum samples, which suggested that antibody raised against this treponemal protein was of higher titer than the other *T. pallidum* immunogens. Coomassie brilliant blue staining of *T. pallidum* proteins indicates that band 1, as well as bands 2 and 3, exist in approximately equal amounts and are similar in concentration to other major treponemal proteins (26). Thus band 1 may prove to be highly immunogenic when compared with other treponemal proteins, possibly because of its association with host cell membranes resulting in preferential and/or prolonged antigenic processing. Whatever the case, *T. pallidum* protein band 1, along with bands 2 and 3, would seem to be appropriate antigens for consideration in the development of a multiple component vaccine.

T. pallidum continues to be an exceedingly difficult microorganism to manipulate experimentally. Two primary deficiencies inherent in experimental studies are the inability to grow *T. pallidum* in vitro and the subsequent dependence upon outbred rabbits for passage of treponemes and for investigation of host response to infection. As demonstrated in this work, humoral response to a multitude of treponemal proteins occurs during syphilis, yet in many instances disease continues to progress. It appears that cell-mediated immunity is altered during early *T. pallidum* infection, thus providing a mechanism by which virulent treponemes avoid selective host defense mechanisms. A detailed review of cell-mediated immunity during syphilis has been published (32). Additional data indicate that serum factors generated during experimental syphilis in rabbits significantly depress normal rabbit lymphocyte response to phytohemagglutinin (11), concanavalin A (12), and periodate oxidation (12). In addition, lymphocytes obtained from various organs of *T. pallidum*-infected rabbits respond differently to plant lectins when incubated with syphilitic rabbit sera (33). Complicating these data is the observation that *T. pallidum* resists phagocytosis (18, 34). The finding of Alderete and Baseman (26) that numerous host proteins are both loosely and avidly associated with the outer envelope of virulent *T. pallidum* provides a mechanism by which treponeme populations might avoid immune surveillance or other immune defenses. Furthermore, the intimate association of host proteins with *T. pallidum* may precipitate autoimmune reactions that result in clinical symptoms of

syphilis and continued progression of disease. Likewise, the fact that *T. pallidum* protein bands 1–3 avidly bind to mammalian cell surfaces raises questions as to the potential consequences of the host response to this antigenic complex, especially in terms of autoimmunity.

Immunostimulatory and immunoinhibitory bacterial components exist in many species of pathogenic microorganisms (35), and the ability to determine the immunologic activities of specific bacterial proteins may circumvent the arbitrary inclusion in vaccine preparations of bacterial fractions of unknown function that may possess immunonegative properties. The identification of antigenic treponemal macromolecules that may serve an essential role in virulence such as mediating surface parasitism is an initial step in the establishment of a rational vaccine candidate. With the techniques described in this study, resolution of biologically functional treponemal immunogens is feasible. This approach should have general application in the establishment of a basic strategy for vaccine development against microbial agents that mediate infection by direct surface interactions with host tissue.

Summary

Receptor binding proteins of *Treponema pallidum* were identified by incubation of [^{35}S]methionine-labeled, soluble *T. pallidum* preparations with formaldehyde-fixed HEp-2 cells. Three major treponemal proteins (bands 1–3) that avidly bound to the eucaryotic cell surface were detected by sodium dodecylsulfate-polyacrylamide gel electrophoresis and fluorography. Brief trypsin treatment of HEp-2 cells before formaldehyde fixation reduced the extent of the interaction of these treponemal macromolecules, which implicated receptor-mediated attachment mechanisms. The presence of unlabeled *T. pallidum* preparations directly competed with radiolabeled *T. pallidum* samples for the available HEp-2 cells, which suggested a limiting number of membrane binding sites. Samples of unlabeled avirulent Reiter treponeme did not compete.

T. pallidum immunogens were examined by radioimmunoprecipitation with human and rabbit syphilitic sera. Of interest were the similarities and extent of the humoral response represented by the detection of antigen-antibody complexes against numerous treponemal proteins, including bands 1–3. *T. pallidum* protein band 1 appeared to be the major antigenic stimulus. Formation of antigen-antibody complexes between ^{35}S-labeled *T. pallidum* proteins and human syphilitic sera was prevented by unlabeled *T. pallidum* but not by *T. phagedenis* preparations, which demonstrated specificity of the reaction. Gel profiles of radioimmunoprecipitation assays using radiolabeled *T. pallidum* antigens and human syphilitic and yaws sera delineated both the similarities and differences in the humoral response to these two spirochetes. The latter suggested both overlapping and distinguishing antigenic properties between *T. pallidum* and *T. pertenue*. Detection in yaws sera of specific antibody against *T. pallidum* protein bands 1–3 further incriminates the role of these three treponemal proteins as virulence determinants.

References

1. Turner, T. B., and D. H. Hollander. 1957. Biology of the treponematoses. Monograph Series No. 35. World Health Organization, Geneva.
2. Cannefax, G. R. 1965. Immunity in syphilis. *Br. J. Vener. Dis.* **41:**260.
3. Perine, P. L., R. S. Weiser, and S. J. Klebanoff. 1973. Immunity to syphilis: passive transfer in rabbits with hyperimmune serum. *Infect. Immun.* **8:**787.
4. Bishop, N. H., and J. N. Miller. 1976. Humoral immunity in experimental syphilis. I. The demonstration of resistance conferred by passive immunization. *J. Immunol.* **117:**191.
5. Bishop, N. H., and J. N. Miller. 1976. Humoral immunity in experimental syphilis. II. The relationship of neutralizing factors in immune serum to acquired resistance. *J. Immunol.* **117:**197.
6. Miller, J. N. 1973. Immunity in experimental syphilis. VI. Successful vaccination of rabbits with *Treponema pallidum*, Nichols strain, attenuated by γ-irradiation. *J. Immunol.* **110:**1206.
7. Friedmann, P. S., and J. L. Turk. 1975. A spectrum of lymphocyte responsiveness in human syphilis. *Clin. Exp. Immunol.* **21:**59.
8. Pavia, C. S., J. B. Baseman, and J. D. Folds. 1977. Selective response of lymphocytes from *Treponema pallidum*-infected rabbits to mitogens and *Treponema reiteri*. *Infect. Immun.* **15:**417.
9. Pavia, C. S., J. D. Folds, and J. B. Baseman. 1977. Selective in vitro response of thymus-derived lymphocytes from *Treponema pallidum*-infected rabbits. *Infect. Immun.* **18:**603.
10. Levene, G. M., J. L. Turk, D. J. M. Wright, and A. G. S. Grimble. 1969. Reduced lymphocyte transformation due to a plasma factor in patients with active syphilis. *Lancet.* **II:**246.
11. Wicher, V., and K. Wicher. 1977. In vitro cell response of *Treponema pallidum*-infected rabbits. II. Inhibition of lymphocyte response to PHA by serum of *T. pallidum*-infected rabbits. *Clin. Exp. Immunol.* **29:**487.
12. Ware, J. L., J. D. Folds, and J. B. Baseman. 1979. Serum of rabbits infected with *Treponema pallidum* (Nichols) inhibits in vitro transformation of normal rabbit lymphocytes. *Cell. Immunol.* **42:**363.
13. Ware, J. L., J. D. Folds, and J. B. Baseman. 1980. Modulation of normal lymphocyte stimulation by serum from *Treponema pallidum*-infected rabbits. *Cell. Immunol.* In press.
14. Hayes, N. S., K. E. Muse, A. M. Collier, and J. B. Baseman. 1977. Parasitism by virulent *Treponema pallidum* of host cell surfaces. *Infect. Immun.* **17:**174.
15. Hu, P. C., A. M. Collier, and J. B. Baseman. 1977. Surface parasitism by *Mycoplasma pneumoniae* of respiratory epithelium. *J. Exp. Med.* **145:**1328.
16. Smith, H. 1977. Microbial surfaces in relation to pathogenicity. *Bacteriol. Rev.* **41:**475.
17. Jones, G. W. 1977. The attachment of bacteria to the surfaces of animal cells. *In* Receptors and Recognition. J. L. Reissig, editor. Chapman & Hall, Ltd., London. **3B:**141.
18. Baseman, J. B., N. S. Hayes, J. K. Spitznagel, and E. C. Hayes. 1979. Virulence determinants among the spirochetes. *In* Microbiology–1979. D. Schlessinger, editor. American Society for Microbiology, Washington, D. C. 203.
19. Baseman, J. B., J. C. Nichols, and S. Mogerley. 1979. Capacity of virulent *Treponema pallidum* (Nichols) for deoxyribonucleic acid synthesis. *Infect. Immun.* **23:**392.
20. Baseman, J. B., J. C. Nichols, and N. S. Hayes. 1976. Virulent *Treponema pallidum*: aerobe or anaerobe. *Infect. Immun.* **13:**704.
21. Kessler, S. W. 1976. Cell membrane antigen isolation with the staphylococcal protein A-antibody adsorbent. *J. Immunol.* **117:**1482.
22. Baseman, J. B., and N. S. Hayes. 1977. Anabolic potential of virulent *Treponema pallidum*. *Infect. Immun.* **18:**857.
23. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46:**83.
24. Moore, P. M. E., E. C. Hayes, S. E. Miller, L. L. Wright, C. E. Machamer, and H. J.

Zweerink. 1978. Measles virus nucleocapsids: large-scale purification and use in radioim-
munoassays. *Infect. Immun.* **20:**842.

25. Fitzgerald, T. J., R. C. Johnson, J. N. Miller, and J. A. Sykes. 1977. Characterization of the
attachment of *Treponema pallidum* (Nichols strain) to cultured mammalian cells and the
potential relationship of attachment to pathogenicity. *Infect. Immun.* **18:**467.

26. Alderete, J. F., and J. B. Baseman. 1979. Surface-associated host proteins on virulent
Treponema pallidum. Infect. Immun. **26:**1048.

27. Kono, T., and F. W. Barham. 1971. The relationship between the insulin-binding capacity
of fat cells and the cellular response to insulin. *J. Biol. Chem.* **246:**6210.

28. Miao, R., and A. H. Fieldsteel. 1978. Genetics of *Treponema*: relationship between *Treponema
pallidum* and five cultivable treponemes. *J. Bacteriol.* **133:**101.

29. Hardy, P. H., Jr. 1976. Pathogenic treponemes. *In* The Biology of the Parasitic Spirochetes.
R. C. Johnson, editor. Academic Press, Inc., New York. 107.

30. Baseman, J. B. 1977. Report of a workshop: the biology of *Treponema pallidum. J. Infect. Dis.*
136:308.

31. Fieldsteel, A. H., J. G. Stout, and F. A. Becker. 1979. Comparative behavior of virulent
strains of *Treponema pallidum* and *Treponema pertenue* in gradient cultures of various mam-
malian cells. *Infect. Immun.* **24:**337.

32. Pavia, C. S., J. D. Folds, and J. B. Baseman. 1977. Cell-mediated immunity during syphilis.
Br. J. Vener. Dis. **54:**144.

33. Maret, S. M., J. B. Baseman, and J. D. Folds. 1980. Cell-mediated immunity in *Treponema
pallidum*-infected rabbits: in vitro response of splenic and lymph node lymphocytes to
mitogens and specific antigen. *Clin. Exp. Immunol.* In press.

34. Brause, B. D., and R. B. Roberts. 1978. Attachment of virulent *Treponema pallidum* to
human mononuclear phagocytes. *Br. J. Vener. Dis.* **54:**218.

35. Schwab, J. H. 1975. Suppression of the immune response by microorganisms. *Bacteriol. Rev.*
39:121.