6. Wash the wells three times with 0.1% Nonidet P-40, PBS–Ca/Mg.
7. Count the filter spots for γ irradiation.

For inhibition experiments, various concentrations of inhibitors are added during the incubation with the labeled tracer protein (i.e., [^{125}I]MBP–Inv479). Figure 4 shows inhibition of invasin binding to the $\alpha_5\beta_1$-integrin by the fibronectin RGD peptide, consistent with the notion that invasin and fibronectin interact with the same site on the $\alpha_5\beta_1$ receptor.[14]

Conclusion

The use of affinity chromatography has allowed the identification of several β_1-chain integrins as receptors for invasin.[10] It is likely that these β_1-chain integrins identified as receptors for invasin, and normally involved in cell attachment to the extracellular matrix, are directly involved in the process of internalization of the bacteria as it appears that high-affinity binding to integrins (i.e., $\alpha_5\beta_1$ or $\alpha_3\beta_1$) is sufficient to promote efficient bacterial internalization.[13] The development of the assays described in this article has allowed us to study the interaction between invasin and the $\alpha_5\beta_1$-integrin receptor isolated from placenta. These studies have shown that invasin binds to the receptor with a much higher affinity than does fibronectin,[14] an important property in discriminating between bacterial internalization and simple extracellular adherence to human epithelial cells.[9]

[22] Identification of Fibronectin as a Receptor for Bacterial Cytoadherence

By JOHN F. ALDERETE, ROSSANA ARROYO, and MICHAEL W. LEHKER

Introduction

The idea that organisms are coated with soluble, host-derived serum proteins may be an obvious one. After all, it is difficult to envision a situation where a microbial pathogen will not, at any time after entry into the host environment and regardless of the site of infection, be in contact, indeed continuously bathed, with host fluids as complex as serum. In support of this view, the literature is replete with reports of specific host

proteins associating with microbial surfaces, and many of these examples have been discussed in numerous reviews.[1,2] From the beginning, though, studies on the associations between bacterial pathogens and certain host proteins likely resulted from serendipitous observations. Such was the case involving fibronectin and *Staphylococcus aureus*.[3] The same could be said of perhaps many other examples of bacterial pathogens and fibronectin associations, and whether or not a bacterial pathogen bound a particular host protein was the result of a rather random screening of a library of commercially available proteins. Not surprisingly, then, there are few reports in the literature where the uniqueness and/or nature of the relationship between the host and a bacterial pathogen has drawn attention to whether a host serum or cellular component might be recognized and bound by the organism.

The significance of the early report on the *S. aureus*–fibronectin association[3] is underscored by the explosion in the number of articles that have appeared showing interactions by pathogenic bacteria,[4-8] protozoa,[9-11] yeast,[12] virus,[13,14] and parasites[2] with extracellular matrix (ECM) proteins. The consequences to the host of being infected by organisms interacting with host serum proteins in general and ECM proteins in particular are numerous, apart from the obvious feature of host parasitism through enhanced cytoadherence. For example, masking of the microbial surface might interfere with antigen presentation or prevent recognition of the foreign organism or antigens leading to an overall immune evasion strategy by the pathogen. Host proteins might impair further the more specialized functions of immune cells whose job is ligand recognition for phagocytosis. Autoimmune reactions might be envisioned, possibly the result of an

[1] M. Höök and L. M. Switalski, *in* "Biology of Extracellular Matrix" (D. Mosher, ed.). Vol. 5, p. 295. Academic Press, San Diego, 1989.
[2] D. J. Wyler, *Rev. Infect. Dis.* **9,** Suppl., S391 (1987).
[3] P. Kuusela, *Nature (London)* **276,** 718 (1978).
[4] K. M. Peterson, J. B. Baseman, and J. F. Alderete, *J. Exp. Med.* **157,** 1958 (1983).
[5] L. M. Switalski, P. Speziale, M. Höök, T. Wadström, and R. Timpl, *J. Biol. Chem.* **259,** 3734 (1984).
[6] S. B. Baloda, A. Faris, G. Fröman, and T. Wadström, *FEMS Microbiol. Lett.* **28,** 1 (1985).
[7] C. Abon-zeid, T. Garbe, R. Lathigra, H. G. Wiker, M. Harboe, G. A. W. Rook, and D. B. Young, *Infect. Immun.* **59,** 2712 (1991).
[8] M. Haapasalo, U. Singh, B. C. McBride, and V. Uitto, *Infect. Immun.* **59,** 4230 (1991).
[9] D. J. Wyler, J. P. Sypek, and J. A. McDonald, *Infect. Immun.* **49,** 305 (1985).
[10] S. T. Pottratz, J. Paulsrud, J. S. Smith, and W. J. Martin, II, *J. Clin. Invest.* **88,** 403 (1991).
[11] M. A. Onaissi, D. Afchain, A. Capron, and J. A. Grimaud, *Nature (London)* **308,** 380 (1984).
[12] A. Kalo, E. Segal, E. Sahar, and D. Dayon, *J. Infect. Dis.* **157,** 1253 (1988).
[13] K. Wang, R. J. Kuhn, E. G. Strauss, S. On, and J. H. Strauss, *J. Virol.* **66,** 4992 (1992).
[14] I. Julkunen, A. Hautanen, and J. Keski-oja, *Infect. Immun.* **40,** 876 (1983).

altered conformation of the host protein and elicitation of autoreactive antibody, and contribute to disease pathogenesis.[15]

From this vantage point, might it be possible to develop a strategy by which investigators could (1) examine the general association between organisms and host serum or cellular components; (2) identify the protein or proteins preferentially enriched onto that microbial surface (as compared with those proteins just loosely associated with the surface); and (3) determine the role that the acquired host protein plays in the biology of the host–parasite interrelationship?

An experimental approach as outlined here would enable the examination of the extent to which microorganisms associate themselves with many serum proteins and might allow for the questions posed above to be answered. Of course, the nature of the host protein–bacteria interactions, whether a loose association of serum proteins with the microorganism (in essence coating or masking the bacterial surface) versus one of a high-affinity, receptor-mediated binding, would be examined in step 1. The identification of the preferentially bound serum protein(s) (step 2) would employ its own substrategy, such that from this information the investigator could then plan experiments to test experimentally the contribution of the host protein to the biology of the host–bacteria relationship (step 3). Considering the complex composition of serum, evidence for a preferential enrichment by the bacterial surface for specific serum proteins would itself be informative, as it is unlikely that receptor-mediated acquisition of a few proteins from serum would occur without relevance.

Therefore, the examination of the interaction between host proteins and microorganisms appears to be central to many questions involving molecular aspects of pathogenesis, from establishment of infection to survival within the adverse environment of the host, through nutrient acquisition and immune evasion, to possible autoimmune manifestations. These types of issues became prominent after the report showing the specific fibronectin binding by *S. aureus,* and it was at this time that the approach mentioned above was attempted for the syphilis spirochete, *Treponema pallidum.* What follows, then, is a description of the approach and brief relevant methodology, except that it is put into a historical context, thereby showing the stepwise nature of the investigation that led to the identication of fibronectin and the highly defined RGD sequence as a receptor for host cytoadherence by this spirochete. Another technique, called the ligand assay and developed by Baseman and Hayes,[16] was used in separate but parallel studies. The importance of this technique is also

[15] R. E. Baughn, *Rev. Infect. Dis.* **9,** Suppl., S372 (1987).
[16] J. B. Baseman and E. C. Hayes, *J. Exp. Med.* **151,** 573 (1980).

addressed here because it initiated the investigation for the fibronectin-binding proteins of *T. pallidum* organisms, which were later verified. Thus, the host protein binding studies and the ligand assay both came together to aid in our understanding of the molecular nature of host parasitism by this infectious agent. Finally, the importance of these approaches has since been affirmed and realized by showing their usefulness in other models of microbial pathogens, including a protozoan parasite, *Trichomonas vaginalis*.

Identification of Fibronectin as a Host Cell Receptor

Loosely Associated Serum Proteins

In 1963 a suggestion was made that the syphilis spirochete was surrounded by a protective, host-derived surface coat.[17] The indication that serum proteins were contaminating the organisms was reinforced in observations made from stained patterns of total proteins after electrophoresis.[18] Prominent bands disappeared during the various washings of the spirochetes in phosphate-buffered saline (PBS). Even more intriguing was the reacquisition of the protein bands by incubation of the washed bacteria with rabbit serum. It is noteworthy that *T. pallidum* was isolated from extractions of infected rabbit testes, as *in vitro* cultivation was and remains a major impediment to the study of this pathogen. Nonetheless, it was fortuitous that these *in vivo*-derived organisms were being examined immediately after isolation, as a feature of the host protein interaction was the rather loose and reversible association of serum proteins with the spirochete. The loss of the loosely associated serum protein was confirmed by comparison of the patterns of bands from stained gels with fluorograms of intrinsically labeled proteins.

Agglutination Assay to Demonstrate Avid Binding of Serum Proteins

At the time, the protein A-bearing *S. aureus* was being used in radioimmunoprecipitation assays (RIPAs),[19] and these fixed bacteria were now adapted in agglutination assays to determine the avid association of serum proteins to the surface of *T. pallidum*. After extensive washing, the freshly extracted organisms were radiolabeled for a brief period before being incubated with commercially available antiserum to specific serum proteins. Binding of antibody to proteins not removed by the extensive wash-

[17] S. Christiansen, *Lancet* **1,** 423 (1963).
[18] J. F. Alderete and J. B. Baseman, *Infect. Immun.* **26,** 1048 (1979).
[19] S. W. Kessler, *J. Immunol.* **117,** 1482 (1976).

ing of the spirochetes with PBS was then monitored by an agglutination assay involving the addition of formaldehyde-fixed *S. aureus*. The staphylococcus–antibody–spirochete complex was stable, highly specific, and readily precipitable and separable from free treponemes by low-speed centrifugation, which allowed for quantitation of radioactivity of the pellet. Agglutination could be abolished only by enzymatic (trypsin) treatment of the treponemes for release of the avidly bound host proteins. As a control, it was important to show the absence of immunoreactivity of the antisera with treponemal proteins, and this was demonstrated by the RIPA.

This agglutination assay was again exploited several years later to show the general feature of avidly associated host serum proteins with *Trichomonas vaginalis*, a sexually transmitted protozoan parasite.[20] Here, too, ultrastructural work showed the appearance of a host-derived "coat" which was lost during *in vitro* cultivation. In this case, *T. vaginalis* organisms grown in a complex medium supplemented with heat-inactivated human serum, a requirement for growth and multiplication of trichomonads, were washed numerous times to remove loosely bound serum proteins and added to a suspension of *S. aureus* that was first pretreated with specific antibodies to individual host proteins. The bacteria–antibody–trichomonad agglutination was extensive, and the complex readily separated itself from *S. aureus* alone or unbound trichomonads.

Identification of Specific Plasma Proteins Bound onto the Bacterial Surface

Preparation of Plasma and Fibronectin for Binding Studies

Plasma proteins were iodinated using lactoperoxidase as described earlier.[4] Radiolabeled proteins were dialyzed for 3 days with numerous changes of PBS at 4° prior to incubation of iodinated plasma with live treponemes. Fractionation of the plasma containing about 2.2 g of protein was performed by cold ethanol precipitation procedures,[4] which fractionated plasma into eight defined Cohn fractions listed in Table I. Fibronectin (Fn) was purified on gelatin–agarose and examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)[21] to ensure the use of only purified Fn fractions for important acquisition experiments. In this case, Fn was obtained from normal human plasma or fraction I + III-3 (Table I).

[20] K. M. Peterson and J. F. Alderete, *Infect. Immun.* **37,** 755 (1982).
[21] D. D. Thomas, J. B. Baseman, and J. F. Alderete, *J. Exp. Med.* **161,** 514 (1985).

TABLE I
COMPARATIVE ACQUISITION OF ^{125}I-RADIOLABELED PLASMA PROTEIN PREPARATIONS BY *T. pallidum* AND *T. phagedenis* BIOTYPE REITER[a]

Sample number	Protein preparation added to treponemes[b]	Composition[c]	cpm avidly bound (% of total cpm added)	
			T. pallidum	*T. phagedenis*
1	Normal human plasma	—	1,900 (0.7)	Same as control[d]
2	I + III-3	Plasminogen, fibronectin, fibrinogen	3,190,000 (7.6)	Same as control[d]
3	II	γ-Globulins	20,800 (0.4)	Same as control[d]
4	III-0	β-Lipoproteins, euglobulins, ceruloplasmin	34,600 (1.4)	Same as control[d]
5	III-1,2	Prothrombin, isoagglutinins	18,000 (0.6)	Same as control[d]
6	IV-1	α-Lipoproteins	19,500 (0.5)	Same as control[d]
7	IV-6,7	β-Metal binding protein, α_2-mucoprotein, choline esterase, α_2-glycoprotein	13,700 (0.3)	Same as control[d]
8	V	Albumin	6,700 (0.5)	Same as control[d]
9	VI	α_1-Glycoprotein, small proteins and peptides	1,411,000 (0.4)	Same as control[d]
10	Fibronectin	—	5,430,000 (1.3)	Same as control[d]

[a] Reproduced from Peterson *et al.*,[4] by copyright permission of the Rockefeller University Press.
[b] 50 μl of radioiodinated plasma, specific Cohn fractions, or purified fibronectin was added to 100 μl containing 5×10^9 organisms and incubated at 37° for 30 min as described under Materials and Methods.
[c] Specific activities for individual protein preparations (cpm/ng protein): (1) 2700, (2) 2800, (3) 900, (4) 800, (5) 520, (6) 700, (7) 750, (8) 500, (9) 5600, (10) 4500. Purified fibronectin was prepared from fraction I + III-3; Cohn fractions were obtained as described[34] from normal human plasma.
[d] Tubes without organisms but handled identically to those with organisms were used to determine the level of nonspecific binding of radioactivity. Values never exceeded 1% the level detected for *T. pallidum* acquisition. *T. phagedenis* yielded cpm values equivalent to those of control tubes.

Avid Binding of Iodinated Plasma Proteins to Freshly Purified Treponemes

The procedure demonstrating the specific association of fibronectin and other plasma proteins with the syphilis spirochete is detailed in the original publication.[4] A brief description of the basic protocol is as follows: The reactions are carried out in siliconized 1.5-ml microfuge tubes, always pretreated with 1% horse serum to reduce nonspecific binding. After the

tubes are washed, a small volume (usually 100 μl) of a suspension containing 5×10^9 freshly harvested treponemes is mixed with 50-μl volumes of iodinated plasma or plasma fractions. The final volume is immediately adjusted to 300 μl with PBS. Tubes are then placed in a 37° incubator and shaken gently about every 5 min to ensure uniform distribution of the organisms. At designated times the treponemes are pelleted at 17,000 *g* and washed twice before finally resuspending in cold PBS and transferring to another microfuge tube. An aliquot of 100 μl is precipitated with trichloroacetic acid (TCA) for preparation of proteins for SDS–PAGE autoradiography.

Figure 1 (part I) illustrates the dramatic, avid binding of several proteins from iodinated plasma onto the surface of *T. pallidum* organisms (lane A). That this represented an enrichment of just a few proteins was readily demonstrated by comparing two-dimensional protein patterns of autoradiograms of the total plasma proteins with those that were bound. A hallmark of this and numerous other experiments was the absence of binding of significant amounts of any plasma protein by the nonpathogenic oral spirochete, *Treponema phagedenis* (lane B). At this point, evaluation of certain biochemical parameters like time, temperature, pH, and saturation kinetics as well as competition experiments provided initial evidence for the ligand–receptor type of interaction between the bacterial surface and the host proteins, something reaffirmed later in studies involving particular proteins and the microorganism.

Establishing the Identity of the Acquired Fn

The use of Cohn fractions[4] seemed a logical next step in attempts to identify the proteins specifically and avidly bound to the treponemal surface. These fractions have been well characterized and have been traditionally used as sources of enriched and practically purified proteins, such as albumin and immunoglobulins. Table I shows that Sample 2 gave levels of binding greater than those seen for the other fractions. Again, the control avirulent Reiter spirochete did not bind proteins of the various fractions. The next step, then, was to examine which of the three enriched proteins in this fraction (Fn, plasminogen, or fibrinogen) might represent the predominantly bound material. It was apparent that Fn purified from this fraction or directly from serum was indeed readily and avidly bound by *T. pallidum*. Proof of specific Fn acquisition was then demonstrated (Fig. 1, part II). In this case incubation of live, freshly extracted organisms with either plasma (lane C), fraction I + III-3 (lane E), or purified Fn (lane G), but not with Fn-free plasma or other fractions without Fn (lane F), readily bound Fn, as evidenced from immunoblots with anti-Fn serum.

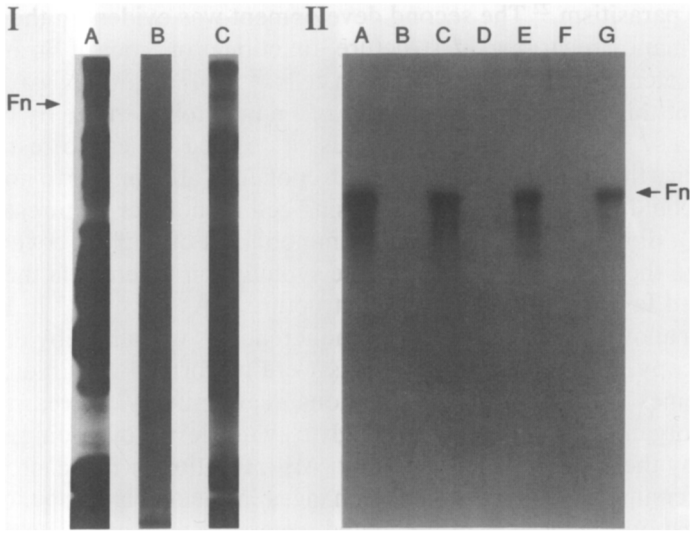

FIG. 1. Left: Representative sodium dodecyl sulfate–polyacrylamide gel electrophoresis/autoradiography of total proteins after incubation of *T. pallidum* with radioiodinated normal human plasma. Lane A represents ^{125}I-labeled plasma proteins avidly bound to *T. pallidum*. Lane B shows the lack of plasma protein acquisition by the avirulent spirochete, *T. phagedenis* biotype Reiter, handled similarly. Lane C is a profile of ^{125}I-labeled normal human plasma used in these acquisition assays. The location of fibronectin (Fn) was established by electrophoresis of ^{125}I-labeled purified human Fn under similar conditions. Right: Electrophoretic transfer and immunodetection of unlabeled fibronectin acquired by motile *T. pallidum* incubated with normal human plasma (lane C), Cohn fraction I + III-3 (lane E), and purified fibronectin (lane G). Lane A shows the immunodetection of purified fibronectin alone. Lane B demonstrates the lack of reactivity of normal goat serum and ^{125}I-labeled protein A with fibronectin alone or with blotted *T. pallidum* proteins (lane D). Lane F shows the lack of detection using antifibronectin antibody and ^{125}I-labeled protein A of *T. pallidum* incubated with plasma depleted of fibronectin. (Reproduced from Peterson *et al.*,[4] by copyright permission of the Rockefeller University Press.)

Fibronectin Involvement in the Cytoadherence Property of T. pallidum

Three reasons motivated the testing of the possibility that Fn was involved in treponemal attachment to host cells. Numerous investigators described the important role in pathogenesis of *T. pallidum* adherence to host cells,[22,23] and the presence of a specialized tip structure on these treponemes was reported as the functional organelle mediating host cell

[22] N. S. Hayes, K. E. Muse, A. M. Collier, and J. B. Baseman, *Infect. Immun.* **17,** 174 (1977).
[23] T. J. Fitzgerald, J. N. Miller, and J. A. Sykes, *Infect. Immun.* **11,** 1133 (1975).

surface parasitism.[22] The second development was evident in the flourish of information regarding the structure–function properties of Fn and other ECM proteins residing on mammalian cells.[24–29] Finally, there was already debate at this time regarding the role of Fn in cytoadherence by bacterial pathogens, including *S. aureus*. Thus, it seemed logical to test for the possibility that Fn was a receptor for treponemal attachment to host cells, which could be performed readily using cell monolayer cultures.

Fn, a dimeric glycoprotein, was immobilized onto glass coverslips to visualize the binding by highly motile syphilis spirochetes via the typical polarized fashion involving the tip structure.[22] As presented in Fig. 2B, only Fn allowed for the tip-oriented adherence as visualized by dark-field microscopy. Subsequent experiments were performed with radiolabeled treponemes to confirm the observations quantitatively. Increasing numbers of organisms gave correspondingly elevated levels of binding as determined by the associated radioactivity. Also, titration of Fn adsorbed onto the coverslips gave corresponding changes in the levels of bound organisms, and only treatment of Fn-coated coverslips with anti-Fn antibodies impaired the treponemal association with immobilized Fn.

Parallel approaches were used to show that Fn on the host cell surface was the target for recognition. Fn was found to be exposed and accessible on the epithelial cells used before to demonstrate cytoadherence.[22] The inhibition of attachment was demonstrated by pretreatment of the host cell surfaces with specific anti-Fn antibodies. Specificity was shown by the lack of inhibition with control antibodies (antialbumin, for example) or with antibodies to other ECM proteins (antilaminin or anticollagen). Dose–response curves showed levels of inhibition as a function of antibody titer. Other parameters that were tested supported the notion of specificity of the spirochete–Fn associations.

Targeting of Fibronectin Cell-Binding Domain and RGD Sequence by Syphilis Spirochete

During these early studies, a wealth of information appeared on the biochemistry of the Fn monomers, so much so that techniques were avail-

[24] M. D. Pierschbacher, E. Ruoslahti, J. Sundelin, P. Lind, and P. A. Peterson, *J. Biol. Chem.* **257,** 9593 (1982).
[25] M. D. Pierschbacher, E. G. Hayman, and E. Ruoslahti, *Cell (Cambridge, Mass.)* **26,** 259 (1981).
[26] M. D. Pierschbacher and E. Ruoslahti, *Nature (London)* **309,** 30 (1984).
[27] K. M. Yamada and K. Olden, *Nature (London)* **275,** 179 (1978).
[28] E. Pearlstein, L. I. Gold, and A. Garcia-Pardo, *Mol. Cell. Biochem.* **29,** 103 (1980).
[29] E. Ruoslahti, E. G. Hayman, E. Engvall, W. C. Cothran, and W. T. Butler, *J. Biol. Chem.* **256,** 7277 (1981).

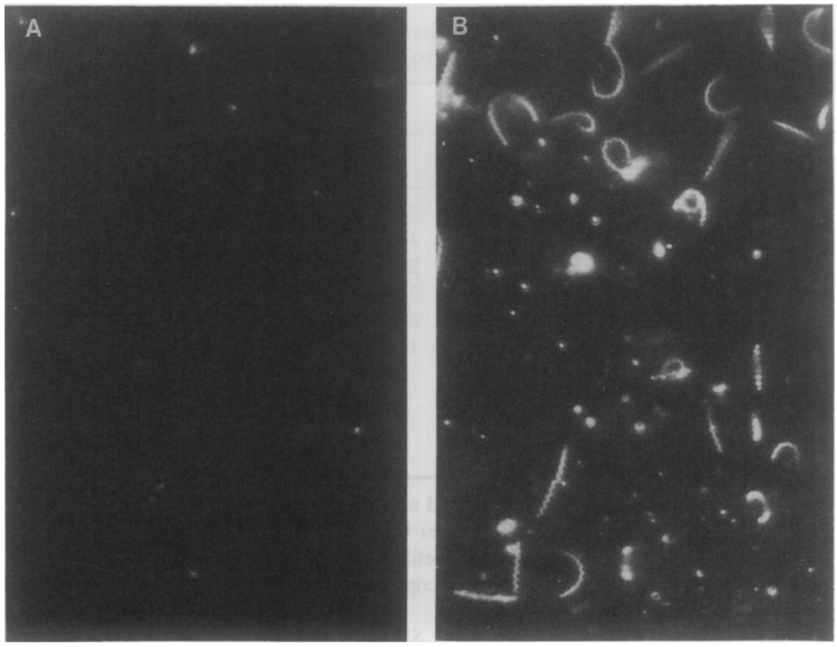

FIG. 2. A representative dark-field view of tip-mediated adherence of freshly harvested *T. pallidum* incubated with fibronectin-coated coverslips (B), compared with the lack of attachment of treponemes from the same extraction to untreated or albumin-coated coverslips (A). (Reproduced from Peterson *et al.*,[4] by copyright permission of the Rockefeller University Press.)

able for purifying the corresponding cell, heparin, and gelatin binding domains.[28,29] In addition, it is noteworthy that monoclonal antibodies (mAbs) directed to amino-terminal, to carboxy-terminal, and to the more central regions of the Fn molecule soon became available,[25] and clearly, these reagents defined the precise domain on Fn being recognized by the organism. Therefore, definition of the structural and functional domain of Fn involved in the tip-oriented treponemal parasitism of host cells was both a natural extension of the earlier work and a requirement for a more molecular understanding of cytoadherence.

The cell and heparin binding domains of purified human Fn were generated through proteolytic digestion of Fn according to the procedure established by Hayashi and Yamada.[30] By use of established protocols, the

[30] M. Hayashi and K. M. Yamada, *J. Biol. Chem.* **258,** 3332 (1983).

TABLE II
INHIBITION OF SYPHILIS SPIROCHETE CYTOADHERENCE[a] BY Fn CELL-BINDING DOMAIN SYNTHETIC PEPTIDES[b]

Expt.	Treatment reagent	μg/ml	Radiolabel recovered[c] HT1080	HEp-2
1	DMEM	—	24,718 ± 2,116 (100)	26,455 ± 3,218 (100)
	GRGDSPC	50	13,077 ± 1,919 (53)	13,761 ± 2,119 (52)
	GRADSPC	50	22,114 ± 1,602 (90)	24,884 ± 2,273 (94)
	GKGDSPC	50	23,325 ± 1,881 (94)	27,043 ± 2,881 (102)
2	DMEM	—	20,565 ± 861 (100)	19,561 ± 580 (100)
	GRGDSPC	25	14,017 ± 1,050 (68)	12,271 ± 261 (63)
	GRGDSPC	50	8,019 ± 164 (39)	8,265 ± 111 (42)
	GRGDSPC	250	7,749 ± 128 (38)	7,712 ± 138 (39)
	GRGDSPC	500	7,318 ± 96 (36)	7,392 ± 165 (38)
	GRGDSPC	750	7,386 ± 186 (36)	7,315 ± 161 (37)

[a] Cytoadherence assays were performed as previously described.[3] Radiolabeled treponemes (7×10^7 cells/reaction volume) were incubated with the indicated synthetic peptide or medium for 30 min at 34° before addition to cultured cell monolayers.

[b] Reproduced from Thomas *et al.*,[33] by copyright permission of the Rockefeller University Press.

[c] Each value represents the mean cpm ± SD of three separate determinations. Numbers in parentheses give data as percentages of control.

gelatin binding domain resulted from thermolysin digestion of Fn.[31,32] The use of these domains in treponemal binding studies has been detailed.[21]

Freshly harvested organisms recognized and bound to the cell binding domain of Fn, results that were further reinforced by the efficient inhibition of attachment to Fn-coated coverslips by specific mAbs. This stage in the studies of Fn targeting was concluded by competition experiments with heptapeptides, reagents that were becoming available following the discovery of the RGD sequence involved in the receptor-mediated binding of Fn by mammalian cells.[26] Only the GRGDSPC peptide, but not three other peptides altered within the RGD sequence, gave concentration-dependent inhibition of cell binding domain acquisition by purified spirochetes. Even more exciting was the quantitative demonstration of a concentration-dependent inhibition of host cytoadherence by *T. pallidum*. Table II shows the inhibitory effect on cell attachment of pretreatment of ^{35}S-labeled organisms with the RGD heptapeptide. Only GRGDSPC, but not the

[31] B. A. Bernard, S. K. Akiyama, S. A. Newton, K. M. Yamada, and K. Olden, *J. Biol. Chem.* **259,** 9899 (1984).

[32] K. Sekiguchi and S. Hakomori, *J. Biol. Chem.* **258,** 3967 (1983).

control peptides, diminished parasitism of two epithelial cell types that have been used by investigators in treponemal binding studies.[33]

This example illustrated the extent of cross-fertilization among scientific disciplines. The cell biology and role of Fn in cell growth and differentiation, the biochemistry of the structure–function properties of Fn regions and defined peptides, the technology for generating mAbs to the various Fn domains, and the *in vitro* manipulation of the syphilis spirochete all came together simultaneously.

Identification of the Treponemal Fn-Binding (Adhesin) Proteins

Ligand Assay

HEp-2 epithelial cells, used as the *in vitro* cell culture model system to study the host cytoadherence property of *T. pallidum*, are resuspended in PBS and washed well before addition of formaldehyde. This is done using 3×10^6 cells/ml placed in a 25-ml siliconized glass flask to which is added 1% (final concentration) formaldehyde. Fixation occurs at 22° for 1 hr, followed by extensive washing in PBS and resuspension in a 150 m*M* NaCl–5 m*M* ethylenediaminetetraacetic acid (EDTA)–50 m*M* Tris (NET) buffer containing phenylmethylsulfonyl fluoride (PMSF), 0.1% SDS, and 1% Triton X-100 as detailed elsewhere.[16] These conditions are optimal for showing the cellular integrity of the HEp-2 cells for the duration of the experiment.

To these fixed host cells is added a detergent extract of ^{35}S-labeled *T. pallidum* organisms. These bacteria are solubilized by taking a suspension containing 5×10^8 treponemes that is already at room temperature (RT) and adding 100 μl of 1% SDS in NET buffer. After 15 min at RT, ovalbumin (10 mg/ml in NET buffer) is added (to bind excess SDS) followed by addition of 100 μl of 10% Triton X-100. This mixture is heated to 37° for 15 min and insoluble material removed by centrifugation. The supernatant is then diluted to a 1-ml final volume, and one-half of this extract is used for incubating with 3×10^5 formaldehyde-fixed HEp-2 cells under conditions described in the first adaptation of this procedure.[16] After incubation at 34° for 1 hr and mixing at 15-min intervals, HEp-2 cells are extensively washed four times in NET–0.05% Triton X-100. The addition of Triton X-100 facilitates the removal of nonspecifically bound treponemal extract components from the fixed cell surfaces. Finally, 1% SDS is added and the pelleted cells are resuspended and stirred vigorously for removal of the avidly bound, radiolabeled treponemal proteins. The supernatant

[33] D. D. Thomas, J. B. Baseman, and J. F. Alderete, *J. Exp. Med.* **162**, 1715 (1985).

is electrophoresed and specific *T. pallidum* proteins are visualized by fluorography.

The employment of this procedure for identifying possible epithelial cell-binding proteins of the syphilis spirochete preceded the studies involving host protein acquisition as described earlier. This ligand assay identified three proteins as the putative adhesins. Two concerns were paramount during this assay. First, the fixation of host cells might destroy the surface-exposed binding sites (receptors) and thereby not allow for accommodation of the bacterial counterparts, the adhesin proteins, if indeed they existed. Second and equally important, the solubilization of the parasite, especially using harsh conditions, might destroy the receptor recognition function of the adhesins.

In the event that this ligand assay might have followed the demonstration of Fn as the receptor, then experiments with anti-Fn antibodies would likely have been performed to show immunoreactivity and, therefore, epitope integrity of Fn on the fixed host cells. In addition, inhibition experiments with anti-Fn antibody labeling of the fixed host cells would have been performed to show possibly diminution of adhesin protein recognition and binding.

Fibronectin-Affinity Purification of Treponemal Proteins

Knowledge of Fn as the likely receptor mediating host parasitism by the live organisms and of putative adhesins paved the way for examining the interaction of treponemal proteins by Fn-affinity chromatography.[4]

About 2×10^{10} ^{35}S-labeled spirochetes, washed well to remove contaminating host material from the bacterial surface, are suspended in 1% Zwittergent 3-12 (Calbiochem-Behring Corp., La Jolla, CA) detergent.[4] The labeled organisms are gently homogenized, and insoluble material is removed by centrifugation at 100,000 *g* for 30 min. The soluble treponemal extract is then diluted further with PBS until the concentration of Zwittergent 3-12 is 0.05%, at which time the extract is passed over a 2 × 10-cm Fn-affinity Sepharose column at the rate of 1 ml/cm^2/hr. Extensive washing of the column is carried out sequentially with PBS, 2 *M* potassium bromide, and 10% SDS. After the initial washing with PBS, the other treatments of the column did not release bound treponemal proteins, illustrating the difficulty in recovering Fn-associated molecules from an affinity column. This material was finally displaced by boiling the beads for 3 min in electrophoresis dissolving buffer. The radioactively labeled proteins released from the column were analyzed and fluorograms revealed three bands, which corresponded to proteins absent or diminished from the total proteins of extract passed over the column.[4] Furthermore, the three

eluted proteins had electrophoretic mobilities and characteristics similar to those detected years earlier by the ligand assay.[16]

Discussion on the Usefulness of the Strategies to Yield Other Important Information and to Work on Other Microbial Pathogens

Nutrient Acquisition by Syphilis Spirochetes and Other Microorganisms

The host protein binding strategy contributed tremendously to knowledge about other aspects of the biology of the host–parasite interrelationship of the syphilis spirochete. The acquisition of host iron-binding proteins (lactoferrin and transferrin)[33] and of lipoproteins[34] showed the nutritional dependency of this organism on its host. This information is important as it may help clarify previously unknown issues regarding survival of the organisms within various host sites and tissues, as well as aid in the delineation of metabolic deficiencies that make the organisms dependent on the host for survival. This information may also explain the inability to cultivate the organism *in vitro*, an experimental problem that must be overcome.

Because these experiments demonstrated the value of the information gained from specific host protein acquisition studies, an attempt was made to examine the usefulness of the strategy on a sexually transmitted *Trichomonas vaginalis* parasite.[20] In the case of this protozoan, some of the avidly associated serum proteins included fibronectin, lipoproteins, iron-binding proteins, α_2-macroglobulin, α_1-antitrypsin, and immunoglobulins. The consequences of binding these and possibly other host proteins onto the parasite surface are numerous and are being dissected. For example, these organisms do not have the biosynthetic capacity for lipid or sterol biosynthesis, so early on the receptor-mediated binding of apoprotein CIII was found to be a mechanism by which the trichomonads obtained lipids, fatty acids, and cholesterol from binding to lipoproteins containing apoprotein CIII.[35] *T. vaginalis* has a very high iron requirement for optimal metabolism and energy generation, and it is now appreciated that a repertoire of parasite receptors for iron-binding and iron-containing proteins of the host satisfy the parasite's demands for iron.[35–37] The trichomonads have also been shown to produce up to 25 distinct cysteine proteinases.[38]

[34] J. F. Alderete and J. B. Baseman, *Genitourin. Med.* **65,** 177 (1989).
[35] K. M. Peterson and J. F. Alderete, *J. Exp. Med.* **160,** 1261 (1984).
[36] K. M. Peterson and J. F. Alderete, *J. Exp. Med.* **160,** 398 (1984).
[37] M. W. Lehker, T. H. Chang, D. C. Dailey, and J. F. Alderete, *J. Exp. Med.* **171,** 2165 (1990).
[38] K. A. Neale and J. F. Alderete, *Infect. Immun.* **58,** 157 (1990).

so that the coating of the surface with host-derived proteinase inhibitors (α_2-macroglobulin and α_1-antitrypsin) may confer resistance against the parasite's own degradative enzymes. In fact, the inhibitory capacity of α_1-antitrypsin, while on the surface of the parasite, was demonstrated.[39]

Ligand Assay for Detection of Microbial Adhesins

The ligand assay, although seemingly straightforward, can be used on other microbial models, but only after empirically testing and optimizing conditions. Problems, as mentioned below and in the next section, can preclude obtaining information about adhesin candidates.

Incubation of glutaraldehyde-fixed HeLa or vaginal epithelial cells with a deoxycholate extract of radiolabeled *T. vaginalis* has shown the existence of four distinct adhesin proteins.[40,41] It is noteworthy that Zwittergent 3-12, used for making treponemal extracts and also used for RIPA of *T. vaginalis*,[42] failed to solubilize the epithelial cell-binding proteins of this protozoan. This selective solubilization highlights the need to test adequately multiple detergent systems for optimal results from the ligand assay. That these proteins are adhesins has been proven through fulfillment of criteria summarized by Beachey.[43]

Problems Demonstrating Extracellular Matrix Proteins as Host Receptors and Existence of Adhesins by the Ligand Assay

Although the host protein binding strategy and the ligand assay proved worthwhile for the examples involving *T. pallidum* and the protozoan *T. vaginalis*, this last section highlights a few examples of problems that might be encountered.

One of the constant hazards in experiments is the *in vitro* cultivation of microbial pathogens. The growth medium likely will not be reflective of the microenvironments of the host during infection. It is reasonable to presume that these environments result in adaptive responses of the invading organisms,[44] which allow for expression of certain virulence factors, possibly adhesins. A recent report showed that laminin and collagen were

[39] K. M. Peterson and J. F. Alderete, *Infect. Immun.* **40,** 640 (1983).
[40] J. F. Alderete and G. E. Garza, *Infect. Immun.* **56,** 28 (1988).
[41] R. Arroyo, J. Engbring, and J. F. Alderete, *Mol. Microbiol.* **6,** 853 (1992).
[42] J. F. Alderete, *Infect. Immun.* **39,** 1041 (1983).
[43] E. H. Beachey, "Molecular Mechanisms of Microbial Adhesion," p. 1. Springer-Verlag, New York, 1989.
[44] C. A. Lee and S. Falkow, *Proc. Natl. Acad. Sci. U.S.A.* **87,** 4304 (1990).

recognized by *Streptococcus gordonii*.[45] This work was significant in that it demonstrated that induction of the laminin-binding protein of *S. gordonii* occurred under positive control. The upregulation of laminin-binding proteins required exposure of the bacteria to laminin.

Equally noteworthy was that the levels of cytoadherence and amounts of adhesins of *T. vaginalis* were regulated by the concentration of iron in the growth medium.[46] Thus, batch cultures of organisms may not allow for expression of ECM-binding receptors, and in fact, it was exceedingly difficult to detect the trichomonad adhesins by the ligand assay in laboratory isolates grown *in vitro* for extended periods, conditions that favor loss of expression of the adhesins. Thus, recent work indicating the ECM proteins may represent host cell receptors which bind the *T. vaginalis* adhesins requires a priori knowledge of the environmental cues which favor expression of cytoadherence of ECM protein targeting. In both cases, the lack of basic knowledge regarding expression of the adhesins, which bind ECM proteins, would preclude the ability to identify them by the ligand assay.

Last, detergent extracts must include inhibitors of proteinases, as pathogenic human trichomonads and undoubtedly numerous other pathogens possess proteinases which may degrade ECM proteins[47] or, alternatively, within minutes after solubilization, degrade adhesins detectable by the ligand assay.[41] This means that basic research into the existence and type of proteinases produced by microbial pathogens is necessary for the analyses presented here. In both scenarios the presence of active microbial proteinases during the assays may result in inaccurate observations or results.

[45] P. Sommer, C. Gleyzal, S. Guerret, J. Etienne, and J. Grimaud, *Infect. Immun.* **60,** 360 (1992).
[46] M. W. Lehker, R. Arroyo, and J. F. Alderete, *J. Exp. Med.* **174,** 311 (1991).
[47] M. Wikström and A. Linde, *Infect. Immun.* **51,** 707 (1986).

[23] Interactions of Bacteria with Leukocyte Integrins

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Introduction

Integrins are a family of cell surface glycoproteins which are known to mediate cell–cell and cell–extracellular matrix adhesion in eukaryotic systems. They consist of an α chain and a β chain which both take part