INFECTION AND IMMUNITY, Oct. 1994, p. 4622–4625
0019-9567/94/$04.00+0
Copyright © 1994, American Society for Microbiology
Vol. 62, No. 10

Virulent *Treponema pallidum* Promotes Adhesion of Leukocytes to Human Vascular Endothelial Cells

BRYAN S. RILEY,[1] NANCY OPPENHEIMER-MARKS,[2] JUSTIN D. RADOLF,[1,2] AND MICHAEL V. NORGARD[1]*

Departments of Microbiology[1] and Internal Medicine,[2] The University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235

Received 18 May 1994/Returned for modification 20 June 1994/Accepted 11 July 1994

Perivasculitis and endothelial cell abnormalities are characteristic histopathologic features of syphilis, a sexually transmitted disease caused by *Treponema pallidum*. To extend earlier studies demonstrating that *T. pallidum* activates endothelial cells, we now show that virulent *T. pallidum*, but not heat-killed *T. pallidum* or nonpathogenic *Treponema phagedenis*, promotes increased adherence of lymphocytes and monocytes to human umbilical vein endothelial cells. Lymphocytes and monocytes are the two cell types prominent in the histopathology of syphilis. Recognition that *T. pallidum* can stimulate endothelial cells to bind leukocytes provides important insights into the early mechanisms of syphilis immunopathogenesis.

Syphilis is a chronic, systemic sexually transmitted disease caused by the spirochetal bacterium *Treponema pallidum* subsp. *pallidum* (*T. pallidum*) (23). For *T. pallidum* to establish systemic infection, it must migrate from the dermis, disseminate hematogenously, and invade vascular beds in target organs. It is plausible that endothelial cells are critically involved during these processes because of the need for *T. pallidum* to traverse endothelial cell barriers and the propensity for *T. pallidum* in vitro to adhere to endothelial cells and invade monolayers via intercellular junctions (6, 7, 19, 25, 26). Endothelial cell abnormalities, perivasculitis, and thrombosis, which characterize the histopathology of syphilis (11), are consistent with the hypothesis that endothelial cell activation results from the close association of *T. pallidum* with vascular endothelium.

When activated, vascular endothelial cells express a broad repertoire of molecules relevant to inflammation, including cytokines, coagulation factors, and leukocyte adhesion molecules (29). Leukocyte adhesion molecules play pivotal roles in the initiation of immune processes by recruiting leukocytes to sites of inflammation (13, 17). In a previous study by Riley et al. (21), expression of intercellular adhesion molecule 1 (ICAM-1), a prominent leukocyte adhesion molecule (12), was used as a marker for demonstrating that human umbilical vein endothelial cells (HUVEC) become activated upon interaction with virulent *T. pallidum*. However, a functional assay for the correlation of *T. pallidum*-mediated endothelial cell activation (i.e., ICAM-1 expression) with leukocyte adhesion thus far has been lacking. Prior to extensive studies examining the role(s) of the numerous leukocyte adhesion molecules expressed by *T. pallidum*-activated endothelial cells (e.g., ICAM-1, vascular cell adhesion molecule 1 [VCAM-1], P-selectin, and E-selectin) (29, 31), it was logical first to confirm physiologically that *T. pallidum* promoted the adhesion of leukocytes to endothelial cells. A second objective of this study was to compare leukocyte populations bound by *T. pallidum*-activated endothelial cells in vitro with the cell types which characterize the histopathology of the disease as a means of establishing the relevance of the in vitro model.

In these studies, *Treponema phagedenis* biotype Reiter (a nonpathogenic commensal treponeme) was maintained and passaged at 34°C in thioglycolate medium (Brewer modified) with 10% normal rabbit serum. Virulent *T. pallidum* (Nichols strain) was maintained by intratesticular inoculation of New Zealand White rabbits (22). Spirochetes were extracted from tissue into RPMI 1640–15% fetal calf serum (FCS) and were isolated by differential centrifugation (22); the supernatant was carefully aspirated to avoid disruption of the bacterial pellet, and spirochetes were resuspended in RPMI 1640–15% FCS. To determine the amount of testis extract (TEx) remaining with the treponemal pellet, ^{125}I-labeled human transferrin (2×10^6 acid-precipitable cpm) was added as a tracer to the treponemal suspension before centrifugation (21); transferrin is a host protein which does not bind to *T. pallidum* (2, 4, 24). The treponemal pellet was solubilized, and the amount of radioactivity remaining with the bacteria was used to calculate the amount of TEx contamination in the spirochetal preparations (21). TEx, used separately in some experiments, was filtered through a 0.2-μm-pore-size membrane filter to remove residual spirochetes (21).

HUVEC were isolated from human umbilical cords and cultured as previously described (21). To establish monolayers, 10^5 HUVEC were seeded onto eight-well plastic slides (coated with bovine fibronectin) in 0.4 ml of RPMI 1640–15% FCS; cultures were incubated at 37°C in an atmosphere of 5% CO_2 until confluent (ca. 16 h) (21). Peripheral blood mononuclear cells (PBMCs) (comprising approximately 85% lymphocytes and 15% monocytes) were isolated from heparinized human whole blood by Ficoll-Hypaque gradient centrifugation (10). Lymphocytes were purified by separation from monocytes by discontinuous Percoll density gradient centrifugation (28). PBMCs, lymphocytes, and monocytes were suspended in RPMI 1640–10% FCS and stored at 37°C until use. The purities of lymphocyte and monocyte populations were assessed by forward light scatter flow cytometric analysis and microscopic examination of hematoxylin-stained cells; purities of lymphocyte and monocyte preparations were greater than 95 and 80%, respectively. Polymorphonuclear leukocytes (PMNs) were isolated from the pellets of Ficoll-Hypaque gradients of whole blood by dextran sulfate sedimentation and

* Corresponding author. Mailing address: Department of Microbiology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-9048. Phone: (214) 648-2160. Fax: (214) 648-7955.

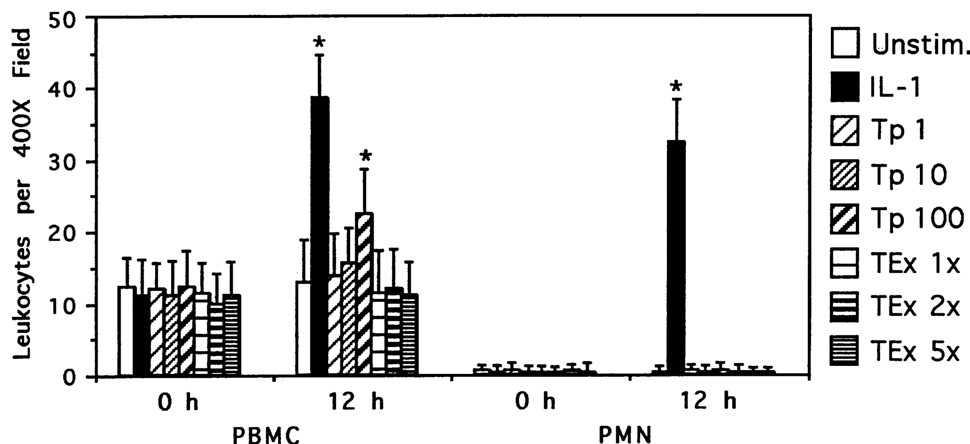

FIG. 1. Adherence of leukocytes (PBMCs or PMNs) to *T. pallidum*-stimulated HUVEC monolayers. HUVEC were either unstimulated (Unstim.), treated with IL-1β (IL-1), or cocultured with 1, 10, or 100 *T. pallidum* organisms per HUVEC (Tp 1, Tp 10, and Tp 100, respectively) for 0 or 12 h. HUVEC also were treated with one-, two-, or fivefold the amount of TEx calculated by radioactive tracer to remain with the sample containing 100 *T. pallidum* organisms per HUVEC (TEx 1×, 2×, and 5×, respectively). Values are expressed as means ± standard deviations. *, significantly greater than the control (Fisher's least significant difference, $n = 30$, $P \leq 0.05$).

lysis of erythrocytes (8). PMNs in physiological saline were stored on ice until just prior to use; they were then pelleted and suspended in RPMI 1640–10% FCS.

Conditions for activation of HUVEC by *T. pallidum* were as previously described (21). Experiments to assess adherence of leukocytes following incubation of HUVEC with *T. pallidum* were modeled after those of Oppenheimer-Marks et al. (16), Picker et al. (18), and Wegner et al. (30). Monolayers were incubated with various quantities of motile *T. pallidum* at 34°C in 4.5% O_2–5% CO_2 for either 0 h or 12 h (21). The wells were then rinsed twice with RPMI 1640–10% FCS, leukocytes (10^6 in 300 μl of RPMI 1640–10% FCS) were added, and the slides were incubated at 37°C for 30 min under 5% CO_2. The slides were then rinsed three times with RPMI 1640–10% FCS and fixed in 10% neutral-buffered formalin for 30 min at room temperature. The cells were permeabilized with 80% methanol and stained with hematoxylin. All experiments were performed in triplicate; leukocytes attached to HUVEC were enumerated by light microscopy (40× objective). Ten microscope fields were counted for each well; counts for the three wells of each group were averaged, and data were expressed as the mean and standard deviation for each group at each time point.

Treatment of HUVEC monolayers for 12 h with interleukin-1β (IL-1β) (10 U/ml), a potent endothelial cell activator (15), markedly increased the adhesiveness of endothelial cells for PBMCs and PMNs (Fig. 1). Incubation with 100 *T. pallidum* organisms per endothelial cell resulted in significantly increased binding of PBMCs but not PMNs to HUVEC (Fig. 1). The failure of PMNs to bind to HUVEC following treponemal stimulation (Fig. 1) was interesting given that PMNs are not prevalent among the cellular infiltrates of syphilitic lesions (11). *T. pallidum* at lower inoculation ratios (i.e., 1 or 10 treponemes per HUVEC) did not promote leukocyte adhesiveness. The requirement for relatively high numbers of spirochetes to induce leukocyte adhesiveness was consistent with our earlier study of endothelial cell activation by *T. pallidum* (21) and likely reflects the well-recognized observation that only a very small proportion of treponemes in a given population cytadhere to HUVEC in vitro (6, 7, 19, 21, 25, 26). Given that *T. pallidum* was prepared from inflamed testicular tissue, it was essential to rule out the possibility that leukocyte adhesion by activated HUVEC was induced by contaminating rabbit cytokines rather than by treponemes. To examine this possibility, HUVEC were treated with fresh TEx at one-, two-, and fivefold the amount which was calculated with radioactive tracer to be present in cocultures of 100 *T. pallidum* organisms per HUVEC. Incubation of HUVEC with up to fivefold (3 μl) the amount of TEx in the coculture containing 100 *T. pallidum* organisms per HUVEC did not result in enhanced binding of leukocytes to the monolayers (Fig. 1).

Having established that PBMCs constituted the adherent leukocyte population for *T. pallidum*-activated endothelial cells, further experiments were performed using purified lymphocytes and monocytes. For these experiments, in addition to the test groups shown in Fig. 1, test samples also included the nonpathogenic *T. phagedenis* and heat-killed (56°C, 30 min) *T. pallidum*, neither of which cytadhere or activate HUVEC (6, 21). Also, to further prove that TEx was not responsible for inducing leukocyte adhesiveness in HUVEC, *T. phagedenis* and heat-killed *T. pallidum* were suspended in fresh TEx (unheated), processed in the same manner used to prepare virulent *T. pallidum*, and then added to cell monolayers (100 spirochetes per HUVEC). As shown in Fig. 2, HUVEC incubated for 12 or 24 h with either IL-1β or 100 viable *T. pallidum* organisms per endothelial cell showed increased binding of both lymphocytes and monocytes. None of the other test samples, including *T. phagedenis* and killed *T. pallidum* (both processed in fresh TEx), promoted lymphocyte or monocyte binding to HUVEC (Fig. 2). Although significant differences between the results for test groups shown in Fig. 2 were statistically determined, the light micrographs in Fig. 3 show that lymphocyte binding to HUVEC monolayers promoted by viable *T. pallidum* also was visually evident.

The combined studies demonstrate that the interaction of virulent *T. pallidum* with HUVEC promotes increased adhesiveness for leukocytes. Data from both radioactive tracer and reconstruction experiments with TEx provided compelling evidence that this phenomenon was not due to rabbit cytokines. Furthermore, inasmuch as syphilitic lesions are composed almost exclusively of macrophages, T cells, and plasma cells (11), the enhanced binding of monocytes and lymphocytes by *T. pallidum*-activated HUVEC correlated closely with those cell populations prominent in the histopathology of syphilis (11).

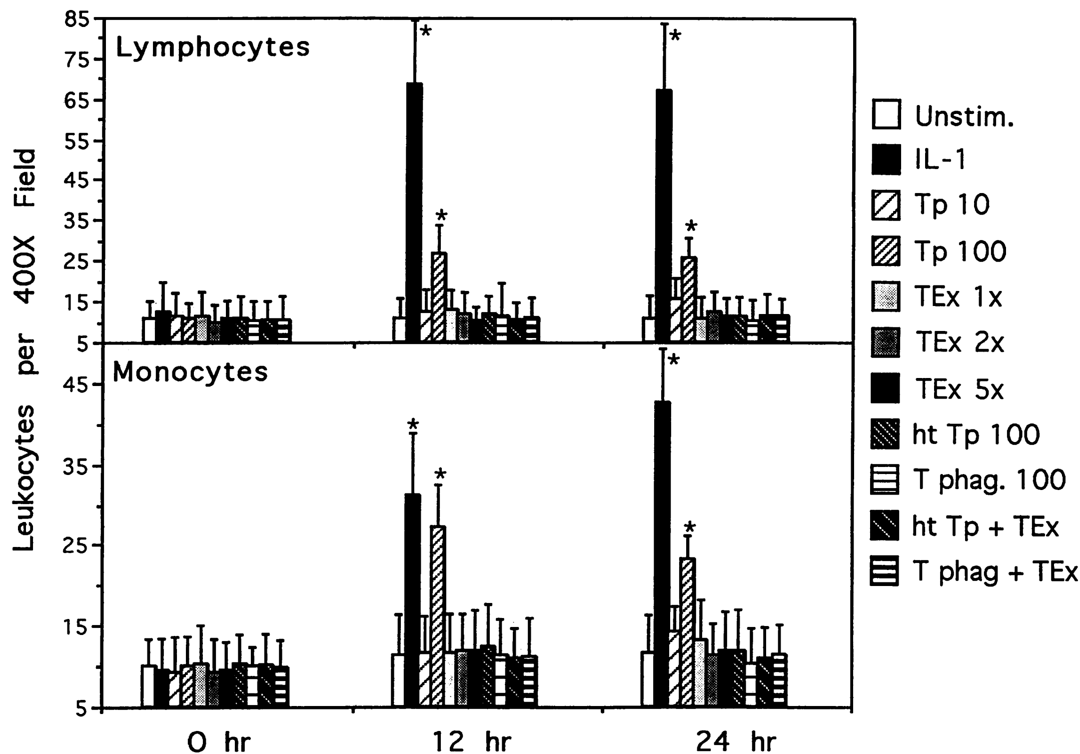

FIG. 2. Lymphocyte and monocyte adherence to *T. pallidum*-stimulated HUVEC. HUVEC were either unstimulated (Unstim.), treated with IL-1β (IL-1), or cocultured with 10 or 100 *T. pallidum* organisms per HUVEC (Tp 10 and Tp 100, respectively). HUVEC also were treated with one-, two-, or fivefold the amount of TEx calculated by radioactive tracer to remain with the sample containing 100 *T. pallidum* organisms per HUVEC (TEx 1×, 2×, and 5×, respectively). In addition, 100 heat-killed *T. pallidum* organisms per HUVEC (ht Tp 100), 100 *T. phagedenis* organisms per HUVEC (T phag. 100), and heat-killed *T. pallidum* or *T. phagedenis* (100 spirochetes per HUVEC) reconstituted with TEx (ht Tp + TEx and T phag + TEx, respectively) were used to stimulate monolayers. Values are expressed as means ± standard deviations. *, significantly greater than the control (Fisher's least significant difference, $n = 30$, $P \leq 0.05$).

Our studies raise three issues relevant to clarifying the mechanism(s) by which *T. pallidum* promotes leukocyte adherence to activated endothelial cells. First, the adhesion molecules responsible for this process are as yet undefined. On the basis of our former study (21), ICAM-1 remains a candidate effector, but VCAM-1 also may be implicated because the receptor for this molecule is expressed by both monocytes and lymphocytes (13, 31). Assays for these and other adhesion molecules, as well as blocking experiments with specific antibodies, will be necessary for more precise identification of the relevant adhesion molecule(s). Second, because *T. pallidum* lacks lipopolysacharride (9), a potent inflammatory mediator of gram-negative bacteria (14), its abundant lipoproteins (3) have been implicated as activators of immune effector cells (1, 5, 20). However, there remains a paucity of information on the ability of treponemal lipoproteins to activate endothelial cells (21). Regardless of the treponemal constituents which may be responsible for endothelial cell activation, it should be noted that killed *T. pallidum* will not cytadhere (6, 19, 21, 26), activate endothelial cells (21), or, as demonstrated in this study, promote leukocyte adhesion. These observations suggest that the treponemal constituents responsible for promoting activation of endothelial cells must be presented in the context of some unique interaction(s), as yet poorly understood, of virulent *T. pallidum* with target endothelial cells. Finally, although *T. pallidum*-activated HUVEC bound significantly increased numbers of leukocytes, the overall effect was not as striking as that induced by the potent inflammatory

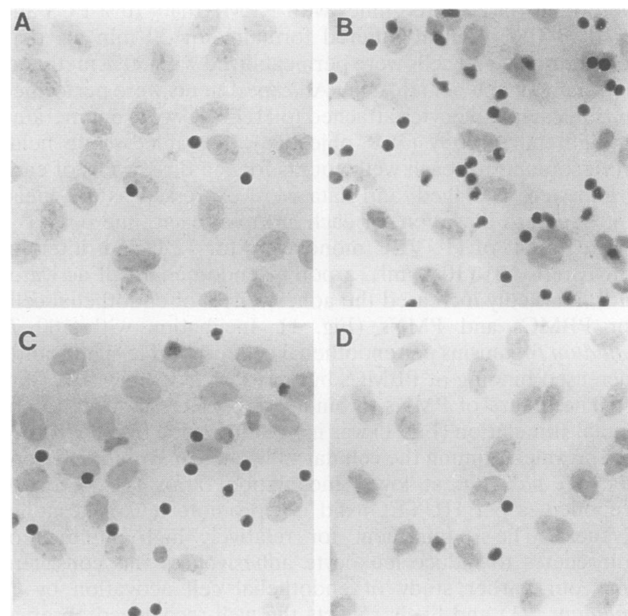

FIG. 3. Hematoxylin-stained lymphocytes attached to HUVEC. Stimulation of monolayers was for 12 h. Only a portion of each 40× microscopic field is shown in each panel. (A) Unstimulated (medium) control; (B) IL-1β; (C) 100 *T. pallidum* organisms per HUVEC; (D) 100 heat-killed *T. pallidum* organisms reconstituted with fresh TEx.

cytokine IL-1β. Modest induction by *T. pallidum* of early inflammatory processes is consistent with the well-recognized fact that inflammation develops slowly at local sites of infection (11). On the other hand, inasmuch as endothelial cell activation correlates with increased vascular leakiness (27), reduction in the integrity of endothelial cell tight junctions resulting from this response may enhance transendothelial passage by *T. pallidum*, thereby facilitating tissue invasion. Eventually, cytokine cascades will intensify the response, culminating in clinical disease. Additional work will be necessary to elucidate more completely the molecular mechanisms by which pathogenic treponemes influence these pivotal inflammatory processes.

We thank George Wendel for umbilical cord material, Louis Picker and Julia Bass for flow cytometry, and Yuenan Shen and Martin Goldberg for excellent technical assistance.

This work was partially supported by Public Health Service grant AI-16692 from the National Institute of Allergy and Infectious Diseases to M.V.N. and by grant-in-aid 91015470 from the American Heart Association to J.D.R. J.D.R. is the recipient of an Established Investigatorship Award from the American Heart Association.

REFERENCES

1. **Akins, D. R., B. K. Purcell, M. Mitra, M. V. Norgard, and J. D. Radolf.** 1993. Lipid modification of the 17-kilodalton membrane immunogen of *Treponema pallidum* determines macrophage activation as well as amphiphilicity. Infect. Immun. **61:**1202–1210.
2. **Alderete, J. F., K. M. Peterson, and J. B. Baseman.** 1988. Affinities of *Treponema pallidum* for human lactoferrin and transferrin. Genitourin. Med. **64:**359–363.
3. **Chamberlain, N. R., M. E. Brandt, A. L. Erwin, J. D. Radolf, and M. V. Norgard.** 1989. Major integral membrane protein immunogens of *Treponema pallidum* are proteolipids. Infect. Immun. **57:**2872–2877.
4. **Cox, D. L., P. Chang, A. McDowall, and J. D. Radolf.** 1992. The outer membrane, not a coat of host proteins, limits the antigenicity of virulent *Treponema pallidum*. Infect. Immun. **60:**1076–1083.
5. **DeOgny, L., B. C. Pramanik, L. L. Arndt, J. D. Jones, J. Rush, C. A. Slaughter, J. D. Radolf, and M. V. Norgard.** 1994. Solid-phase synthesis of biologically active lipopeptides as analogs for spirochetal lipoproteins. Peptide Res. **7:**91–97.
6. **Fitzgerald, T. J.** 1983. Attachment of treponemes to cell surfaces, p. 195–228. *In* R. F. Schell and D. M. Musher (ed.), Pathogenesis and immunology of treponemal infections. Marcel Dekker, Inc., New York.
7. **Fitzgerald, T. J., J. N. Miller, and J. A. Sykes.** 1975. *Treponema pallidum* (Nichols strain) in tissue cultures: cellular attachment, entry, and survival. Infect. Immun. **11:**1133–1140.
8. **Gorman, R. R., and A. H. Lin.** 1987. Assay for the leukotriene B_4 receptor. Methods Enzymol. **141:**373–374.
9. **Hardy, P. H., Jr., and J. Levin.** 1983. Lack of endotoxin in *Borrelia hispanica* and *Treponema pallidum*. Proc. Soc. Exp. Biol. Med. **174:**47–52.
10. **Kanof, M. E., and P. D. Smith.** 1991. Isolation of whole mononuclear cells from peripheral blood, p. 7.1. *In* J. E. Colgan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober (ed.), Current protocols in immunology. John Wiley and Sons, New York.
11. **Lukehart, S. A., and K. K. Holmes.** 1991. Syphilis, p. 651–661. *In* E. Braunwald, K. J. Isselbacher, R. G. Petersdorf, J. D. Wilson, J. B. Martin, and A. S. Fauci (ed.), Harrison's principles of internal medicine. McGraw Hill Book Company, New York.
12. **Marlin, S. D., and T. A. Springer.** 1987. Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). Cell **51:**813–819.
13. **McEver, R. P.** 1991. Selectins: novel receptors that mediate leukocyte adhesion during inflammation. Thromb. Haemostasis **65:**223–228.
14. **Morrison, D. C., and R. J. Ulevitch.** 1978. The effects of bacterial endotoxins on host mediation systems. Am. J. Pathol. **93:**527–617.
15. **Moser, R., B. Schleiffenbaum, P. Groscurth, and J. Fehr.** 1989. Interleukin 1 and tumor necrosis factor stimulate human vascular endothelial cells to promote transendothelial neutrophil passage. J. Clin. Invest. **83:**444–455.
16. **Oppenheimer-Marks, N., L. S. Davis, and P. E. Lipsky.** 1990. Human T lymphocyte adhesion to endothelial cells and transendothelial migration. Alteration of receptor use relates to the activation status of both the T cell and the endothelial cell. J. Immunol. **145:**140–148.
17. **Osborn, L.** 1990. Leukocyte adhesion to endothelium in inflammation. Cell **62:**3–6.
18. **Picker, L. J., T. K. Kishimoto, C. W. Smith, R. A. Warnock, and E. C. Butcher.** 1991. ELAM-1 is an adhesion molecule for skin-homing T cells. Nature (London) **349:**796–799.
19. **Quist, E. E., L. A. Repesh, R. Zeleznikar, and T. J. Fitzgerald.** 1983. Interaction of *Treponema pallidum* with isolated rabbit capillary tissues. Br. J. Vener. Dis. **59:**11–20.
20. **Radolf, J. D., M. V. Norgard, M. E. Brandt, R. D. Isaacs, P. A. Thompson, and B. Beutler.** 1991. Lipoproteins of *Borrelia burgdorferi* and *Treponema pallidum* activate cachectin/tumor necrosis factor synthesis: analysis using a CAT reporter construct. J. Immunol. **147:**1968–1974.
21. **Riley, B. S., N. Oppenheimer-Marks, E. J. Hansen, J. D. Radolf, and M. V. Norgard.** 1992. Virulent *Treponema pallidum* activates human vascular endothelial cells. J. Infect. Dis. **165:**484–493.
22. **Robertson, S. M., J. R. Kettman, J. N. Miller, and M. V. Norgard.** 1982. Murine monoclonal antibodies specific for virulent *Treponema pallidum* (Nichols). Infect. Immun. **36:**1076–1085.
23. **Sell, S., and S. J. Norris.** 1983. The biology, pathology, and immunology of syphilis. Int. Rev. Exp. Pathol. **24:**203–276.
24. **Staggs, T. M., M. K. Greer, J. B. Baseman, S. C. Holt, and V. V. Tryon.** 1994. Identification of lactoferrin-binding proteins from *Treponema pallidum* subspecies *pallidum* and Treponema denticola. Mol. Microbiol. **12:**613–619.
25. **Thomas, D. D., A. M. Fogelman, J. N. Miller, and M. A. Lovett.** 1989. Interactions of *Treponema pallidum* with endothelial cell monolayers. Eur. J. Epidemiol. **5:**15–21.
26. **Thomas, D. D., M. Navab, D. A. Haake, A. M. Fogelman, J. N. Miller, and M. A. Lovett.** 1988. *Treponema pallidum* invades intracellular junctions of endothelial cell monolayers. Proc. Natl. Acad. Sci. USA **85:**3608–3612.
27. **Tracey, K. J., B. Beutler, S. F. Lowry, J. Merryweather, S. Wolpe, I. W. Milsark, R. J. Hariri, T. J. Fahey III, A. Zentella, and J. D. Albert.** 1986. Shock and tissue injury induced by recombinant human cachectin. Science **234:**470–474.
28. **Wahl, L. M., and P. D. Smith.** 1991. Isolation of monocyte/macrophage populations, p. 7.6. *In* J. E. Colgan, A. M. Kruisbeek, D. H. Margulies, and W. Strober (ed.), Current protocols in immunology. John Wiley and Sons, New York.
29. **Wallis, W. J., and J. M. Harlan.** 1986. Effector functions of endothelium in inflammatory and immunologic reactions. Pathol. Immunopathol. Res. **5:**73–103.
30. **Wegner, C. D., R. H. Gundel, P. Reilly, N. Haynes, L. G. Letts, and R. Rothlein.** 1990. Intercellular adhesion molecule-1 (ICAM-1) in the pathogenesis of asthma. Science **247:**456–459.
31. **Williams, T. J., and P. G. Hellewell.** 1992. Endothelial cell biology. Adhesion molecules involved in the microvascular inflammatory response. Am. Rev. Respir. Dis. **146:**S45–S50.