Entry of *Borrelia burgdorferi* into Macrophages Is End-On and Leads to Degradation in Lysosomes

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The Lyme disease spirochete, *Borrelia burgdorferi*, is ingested rapidly by mouse macrophages in vitro. Spirochetes attach by their ends and become progressively coiled as they move deeper into cells. From the earliest measurements, spirochetes colocalize with a marker of endosomes and lysosomes, and degradation of spirochetes occurs within lysosomes.

Lyme disease, caused by the spirochete *Borrelia burgdorferi*, is characterized by a transient rash, erythema chronicum migrans, and subsequent inflammatory processes in the joints, nervous system, and heart (3). While spirochetes become more difficult to find as the disease progresses, rare ones may be sighted in affected organs (3). Many spirochetes are removed by the host's phagocytic cells (3, 17), but clearance is incomplete; the mechanisms of spirochetal persistence are the subject of vigorous investigation.

A common mechanism of pathogenesis used by parasites is persistence in immunoprivileged locations, such as intracellular sites. Although this has been observed for *B. burgdorferi* in vitro in experiments using large numbers of infecting organisms (21, 27), the clinical relevance of these observations re-

mains unknown. For *Toxoplasma gondii*, the outcome—whether it will persist and establish an intracellular phase or be eliminated by the phagocytic cell (19)—is determined on entry into the host cell (18). Thus, the pathway of entry may enhance the persistence of a given microorganism or lead to its elimination. Here we examine the general mechanism by which spirochetes enter the cell and are processed.

The usual interaction of the Lyme spirochete with phagocytes in vitro follows a well-recognized pattern of attachment, stimulation of release of inflammatory mediators, ingestion, and killing (25). We have recently shown the rapid ingestion of large numbers of spirochetes, with or without opsonization and participation of Fc receptors (28); ingested spirochetes arrive in lysosomes with a half-life of about 20 min (27). Some ultra-

FIG. 1. Attachment of spirochetes to the macrophage cell surface. Adherent macrophages were visualized at room temperature with Nomarski optics, and video images were recorded at ×63 with an optical disc recorder. (A) Macrophage before spirochete attachment. (B through D) Spirochetal attachment to macrophages occurs via the ends. While the spirochete is joined to the macrophage at one end, its other end moves freely. Time elapsed is 20 min.

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FIG. 2. Entry of *B. burgdorferi* into macrophages. Macrophages bound spirochetes on ice and ingested them for 10 min at 37°C; uningested microorganisms were then removed. Samples were fixed at intervals and labeled with *B. burgdorferi*-specific antisera. The process is shown at 0 min, 20 min, and 180 min. Note that elongated spirochetes become progressively bundled in the perinuclear region of the engulfing macrophage.

structural studies have indicated that *B. burgdorferi* enters phagocytes preferentially by "coiling phagocytosis" (32, 35), in which the organism is wrapped in a pseudopod coil of cell membrane (15). The significance of this unusual method of uptake has not been determined for borrelias.

While the particular pathway of entry into eukaryotic cells is not yet known for unopsonized *B. burgdorferi*, entry may occur via specific receptor-mediated uptake: *B. burgdorferi* binds to integrins (7, 22), which have been shown to mediate cellular adherence of another spirochete, *Treponema pallidum* (36). In the present study, we have used video, immunofluorescent, and immunoelectron microscopy to analyze in detail the uptake of

B. burgdorferi into macrophages. We have demonstrated that spirochetes enter macrophages end-on, are quickly localized in endosomes and lysosomes, and are degraded in the lysosomes. This clarification of initial events in *B. burgdorferi* ingestion by macrophages extends our previous work to the ultrastructural level (27) but does not yet explain the occasionally observed cell-associated persistence.

Cell culture and antibodies. The J774 mouse macrophage cell line was cultured in suspension as described previously (27) and plated overnight before experiments in modified essential medium (MEM)–10% fetal bovine serum on glass coverslips or in 35-mm tissue culture dishes. Low-passage-number, virulent *B. burgdorferi* (strain N40) was maintained in Barbour-Stoenner-Kelley liquid medium at 33°C. Spirochetes were washed by pelleting in an Eppendorf microcentrifuge for 2 min at room temperature and resuspended in MEM without antibiotics, with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.2) (27). Rabbit polyclonal anti-*B. burgdorferi* (12) was kindly supplied by Fred S. Kantor, Department of Internal Medicine, and monoclonal antibodies directed against mouse lysosomal glycoprotein 120 (Igp120) were supplied by Ira Mellman (16), Department of Cell Biology, both of Yale University School of Medicine, New Haven, Conn.

Microscopy. For video microscopy, macrophages were plated onto fragments (3 by 3 mm) of glass coverslips overnight and were examined at room temperature by Nomarski optics with a Zeiss IM35 microscope. *B. burgdorferi* was added at 10^7 /ml of MEM–10 mM HEPES (pH 7.2), and cultures were recorded live every 3 s with a Panasonic TQ-2026F optical disc recorder. For fluorescent microscopy, as described previously, macrophages in MEM–10% fetal bovine serum and adherent to 12-mm glass coverslips were allowed to bind spirochetes (10^7 /ml) on ice, warmed to 37°C for 10 min to allow ingestion, rinsed in water to remove uningested microorganisms, and recultured in fresh medium until fixation at chase times of 0, 20, 40, and 60 min and 3 h (27). Labeled samples were examined with a Bio-Rad MRC 600 Confocal Imaging System mounted on a Zeiss Axiovert 10 microscope (27). For electron microscopy, cells were fixed in 3% paraformaldehyde–0.1% glutaraldehyde at room temperature before scraping and pelleting. Cell pellets were cryoprotected with 2.3 M sucrose for 15 min, frozen in liquid N_2 , freeze-substituted with methanol–1% OsO_4 , embedded in Lowicryl, and polymerized under UV light, all at $-80^\circ C$. Sixty-nanometer sections were stained with anti-*B. burgdorferi* and anti-Igp monoclonal antibodies and visualized with 5- and 10-nm-diameter protein A-gold particles (provided by Jan Slot, Utrecht, The Netherlands) (see the legend to Fig. 3). Conventional embedding in Epon followed postfixing with 1% OsO_4 and uranyl acetate (29).

End-on entry into macrophages. Previous studies of *B. burgdorferi* report attachment to eukaryotic cells via the sides of spirochetes as well as by the ends, suggesting that *B. burgdorferi* has a uniform surface (37). However, recent studies of spirochetes have shown a specialization of their tips; the ends are able to bind a variety of host proteins, which in turn may equip the organisms for entry into cells or dissemination through tissues (9, 10, 20). End-on attachment has been described for the entry of *T. pallidum* into phagocytes (5). To determine whether spirochete tips or sides initiate entry into phagocytes, we examined the live interaction of spirochetes with macrophages by using Nomarski optics to enhance visualization of the phagocyte surface. Macrophages at room temperature were recorded before the addition of low-passage-number, virulent *B. burgdorferi* (10^7 /ml) on the microscope stage. Images were recorded every 3 s for up to 30 min. As the spirochetes

FIG. 3. Colocalization of *B. burgdorferi* in macrophage endosomes and lysosomes. (A) Time (chase), 0 min. Spirochetes near the surface of the macrophage are labeled with 5-nm gold particles, and IgG is labeled with 10-nm gold particles. Arrows indicate examples in which both sizes of particles can be seen together. (B) Time (chase), 30 min. Entwined spirochetes are seen enclosed within a vacuole and labeled with 10-nm gold particles; IgG is labeled with 5-nm gold particles. Arrows indicate examples in which both sizes of particles can be seen together.

FIG. 4. Degradation of spirochetes within a macrophage. Spirochetes were incubated with macrophages as above, fixed, and postfixed with OsO_4 and uranyl acetate before being embedded in Epon 512. As spirochetes within vesicles move from the surface of the cell (20 min) (A) to a more perinuclear location (1 h) (B), their structure becomes less distinct and finally unrecognizable (5 h) (C). N, nucleus; G, Golgi apparatus. Arrows indicate examples of vesicles containing organisms at various stages of degradation.

encountered adherent macrophages, they contacted them initially by their ends (Fig. 1A and B). As the interaction proceeded, the spirochete remained attached to the macrophage by one end while its free end rotated vigorously; note the different positions of the spirochete over time in relation to the stationary position of the macrophage (Fig. 1B through D). The high degree of fluidity of the spirochete membrane suggests that even an initial lateral contact may promptly reorient, resulting in an eventual tip attachment (6); in that case, end-on entry of spirochetes into macrophages would not require specialized tip structures. Spirochetes appeared to be pulled into the cells in an active process.

Progressive engulfment of spirochetes. We employed a pulse-chase design to introduce a cohort of spirochetes to the macrophages and follow their uptake and disposition within the cells. Samples were fixed and labeled for *B. burgdorferi* by using rabbit antisera directed against whole N40 organisms. At 0 min, spirochetes near the periphery of the cell were still elongated (Fig. 2, 0 min). As the time course progressed, recognizable spirochetes became bundled within the macrophages and were found in a more perinuclear position in the cells (Fig. 2, 20 min and 180 min). The spirochetes appeared coiled up within the phagocytes, as though reeled in from one end into the phagosomes, rather than randomly bunched together. This progressive engulfment supports the end-on entry recorded with live cells.

Lysosomal colocalization and degradation of spirochetes. To localize spirochetes in particular intracellular compartments definitively, we employed double labels following ingestion. After internalization (as described above) we fixed samples for immunoelectron microscopy. The cells were labeled with antibodies directed against *B. burgdorferi* and against IgGs, which are well-characterized markers of the endocytic pathway (16). The initial time points show spirochetes near the periphery of cells, apparently in close-fitting vesicles. From the earliest fixation time, spirochete markers colocalized with markers of endosomes and lysosomes (Fig. 3A). Over time, recognizable spirochetes were seen twisted up within macrophage lysosomes and were found in a perinuclear position (Fig. 3B). Later, *B. burgdorferi* epitopes began to disappear, as indicated by decreased labeling by specific antibody, suggesting degradation of spirochetal proteins; the observed kinetics are similar to lysosome delivery times observed by us (27) and others (23).

Degradation of spirochetes within macrophages was apparent from early in their cellular course. As the spirochetes were transported deeper into the cell, they ceased being elongated and were observed wrapped around themselves within the lysosomes (Fig. 4A). Gradually, the core of the spirochete became less distinct, membrane blebs appeared (Fig. 4B), and morphologically distinct spirochetes became rare (Fig. 4C).

Aside from specific antiphagocytic mechanisms, such as have been characterized for *Salmonella* spp. (11) and for streptococcal M proteins (13), most infectious agents are ingested by phagocytes and dispatched by conventional lysosomal degradation (8, 14); this pathway has been clearly characterized by specific-antibody studies (23). *B. burgdorferi* has been reported in neutrophil phagosomes in vitro (2, 30) and in an intracellular vacuole in Langerhans cells from the skin of Lyme disease patients (17); intravacuolar destinations are also documented for other spirochetes, including *T. pallidum* (24, 34). We have localized spirochetes within macrophage lysosomes and demonstrated their concomitant degradation within lysosomes by ultrastructural analysis. Thus, our studies lend no support to an unknown nonlysosomal mechanism that has been invoked for degradation of spirochetes within macrophages (31).

It remains for further exploration to explain the puzzling

coexistence in vivo of intact extracellular spirochetes and phagocytes in chronically infected tissues. Spirochetes may evade phagocytic clearance by varying recognizable outer surface proteins, as we have recently demonstrated (26), or by eliminating them from their surfaces entirely, as has been observed in mutant spirochetes in vitro (33). Many other infectious microorganisms employ these effective strategies, including *Borrelia hermsii*, the agent of relapsing fever (4). Alternatively, or in addition, Lyme infection may impede macrophage function via the action of cytokines, resulting in reduced phagocytic clearance of infectious agents, as is seen, for example, in leishmaniasis (1).

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REFERENCES

1. Barral-Netto, M., A. Barral, C. E. Brownell, Y. A. W. Skeiky, L. R. Elingsworth, D. R. Twardzik, and S. R. Reed. 1992. Transforming growth factor- β in leishmanial infection: a parasite escape mechanism. *Science* **257**: 545-548.
2. Benach, J. L., H. B. Fleit, G. S. Habicht, J. L. Coleman, E. M. Bosler, and B. P. Lane. 1984. Interactions of phagocytes with the Lyme disease spirochete: role of the Fc receptor. *J. Infect. Dis.* **150**:497-507.
3. Bockenstedt, L. K., and S. E. Malawista. 1995. Lyme disease, p. 1234-1249. In R. R. Rich (ed.), *Clinical immunology*. Mosby-Year Book, St. Louis.
4. Borst, P., and D. R. Greaves. 1987. Programmed gene rearrangements altering gene expression. *Science* **235**:658-667.
5. Brause, B. D., and R. B. Roberts. 1978. Attachment of virulent *Treponema pallidum* to human mononuclear phagocytes. *Br. J. Vener. Dis.* **54**:218-224.
6. Charon, N. W., C. W. Lawrence, and S. O'Brien. 1981. Movement of antibody-coated latex beads attached to the spirochete *Leptospira interrogans*. *Proc. Natl. Acad. Sci. USA* **78**:7166-7170.
7. Coburn, J., S. W. Barthold, and J. M. Leong. 1994. Diverse Lyme disease spirochetes bind integrin $\alpha_{IIb}\beta_3$ on human platelets. *Infect. Immun.* **62**:5559-5567.
8. Cohn, Z. A. 1982. The macrophage—versatile element of inflammation. *Harvey Lect.* **77**:63-80.
9. Coleman, J. L., T. J. Sellati, J. E. Testa, R. R. Kew, M. B. Furie, and J. L. Benach. 1995. *Borrelia burgdorferi* binds plasminogen, resulting in enhanced penetration of endothelial monolayers. *Infect. Immun.* **63**:2478-2484.
10. Dawson, J. R., and R. P. Ellen. 1994. Clustering of fibronectin adhesins toward *Treponema denticola* tips upon contact with immobilized fibronectin. *Infect. Immun.* **62**:2214-2221.
11. Fields, P. I., E. A. Groisman, and F. Heffron. 1989. A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. *Science* **243**:1059-1062.
12. Fikrig, E., S. W. Barthold, F. S. Kantor, and R. A. Flavell. 1990. Protection of mice against the Lyme disease agent by immunizing with recombinant OspA. *Science* **250**:553-556.
13. Fischetti, V. A. 1989. Streptococcal M protein: molecular design and biological behavior. *Clin. Microbiol. Rev.* **2**:285-314.
14. Hall, B. F., and K. A. Joiner. 1991. Strategies of obligate intracellular parasites for evading host defences. *Immunol. Today* **12**:A22-A27.
15. Horwitz, M. A. 1984. Phagocytosis of the Legionnaire's disease bacterium (*Legionella pneumophila*) occurs by a novel mechanism: engulfment with a pseudopod coil. *Cell* **36**:27-33.
16. Howe, C. L., B. L. Granger, M. Hull, S. A. Green, C. A. Gabel, A. Helenius, and I. Mellman. 1988. Derived protein sequence, oligosaccharides, and membrane insertion of the 120-kDa lysosomal membrane glycoprotein (lgp): identification of a highly conserved family of lysosomal membrane glycoproteins. *Proc. Natl. Acad. Sci. USA* **85**:7577-7581.
17. Hulska, D., P. Bartak, J. Hercogova, J. Hancil, J. Basta, and J. Schramlova. 1994. Electron microscopy of Langerhans cells and *Borrelia burgdorferi* in Lyme disease patients. *Int. J. Med. Microbiol. Virol. Parasitol. Infect. Dis.* **280**:348-359.
18. Joiner, K. A., S. A. Fuhrman, H. M. Miettinen, L. H. Kasper, and I. Mellman. 1990. *Toxoplasma gondii*: fusion competence of parasitophorous vacuoles in Fc receptor-transfected fibroblasts. *Science* **249**:641-646.
19. Jones, T. C., S. Yeh, and J. G. Hirsch. 1972. The interaction between *Toxoplasma gondii* and mammalian cells. I. Mechanism of entry and intracellular fate of the parasite. *J. Exp. Med.* **136**:1157-1172.

20. Klemmner, M. S., R. Noring, M. P. Epstein, B. McCloud, R. Hu, S. A. Limentani, and R. A. Rogers. 1995. Binding of human plasminogen and urokinase-type plasminogen activator to the Lyme disease spirochete, *Borrelia burgdorferi*. J. Infect. Dis. **171**:1258–1265.
21. Klemmner, M. S., R. Noring, and R. A. Rogers. 1993. Invasion of human skin fibroblasts by the Lyme disease spirochete, *Borrelia burgdorferi*. J. Infect. Dis. **167**:1074–1081.
22. Kopp, P. A., M. Schmitt, H.-J. Wellensiek, and H. Blobel. 1995. Isolation and characterization of fibronectin-binding sites of *Borrelia garinii* N34. Infect. Immun. **63**:3804–3808.
23. Lang, T., M.-T. Tassin, and A. Ryter. 1988. Bacterial antigen immunolabeling in macrophages after phagocytosis and degradation of *Bacillus subtilis*. Infect. Immun. **56**:468–478.
24. Lukehart, S. A., and J. N. Miller. 1978. Demonstration of the *in vitro* phagocytosis of *Treponema pallidum* by rabbit peritoneal macrophages. J. Immunol. **121**:2014–2023.
25. Montgomery, R. R., and S. E. Malawista. 1994. *Borrelia burgdorferi* and the macrophage: routine annihilation but occasional haven? Parasitol. Today **10**:154–157.
26. Montgomery, R. R., S. E. Malawista, K. J. M. Feen, and L. K. Bockenstedt. 1996. Direct demonstration of antigenic substitution of *Borrelia burgdorferi* *ex vivo*: exploration of the paradox of the early immune response to outer surface proteins A and C in Lyme disease. J. Exp. Med. **183**:261–270.
27. Montgomery, R. R., M. H. Nathanson, and S. E. Malawista. 1993. The fate of *Borrelia burgdorferi*, the agent for Lyme disease, in mouse macrophages: destruction, survival, recovery. J. Immunol. **150**:909–915.
28. Montgomery, R. R., M. H. Nathanson, and S. E. Malawista. 1994. Fc and non Fc-mediated phagocytosis of *Borrelia burgdorferi* by macrophages. J. Infect. Dis. **170**:890–893.
29. Montgomery, R. R., P. Webster, and I. Mellman. 1991. Accumulation of indigestible substances reduces fusion competence of macrophage lysosomes. J. Immunol. **147**:3087–3095.
30. Peterson, P. K., C. C. Clawson, D. A. Lee, D. J. Garlich, P. G. Quie, and R. C. Johnson. 1984. Human phagocyte interactions with the Lyme disease spirochete. Infect. Immun. **46**:608–611.
31. Rittig, M. G., T. Häupl, A. Krause, M. Kressel, P. Groscurth, and G. R. Burmester. 1994. *Borrelia burgdorferi*-induced ultrastructural alterations in human phagocytes: a clue to pathogenicity? J. Pathol. **173**:269–282.
32. Rittig, M. G., A. Krause, T. Häupl, U. E. Schaible, M. Modolell, M. D. Kramer, E. Lütjen-Drecoll, M. M. Simon, and G. R. Burmester. 1992. Coiling phagocytosis is the preferential phagocytic mechanism for *Borrelia burgdorferi*. Infect. Immun. **60**:4205–4212.
33. Sadziene, A., D. D. Thomas, and A. G. Barbour. 1995. *Borrelia burgdorferi* mutant lacking Osp: biological and immunological characterization. Infect. Immun. **63**:1573–1580.
34. Sell, S., S. Baker-Zander, and H. C. Powell. 1982. Experimental syphilitic orchitis in rabbits: ultrastructural appearance of *Treponema pallidum* during phagocytosis and dissolution by macrophages *in vivo*. Lab. Invest. **46**:355–364.
35. Szczepanski, A., and H. B. Fleit. 1988. Interaction between *Borrelia burgdorferi* and polymorphonuclear leukocytes: phagocytosis and the induction of the respiratory burst. Ann. N. Y. Acad. Sci. **539**:425–428.
36. Thomas, D. D., J. B. Baseman, and J. F. Alderete. 1985. Fibronectin tetrapeptide is target for syphilis spirochete cytoadherence. J. Exp. Med. **162**:1715–1719.
37. Thomas, D. D., and L. E. Comstock. 1989. Interaction of Lyme disease spirochetes with cultured eucaryotic cells. Infect. Immun. **57**:1324–1326.

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