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Coiling Phagocytosis Is the Preferential Phagocytic Mechanism for *Borrelia burgdorferi*

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The uptake mechanism for the spirochete *Borrelia burgdorferi*, the causative agent of Lyme disease, was investigated by electron microscopy for human and murine phagocytes. Spirochetes of both a low- and a high-passage strain were preferentially internalized by coiling rather than by conventional phagocytosis. The spirochetes engulfed by coiling phagocytosis were found to disintegrate in an organelle exclusion zone without evident participation of lysosomes. Preincubation of *B. burgdorferi* with monoclonal antibody to the spirochetal OspA enhanced phagocytosis in general but did not consistently influence the uptake mechanism. Quantitative and kinetic differences concerning the phagocytic rate and mechanism were evident between cells from different lineages, different human individuals, and mice and humans. In general, when few phagocytes participated in spirochete uptake, the active cells displayed a high ratio of coiling versus conventional phagocytosis. These results suggest that coiling phagocytosis of *B. burgdorferi* plays a critical role in the control of spirochetal infection. More detailed studies on the molecular basis of this phagocytic mechanism may lead to new insights into the pathogenesis of Lyme borreliosis, a disease which is frequently characterized by the host's inability to eliminate the pathogenic spirochete.

The spirochete *Borrelia burgdorferi* is the causative agent of Lyme disease. This illness is clinically characterized by acute and chronic stages with multiple manifestations, primarily affecting the skin, the nervous system, and the joints. Compared with the marked tissue reactions, the number of spirochetes in the organs affected is low (3, 28). Although patients suffering from Lyme disease show a vigorous specific immune response to *B. burgdorferi* (19, 30), spirochetes appear to persist in chronic stages. Insufficient phagocytosis could contribute to this inability of the defense system to control infection with *B. burgdorferi*. Previous studies on the phagocytosis of *B. burgdorferi* have consistently described conventional phagocytic mechanisms (5, 6, 23). There is one report, however, that human neutrophil pseudopods were observed to wrap *B. burgdorferi* in a coillike fashion (31).

This unusual uptake mechanism of coiling phagocytosis has already been observed with *Candida* species (20), *Trypanosoma brucei* (29), *Leishmania donovani* promastigotes (7), *Legionella pneumophila* (14), and silicogenic crystalline quartz dust (4). However, only little is known about its frequency and the underlying mechanisms. Also, the difference in the elimination potency of coiling versus conventional phagocytosis is not clear.

To further address these questions we have performed a comprehensive study by electron microscopy on the uptake of *B. burgdorferi* by human and murine phagocytes of different lineages. Our results demonstrate that coiling phagocytosis is the preferential uptake mechanism for *B. burgdorferi*. Our observations suggest that the spirochetes internalized by this mechanism undergo an intracellular processing distinct from that following conventional phagocytosis.

MATERIALS AND METHODS

Phagocyte preparations. Human phagocytes were isolated from heparinized peripheral blood of 5 healthy adult volunteers. The monocytes (hMϕ) were prepared as described by Krause et al. (19). Briefly, an hMϕ-enriched cell fraction was obtained by combining two density gradient centrifugation procedures. Cells were applied first to Ficoll-Hypaque (Nycomed, Oslo, Norway), followed by a second gradient over a 1.065-g/ml Percoll solution (Biochrom, Berlin, Germany) previously diluted with phosphate-buffered saline. The remaining T lymphocytes were removed by an E-rosetting procedure with neuraminidase-treated sheep erythrocytes. These steps finally resulted in a preparation containing 85 to 90% hMϕ as determined by light microscopy and fluorescence-activated cell sorter analysis using an anti-CD14 monoclonal antibody (MAb) (19). The remaining cells were either mature T cells or B lymphocytes, as signified by their expression of the CD3 or the CD20 antigen, respectively. Polymorphonuclear leukocytes were prepared by a double density-gradient centrifugation procedure over Percoll (8). Briefly, after centrifugation the neutrophilic granulocytes (hNG) were enriched in the interphase between a 1.077-g/ml and a 1.087-g/ml Percoll solution. The remaining erythrocytes were removed by osmotic lysis.

For murine phagocytes, adult mice of inbred strains C.B-17 ($n = 2$) and AKR/N ($n = 1$) were used, obtained from the animal colony maintained at the Max-Planck-Institut für Immunbiologie, Freiburg, Germany. These mice had been kept under pathogen-free conditions. Bone marrow-derived macrophages (mMa) were prepared and generated as described previously (21). Briefly, bone marrow cells were

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cultivated in Teflon bags for 11 days in Dulbecco's modified Eagle's medium (GIBCO, Eggenstein, Germany) supplemented with 10% (vol/vol) fetal calf serum, 5% (vol/vol) horse serum, and 15% (vol/vol) supernatant from the mouse fibroblast line L929S as the granulocyte-macrophage colony-stimulating factor supply.

Bacterial strains and cultural conditions. *B. burgdorferi* PKo 2-85 was kindly provided by Vera Preac-Mursic, Max-von-Pettenkofer-Institut, Munich, Germany. It is a high-passage isolate from the skin of a southern German resident suffering from erythema chronicum migrans. The spirochetes were grown in modified Kelly medium (BSK medium [2]) and subcultured as described elsewhere in detail (11). *B. burgdorferi* ZS7 is a low-passage tick isolate from the Freiburg area in southwestern Germany. It was grown in BSK medium and harvested as previously described (25). *Escherichia coli* NM538 (Genofit, Heidelberg, Germany) was subcultured in Luria-Bertani medium (GIBCO).

Incubation and experimental conditions. Phagocytes and pathogens were mixed in ratios of either 10 *B. burgdorferi* or 100 *E. coli* cells per single phagocyte for 15 to 120 min. Incubation took place under gentle rotation at 37°C in the presence of 5% CO_2 in polyethylene microtubes (Eppendorf, Hamburg, Germany). With hMϕ and hNG a modified RPMI 1640 culture medium (Biochrom) was used, supplemented with 10% (vol/vol) autologous donor serum (19). In some preparations 100 IU of penicillin per ml and 100 μg of streptomycin per ml (both GIBCO) were added to this culture medium. Dulbecco's modified Eagle's medium without addition of antibiotics was used with mMa.

Prior to the incubation with phagocytes, some of the *B. burgdorferi* preparations were treated with supernatant from a mouse hybridoma cell culture containing immunoglobulin G2a MAb LA-2 specific for the outer surface protein A (OspA; 31-kDa protein) from *B. burgdorferi* preparations (18). For subsequent incubation with hMϕ, 1 ml of medium containing 2×10^8 spirochetes and 1 ml of supernatant containing 75 pg of MAb were mixed in polyethylene microtubes for 45 min under gentle rotation at 37°C in the presence of 5% CO_2. For experiments with mMa the spirochetes were opsonized in essentially the same way as described previously (24).

At the end of the incubation period, the suspensions were centrifuged at $500 \times g$ for 10 min. The pellets were resuspended in Ito's fixative (17), supplemented with 2 mM $MgCl_2$, and kept at 4°C until further processing. With some of the preparations either 0.1% (wt/vol) ruthenium red or 1% (wt/vol) tannic acid was added to the fixative solution in order to stain the cell membranes (10). Alternatively, following washes in 0.1 M cacodylate buffer, pH 7.2, the suspensions were incubated with concanavalin A conjugated to colloidal gold particles 10 nm in diameter (100 μg of protein per ml of buffer [12]).

Transmission electron microscopy. The suspensions stored were centrifuged again. After the pellets were transferred into 2% agar, the samples were thoroughly rinsed with cacodylate buffer at 4°C overnight, postfixed with 1% aqueous osmium tetroxide for 120 min, and again rinsed with cacodylate buffer overnight. Subsequently, the samples were dehydrated with a graded ethanol series completed by dry acetone and finally embedded in Epon 812 by following standard techniques (10).

Semithin sections were cut from the plastic blocks for light microscopical survey. From selected areas thin sections were cut and placed onto 200-mesh standard square copper grids (Polysciences, St. Goar, Germany). After counterstaining first with 10% uranyl acetate followed by 2.8% lead citrate, sections were investigated with electron microscope types Siemens EM 101 and Zeiss EM 902. One grid mesh per section of the different preparations was selected for semiquantification. The following events were semiquantified: (i) the proportions of phagocytes participating in the uptake of *B. burgdorferi*, regardless of the phagocytic mechanism, (ii) the proportions of coiling phagocytosis used for the uptake of *B. burgdorferi*, and (iii) the maximum number of *B. burgdorferi* organisms engulfed as seen in any phagocyte, regardless of the phagocytic mechanism.

RESULTS

Ultrastructural characteristics of coiling phagocytosis. The ultrastructural characteristics of coiling phagocytosis of *B. burgdorferi* cells were already evident upon the initial contact between phagocytes and spirochetes, though it can be difficult to distinguish between early coiling phagocytosis and conventional phagocytosis. Thus, while in conventional phagocytosis cellular protrusions symmetrically enclosed the spirochetes (see Fig. 3), in coiling phagocytosis a single phagocyte pseudopod bent around the spirochetes in a hooklike fashion (Fig. 1a). When the pseudopod tips turned back and attached to their base, the pseudopods appeared to slide along the cell membrane, leading to characteristic ultrastructural figures. In transverse sections, up to five pseudopod rotations wrapped the spirochetes (Fig. 1b), whereas in longitudinal sections the spirochetes were packed between pseudopod piles (Fig. 1c). Combining these two images, the wrapping pseudopods appeared as sheathlike protrusions measuring up to 6 μm in width, often more than 15 μm in length, and 0.2 to 0.5 μm in transverse diameter.

These entire phagocytic complexes were subsequently internalized into the phagocytes (Fig. 1d), leading to a deformation of the regular pseudopod coil (Fig. 2a). The adjacent coiled membranes fused, turned to electron-dense hyaline figures, and finally dissolved. As this event started at various spots along the line of the pseudopod coils, different stages of membrane dissolution were seen in sequence (Fig. 2a and b). The wrapped *B. burgdorferi* organisms were first situated in vacuoles with tight-fitting membranes except for the central cisterns created by the diverging pseudopod membranes (Fig. 2a). In the course of processing, the membranes of both the cellular vacuoles and the enclosed spirochetes became undistinguishable and dissolved. The spirochetes turned into electron-dense areas with nondefinable borders, surrounded by granular cytoplasmic areas devoid of any cellular organelles, particularly lysosomes (Fig. 2b and c). This accumulation of membrane remnants, organelle exclusion zone, and disintegrating spirochete constituted the intracellular residual figure of coiling phagocytosis (Fig. 2b and c). Occasionally, vacuoles which contained both spirochetes and pseudopods or spirochetes alone were observed (Fig. 4). Vacuoles of the latter type obviously were not derived from pseudopod coils but rather were a follow-up of conventional phagocytosis (Fig. 3). Also, in contrast to the organelle exclusion zone of coiling phagocytosis, lysosomes were frequently present in close vicinity to these vacuoles (Fig. 4).

Experiments using the cell surface markers concanavalin A-gold, ruthenium red, and tannic acid demonstrated that the gaps between the adjacent coiled membranes including the central vacuole bearing the spirochete were continuous with the extracellular space until the membranes fused (data not shown). With the other types of vacuoles, part displayed

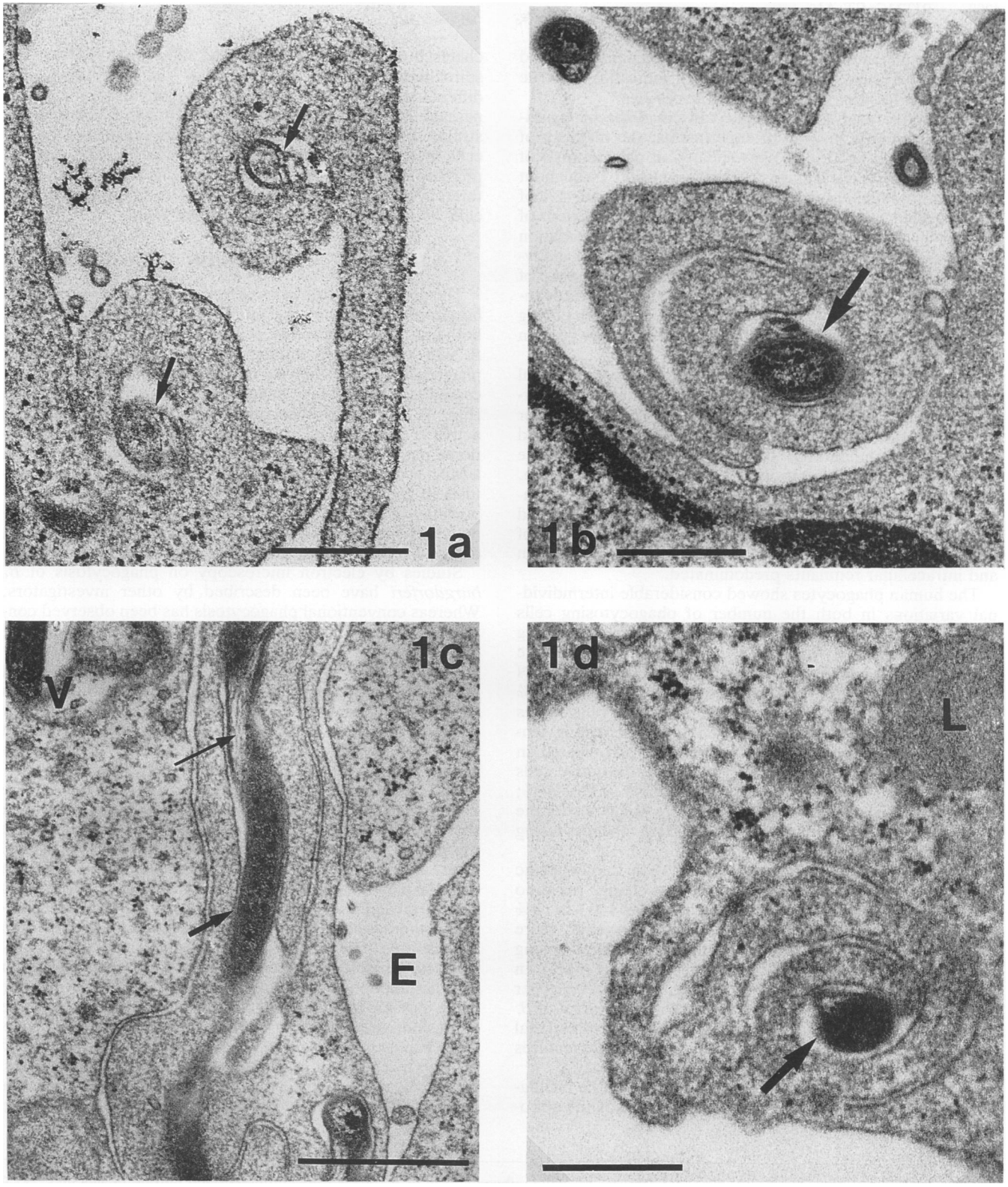


FIG. 1. Electron micrographs showing early features of coiling phagocytosis of *B. burgdorferi* by human and murine phagocytes. (a) Two single pseudopods of a human peripheral blood neutrophil bend around spirochetes (arrows) in a hooklike fashion (incubation period, 45 min; magnifications, ×50,000; bar = 0.5 μm). (b) A spirochete which is still located in the extracellular space is wrapped by the pseudopod of a human peripheral blood neutrophil (incubation period, 45 min; magnification, ×90,000; bar = 0.25 μm). Sectioned in the transverse plane, the spirochete (arrow) appears to be enrolled in a pseudopod coil. (c) A spirochete wrapped by the pseudopod of a peripheral blood hMϕ (incubation period, 45 min; magnification, ×30,000; bar = 1 μm). Sectioned in the longitudinal plane, the spirochete (thick arrow) appears to be sandwiched between pseudopod piles. Some spirochetal endoflagella are visible (thin arrow). V, vacuole containing spirochetes; E, extracellular space. (d) A phagocytic complex consisting of the pseudopod coil and the trapped spirochete (arrow) has been internalized into a bone marrow-derived mMa (C.B-17 mouse; incubation period, 45 min; magnification, ×50,000; bar = 0.5 μm). L, lysosome.

membrane labeling (Fig. 4) whereas the remaining part had unlabeled vacuolar membranes (Fig. 4), indicating the true intracellular location of these vacuolar cavities.

Quantitative analysis of phagocytosis in different experimental conditions. In 7 of 10 experiments, the majority of hMϕ (59%) and hNG (62%) participated in phagocytosis of *B. burgdorferi*. This feature remained constant throughout the different incubation periods. In contrast, 20 and 41% of mMa participated in phagocytosis after incubation periods of 30 and 60 min, but 63% participated after an incubation period of 120 min. In 9 of 10 experiments, coiling phagocytosis was the preferential mechanism used for the uptake of *B. burgdorferi* (hMϕ, 61%; hNG, 63%; mMa, 70%) throughout the different incubation periods. Features of both coiling and conventional phagocytosis could simultaneously be seen in the same phagocyte (data not shown).

Whereas the proportions of coiling and conventional phagocytosis did not change considerably within the different incubation periods (9% for hMϕ; 10% for hNG; 8% for mMa), the ratio between extracellular pseudopod coils and intracellular remnants of coiling phagocytosis was time dependent. After incubation periods of 15 and 30 min, extracellular pseudopod coils were abundant. After incubation periods of 45 and 60 min, their number decreased and intracellular remnants became visible. After 120 min of incubation, extracellular pseudopod coils were rarely seen and intracellular remnants predominated.

The human phagocytes showed considerable interindividual variations in both the number of phagocytosing cells (hMϕ, 40 to 87%; hNG, 45 to 80%) and the use of coiling phagocytosis (hMϕ, 48 to 71%; hNG, 53 to 69%). These variations were less distinct between the mMa from AKR/N and C.B-17 mice (39 to 43% phagocytosing cells; 63 to 71% use of coiling phagocytosis). In comparing the phagocytes obtained from each human individual, an interesting tendency became apparent. When few cells participated in phagocytosis (40% for hMϕ; 46% for hNG), the active cells displayed a high ratio of coiling phagocytosis (hMϕ, 64%; hNG, 69%). This tendency was even more obvious with the mMa after short incubation periods (31% phagocytosing cells; 67% coiling phagocytosis).

Preincubation of *B. burgdorferi* with MAb LA-2 specific for the spirochetal OspA virtually did not change the ratio between coiling and conventional phagocytosis (53 to 54% with the hMϕ; 67 to 68% with the mMa). However, there was a significant increase in the number of phagocytosing cells (56 to 68% with the hMϕ; 41 to 88% with the mMa). In addition, the number of ingested *B. burgdorferi* particles per mMa increased from 5 to 22. In contrast, pretreatment of *B. burgdorferi* with MAb LA-10 specific for the spirochetal flagellin (18) had no considerable effect upon these features (data not shown).

The addition of penicillin and streptomycin to the incubation media did not influence the phagocytosis of the spirochetes but caused outer envelope blebs with 30% of the spirochetes in the extracellular space (data not shown). No differences were observed in the phagocytosis of the low-passage ZS7 and the high-passage PKo 2-85 *B. burgdorferi* strains. In contrast to the *B. burgdorferi* organisms, *E. coli* cells were almost exclusively internalized by conventional phagocytosis (Fig. 5a). Among all sections investigated, only three hNG and one hMϕ were found to internalize *E. coli* cells by use of a pseudopod coil (Fig. 5b).

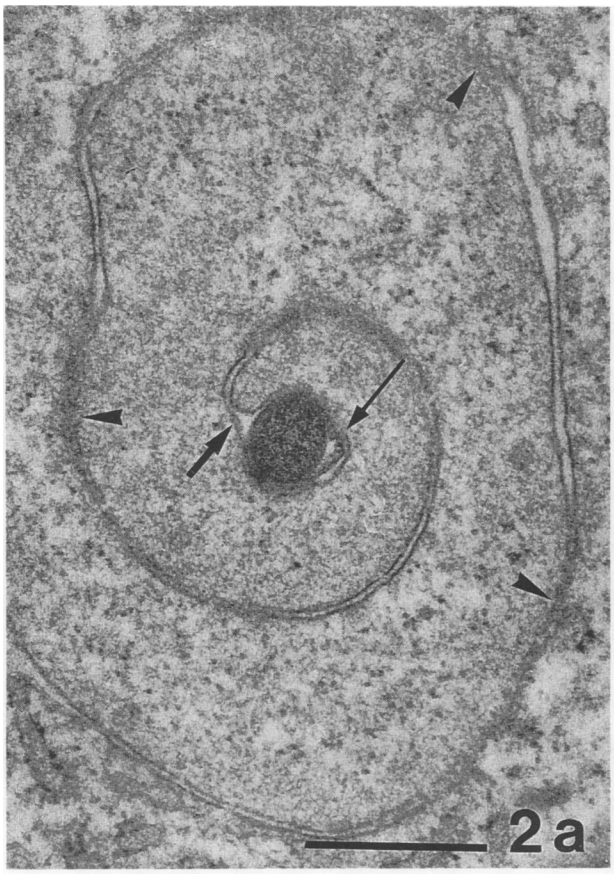
DISCUSSION

The present electron microscopic study of the uptake of *B. burgdorferi* by human and murine phagocytes revealed that both low- and high-passage *B. burgdorferi* strains, but not an *E. coli* strain, were internalized preferentially by coiling phagocytosis. The thereby engulfed spirochetes were processed differently as compared with conventional uptake (Fig. 6). When few cells participated in phagocytosis, the active cells displayed a high ratio of coiling versus conventional phagocytosis. Preopsonization of *B. burgdorferi* with a MAb specific for the spirochetal OspA enhanced phagocytosis in general but did not consistently influence the proportions of both uptake mechanisms. Quantitative and kinetic differences were evident between various phagocytes, different human individuals, and different species.

Studies by electron microscopy on phagocytosis of *B. burgdorferi* have been described by other investigators. Whereas conventional phagocytosis has been observed consistently (5, 6, 23), coiling phagocytosis was reported only once as an occasional feature in phagocyte-spirochete interaction (31). The present investigation demonstrates that coiling phagocytosis is the preferential uptake mechanism for two independent *B. burgdorferi* strains. The discrepancy between both results may be caused by use of different incubation periods and different spirochete strains or by evaluation of only a small number of ultrathin sections.

The spirochetes engulfed by coiling phagocytosis were degraded in distinct cytoplasmic areas. Cell organelles other than contractile fibers, and lysosomes in particular, were missing in the vicinity of the disintegrating spirochetes. These findings indicate that coiling phagocytosis results in a cytoplasmic area which is apparently derived from the peripheral hyaline cortex of the phagocyte (27). Furthermore, the spirochetes in this area appear to be killed with mechanisms independent from lysosomal activities. However, further studies are required to definitely prove whether this assumption is correct. In comparison, following the same uptake by coiling phagocytosis a similar organelle exclusion zone was reported for *L. donovani* promastigotes (7), whereas two different intracellular pathways were found for two independent *L. pneumophila* strains (13, 16). Thus, the identical uptake mechanism of coiling phagocytosis does not necessarily determine identical intracellular processing.

FIG. 2. Electron micrographs showing late features of coiling phagocytosis of *B. burgdorferi* by human and murine phagocytes. (a) A phagocytic complex internalized in a peripheral blood hMϕ (incubation period, 60 min; magnification, ×50,000; bar = 0.5 μm). The pseudopod membranes of the deformed coil fuse discontinuously (arrowheads). The short arrow points to the central cistern of the coil created by the diverging pseudopod membranes. Note the spirochetal endoflagella in the periplasmic space (long arrow). (b) A residual figure of coiling phagocytosis in a bone marrow-derived mMa (AKR/N mouse; incubation period, 60 min; magnification, ×30,000; bar = 1 μm). The disintegrating spirochete (arrows) is surrounded by an organelle exclusion zone. Remnants of the pseudopod membranes (arrowheads) mark the path of the strongly deformed coil. N, nucleus; E, extracellular space. (c) A final stage of coiling phagocytosis in a bone marrow-derived mMa (C.B-17 mouse; incubation period, 120 min; magnification, ×20,000; bar = 1 μm). The engulfed spirochete has vanished, and only the organelle exclusion zone (star) and remnants of the pseudopod membranes (arrowheads) remain. V, vacuole containing damaged spirochetes. N, nucleus.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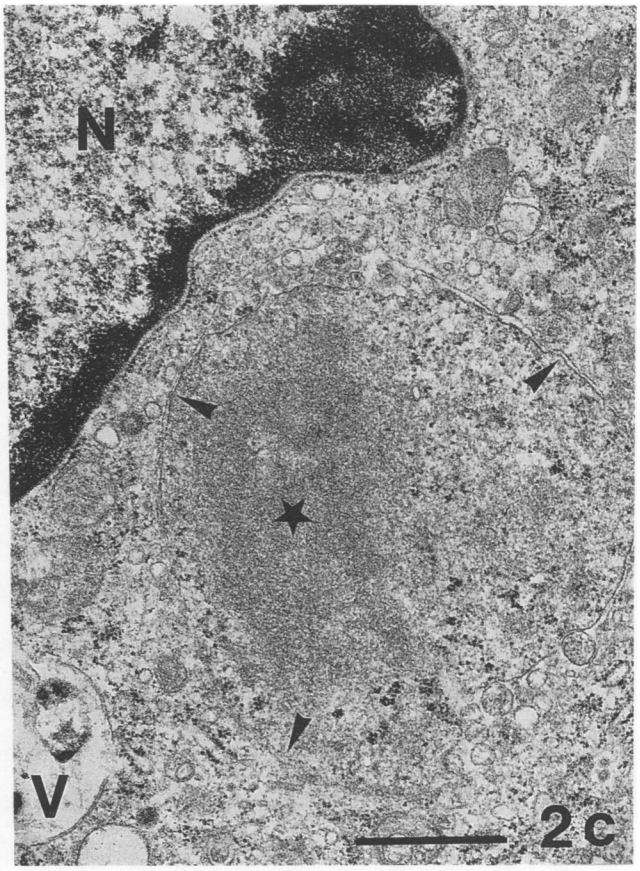
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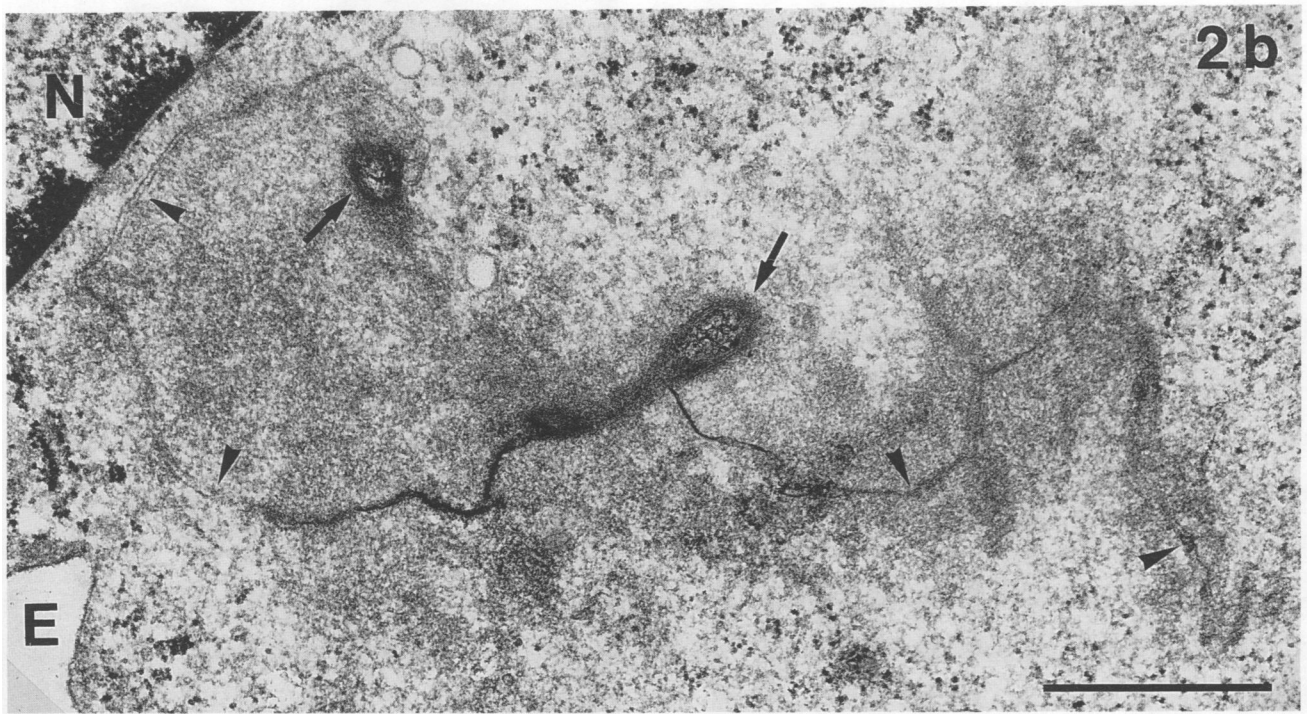
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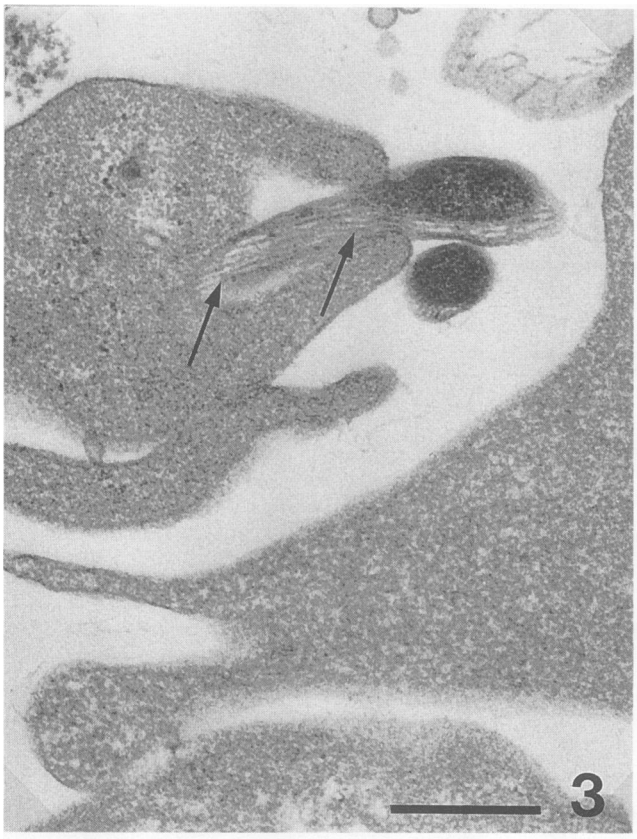

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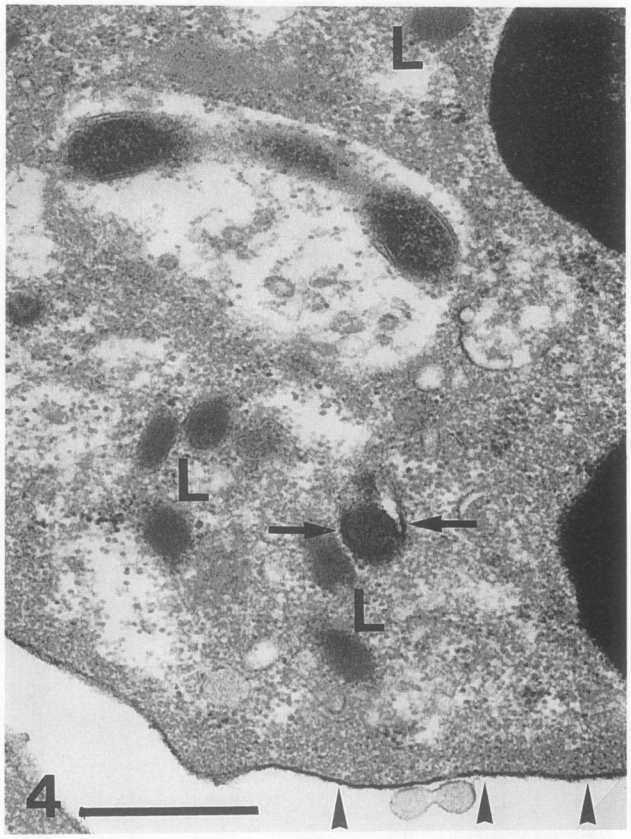

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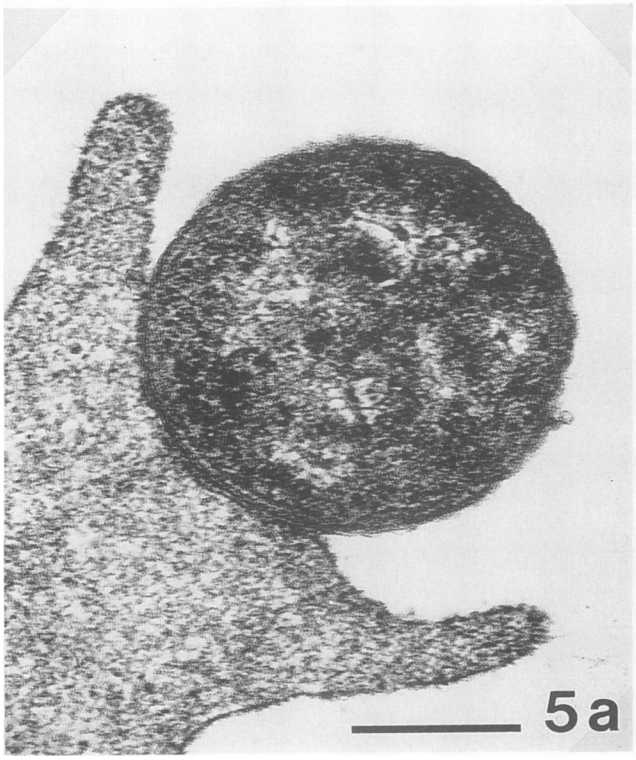

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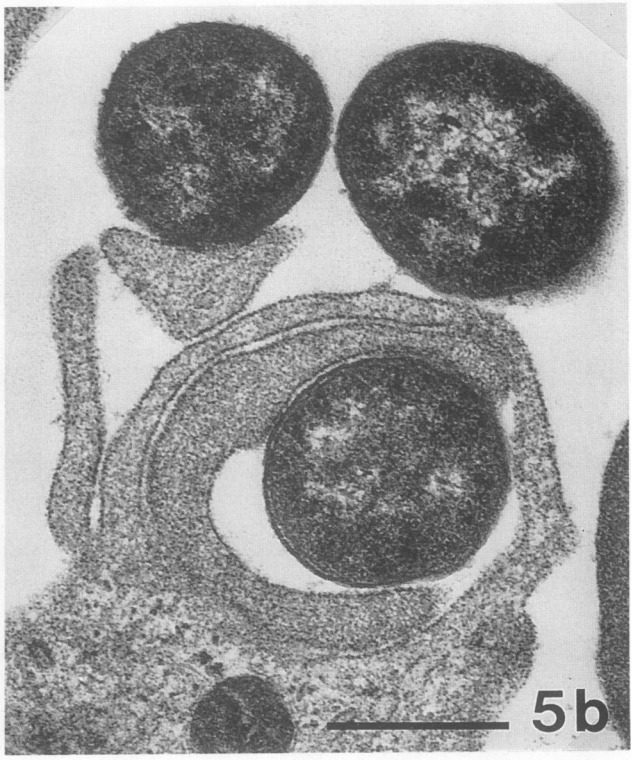

5b

FIG. 3. The electron micrograph shows the uptake of a *B. burgdorferi* organism by a peripheral blood hMϕ (incubation period, 60 min; magnification, ×70,000; bar = 0.3 μm). The arrows point to the spirochetal endoflagella sectioned in the tangential plane. The phagocyte engulfs the spirochete by use of symmetrical protrusions, a characteristic feature of conventional phagocytosis.

FIG. 4. The electron micrograph shows spirochete-containing vacuoles in a human peripheral blood neutrophil (incubation period, 30 min; magnification, ×50,000; bar = 0.5 μm). The extracellular surface (arrowheads) has been labeled with ruthenium red. Compared with the cellular surface, the membrane lining the big vacuole is unlabeled, whereas at the membrane of the small vacuole some marker is present (arrows). Apparently, both vacuoles are not derived from pseudopod coils. L, lysosomes.

FIG. 5. Electron micrographs showing conventional and coiling phagocytosis of *E. coli* by human peripheral blood phagocytes. (a) Neutrophil pseudopods symmetrically enclose an *E. coli* cell (incubation period, 45 min; magnification, ×90,000; bar = 0.25 μm). Conventional phagocytosis represents the usual uptake mechanism for *E. coli*. (b) A monocyte pseudopod repeatedly wraps around an *E. coli* cell (incubation period, 45 min; magnification, ×50,000; bar = 0.5 μm). Coiling phagocytosis is an exceptional uptake mechanism for *E. coli*.

In contrast to *B. burgdorferi*, virtually all *E. coli* cells were internalized by conventional phagocytosis. This result is in accordance with previous data on two independent *E. coli* strains (14). However, our rare findings of *E. coli* cells being wrapped by pseudopod coils suggest that this uptake mechanism is a basic event during phagocytosis. The fact that coiling phagocytosis has been observed with phagocytes from different mammals (4, 7, 14, 29, 31) as well as from a holostean fish (20) supports this view.

Motility was favored as a trigger mechanism for coiling phagocytosis, since exoflagella of both African trypanosomes (29) and only viable but not killed *L. donovani* promastigotes (7) were found to be wrapped by pseudopod coils. Indeed, *B. burgdorferi*, like *L. donovani* and *T. brucei*, is a vividly motile organism, but in our study antibiotic-induced immobility of the spirochetes did not influence the uptake mechanism. In comparison, nonmotile *L. pneumophila* (14) and *Candida* sp. (20) organisms and even inanimate quartz crystals (4) are internalized by pseudopod coils.

Surface components have been considered to induce coiling phagocytosis, since preopsonization of *L. pneumophila* with human immune serum abolished this uptake mechanism (14) and complement receptors CR1 and CR3 mediated engulfment of *L. pneumophila* (15, 22). In comparison, the presence of antibodies or complement did not influence the uptake mechanism of *T. brucei* (29). In our study, preopsonization of *B. burgdorferi* with a MAb to the spirochetal OspA did not abolish or even consistently influence the phagocytic mechanism but enhanced phagocytosis in general. The latter finding supports and extends previous studies demonstrating that elimination of *B. burgdorferi* organisms was increased when opsonized spirochetes were used (1, 5, 6, 23).

In a recent study AKR/N mice were susceptible but C.B-17 mice were resistant to *B. burgdorferi*-induced arthritis (26). In our study mMa from both these strains interacted similarly with *B. burgdorferi* organisms, suggesting that differential pathogenesis of borreliosis is not caused by differential uptake or processing of the pathogen. This consideration could also be true for differential virulence of *B. burgdorferi* strains. In our study both a low- and a high-passage *B. burgdorferi* strain were engulfed and processed identically, and in other studies pathogenic low-passage and apathogenic high-passage *B. burgdorferi* strains equally stimulated neutrophil oxidative burst (9) and were eliminated to similar extents (23).

In conclusion, *B. burgdorferi* appears to trigger an unusual phagocytic mechanism followed by a distinct intracellular processing apparently different from that following conventional phagocytosis. Further studies of phagocytosis will have to address the questions of how coiling phagocytosis is induced by *B. burgdorferi* and what role this phagocytic mechanism plays for the incomplete elimination of spirochetes from the host.

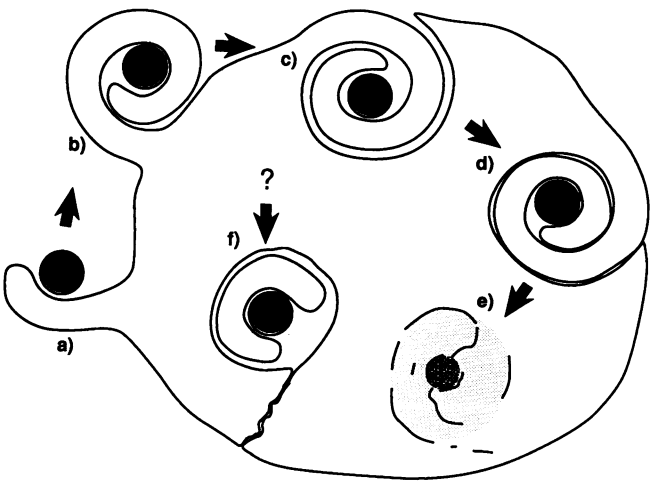

FIG. 6. Diagram summarizing the sequence of cellular events observed with coiling phagocytosis of *B. burgdorferi* as a possible interpretation of the electron micrographs. (a) A single phagocyte pseudopod bends around an adjacent spirochete in a hooklike fashion. (b) The tip of the pseudopod turns back and slides along the cell membrane, leading to the characteristic coillike figure. (c) The phagocytic complex is internalized. (d) The adjacent membranes of the coil fuse discontinuously. (e) The engulfed spirochete, surrounded by granular cytoplasma, disintegrates without evident participation of lysosomes. (f) Occasionally, vacuoles are observed containing both spirochetes and pseudopods.

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