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Interaction of *Borrelia burgdorferi* with Peripheral Blood Fibrocytes, Antigen-Presenting Cells with the Potential for Connective Tissue Targeting

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Abstract

Background: *Borrelia Burgdorferi* has a predilection for collagenous tissue and can interact with fibronectin and cellular collagens. While the molecular mechanisms of how *B. burgdorferi* targets connective tissues and causes arthritis are not understood, the spirochetes can bind to a number of different cell types, including fibroblasts. A novel circulating fibroblast-like cell called the peripheral blood fibrocyte has recently been described. Fibrocytes express collagen types I and III as well as fibronectin. Besides playing a role in wound healing, fibrocytes have the potential to target to connective tissue and the functional capacity to recruit, activate, and present antigen to $CD4^+$ T cells.

Materials and Methods: Rhesus monkey fibrocytes were isolated and characterized by flow cytometry. *B. burgdorferi* were incubated with human or monkey fibrocyte cultures in vitro and the cellular interactions analyzed by light and electron microscopy. The two strains of *B. burgdorferi* studied included JD1, which is highly pathogenic for monkeys, and M297, which lacks the cell surface OspA and OspB proteins.

Results: In this study, we demonstrate that *B. burgdorferi* binds to both human and monkey (rhesus) fibrocytes in vitro. This process does not require OspA or OspB. In addition, the spirochetes are not phagocytosed but are taken into deep recesses of the cell membrane, a process that may protect them from the immune system.

Conclusions: This interaction between *B. burgdorferi* and peripheral blood fibrocytes provides a potential explanation for the targeting of spirochetes to joint connective tissue and may contribute to the inflammatory process in Lyme arthritis.

Introduction

Lyme disease is a tick-transmitted multisystemic disorder in humans (and other mammals) caused by the spirochete *Borrelia burgdorferi* (1). The bacteria are transmitted to humans by the bite of infected ticks of the *Ixodes ricinus* complex. In North America, Lyme disease is the most frequently reported arthropod-borne infection accounting for over 80% of all vector-borne infections in the United States (2). If left untreated, one major long-term manifestation of Lyme disease is chronic arthritis. Little is known about the mechanisms used by *B. burgdorferi* to target connective or other tissues. Although some spiro-

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chetes inevitably reach tissues passively via the circulatory system, other perhaps more specific targeting mechanisms may be involved.

B. burgdorferi bind or produce proteolytic enzymes, and several reports describe the ability of *B. burgdorferi* to bind to host-derived plasminogen. Although the bacteria do not directly activate bound plasminogen, bound plasminogen can be converted to a potent serine protease, plasmin, by host-derived plasminogen activators (3–7). *B. burgdorferi* possess endogenous collagenase(s) (8) and proteoglycanase (9) activities and there is also evidence to suggest that the spirochetes possess hemolytic activity (10). These enzymatic activities are capable of degrading the extracellular matrices of cells and tissues and they may play an important role in the process of bacterial dissemination and tissue invasion. Nevertheless, the tropism of spirochetes for joints remains poorly understood.

Borrelia burgdorferi interacts with a variety of cells, including fibroblasts (11–13). For example, it has been postulated that the binding of *B. burgdorferi* to activated platelets might favor the concentration of spirochetes to regions of endothelial damage, thereby targeting the spirochetes to the arthropod vector at the site of the tick bite and tick attachment (14). A novel leukocyte subpopulation with fibroblast-like properties called "fibrocytes" has been described recently (15). Both human and mouse fibrocytes are negative for many B and T lymphocyte, macrophage, and dendritic cell markers (i.e., CD3, CD4, CD8, CD16, CD19, CD25, CD33, CD38, CD44) but are positive for the hematopoetic progenitor cell marker CD34 (15). In addition, fibrocytes express the common leukocyte marker CD45 as well as the fibroblast products collagen type I, collagen type III, vimentin, and fibronectin (15). Fibrocytes are present in connective tissue scars and have the ability to rapidly enter from blood into subcutaneously implanted wound chambers (15). Because of the known interaction of *B. burgdorferi* with fibroblasts (11–13) and fibronectin (16,17), we predicted that the spirochete would also interact with fibrocytes. The predilection of both *B. burgdorferi* (13,18,19) and fibrocytes (15) for connective tissues led us to speculate that the spirochete may utilize the fibrocyte as a means to reach joints from the peripheral circulation. We provide here morphologic evidence for spirochete/fibrocyte interactions, an important prerequisite for fibrocyte-mediated spirochete targeting.

Materials and Methods

Materials

Rabbit anti-human collagen immunoglobulin G (IgG) (No. T61554R: a mixture of anti-human type I, II, III, IV, and V IgGs) was purchased from Biodesign (Kennebunk, ME). Fluorescein (FITC)-conjugated sheep anti-rabbit IgGAM (No. PF310) was obtained from The Binding Site (San Diego, CA), Phycoerythrin-conjugated anti-CD34 monoclonal antibody (MAb) was from Becton Dickinson (Bedford, MA), and FITC-conjugated anti-collagen type I MAb was from Chemicon (Temecula, CA). Rabbit serum was obtained from Pel-Freeze Biologicals (Rogers, AR). Histopaque-1077, gelatin-free BSK H medium, dextran sulfate, and all other biochemicals were from Sigma (St. Louis, MO).

Analysis of Collagen-Positive Lymphocyte-like Cells of Rhesus Monkey Mononuclear Cells by Flow Cytometry

Monkey mononuclear cells were isolated from normal male Rhesus monkey blood on Histopaque-1077 according to the manufacturer's instructions. The cells were washed with phosphate-buffered saline (PBS) and incubated for 10 min at 37°C in PBS, 0.1% NaN_3 containing rabbit anti-human collagen IgG as the primary antibody. The cells then were washed in PBS-azide and incubated for 30 min at 37°C with FITC-conjugated sheep anti-rabbit IgG. The cells were fixed with 1% paraformaldehyde and resolved on an EPICS 541 flow cytometer. In all experiments at least 10,000 cells were analyzed.

In Vitro Cultivation of Spirochetes

Low-passage (<10) *B. burgdorferi* strain JD1 was obtained from the Centers of Disease Control (CDC). *B. burgdorferi* M297 was obtained from Dr. Russell Johnson (University of Minnesota). The spirochetes were cultured according to Barbour (20) at 34°C (in 5% CO_2, 3% O_2, and 92% N_2) in gelatin-free BSK-H medium containing 10% young-rabbit serum. The bacteria were examined with a dark-field microscope to verify that the organisms were thoroughly dispersed at the start of all assays.

In Vitro Cultivation of Human and Rhesus Monkey Peripheral Blood Fibrocytes

Fibrocytes were harvested and cultured from human or rhesus monkey blood peripheral blood

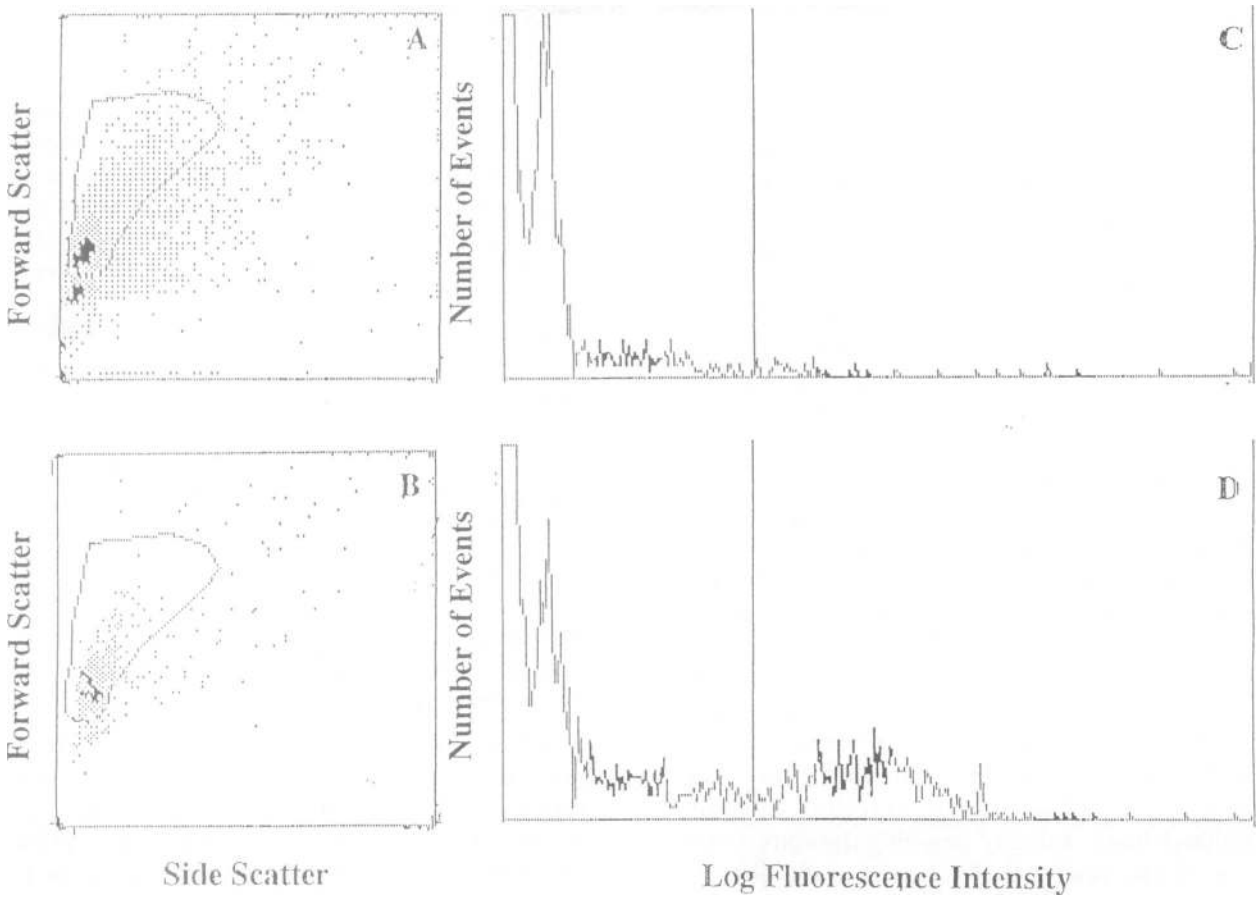

Fig. 1. Flow cytometry analysis of collagen-positive lymphocyte-like cells of rhesus monkey peripheral blood mononuclear cells by flow cytometry. (A) Lymphocyte and monocyte region of light scatter plot of the stained cell preparation selected for analysis. (B) Brightly stained cells shown in (D) exhibited light scatter properties of lymphocytes. (C) Fluorescence intensity of the control cells incubated with the conjugated second antibody alone. (D) Fluorescence intensity of the stained cells incubated with the sheep anti-collagen followed by conjugated anti-sheep IgG. In this experiment, specifically stained cells represented 8.6% of the cells gated in (A).

mononuclear cell preparations as previously described (15). Following 10 days of continuous culture, most of the attached human or monkey cells become morphologically transformed into elongated fibroblast-shaped cells. In addition to morphology, the purity of the fibrocyte (human) cultures was verified by flow cytometry analysis as previously described (15) using both phycoerythrin-conjugated anti-CD34 and FITC-conjugated anti-collagen type I MAb.

Interaction of B. burgdorferi *with Peripheral Blood Fibrocytes and Mononuclear Cells*

B. burgdorferi (approximately 10^7 to 10^8 cells/ml in BSK-H medium), were directly added to a human peripheral blood mononuclear cell preparation or human or monkey fibrocyte cultures, usually at a 1:2 ratio (v:v), and the cells were co-cultured at 34°C (in 5% CO_2, 3% O_2, and 92% N_2). *B. burgdorferi* binding to fibrocytes was monitored by dark-phase contrast microscopy using a Zeiss Axiovert 100 inverted light microscope.

For electron microscopy, the cells were fixed in situ with 2% (v/v) glutaraldehyde in 0.1 M Na-cacodylate-HCl buffer at pH 7.3. After fixation, the cells were removed from the T flasks with a cell scraper, and further processing was done with 2% (w/v) OsO_4 and 0.5% (w/v) uranyl acetate. After ethanol dehydration, the cells were embedded in EMBed 812 via propylene-oxide. Thin sections were examined with a JEOL 1200EX II electron microscope.

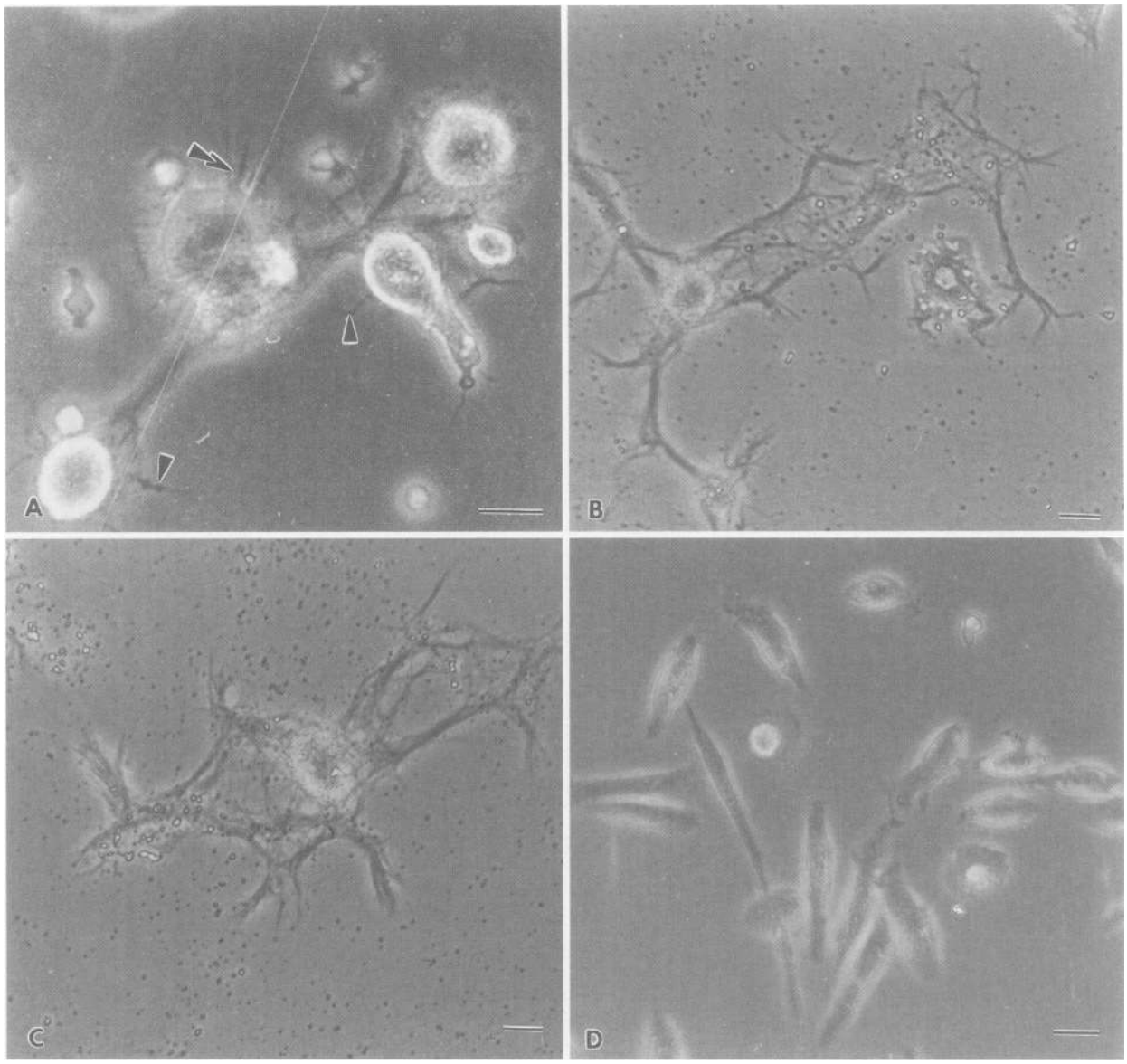

Fig. 2. Binding of *B. burgdorferi* to human and monkey peripheral blood fibrocytes. The binding of *B. burgdorferi* JD1 to human fibrocytes after 30 min (A) or monkey fibrocytes after 30 min (B) or 45 min (C) coculture is shown. Control human fibrocytes are also shown (D). Incubation with M297 gave essentially similar results. In (A), single (arrowhead) and groups of (double arrowhead) spirochetes showed binding. (Bars = 10 μm).

Results and Discussion

The rhesus monkey is an important animal model for human Lyme disease (21,22). Accordingly, we first purified and characterized circulating fibrocytes from the rhesus monkey. Rhesus monkey mononuclear cells were isolated and analyzed for fibrocyte content by flow cytometry (Fig. 1). The lymphocyte and monocyte region of the light scatter plot was selected for fibrocyte analysis using anti-collagen IgG. On the basis of experimental data obtained from 4 animals (1 female and 3 males), 6.1 ± 3.3% (mean ± standard deviation) of the cells with light scatter properties of lymphocytes stained positively for collagen. As in the human, where fibrocytes have been estimated to account for up to 0.5% of circulating leukocytes (23,24), peripheral blood fibrocytes constitute an appreciable circulating cell population in the rhesus monkey.

We tested the ability of *B. burgdorferi* to interact with human (Fig. 2A) and with monkey peripheral blood fibrocytes (Fig. 2B, C). From

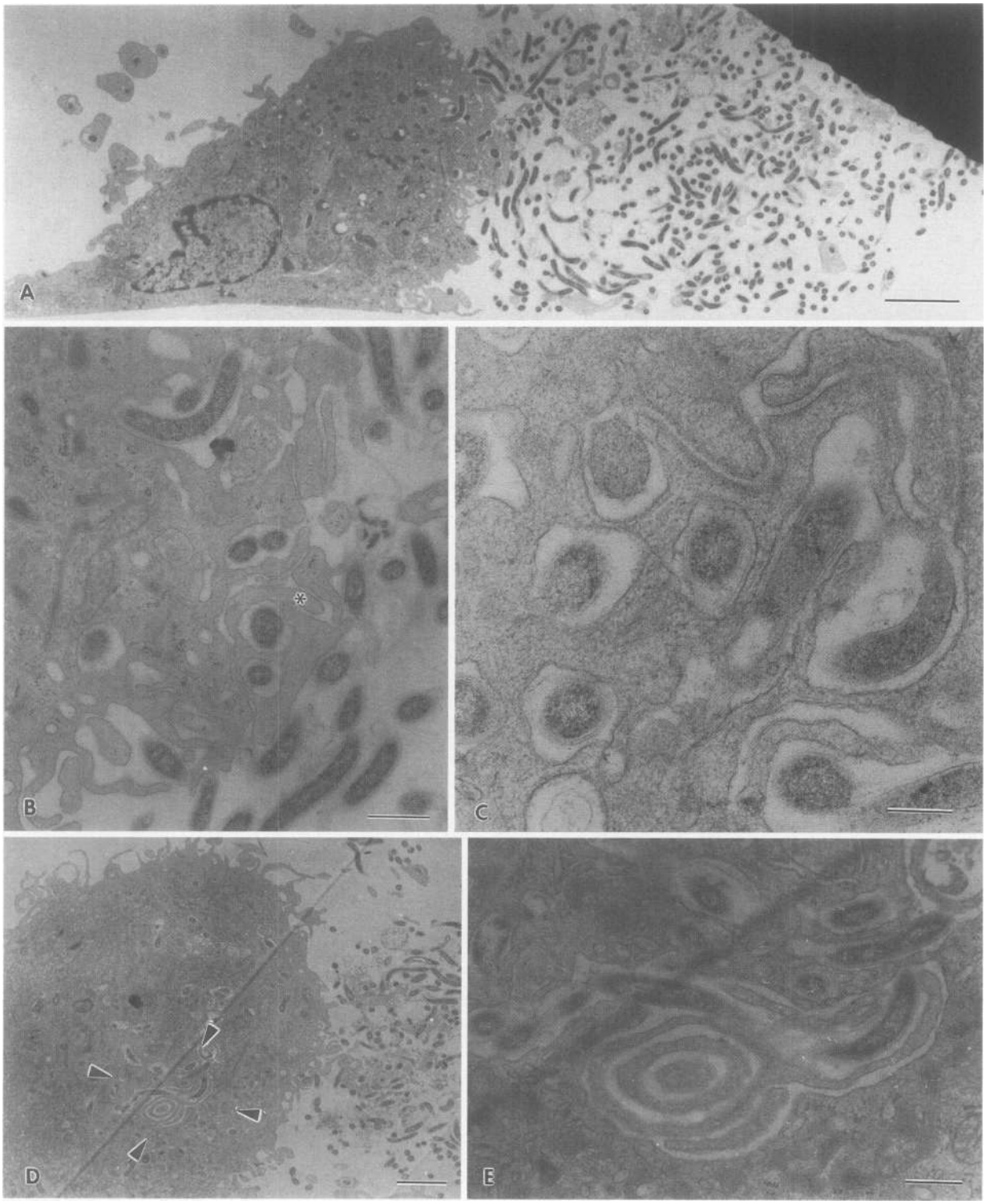

Fig. 3. Interaction of *B. burgdorferi* with peripheral blood fibrocytes. The figures represent fibrocyte/*B. burgdorferi* interactions of approximately 1–2 hr. (A) Fibrocyte sectioned perpendicular to substrate and showing polarized binding of spirochetes (M297). Bar = 4 μm. (B) Periphery of fibrocyte showing uptake of spirochetes. Some of the pseudopodial profiles resemble coiled phagocytosis (asterisk). Bar = 500 nm. (C) Location of *B. burgdorferi* within recesses of the cell membrane, deep within the cytoplasm of a fibrocyte. Bar = 200 nm. (D) Rounded fibrocyte. The arrowheads delineate the area enlarged in (E). Bar = 2 μm. (E) Enlargement from (D), delineated area. Spirochetes are located in deep invaginations of the cell membrane, deep within the cytoplasm. Bar = 500 nm.

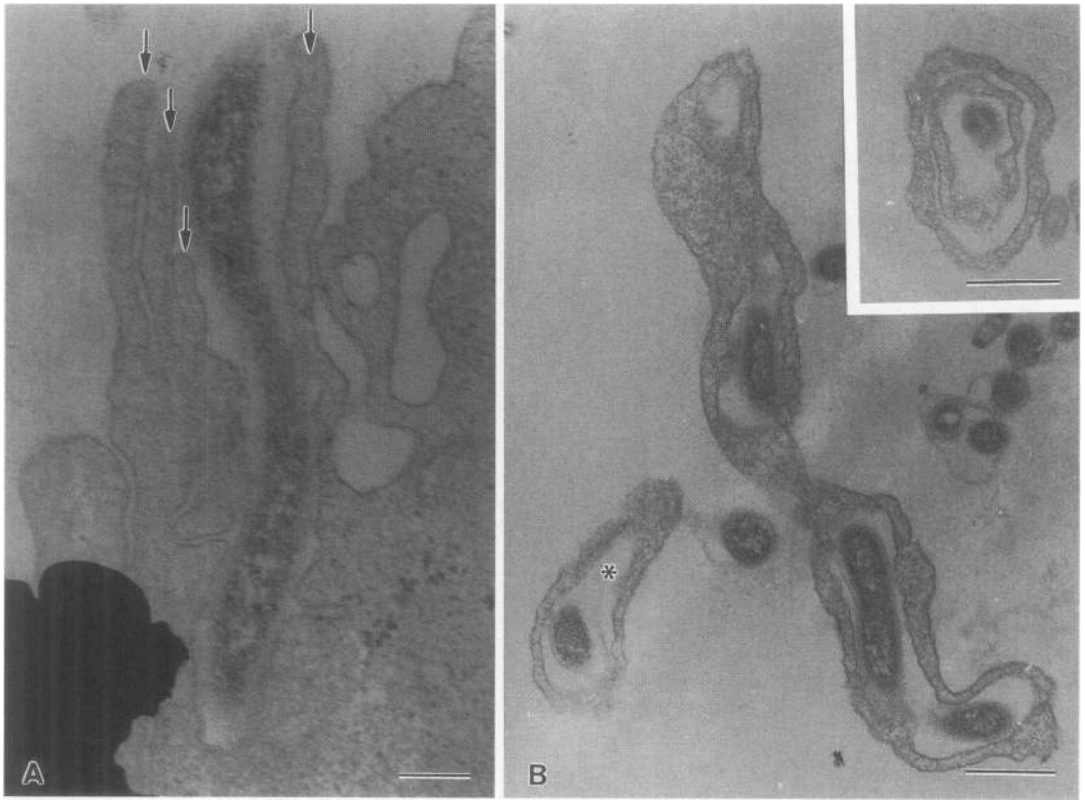

Fig. 4. Tube phagocytosis of *B. burgdorferi* by fibrocytes. (A) Longitudinal section through a spirochete cell being taken up by a fibrocyte. During uptake, the spirochete cell is surrounded by straight cell extensions (arrows). The appearance of these finger-like extensions is due to the section plane. These pseudopodial cell extensions actually form cuffs that surround the spirochete. Bar = 200 nm. (B) Phagocytic tube in longitudinal section. A *B. burgdorferi* cell is sectioned in several places. The asterisk marks a phagocytic tube in cross section. (Insert) *B. burgdorferi* surrounded by two concentric phagocytic tubes in cross section. Bars (B and insert) = 500 nm.

light-microscopic observations it appears that *B. burgdorferi* spirochetes are able to bind via their tips to the surface of fibrocytes. The binding of single spirochetes (single arrowhead) or groups of spirochetes (double arrowhead) is marked in Figure 2A. Human fibrocyte controls are shown in Figure 2D. *Borrelia* interactions were the same whether we used *B. burgdorferi* JD1, a strain highly pathogenic in monkeys (21,22), or *B. burgdorferi* M297, a strain lacking OspA and OspB (25). This implies that OspA and OspB do not play a role in *Borrelia*/fibrocyte interactions. From electron microscopic observations it appears that the binding of *B. burgdorferi* to fibrocytes is polarized to one side of the cell (Fig. 3A).

B. burgdorferi invades B cells through endocytotic pits into vacuoles (26). However, the entry of *B. burgdorferi* into B lymphocytes differs markedly from the phenomenon we observed for the interaction of spirochetes with peripheral blood fibrocytes. It appears that this uptake involves mechanisms that are different from those in conventional phagocytosis or coiling phagocytosis. The active uptake process known as "coiling" phagocytosis used by human phagocytic cells (i.e. monocytes, macrophages, polymorphonuclear leukocytes, dendritic cells, and synovial macrophages) for *B. burgdorferi* and other spirochetes has been documented recently (27–31). From the work by Rittig and co-workers it is clear that morphologically similar spirochetes can induce different frequencies of coiling phagocytosis (31). The frequency of coiling phagocytosis of different viable or killed high- and low-passage strains of *B. burgdorferi* sensu strictu, *B. garinii* and *B. afzelii*, were reported to be within the same range of 40–60%. Different strains of *Treponema* and *Leptospira* as well as relapsing fever *Borrelia* displayed a much lower frequency of coiling phagocytosis (from 30% to <1%).

As a first visible response to *B. burgdorferi*, we

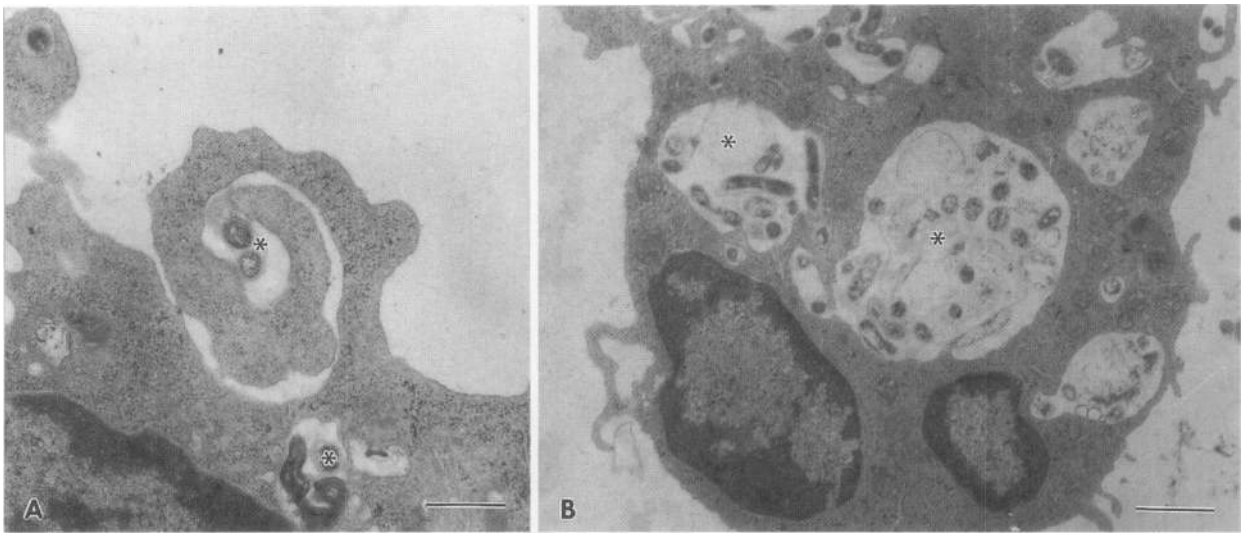

Fig. 5. Interaction of *B. burgdorferi* with human peripheral blood mononuclear cells. Human polymorphonuclear leukocytes and isolated peripheral blood fibrocytes were incubated with *B. burgdorferi* as described in Figures 2 and 3. (A) Coiling phagocytosis of peripheral blood mononuclear cells incubated with *B. burgdorferi* JD1 for 2 hr. Two spirochetes (asterisks) are enveloped by a coiling pseudopodial cell extension. Bar = 500 nm. (B) Mononuclear cell with large phagocytic vacuoles (asterisks) containing spirochetes. Bar = 2 μm.

observed that normally elongated or spindle-shaped fibrocytes (Fig. 2D) become round (Figs. 2A–C, 3A). This morphologic change may occur for the fibrocyte to recruit sufficient plasma membrane to effect spirochete uptake. Indeed, it has been shown by scanning electron microscopy that peripheral blood fibrocytes display from the cell surface projections that are intermediate in size between pseudopodia and microvilli (15) (see also Fig. 3B). We believe that this is a specific response to the presence of spirochetes for the following reasons. The change in fibrocyte cell shape was not induced by incubating the cells at 34°C in the tri-gas mixture favored by the spirochetes, nor by addition of BSK-H medium. The binding itself is polarized to one side of the cell (Fig. 3A). Whether this is an induced receptor capping phenomenon or it has another molecular basis remains to be investigated.

Once binding has occurred, the spirochetes are not internalized into the fibrocyte cytoplasm but are first surrounded by slender, pseudopod-like extensions of the cell (Figs. 3B and 4A). Recently, the term "tube phagocytosis" was introduced to describe how *B. burgdorferi* invade neutrophils (32). We also see 'tube-like' structures extruding from fibrocytes and containing *B. burgdorferi* cells (Fig. 4B). These processes are formed on that side of the cell where spirochetes are bound (cf. Fig. 3A, the right side of the cell with the spirochete-free left side of the pictured cell). We conclude that these structures are a cellular response to *B. burgdorferi* spirochetes, perhaps as a consequence of receptor capping. The *Borelliae* are then enveloped into recesses of the cell membrane projecting deep into the cytoplasm (Fig. 3C, D, E). These recesses actually form an anastomosing coiled and twisted network within the fibrocyte with *B. burgdorferi* cells interspersed (Fig. 3C, E). Occasionally we found profiles that resemble to some degree classical coiling phagocytosis (Fig. 3B, asterisk), but these are probably accidental configurations. Further studies using other *B. burgdorferi* strains will determine the universality of spirochete entry into fibrocytes.

When incubated with a human peripheral blood mononuclear cell preparation, we find many profiles of phagocytic cells where the *Borreliae* are internalized by coiling phagocytosis, as illustrated in Figure 5A. Following this process, they are taken up into large intracellular vacuoles (Fig. 5B). This confirms the significant findings by Rittig and co-workers (28–31) and illustrates the difference between *B. burgdorferi* uptake into leukocytes and into fibrocytes.

In conclusion, we found that *B. burgdorferi* binds to fibrocytes and resides within deep in-

vaginations on the cell surface. It has been reported that fibrocytes display prominent cell surface projections, intermediate in size between microvilli and pseudopodia (15). Because of the spirochetes' corkscrew movement, it is possible that the spirochetes wrap themselves up within these membrane projections without actually being endocytosed. This kind of "internalization" within the peripheral blood fibrocyte may protect *B. burgdorferi* not only from the host immune system but also from the fibrocyte's lysosomal-digestive system. It recently has been shown that *B. burgdorferi* envelope themselves with layers of lymphocyte membrane as they exit some spirochete-infected lymphocytes and that this membrane-cloaking mechanism may protect the spirochete from humoral and cellular recognition (26). The process by which *B. burgdorferi* can exit the peripheral blood fibrocyte remains to be determined. We hypothesize that the fibrocytes carry the spirochetes to the connective tissues. With respect to the tick vector, it is possible that spirochete-infected fibrocytes have the ability to migrate to the site of tick attachment and infect the arthropod vector. The observation that under the influence of certain physiological signals, fibrocytes also possess the functional capacity to recruit, activate, and present antigen to $CD4^+$ T cells (23) suggests that perhaps other modes of spirochete entry into these cells are possible. Finally, our findings, in combination with these recent discoveries, suggest that peripheral blood fibrocytes have the potential to play an important role in the immunopathology of Lyme arthritis.

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