Adherence and entry of *Borrelia burgdorferi* in Vero cells

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Summary. Adherence to and entry of the parasite into the host is one of the essential elements of microbial pathogenicity. We investigated the adherence to and entry into primate kidney epithelial (Vero) cells of *Borrelia burgdorferi* by radiolabelling techniques, immunofluorescence and electronmicroscopy. The attachment to and subsequent entry of both untreated and heat (50°C)-treated *B. burgdorferi* into Vero cells occurred at cell-surface sites associated with aggregated coated pits. In contrast, there was minimal attachment of spirochaetes heated at 60°C. Radiometric studies showed that, with untreated cells, there was incorporation of both ¹⁴C-glucose-1-phosphate and ¹⁴C-thymidine, whereas with the 50°C-treated spirochaetes only glucose-1-phosphate was incorporated, and with the 60°C-treated spirochaetes neither radionuclide was incorporated. Spirochaetes heated at 50°C or 60°C did not grow at 35°C in culture medium. These results suggest that the presence of certain metabolic activities of the spirochaete but not viability (ability to grow) are necessary for the attachment process. After entry of untreated *B. burgdorferi*, most of the spirochaetes were either free in the cytoplasm or tightly bound to the host membrane. In contrast, 50°C-treated spirochaetes remained bound to host membrane in large phagosome-like vesicles.

Introduction

The spirochaete *Borrelia burgdorferi*,¹ the Lyme disease agent, is transmitted primarily by ticks of the genus *Ixodes*. The inflammatory disease is characterised by a wide spectrum of clinical manifestations affecting multiple organ systems.²

A fundamental feature of the host-parasite relationship is the underlying mechanism by which pathogenic bacteria colonise living surfaces to initially gain a foothold at the potential site of infection. The entry of pathogenic organisms into host cells to evade the immune system allows persistence of the organism and is a feature of organisms that cause chronic infections.³ Pathogenic spirochaetes have been shown to adhere more efficiently than non-pathogenic spirochaetes to host cells.^{4,5} Several reports describe the *in vitro* adherence of *B. burgdorferi* to various mammalian cells of the immune system,^{6–8} non-immune cell lines,^{9–11} and tick-tissue cell cultures.¹² Although it has been shown recently that the spirochaete appears to pass through cultured cells within a host-derived membrane,¹³ there is little information about the initial events of the cell-spirochaete interaction.

We have recently established co-cultivation conditions that allow *B. burgdorferi* to grow and attach to mammalian cells lines.¹⁰ In this study, we report the

early events seen when *B. burgdorferi* spirochaetes interact with and enter Vero cells. We used Vero cells because they are kidney epithelium cells derived from primates and it has been shown that the Lyme spirochaete¹⁴ and associated antigens¹⁵ are readily isolated from or detected in renal tissue and urine of infected animals.

Materials and methods

Growth and treatment of *B. burgdorferi*

B. burgdorferi strain B31 (ATCC 35210) was used. The infectious nature of this strain was demonstrated by its recovery from blood of 3-day-old Wistar rats 48 h after inoculation. For the attachment experiments, *B. burgdorferi* was grown in BSK II medium¹⁶ at 35°C for 2–3 days. The suspension was then centrifuged at 5000 *g* and the pellet was washed in BSK II twice by centrifugation. The spirochaetes were resuspended in BSK II to the original suspension volume and divided into three aliquots; one was left untreated and two were heat treated. Minimal conditions for heat inactivation were found to consist of treating the spirochaetal suspension at 50°C for 20 min in a water bath. The spirochaetes were also inactivated by heating at 60°C for 1 h. Each spirochaetal suspension was then washed and resuspended in BSK II medium as above.

Radiolabelling of *B. burgdorferi*

(i) For quantitative measurements of *B. burgdorferi* attachment, the spirochaetes were intrinsically radiolabelled by incubation in BSK II containing ^{14}C -N-acetyl glucosamine $4.17\ \mu\text{Ci/ml}$ (specific activity, $0.36\ \mu\text{Ci}/\mu\text{mol}$; ICN Biomedicals, Inc., Costa Mesa, CA, USA) at 35°C for 3 days. The labelled spirochaetes were then washed three times in BSK II, resuspended and treated as described above.

(ii) For the metabolic studies, treated and untreated spirochaetes ($4 \times 10^7/\text{ml}$) were also intrinsically radiolabelled by incubation in BSK II containing ^{14}C -thymidine $2.27\ \mu\text{Ci/ml}$ (specific activity $48\ \mu\text{Ci}/\mu\text{mol}$), or ^{14}C -glucose-1-phosphate $2.27\ \mu\text{Ci/ml}$ (specific activity, $294\ \mu\text{Ci}/\mu\text{mol}$). Triplicate samples of $50\ \mu\text{l}$ each were removed after 0, 6 and 26 h and pipetted on to 3-mm filter-paper disks (Whatman International Ltd, Maidstone). Excess unincorporated radiolabels were removed by washing with trichloroacetic acid (TCA)/ethanol/ether¹⁷ and the radioactivity (disintegration/min, dpm) of the TCA precipitate on the disk was counted in a Beckman LS 250 liquid scintillation system.

Vero cells

Vero cells (ATCC CCL 81, obtained from the American Type Culture Collection) were propagated in plastic flasks containing antibiotic-free Eagle's Minimal Essential Medium with fetal calf serum 5% (MEM) at 35°C for 2 days. Before the attachment experiments, the MEM was decanted and the Vero cells were washed twice in BSK II. Viability of Vero cells was measured before and after each experiment by trypan blue exclusion.

Immune sera

Lyophilised spirochaetes (1 mg dry weight), prepared as previously described,²⁰ were suspended in aluminium hydroxide adjuvant and injected intramuscularly (i.m.) into rabbits. This was repeated twice at weekly intervals without adjuvant. The rabbits were then given Ketaset (Veterinary Products, Bristol Laboratories, Syracuse, NY) by i.m. injection and bled out by cardiac puncture. The enzyme-linked immunoassay²⁰ (ELISA) and micro-immunofluorescence²¹ tests (micro-IF) were performed as described previously. The rabbit immune response to the antigens of *B. burgdorferi* was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.¹⁰

Attachment of *B. burgdorferi*

Attachment of *B. burgdorferi* to Vero cells was studied as described previously.¹⁰ Briefly, 2×10^8 spirochaetes (untreated or heat-treated) were co-incubated with Vero cells in BSK II medium at 35°C . Spirochaete-Vero cell samples were taken after 0, 6, 24 and 44 h. The media were decanted and the flasks

were washed twice with BSK II medium to remove non-adherent spirochaetes. Spirochaete-Vero cell combinations on cover slips were processed for immunofluorescent light microscopy or for radiometric quantitation, and those in flasks were processed for transmission electron microscopy.

Radiometric quantitative measurements. The spirochaetes were counted by dark field microscopy as described previously.¹⁸ Briefly, the surface area of the observed microscopic field of a $40\times$ objective was determined with a counting chamber with a Neubauer ruling. The surface area of the microscopic field was calculated at $9.36 \times 10^{-2}\ \text{mm}^2$ and the surface area of the cover slips used in the counting of spirochaetes was $484\ \text{mm}^2$. A factor was then derived by dividing the area of the cover slip by the area of microscopic field, $484\ \text{mm}^2/9.36 \times 10^{-2}\ \text{mm}^2 = 5171$. To count unattached spirochaetes, $5\ \mu\text{l}$ of diluted growth medium containing spirochaetes (9–16/field) was placed on a microscope slide. A cover slip of known surface area was placed on the $5\text{-}\mu\text{l}$ drop and gently pressed to evenly spread the drop. The spirochaetes present in 20 microscopic fields from several areas were counted and the number of spirochaetes/ μl was then derived from the following formula:

$$\frac{\text{Number of spirochaetes in 20 fields}}{20 \times 5\ \mu\text{l}} \times \frac{1}{\text{dilution}} \times 5171$$

The total radioactivity of unattached spirochaete suspensions at the various sampling times was also determined. Spirochaete samples were pelleted by centrifugation, resuspended in phosphate-buffered saline (PBS; pH 7.2) to their original volume and the radioactivity was measured. The specific radioactivity/spirochaete (dpm/spirochaete) was then determined for each sample.

To quantitate Vero cells, cells grown on cover slips for 0 and 24 h were trypsinised¹⁰ and suspended in PBS. Vero cell suspensions were counted with a haemocytometer. The average cell count per cover slip was 4.40×10^5 (SD 1.09×10^5) Vero cells. Cell counts at all sampling times were not significantly different throughout the experiments (Student's *t* test,¹⁹ $p > 0.05$).

At each sampling time, the radioactivity of each of three cover slips was counted and the radioactivity was averaged. The number of spirochaetes was then derived by dividing the radioactivity by the specific activity (dpm/spirochaete) for each sample. Unless stated otherwise, the radioactive and cell counts of attached spirochaetes are expressed/ 4.40×10^5 cells.

Immunofluorescent microscopy. The cover slips were edge-blotted to drain excess medium and then washed gently for 15 s with PBS and air dried. Cells were fixed to cover slips with acetone for 10 min, air dried, and then incubated with rabbit immune serum to *B. burgdorferi*, diluted 1 in 400, at 37°C for 30 min. After washing three times in PBS, cover slips were incubated with FITC-goat anti-rabbit globulins for 30 min at 37°C . Cover slips were washed again as above,

Fig. 1. Immunofluorescent microscopy of untreated and heat-treated *B. burgdorferi* attached to Vero cells at various times; $\times 840$.

Table. Effect of heat treatments on *B. burgdorferi* uptake of ^{14}C thymidine and ^{14}C glucose-1-phosphate

Treatment	Number of growth doublings in 26 h	Uptake (DPM*/50 μl)	
		^{14}C thymidine	^{14}C glucose-1-phosphate
Untreated	2.5–3	774	256
50°C; 20 min	0†	11	335
60°C; 1 h	0†	0	13

* Net DPM after subtracting the radioactivity of 0 time. Average counts of triplicate aliquots.

† No change in total number.

counterstained with Evans blue 1% for 5 min, washed twice in PBS followed by distilled water, and air dried. The cover slips were mounted in glycerol-PBS on glass slides with the spirochaete-Vero cells side facing down.

Transmission electronmicroscopy. The spirochaete-Vero cell monolayers were fixed with formaldehyde 4% and glutaraldehyde 1% in phosphate buffer, pH 7.2, for 1 h, gently scraped off the plastic flasks, washed in 0.1 M cacodylate buffer, pH 7.2, and post-fixed with osmium tetroxide 1% in 0.1 M cacodylate. After washing in buffer, the material was centrifuged, encapsulated in agar 2%, dehydrated in a graded ethanol series, and embedded in Epon-Araldite resin. Thin sections were stained with uranyl and lead salts.

To quantitate the number of Vero cells with spirochaetes, 0.25- μm thick cell sections at each sampling were examined by electronmicroscopy and 200 cell profiles for each were counted. The results

Fig. 2. Radiometric quantitation (mean, ISD) of adherence of spirochaetes to Vero cells: untreated spirochaetes (●); 50°C-treated spirochaetes (○).

Fig. 3. Percentage of Vero cells ($n=200$) with (▨) and without (□) attached spirochaetes, and with internalised spirochaetes (▤) determined by transmission electronmicroscopy. Vero cells with both internalised and attached spirochaetes were scored as internalised.

were scored as follows: no spirochaetes seen (no spirochaetes were seen in association with the Vero cell); spirochaetes attached (if spirochaetes were seen attached to or penetrating the Vero cell); spirochaetes internalised (if a spirochaete was totally within the Vero cell profile, whether or not additional spirochaetes were attached or penetrating the cell surface). We noted also whether or not coated pits and vesicles or their aggregates could be seen at the site of spirochaete attachment or penetration.

Results

Growth of *B. burgdorferi* in cell culture

Examination of the growth of untreated *B. burgdorferi* in the Vero-cell cultures by dark-field microscopy indicated that the spirochaete had grown well. The generation time of the spirochaete co-cultivated with Vero cells during the log phase (2–8 h) was 3.87 h. This is in contrast to the generation time of 12 h when

B. burgdorferi was cultured in BSK II alone.¹⁶ Both free and cell-attached spirochaetes were motile. They did not appear to elicit a significant cytopathic effect on Vero cells (only 5% of the cells were permeable to trypan blue). The monolayer remained intact throughout the 44 h of co-incubation.

Immune sera

The ELISA and micro-IF anti-*B. burgdorferi* titres with the *B. burgdorferi* antigens were 20, 480 and 1024 respectively. The immune response was against a minimum of 31 bands, including flagellar, Osp A, and Osp B antigens.

Heat treatment of *B. burgdorferi*

The minimum conditions for loss of viability (determined by failure of growth) of *B. burgdorferi*

were 50°C for 20 min. Loss of viability was assessed by (i) loss of motility when examined by darkfield microscopy; (ii) failure of growth of spirochaetes after incubation in BSK II for 48 h, as indicated by the static optical density; and (iii) the effect on the uptake of radionuclides (table). There was incorporation of radiolabelled thymidine only in the control, untreated spirochaetes, whereas radiolabelled glucose-1-phosphate was incorporated in both untreated and 50°C-treated spirochaetes, but not in spirochaetes heated at 60°C for 1 h.

Attachment of *B. burgdorferi*

Immunofluorescence microscopy. Untreated and 50°C-treated spirochaete suspensions attached to Vero cells (fig. 1) and the degree of specific fluorescence due to spirochaetes attachment increased with the length

Fig. 4. Attachment of *B. burgdorferi* to Vero cells. Coated pits and vesicles (►) are seen near the attachment site of the spirochaete to the Vero cell (A). Attachment was not limited to the apical tips of the spirochaetes, as spirochaetes were often seen attached laterally to the Vero cell membrane which appeared to be a fusion of several coated pits (B, ►); these structures surrounded the spirochaete (C, stereo pair); electron-dense material between the Lyme spirochaete and the coated-pit regions of the Vero cell was frequently seen (D, ►).

of incubation. Attachment of 60°C-treated spirochaetes was minimal.

Quantitative measurements. As a result of growth of untreated radiolabelled spirochaetes during co-incubation with Vero cells, the specific activity decreased with incubation time from 1.93×10^{-4} dpm/spirochaete at 0 h to 2.63×10^{-5} dpm/spirochaete at 44 h. In contrast, the specific activity in suspensions of the 50°C-treated spirochaetes remained constant, further indicating that there was no growth. The average specific activity of eight samples of 50°C-treated spirochaetes was 1.78×10^{-4} dpm/spirochaete (SD 3.8×10^{-5}); the specific activities of the 50°C-treated spirochaetes at all sampling times were not significantly different from the zero-time sampling ($p > 0.05$, Student's *t* test). The radiometric measurements of adherence (fig. 2) to the Vero cells of untreated and 50°C-treated spirochaetes were at background level

for the first hour, then increased logarithmically (as indicated by the increase in radioactivity) for the next 8 h. The increase in attachment of the untreated spirochaetes continued but at a lower rate for the duration of the experiment. In contrast, the attachment of the 50°C-treated spirochaetes appeared to level off after 20 h. The radioactivity counts with 60°C-treated spirochaetes remained at background level.

Transmission electronmicroscopy. To elucidate the relationship between spirochaetes and Vero cells, all samples were examined by thin (70 nm) and thick (250 nm) partial serial sections. In samples taken at 9 h or earlier, electronmicroscopy results showed few Vero cells with associated spirochaetes (<1.5% for all samples). At 24–48 h, in experiments with untreated or 50°C-treated spirochaetes, most Vero cells had spirochaetes either attached at their surface or

Fig. 5. Engulfment of *B. burgdorferi* by Vero cells. After attachment at coated pits of the Vero cell (A, $\blacktriangleright$), the spirochaete is progressively engulfed by the Vero cell membrane, which appeared to consist of several merged coated pits and vesicles (B–E, $\blacktriangleright$); a tangential section through this region revealed the typical subunit structure associated with clathrin-containing coated vesicles (C, $\rightarrow$). Once inside the cell, it was difficult to determine whether the host membrane remained tightly bound to the spirochaete or if the spirochaete was free in the cytoplasm (F).

internalised; there was no attachment of 60°C-treated spirochaetes (fig. 3). Numerous coated pits and vesicles (average diameter 120–150 nm, fig. 4A) and aggregates of these were seen in association with the attached or penetrating spirochaetes (56% of profiles seen with Vero cells with untreated spirochaetes, and 70% of profiles with the 50°C-treated spirochaetes). The interaction of *B. burgdorferi* at the Vero cell surface was not limited to the apical region of the spirochaete. We often observed long, 800–1000 nm, fused aggregates of coated vesicles along the outer surface of the Vero cell coincident with the lateral bacterial surface (fig. 4B). During engulfment of a spirochaete, the aggregates of coated pits surrounded the bacterium (fig. 4C, the stereo pair) as it entered the cell; in cross-sectional views, these aggregates had a diameter of 250–300 nm and fine fibrous material was seen frequently extending between them and the spirochaete (fig. 4D). This material did not stain with ruthenium red (not shown).

Fig. 5 is a montage of micrographs that illustrate and summarise our ultrastructural observations associated with the penetration of *B. burgdorferi* into Vero cells. During the initial phases of internalisation, we could clearly observe the host membrane surrounding the spirochaete (fig. 5E), but at later stages, within the cell cytoplasm, it was difficult to ascertain whether the spirochaete was free or tightly bound to the host membrane (figs. 5F, 7A).

Similar results were encountered with 50°C-treated spirochaetes during the initial attachment and engulfment processes. The spirochaetes were associated with Vero cell coated vesicles (fig. 6A) and the internalisa-

tion steps appeared the same (not shown). However, in contrast to the untreated *B. burgdorferi*, the engulfed 50°C-treated spirochaetes remained surrounded by a sac-like, host-derived membrane structure containing non-electron-dense regions (figs. 6B, 7B).

Discussion

B. burgdorferi is sensitive to antibiotics *in vitro* and the disease can be treated successfully during the early stages. However, during the later stages, the disease has proved difficult to treat. The presence of the spirochaete in the host at this stage has not been established. If present, it appears to evade the host immune system. One of several hypotheses proposed is that the spirochaete may have an intracellular phase where it is protected from antibiotics and from host defence mechanisms. As a preliminary step to test this hypothesis, we have investigated, in an *in vitro* model, the initial interaction between *B. burgdorferi* and the host eukaryotic cell. We have shown previously¹⁰ that, while the untreated spirochaete adhered to a number of eukaryotic cell lines, it did not adhere preferentially (qualitatively or quantitatively) to any given cell line. In the present investigation, we monitored the adherence of untreated and heat-treated *B. burgdorferi* to Vero cells.

Receptor-mediated endocytosis (RME), a pathway by which extracellular macromolecules^{22–27} and particles, e.g., viruses,²⁵ gain entrance to the intracellular milieu, has recently received much attention. Ligands

Fig. 6. Attachment of 50°C-heated *B. burgdorferi* to Vero cells. Although heat-inactivated, the spirochaete appeared to attach to Vero cells at regions of coated pits and vesicles (A). Internalised 50°C-treated spirochaetes were loosely surrounded by a distinct host membrane within structures that contained non-electron-dense material (B).

appear to bind to the receptor cell surface within specialised regions of the plasma membrane ("coated pits") of the eukaryote host cells.²⁷ These coated pits are mainly composed of a single protein, clathrin.²⁶ Several hypotheses have also been developed to describe mechanisms whereby prokaryotes enter and exit cells. Finlay *et al.*²⁸ used the term "transcytosis", originally described as a form of RME by which macromolecules are transported through cells,^{29,30} to describe how *Salmonella choleraesuis*, a free-living bacterium with an intracellular phase, passes through

a monolayer of kidney cells. However, they did not demonstrate any morphological evidence of coated pits or vesicles. Another hypothesis, "parasite-directed endocytosis", has recently been described by McGee *et al.*³¹ to characterise the successful invasion of host cells by a number of human pathogens. They suggested that this endocytosis is mediated directly by the organisms or by their products, the latter indicating that organisms need not be viable to enter the host cell. Transmission electronmicroscopy evidence was presented suggesting the participation of clathrin-

Fig. 7. Thick sections (250 nm) of untreated (A) and 50°C-treated (B) *B. burgdorferi* within Vero cells. It was not possible to determine if the untreated spirochaete was tightly bound by host membrane or was free in the Vero cell cytoplasm. In contrast, 50°C-treated spirochaetes consistently appeared within host-derived membranes.

coated membranes during the endocytosis of an obligate intracellular parasite, *Chlamydia trachomatis*, cultured in McCoy cells. Recently, Clerc and Sansonetti³² have shown, by potassium ion depletion studies, evidence for clathrin mobilisation during directed phagocytosis of *Shigella flexneri* by HEp2 cells; morphological evidence for clathrin coated pits was, however, not presented.³² Our transmission electronmicroscopy results show the presence of "clathrin-like" coated pits and vesicles at the attachment of *B. burgdorferi* on cell-surface sites of the Vero cells. The coated pits were still observed around the spirochaetes soon after their engulfment by the host cells, although they were not observed in the later stages. Presumably, they were recycled to the region of the plasma membrane.²⁷

Adherence of *B. burgdorferi* was not limited to the tips of the cells, as we often found specific attachment mediated by what appeared to be clathrin-coated membranes in association with lateral profiles of *B. burgdorferi* cells (fig. 4B). The lateral attachment was similar to results recently reported³³ for *Treponema pallidum*, although, in this latter study, no coated pits were observed. This contrasts with previous reports^{4,5,34} on *T. pallidum* adherence, which indicated only polar attachment of this spirochaete to the host cell.

We observed no differences in the early steps of attachment and the endocytic process between untreated or 50°C-treated spirochaetes presented to Vero cells; however, the 60°C-treated spirochaetes did not

bind. This observation is similar to other bacterial attachment patterns where viability or integrity of the bacteria or integrity of component(s) that mediate attachment is essential.³⁵⁻³⁷ As indicated by our studies, it is possible that the milder, 50°C treatment was not harsh enough to destroy the integrity of the spirochaete, or the spirochaetal ligand or metabolic product that initiates binding. We have obtained similar attachment results with ultra violet-treated spirochaetes (data not shown). As with 50°C treatment, ultraviolet treatment affected ¹⁴C thymidine but not ¹⁴C glucose-1-phosphate incorporation into spirochaetes, further indicating the significance of the "integrity of certain metabolic activities" of the organism for adherence and entry.

Preliminary tests indicated that the fate of the enclosed spirochaetes may depend upon their viability. Untreated spirochaetes appeared to be either "enclosed" by a close-fitting host membrane or "free" in the cytoplasm. In contrast, the 50°C-treated spirochaetes invariably appeared in large vacuoles, filled with non-electron-dense material, that resembled degraded lysosomes.

In summary, *B. burgdorferi* attached to coated pits on the plasma membrane and then penetrated into the host while enveloped by the host membrane. Biochemical studies are now being undertaken to determine whether the coated pits apparently involved in the adherence process contain clathrin. The fate of the intracellular untreated spirochetes is also being investigated.

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