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Invasion and Cytopathic Killing of Human Lymphocytes by Spirochetes Causing Lyme Disease

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Lyme disease is a persistent low-density spirochetosis caused by *Borrelia burgdorferi sensu lato*. Although spirochetes causing Lyme disease are highly immunogenic in experimental models, the onset of specific antibody responses to infection is often delayed or undetectable in some patients. The properties and mechanisms mediating such immune avoidance remain obscure. To examine the nature and consequences of interactions between Lyme disease spirochetes and immune effector cells, we coincubated *B. burgdorferi* with primary and cultured human leukocytes. We found that *B. burgdorferi* actively attaches to, invades, and kills human B and T lymphocytes. Significant killing began within 1 hour of mixing. Cytopathic effects varied with respect to host cell lineage and the species, viability, and degree of attenuation of the spirochetes. Both spirochetal virulence and lymphocytic susceptibility could be phenotypically selected, thus indicating that both bacterial and host cell factors contribute to such interactions. These results suggest that invasion and lysis of lymphocytes may constitute previously unrecognized factors in Lyme disease and bacterial pathogenesis.

Following its discovery as the agent of Lyme disease [1], *Borrelia burgdorferi sensu lato* was subdivided into several genospecies, including the known human pathogens *B. burgdorferi sensu stricto* (hereinafter *B. burgdorferi*), *Borrelia garinii,* and *Borrelia afzelii* (formerly group VS461) [2, 3]. Human infection by these tick-borne agents progresses slowly from localized dermatologic involvement to a persistent low-density multisystemic spirochetosis. Because spirochetes causing Lyme disease can establish chronic infections in otherwise healthy and immunocompetent individuals, many investigators believe that these bacteria can occupy immune-privileged niches or otherwise modify and evade immune responses. Although the mechanisms of immune evasion and modifications are not fully understood, interactions between *B. burgdorferi* and several types of mammalian cells and factors have been described.

Previous studies have shown that *B. burgdorferi* can attach to a variety of mammalian cells [4–6], invade fibroblasts [4], bind host proteins onto their surfaces [7–10], and alter secretion of host cytokines [11–14] and antibodies [15]. Invasion of fibroblasts is relatively benign, involving intracellular penetration and possible long-term intracellular survival [4]. Invasion of endothelial cells with nonlytic escape was reported [6], but this finding was later disputed [7]. It is believed that the intracellular survival of *B. burgdorferi* may function in chemotherapeutic resistance and interference with immune clearance [4, 6, 16].

In a recent study by Schwan and co-workers [17], it was also reported that antigenic phase changes involving surface-exposed lipoproteins may occur. Using infected ticks, these researchers found that upregulation of the expression of *B. burgdorferi* outer-surface protein C occurred while ticks fed on mammalian blood. Temperature was believed to mediate this change. Furthermore, they reported a concurrent decrease in the apparent expression of the known protective immunogen outer-surface protein A on spirochetal surfaces.

Cell-surface binding of host proteins such as naive IgM antibodies [8], urokinase, and fibrinogen [9, 10] may also interfere with immune clearance by providing immunologic "camouflage" and by facilitating migration of the spirochetes through interstitial spaces. Induction of inflammatory cytokines [11–13] and mitogenesis of polyclonal B cells [14, 15] suggest that Lyme disease spirochetes can manipulate and modify the immune response to infection. However, current understanding of direct interactions between spirochetes and lymphocytes is limited. In this study, we examined interactions between *Borrelia* species and human leukocytes and assessed cytopathic effects. We found that *B. burgdorferi* and *B. garinii* specifically attached to, invaded, and killed significant proportions of human B and T cells.

Methods

Unless otherwise specified, all reagents were obtained from the Sigma Chemical Company in St. Louis. The spirochetes and eukaryotic cells used in this study are described in table 1. All spirochetes were cultured in modified Barbour-Stoenner-Kelly medium as previously described [18]. Human cell lines were obtained from the American Type Culture Collection

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Table 1. *Borrelia* species and eukaryotic cells used in a study on the interactions and cytopathic effects.

Borrelia species or cells (description)	Strain or cell line (description)	Source	No. of passages	Infectious in mice
B. burgdorferi (low-passage)	Sh-2-82	Tom Schwan, Rocky Mountain Laboratories, Hamilton, MT	4–7	Yes
B. burgdorferi (high-passage)	Sh-2-82	Tom Schwan, Rocky Mountain Laboratories, Hamilton, MT	>30	No
B. burgdorferi (enriched)	Sh-2-82	This study	3	ND
B. hermsii	MAN	Tom Schwan, Rocky Mountain Laboratories, Hamilton, MT	<8	Yes
B. garinii	IP90	Alan Barbour, University of Texas, San Antonio, TX	5	Yes
B. afzelii	ACA1	Alan Barbour, University of Texas, San Antonio, TX	2	Yes
B cells	SKW 6.4	ATCC (Rockville, MD)	>100	NA
B cells (enriched)	SKW 6.4	This study	3–4	NA
B cells ($CD19^+$)	Primary human	Volunteers, this study	0	NA
T cells	H9	ATCC (Rockville, MD)	>100	NA
T cells ($CD4^+$ or $CD8^+$)	Primary human	Volunteers, this study	0	NA
Mononuclear cells	Primary human	Volunteers, this study	0	NA

NOTE. For this study, low and high passages were defined as less than eight and more than 30 in vitro passages, respectively. Spirochetal isolates were not cloned for these experiments. Enriched *B. burgdorferi* refers to spirochetes that were recovered in association with SKW 6.4 cells after three sequential cycles of a 90-minute coincubation period and differential centrifugation. Enriched B cells were lymphocytes selected by survival after three sequential 24-hour coincubation periods with a 100-fold excess of low-passage *B. burgdorferi* spirochetes. ATCC = American Type Culture Collection; NA = not applicable; ND = not determined.

(Rockville, MD) and propagated according to their instructions. Media for tissue cultures and certified fetal bovine serum were provided by Life Technologies (Gaithersburg, MD). Primary human mononuclear cells were obtained from laboratory volunteers and prepared by centrifugation in lymphocyte separation medium (Organon Teknika, Durham, NC) according to the manufacturer's recommendations. Primary B or T cells were purified from mononuclear cell preparations by using anti B cell (CD19) or anti T cell (CD4 and CD8) immunomagnetic beads (Dynal, A. S., Oslo), respectively. Eukaryotic cells were quantified by counting in Petroff-Hausser counting chambers. The number of spirochetes was estimated by means of absorbance at 600 nm as previously described [19].

Coincubation mixtures typically contained 2×10^6 host cells and 2×10^8 spirochetes per milliliter of medium. Experimental parameters that were varied included the species, concentration, and in vitro passage number of spirochetes; the coincubation period; and the number of sequential "reinfections" with a single lymphocytic population or a single spirochetal population. Low and high passages were defined as less than eight or more than 30 in vitro passages, respectively.

For some experiments, B cells resistant to attachment and killing by Lyme disease spirochetes were enriched by mixing with fresh low-passage *B. burgdorferi* every 24 hours for three consecutive days. Similarly, spirochetes with affinity for B cells were enriched by using differential centrifugation to recover B cells from coincubation mixtures maintained for 1.5 hours. Cell pellets containing cell-associated spirochetes were washed in and finally resuspended in Barbour-Stoenner-Kelly medium. Host cell viability was assessed microscopically by trypan blue exclusion or by flow cytometric analysis with use of propidium iodide staining [20].

Attachment and invasion by spirochetes were monitored by light and electron microscopy. Light microscopy was performed on wet mounts by using a Nikon FXA photomicroscope equipped with a Nomarski differential interference contrast condenser (Nikon, Tokyo). Digital micrographs were recorded by means of a Dage-MTI CCD 72 camera and a DSP2000 image processor (Dage-MTI, Michigan City, IN).

For scanning electron microscopy, lymphocytes, bacteria, or coincubation mixtures were concentrated by centrifugation at 1,000*g* for 3 minutes in a microfuge. Pellets were gently resuspended and washed once in Tyrode's buffer (pH, 7.2) [21], and 70 μL of cell suspension was allowed to settle onto coverslips previously coated with 0.01% poly-L-lysine in water. After 5 minutes, much of the buffer was removed, and the cells were fixed in place by adding 50 μL of 2.5% glutaraldehyde in 0.2 *M* cacodylate (pH, 7.2). Such coverslips were processed further by standard procedures [22]. Samples were examined with a Hitachi S-4500 field emission scanning electron microscope (Hitachi, Tokyo) operated at 5 kV.

For transmission electron microscopy, equivalent samples were collected by centrifugation and washed as above. Cell pellets were fixed with 2.5% glutaraldehyde and 4% *p*-formaldehyde in 0.1 *M* cacodylate (pH, 7.2) containing 0.1 *M* sucrose. Pellets were postfixed in 1% OsO_4, dehydrated, embedded in Spurr's resin, and prepared for thin sectioning according to standard procedures [23]. Sections were examined with a Philips CM10 transmission electron microscope (Philips Electron Optics, Eindhoven, The Netherlands) operated at 80 kV.

Host cells and coincubation mixtures were prepared for fluorescence-activated cell sorting according to previously reported procedures [20]. Primary leukocyte lineage was determined by flow cytometric analysis of mixed mononuclear cells

labeled with fluorescein isothiocyanate or phycoerythrin conjugates of monoclonal antibodies to CD19 (pan B cells), CD5 (pan T cells and activated B cells), CD14 (monocytes), or CD15 (granulocytes) cell-surface markers according to the manufacturer's instructions (Immunotech, Westbrook, ME).

Results

To examine the interactions between Lyme disease spirochetes and primary and cultured human lymphocytes, *Borrelia* species and host cells were mixed at a multiplicity of infection of 100; the mixtures were incubated for varying periods, and changes to the bacteria and cells were assessed by light and electron microscopy (figure 1). The micrographs in figure 1 are representative of structures and events that were observed repeatedly in such preparations.

Adherence to and invasion of lymphocytes occurred within 1–2 hours of coincubation. As previously reported for other cell lines [4–7], light microscopy with use of a differential interference contrast condenser showed that low-passage *B. burgdorferi* strain Sh-2-82 attached (via the tips) to lymphocytic surfaces (figure 1a). Scanning electron microscopy showed adherent spirochetes on >90% of both cultured and primary B and T cells (data not shown). Host cell penetration appeared to occur at sites of endocytotic pits (figure 1b).

Spirochetes protruding into invaginations, consistent with endocytotic pits, were also observed by transmission electron microscopy of thin sections (data not shown). No structural perturbations were observed on adherent spirochetes. In contrast, evidence of surface penetration by low-passage *B. burgdorferi* corresponded with loss of filopodia and other surface projections on lymphocytes.

Transmission electron microscopy revealed intracellular spirochetes contained within vacuoles (figure 1c). No evidence of lysosomal fusion with such vacuoles was observed. Video microscopy demonstrated marked motility of spirochetes confined within vacuoles of invaded SKW 6.4 cells (data not shown). However, we found no spirochetes that were clearly free within the cytosol of intact cells. Numerous lymphocytes with disrupted cell membranes were evident in coincubation mixtures with low-passage *B. burgdorferi* (figure 1d). No significant cytopathic effects were observed with lymphocytes incubated with high-passage bacteria (data not shown) or uninfected lymphocytes (figure 1e).

The lineages of primary human mononuclear cells that were killed by *B. burgdorferi* were identified by flow cytometry. Averaging the findings for two independent experiments involving coincubation mixtures with low-passage *B. burgdorferi* and mixed mononuclear cells that were maintained for 2 hours showed that 53.0% of $CD19^+$ B cells and 21.0% of $CD5^+$ T cells were permeable by propidium iodide, compared with 8.3% and 2.8%, respectively, in uninfected mononuclear cell preparations. An average of 10.4% of $CD19^+$ B cells and 13.3% of $CD5^+$ T cells were permeable in coincubation mixtures with high-passage *B. burgdorferi*. Dual fluorescence between propidium iodide and either $CD14^+$ monocytes or $CD15^+$ granulocytes was not significantly greater than background levels observed in uninfected cells.

The kinetics of killing of purified primary lymphocytes, as assessed by trypan blue exclusion, are shown in figure 2. Primary B cells and T cells were fractionated by using either antibodies to CD19 immunomagnetic beads or pooled antibodies to both CD4 and CD8 immunomagnetic beads. Flow cytometry showed that >99% of cells in each B or T cell fraction expressed either CD19 or CD5 cell-surface markers, respectively (data not shown). Low-passage *B. burgdorferi* killed proportions of both classes of lymphocytes. Spirochetes appeared to kill B cells more rapidly than T cells. The percentages of dead lymphocytes in mixtures containing high-passage spirochetes were not significantly greater than those found in uninfected control lymphocytes.

The effects of coincubation on cultured SKW 6.4 (Burkitt's lymphoma) cell lines are shown in figure 3. Up to 40% of SKW 6.4 cells were lysed after coincubation with low-passage *B. burgdorferi*. Killing of lymphocytes peaked during the first day of coincubation. Killing was reduced at infection ratios of 10:1 and 1:1 and was insignificant at lower ratios (data not shown). No significant killing occurred in mixtures of H9 cells and low-passage spirochetes (not shown), mixtures of SKW 6.4 cells and high-passage *B. burgdorferi*, or mixtures of SKW 6.4 cells and low-passage *Borrelia hermsii*, an agent of tick-borne relapsing fever. Reduced killing at later times reflected continued growth by SKW 6.4 cells that survived the initial infection and exhibited significant resistance to subsequent reinfection (see below).

By sequentially reinfecting B cells with low-passage *B. burgdorferi* and, conversely, sequentially enriching for attached or intracellular spirochetes by differential centrifugation, we obtained populations of both SKW 6.4 cells that resisted attachment and killing and Lyme disease spirochetes that exhibited enhanced virulence against SKW 6.4 cells (figure 3, right). In coincubations of enriched spirochetes and enriched lymphocytes, the spirochetes appeared to overcome much of the resistance exhibited by lymphocytes.

The qualitative results of coincubation and killing by spirochetes are summarized in table 2. Only coincubations with low-passage or enriched Lyme disease spirochetes resulted in significant killing of lymphocytes. Although killing occurred with *B. burgdorferi* and *B. garinii* (IP90), killing was not observed in coincubations with either *B. hermsii* or an infectious isolate of *B. afzelii* (ACA1). It was also notable that primary T cells and H9 cells differed in terms of susceptibility. Preliminary experiments suggest that the rates of initial attachment to lymphocytes and subsequent killing of lymphocytes may correlate with the expression of a cell-surface integrin (D. W. Dorward and E. R. Fischer, unpublished data).

Discussion

Our results indicate that Lyme disease spirochetes can selectively attack and kill purified peripheral human lymphocytes

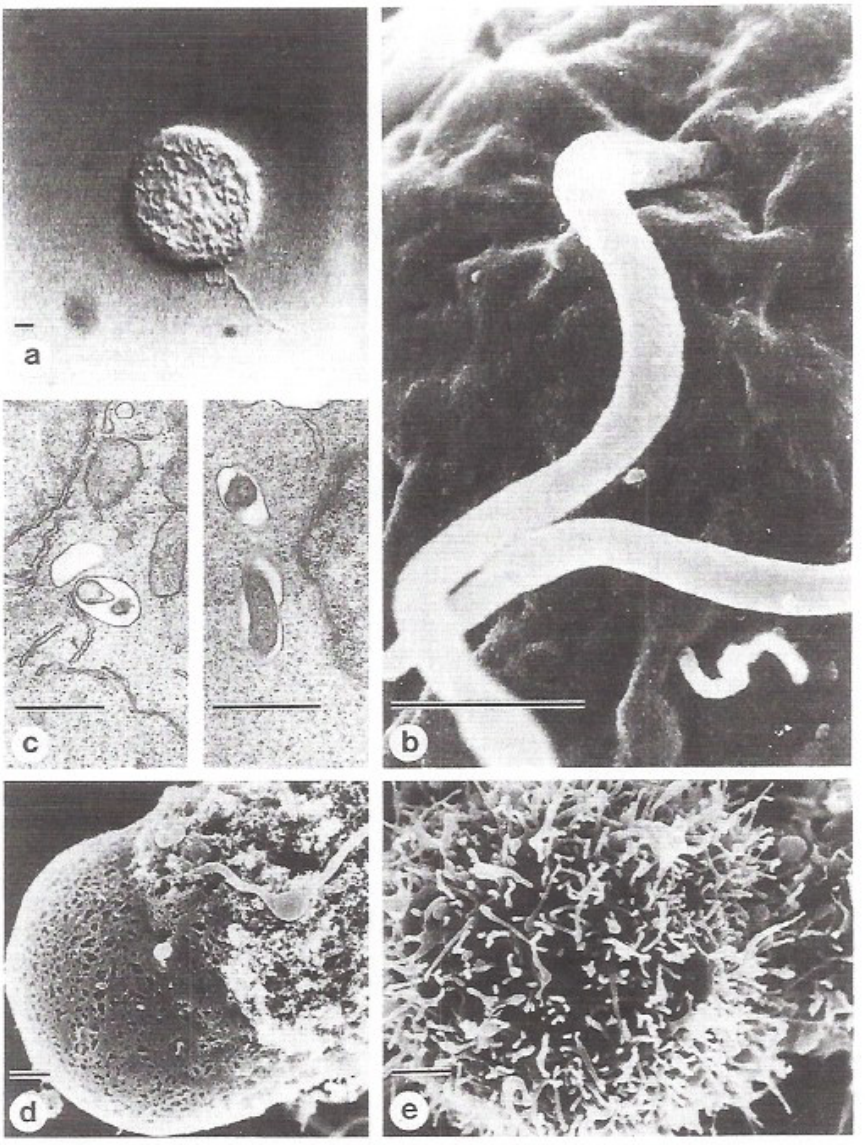

Figure 1. Micrographs revealing attachment to and invasion of lymphocytes by *Borrelia burgdorferi*. Cultured SKW 6.4 cells and primary human peripheral B cells were coincubated with virulent or attenuated *B. burgdorferi* for 1 hour; the mixtures were then prepared for and examined by light or electron microscopy. The micrographs are representative of each susceptible host cell population. *a:* Light microscopy revealed attachment of spirochetal tips to SKW 6.4 cells; attached spirochetes remained highly motile yet anchored to host cells. *b:* Scanning electron microscopy revealed that adherent spirochetes penetrated lymphocytes through endocytotic pits; penetrated lymphocytes exhibited a noticeable loss of filopodia and other surface projections. *c:* Transmission electron microscopy showed that intracellular spirochetes were retained within vacuoles; no fusion of lysosomes to endocytotic vacuoles was observed. *d:* Continued coincubation with virulent spirochetes resulted in numerous lymphocytes with disrupted cell membranes. *e:* No such cytopathic changes were observed with uninfected control lymphocytes. No such cytopathic changes were observed with lymphocytes incubated with *Borrelia hermsii* or attenuated *B. burgdorferi* (not shown). Bars = 1 μm.

and SKW 6.4 B cells. Although internalization of *Chlamydia trachomatis* into vacuoles by certain lymphocytic cell lines has been reported, chlamydiae neither killed nor grew within host lymphocytes [24]. We could find no previous reports of aggressive, invasive cytopathology in lymphocytes caused by bacteria.

Interactions between lymphocytes and *B. burgdorferi* involved tip-directed attachment. It was unclear whether attachment was solely initiated at the tip or whether sites of adherence on the spirochetes could migrate to the tips after attachment. Invasion progressed through endocytotic pits into vacuoles. Invaded lymphocytes exhibited dramatic morphological changes such as loss of surface projections and disruption of the cell membrane. Although supernatants from cultures of low-passage *B. burgdorferi* and heat-killed spirochetes do not appear to kill lymphocytes in vitro (D. W. Dorward and E. R. Fischer, unpublished data), it is unclear whether cellular invasion is a prerequisite for the cytopathology observed in this study. Furthermore, because of the severe cytopathic effects observed, we could not rule out the possibility that spirochetes may also penetrate host cell cytosol. Clearly, the dynamics and mechanisms of attachment to, invasion of, and killing of lymphocytes warrant further investigation.

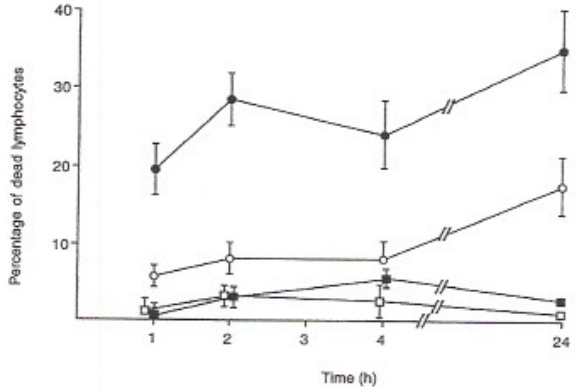

Figure 2. Effects of *Borrelia burgdorferi* on purified primary human lymphocytes. Killing of these lymphocytes during coincubations with high-passage (squares) or low-passage (circles) spirochetes was compared by trypan blue exclusion. Value ranges shown represent 2 SEs. The percentages of lysed B cells (solid symbols) and T cells (open symbols) varied significantly, as did the time of onset of killing (which was relatively delayed for T cells).

Flow cytometry of mixed mononuclear cell preparations showed that *B. burgdorferi* killed significant numbers of cells expressing CD5 and CD19, but not CD14 or CD15, cell-surface markers. These markers are characteristic for pan T cells and activated B cells, pan B cells, monocytes, and granulocytes, respectively. Possible pathogenic effects in subsets of these cells have yet to be determined. The finding that neither monocytes nor granulocytes were susceptible is consistent with previous reports that *B. burgdorferi* is effectively internalized and destroyed by phagocytes [25, 26]. In kinetic experiments with purified primary lymphocytes, we found that $CD19^+$ B cells were killed significantly faster than $CD4^+$ and $CD8^+$ T cells. Whether differing rates of killing by spirochetes reflected differences in expression of factors mediating attachment or internalization, procedural manipulations, or other factors remains to be determined. However, variable lymphocytic susceptibility and passage-dependent spirochetal virulence indicated that the process of attachment, invasion, and killing involves both lymphocytic and bacterial factors.

Coincubation experiments with SKW 6.4 B cells demonstrated that spirochetal virulence and lymphocytic resistance could be phenotypically selected. The nature of these factors has not been determined. However, since killing was not evident in coincubations with attenuated spirochetes, the process of attachment to, invasion of, and lysis of lymphocytes apparently involves factors that are nonessential for growth in vitro.

Differences in killing of lymphocytes by different species of *Borrelia* were noted in this study. Although both the *B. hermsii* isolate (tick-borne relapsing fever agent) and the *B. afzelii* isolate (Lyme borreliosis agent) that were used were infectious in mice [23], neither isolate caused an increase in the number of killed SKW 6.4 cells. *B. hermsii,* like other relapsing fever agents, can repeatedly reach levels of 10^{6-7} per milliliter of

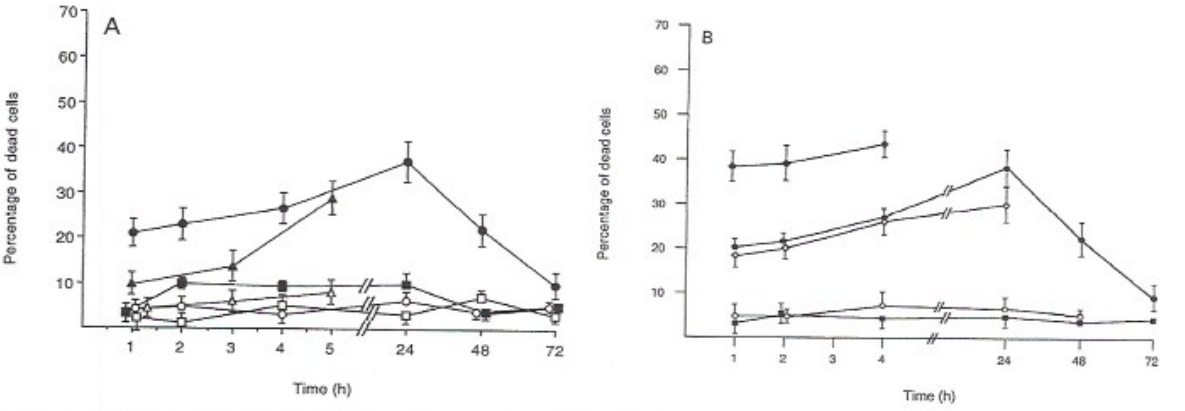

Figure 3. Effects of spirochetes causing Lyme disease on the viability of cultured B and T cells. Mixtures of SKW 6.4 or H9 cells and spirochetes were examined for viability by trypan blue exclusion. Ranges shown are 2 SEs. *A*: Low-passage *Borrelia burgdorferi* (solid circles) and *Borrelia garinii* (solid triangles) killed up to 40% of SKW 6.4 cells. Killing began within 1 hour of mixing and peaked within 24 hours. Surviving lymphocytes continued replicating, thus resulting in recovery of the B cell population at later times. Levels of killing in mixtures of SKW 6.4 cells and low-passage *Borrelia hermsii* (open circles) or *Borrelia afzelii* (open triangles), or SKW 6.4 cells and high-passage *B. burgdorferi* (solid squares) were not statistically different than those observed in unperturbed SKW 6.4 cell cultures (open squares). *B*: Rates of killing observed in mixtures containing normal (solid symbols) or enriched (open symbols) SKW 6.4 cells and *B. burgdorferi.* After three sequential enrichments, killing of normal and resistant SKW 6.4 cells by high-passage (squares), low-passage (circles), and enriched (diamonds) *B. burgdorferi* was compared. Enriched spirochetes killed significantly more B cells than did low-passage spirochetes at early times. Decreases in observed killing that were apparent later may have reflected destruction of dead cells. Normal B cells were also significantly more susceptible to killing by spirochetes than were enriched B cells.

Table 2. Susceptibility of leukocytes to killing by spirochetes causing Lyme disease.

Host cells (description)	Result of killing by indicated spirochete (description)					
	Borrelia burgdorferi (low-passage)	*B. burgdorferi* (high-passage)	*Borrelia hermsii* (low-passage)	*Borrelia garinii* (low-passage)	*Borrelia afzelii* (low-passage)	*B. burgdorferi* (enriched)
H9 cells	−	−	−	−	−	−
SKW 6.4 cells	+	−	−	−	+	+
SKW 6.4 cells (enriched)	−	−	ND	ND	ND	ND
Primary B cells*†	+	−	ND	ND	ND	ND
Primary T cells*†	+	−	ND	ND	ND	ND
Primary monocytes*	−	−	ND	ND	ND	ND
Primary granulocytes*	−	−	ND	ND	ND	ND

NOTE. Killing was defined as a mean percentage of cells stained with propidium iodide or trypan blue that was greater than that of uninfected controls by >2 SEs after a 2-hour coincubation period with a 100-fold excess of spirochetes. See definitions for low and high passages and enriched in the NOTE for table 1. ND = not done; + = positive; − = negative.

* Assessed in mixed mononuclear cell preparations.

† Assessed in purified cell fractions.

peripheral blood in patients [27]. At those levels, if lymphocytes were susceptible to killing by spirochetes causing relapsing fever, severe immune deficiencies in patients could be expected. Such consequences are inconsistent with typical clinical manifestations. We also found differences in killing of lymphocytes by Lyme disease spirochetes. Further investigation with use of additional isolates should elucidate whether such differences correlate with observed predilections of *B. afzelii* for dermatologic manifestations and of *B. burgdorferi* and *B. garinii* for disseminated disease [28].

It is currently unknown whether invasion and killing of lymphocytes occur during natural infections. However, although it is unlikely that the low numbers of Lyme disease spirochetes in infected mammals would cause significant lymphopenia or noticeable immune deficiencies, the ability to invade lymphocytes could provide an effective niche for avoiding immune detection and clearance. Humoral immunity is protective in laboratory animals [29]; in contrast, humoral responses are typically delayed in patients. In vitro spirochete-leukocyte interactions may provide an effective model for understanding the delayed immune response in humans when they are infected by Lyme disease spirochetes.

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