NOTES

Human Phagocyte Interactions with the Lyme Disease Spirochete

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The interaction between human polymorphonuclear leukocytes and monocytes and the Lyme disease spirochete was investigated by incubating phagocytes with microorganisms adherent to plastic or glass surfaces. Both cell populations readily phagocytized and killed spirochetes, and antibodies facilitated but were not essential for phagocytosis.

Lyme disease is a recently recognized inflammatory disease characterized by distinctive skin lesions and involvement of the joints, nervous system, and heart (13). Epidemiological studies early on linked the disease with the bite of the ixodid tick, *Ixodes dammini*, and convincing evidence now has been provided that a spirochete harbored by this tick is the etiological agent of Lyme disease (3, 12). Although a great deal has been learned about the cause, clinical manifestations, and therapy of Lyme disease, relatively little is known about the host and bacterial factors involved in its pathogenesis. In this study we evaluated the interaction between peripheral blood phagocytes and the Lyme disease spirochete, and our findings suggest that these cells may play a role in host defense against this microorganism.

The Lyme disease spirochete used for these studies, kindly provided by A. C. Steere, Yale University, New Haven, Conn., was isolated from cerebrospinal fluid of a patient and was stored at −70°C. A 0.5-ml sample of thawed spirochetes was inoculated into 7 ml of modified Kelly medium (2) and cultivated in 30°C for 6 days. Radiolabeled spirochetes were obtained by cultivation in the presence of 0.1 nmol of [3H]thymidine (specific activity, 90 Ci/mmol; New England Nuclear Corp., Boston, Mass.). Human polymorphonuclear leukocytes (PMNs) and monocytes were obtained from venous blood of healthy donors by previously described techniques (6, 10).

For the purposes of this study, we modified a recently described assay system (8) similar in design to the "surface phagocytosis" methodologies first described by Wood et al. (14) that permit investigation of opsonin-independent (nonspecific) and opsonin-mediated phagocytosis. PMNs or monocytes (2.5×10^6 cells in 0.5 ml of Hanks balanced salt solution containing 0.1% gelatin [GHBSS]) were added to each well of a 24-well tissue culture plate that contained about 7.5×10^6 adherent [3H]-labeled spirochetes. After 5, 15, and 60 min of incubation at 37°C in a stationary position, extracellular bacteria were separated from phagocyte-associated organisms by three cycles of differential centrifugation, and phagocytosis was expressed as the percentage uptake of the total population of bacteria in each well (8). In some wells, 50 μl of normal human serum (pooled from three healthy donors and stored at −70°C until used), preimmune rabbit serum, or immune rabbit serum was added, giving a final concentration of 10% serum. The immune rabbit serum

was obtained from New Zealand white rabbits after five weekly intravenous injections of 10^8 live spirochetes. After 60 min of incubation, >50% of the unopsonized spirochetes were phagocytized by PMNs, and of the serum opsonins tested, only immune rabbit serum increased the rate of phagocytosis (Fig. 1A). Heat inactivation (56°C, 30 min) of the immune rabbit serum did not appreciably alter its opsonic activity, and the substitution of 0.1 mg of purified immunoglobulin G per ml, isolated from immune serum by standard methods (7), resulted in equivalent enhancement of phagocytosis (data not shown). Similar results were obtained with monocytes recovered from the same PMN donors (data not shown).

In all of these experiments, we used spirochetes that had been repeatedly passaged in vitro. To test whether virulent spirochetes would pose a different challenge to phagocytic cells, we studied organisms recovered from infected hamsters. After passage four times in Syrian hamsters, spirochetes were isolated from the spleens, and the first subculture from these spleen isolates was used. In addition, other sources of immune opsonins were used. Immune human serum (kindly provided by H. W. Wilkinson, Centers for Disease Control) and immune synovial fluid (a gift of W. Burgdorfer and A. Barbour, Rocky Mountain Laboratories) were from patients with Lyme disease. Control synovial fluid (kindly provided by D. Zoschke, University of Minnesota) was from a patient with degenerative joint disease. Antibody titers to the Lyme disease spirochete were determined by indirect immunofluorescence and are expressed as reciprocal values. When virulent spirochetes were studied, again ≥40% of the unopsonized spirochetes were phagocytized after 60 min of incubation with PMNs or monocytes, and opsonins that contained high titers of antibodies to the Lyme disease spirochete promoted phagocytosis by both cell types (Table 1).

Morphological assessment of phagocytosis was carried out by scanning electron microscopy and transmission electron microscopy. For these studies, 2.0 ml of phagocyte suspensions (10^5 cells) was added to Falcon 3006 Optical tissue culture dishes fitted with polyester film liners containing about 10^7 adherent spirochetes. After 2 or 30 min of incubation, the film liners with adherent cells were prepared for electron microscopy (D. A. Lee, J. R. Hoidal, D. J. Garlich, C. C. Clawson, P. G. Quie, and P. K. Peterson, J. Leukocyte Biol., in press). These studies confirmed that both PMNs and monocytes readily phagocytized Lyme disease spirochetes and that an opsonin was not required for
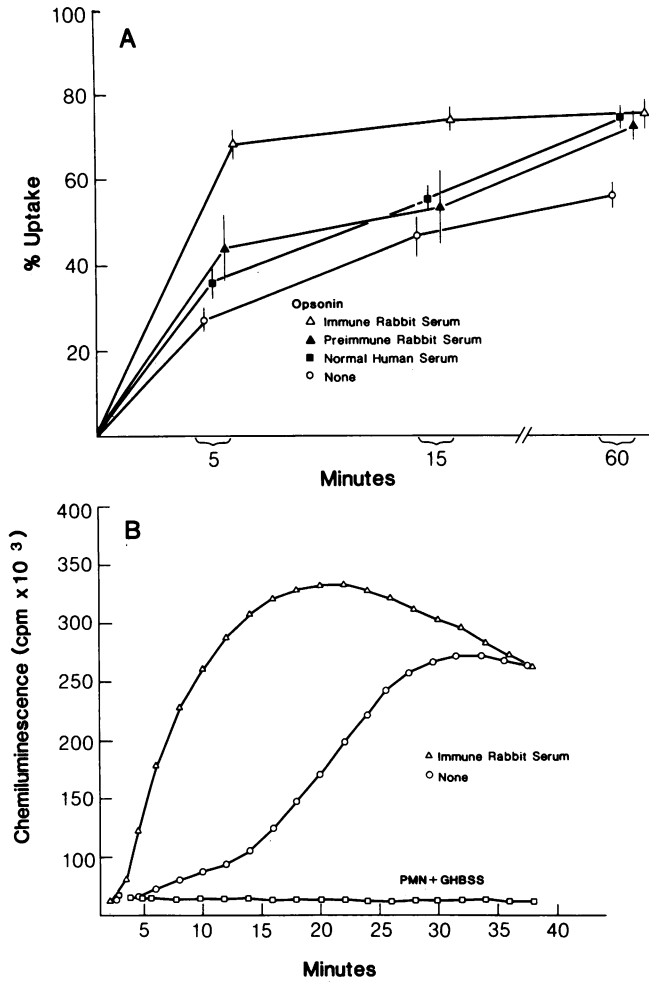
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FIG. 1. (A) Phagocytosis of the Lyme disease spirochete by PMNs. PMNs were added to wells of plastic tissue culture plates containing adherent radiolabeled spirochetes. Results represent the mean ± standard error of the mean of at least three separate experiments with different donor samples. Uptake at 5 and 15 min was significantly increased by immune rabbit serum ($P < 0.05$, Student's t test). (B) CL response of PMNs to Lyme disease spirochetes. Spirochetes were either unopsonized or preopsonized with 10% immune rabbit serum before PMNs were added. PMNs were also tested in the absence of spirochetes (PMN + GHBSS). Results represent averages of three separate experiments.

recognition of surface-adherent bacteria. Representative findings with PMNs and unopsonized spirochetes are shown in Fig. 2; similar results were obtained with monocytes (not shown).

A modified luminol-enhanced chemiluminescence (CL) assay (1, 8) was used to determine whether the interaction of phagocytes with spirochetes would trigger a PMN respiratory burst. PMN suspensions (5×10^4 cells in 1 ml of GHBSS) were added to dark-adapted glass scintillation vials containing about 7.5×10^7 adherent bacteria, 20 μl of luminol, and 4.5 ml of GHBSS. Although unopsonized bacteria stimulated PMN CL, when spirochetes were preopsonized with 10% immune rabbit serum CL was enhanced (Fig. 1B) in a manner paralleling the results of the phagocytosis experiments (see above and Fig. 1A).

Since the oxygen metabolites responsible for PMN CL participate in the process of microbial killing (4, 11), we investigated whether phagocytized spirochetes were also killed. A modification of a fluorochrome microassay (8, 9) was used to assess intracellular survival. Onto acid-washed glass cover slips, 250 μl of a bacterial suspension (5×10^6 spirochetes) was added, and the cover slips were incubated for 2 h at 37°C. After gentle rinsing, 200-μl suspensions of phagocytic cells (10^5 cells in GHBSS) were added to each cover slip and incubated at 37°C for 30 min. The nonadherent cells were then decanted, and acridine orange (1:10,000 in 0.87% NaCl) was added for 30 s. In this assay, live organisms appear green and dead organisms appear red when examined with a UV fluorescence microscope (9). After 30 min of incubation, we determined that more than 50% of the cell-associated bacteria were dead (red). Similar results were obtained with PMNs and monocytes from three normal donors and with spirochetes opsonized with immune rabbit serum. Non-cell-associated spirochetes and control spirochetes (opsonized and unopsonized) that had been incubated similarly but in the absence of phagocytes uniformly appeared green. PMNs were also obtained from a patient with chronic granulomatous disease, a disease associated with a defect in the generation of reactive oxygen species (5), and these cells also killed more than 50% of the cell-associated spirochetes. These findings indicate that PMNs and monocytes are capable of killing intracellular spirochetes and that although phagocytosis is associated with stimulation of oxidative metabolism, oxygen-independent mechanisms of killing may also participate in the spirocheticidal activity of these cells. It should be pointed out, however, that the isolate used in these studies was determined to be catalase

TABLE 1. Phagocytosis of hamster-passaged Lyme disease spirochetes

Opsonin source (antibody titer[a])	% Uptake[b]			
	PMNs		Monocytes	
	5 min	60 min	5 min	60 min
None	21 ± 1.7	51 ± 4.6	16 ± 0.8	41 ± 7.2
Preimmune rabbit serum (<4)	22 ± 3.2	51 ± 2.1	16 ± 1.2	43 ± 9.3
Immune rabbit serum (1,024)	40 ± 5.1[c]	65 ± 1.8[c]	44 ± 3.4[c]	61 ± 7.9
Normal human serum (<4)	20 ± 1.4	56 ± 2.9	20 ± 1.0	40 ± 7.4
Immune human serum (2,048)	56	68	42	61
Control synovial fluid (<4)	28	58	19	49
Immune synovial fluid (4,096)	65 ± 1.1[c]	71 ± 3.3[c]	41 ± 5.2[c]	54 ± 4.0

[a] Serum sources were used at a final concentration in the phagocytosis assay of 10%; synovial fluids were used at a final concentration of 2%. All opsonins were heat activated (56°C, 30 min) before use.

[b] Phagocytosis of surface-adherent spirochetes after 5 and 60 min of incubation with PMNs or monocytes. Values are the mean ± standard error of the mean of at least three separate experiments, except for immune human serum and control synovial fluid, which could be tested only once due to limited supply.

[c] $P < 0.05$ when values with immune rabbit serum or immune synovial fluid are compared with the values with preimmune rabbit serum, normal human serum, or no opsonin.
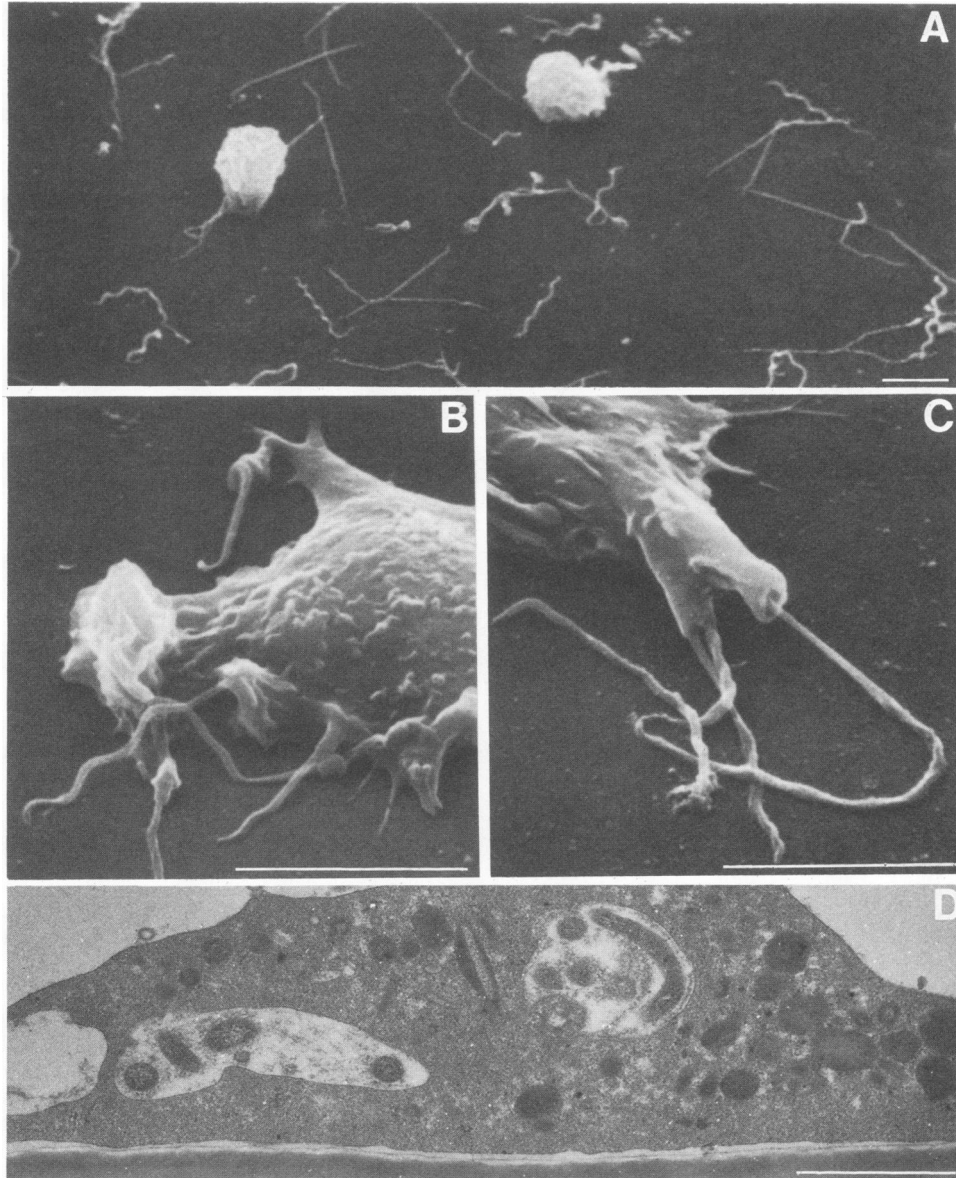
FIG. 2. (A) Scanning electron micrograph of Lyme disease spirochetes adherent to a plastic substrate at the beginning of the surface phagocytosis assay. Two PMNs have settled on this surface but have not yet begun to spread or ingest the spirochetes. (B and C) At 30 min, several spirochetes are seen in the stage of partial engulfment. (D) Transmission electron micrograph of a PMN at 30 min. Segments of the ingested spirochetes are seen in typical phagosomes. Bars: A, B, and C, 5 μm; D, 1 μm.

negative, raising the possibility that the oxidative metabolism of the spirochete itself may contribute to its intracellular demise. Further studies therefore will be necessary to define the susceptibility of the Lyme disease spirochete to oxygen metabolites as well as to phagocyte-derived oxygen-independent microbicidal products.

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