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Interaction of Leptospires with Human Polymorphonuclear Neutrophils

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The role of the polymorphonuclear neutrophils (PMN) in defense against leptospires has not been adequately studied, in part, because of difficulty in quantitating pathogenic leptospires. By using pour plates to quantitate nonpathogenic leptospires and the most-probable-number procedure to quantitate the pathogenic leptospires, we examined the interactions of nonpathogenic *Leptospira biflexa* and pathogenic *Leptospira interrogans* serovar *icterohemorrhagiae* with human neutrophils. Phase-contrast, scanning electron, and transmission electron microscopic observations were made. Leptospires were incubated with PMN at 37°C and tumbled together. There was no ingestion or killing of nonpathogenic leptospires (with no serum) or of pathogenic organisms (with 10% normal serum). However, when leptospires were incubated with PMN (without serum) in a pellet and then resuspended, 91 ± 6% of nonpathogenic leptospires were removed from the supernatant, and 93 ± 4% of these organisms were killed. The pathogenic leptospires became cell associated in a pellet, but were not killed by PMN even in the presence of 10% normal serum. Observations of morphological interactions indicated that PMN phagocytized the nonpathogenic leptospires in the absence of serum and that the pathogenic leptospires attached to but were not ingested by neutrophils in the presence of 10% normal serum. PMN do not seem to be an efficient defense factor for pathogenic leptospires in nonimmune hosts. The virulence of leptospires appears to be related to their ability to resist killing by serum and to resist ingestion and killing by neutrophils.

The role of polymorphonuclear neutrophils (PMN) in resistance against infection with leptospires has not been adequately studied, in part, because pathogenic leptospires grow poorly on solid medium and thus are difficult to quantitate (6, 23). In the 1960's, Faine (11) reported that both virulent and avirulent leptospires were phagocytized by reticuloendothelial-fixed phagocytes in immunized and unimmunized mice. Subsequently, Faine et al. (14) demonstrated phagocytosis of leptospires by macrophages and PMN from different animal species such as mice, guinea pigs, and rabbits. Specific opsonization with whole leptospiral antiserum increased the rate of phagocytosis. However, evidence was based solely on morphological observations through light microscopy. Recently, Cinco et al. (8) and Banfi et al. (5), using differential centrifugation and pour plate techniques, have reported that guinea pig macrophages exert no bactericidal activity against nonopsonized, nonpathogenic or pathogenic strains of leptospires during a 120-min incubation period, although some ingestion was observed. They have found that, when opsonized with specific immunoglobulin G, both the pathogenic and nonpathogenic leptospires become susceptible to phagocytosis and killing by macrophages.

We examined the interactions between leptospires and human PMN quantitatively and morphologically. Serial dilutions and pour plates were used for quantitating viable nonpathogenic leptospires, and the most-probable-number procedure was used for quantitating pathogenic organisms. Morphological observations with phase-contrast, scanning electron, and transmission electron microscopy also were undertaken.

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MATERIALS AND METHODS

Leptospires. Nonpathogenic *Leptospira biflexa* serovar *Patox*, strain Patoc I and pathogenic *Leptospira interrogans* serovar *icterohemorrhagiae*, strain 23581 used in this study were obtained from American Type Culture Collection, Rockville, Md. The organisms were grown at 30°C for 5 to 7 days in Korthof liquid medium (4) containing 10% heat-inactivated rabbit serum. The organisms were harvested by centrifugation at 4,000 × *g* for 20 min, washed with phosphate-buffered saline (pH 7.3), and resuspended in Hanks balanced salt solution (HBSS). The organisms were counted in a Petroff-Hausser counting chamber and adjusted to a density of 5×10^6 to 12×10^6 organisms per ml.

PMN. PMN (95% pure) were obtained from normal heparinized (10 U/ml) human venous blood by Ficoll-Hypaque separation (7), followed by dextran sedimentation and hypotonic lysis of the remaining erythrocytes (10). The cells were counted in a standard hemocytometer and adjusted to a concentration of 3×10^6 to 7×10^6 PMN per ml. Viability was 95% as determined by trypan blue exclusion.

Quantitation of viable leptospires. Two methods were used. (i) Spread plates for nonpathogenic *L. biflexa*. Serial dilutions (10-fold) of samples were made in phosphate-buffered saline (pH 7.3). Two 0.1-ml samples of each dilution were evenly spread on Cox agar plates (9) and then incubated aerobically at 30°C. Visible colonies of organisms developed after 5 days. From the mean number of CFU present in each dilution, the mean number of viable leptospires per ml was calculated.

(ii) The most-probable-number procedure (16, 23, 24) for pathogenic *L. interrogans*. Attempts at quantitative recovery of pathogenic serovars of *L. interrogans* such as *icterohemorrhgiae*, *grippotyphosa*, *canicola*, and *pomona* on solid media, e.g., Cox medium or PLM5 medium (8), with incubation in room air or in 1% CO_2 (27) produced erratic results. Therefore, the most-probable-number procedure was used in

TABLE 1. Antileptospiral activity of normal human serum. Survival of viable organisms compared with that of control (without serum)[a]

% Serum	% Survival (Mean ± SEM) of:	
	L. biflexa	*L. interrogans*
50	0.0	44.2 ± 12.4
10	0.0	109.4 ± 25.0
1	0.6 ± 0.3	106.8 ± 16.6

[a] Leptospires (10^7) with or without indicated serum were tumbled at 12 rpm at 37°C for 1 h. Quantitation was by pour plates for nonpathogenic *L. biflexa* and by the most-probable-number procedure for pathogenic *L. interrogans* (n = 3).

this study for quantitating viable pathogenic leptospires. Dilution (10-fold) samples were transferred to series of five tubes, each containing 3.0 ml of Fletcher semisolid medium (22) supplemented with (per liter): 100 ml of heat-inactivated rabbit serum, 10 ml of sheep hemoglobin preparation, and 10 mg of thiamin hydrochloride. Samples were incubated at 30°C for up to 17 days. Growth of leptospires was indicated by the characteristic Dinger zone (22), a turbid suspension in the upper one-third of the tube. Dark-field microscopy was used to confirm growth. Most-probable-number values were obtained by a computer program by using the calculations of Halvorson and Ziegler (16).

To prevent fungal or bacterial contamination, amphotericin B (20 μg/ml) and 5-fluorouracil (200 μg/ml) (20) were added to both Cox and Fletcher medium. These did not inhibit the growth of either pathogenic or nonpathogenic leptospires.

Assay for antileptospiral activity of normal serum. Human serum for each experiment was obtained from a normal donor and used fresh or stored at −70°C for future use. Leptospiral suspension (0.5 ml containing 10^7 leptospires per ml) plus test serum and diluent were combined in a final volume of 1 ml, incubated at 37°C, and tumbled at 12 rpm for 1 h. Pour plates for quantitating viable nonpathogenic leptospires and dilutions for the most-probable-number procedure for pathogenic organisms were made as described above.

Assessment of phagocytic and bactericidal activity of PMN against leptospires. Two methods were used. (i) Tumbling system. The method of Cinco et al. (8) was used with a few modifications. For each experiment, equal volumes of 5×10^6 to 12×10^6 leptospires per ml and 3×10^6 to 7×10^6 PMN per ml (usually, 1 ml each) were suspended in a polypropylene tube (for pathogenic leptospires, 1.8 ml of PMN-leptospira mixture plus 0.2 ml of normal serum). The control tube contained equal volumes of leptospiral suspension and HBSS. Samples were taken after 0, 30, 60, and 120 min of incubation.

For assessment of phagocytic activity, differential centrifugation was used. The phagocyte-bacteria mixture (0.2 ml) was washed twice in 5 ml of cold HBSS and centrifuged at 250 × g at 4°C for 7 min to separate cell-free from cell-associated leptospires. The supernatants from the two washings were pooled, and serial 10-fold dilutions were made. The pellet was suspended in 0.1 ml of HBSS plus 0.9 ml of 0.5% (wt/vol) sterile saponin to lyse the PMN and release the intracellular leptospires. We found this concentration of saponin to be nontoxic to leptospires. After 15 min at room temperature, 10-fold serial dilutions were made again. The number of viable cell-free and PMN-associated leptospires were assayed by using pour plates for nonpathogenic leptospires or the most-probable-number procedure for pathogenic organisms as described above.

For assessment of bactericidal activity, 0.1 ml of the PMN-bacteria mixture was added to 0.9 ml of 0.5% (wt/vol) sterile saponin to lyse the PMN and release intracellular leptospires. After 15 min, 10-fold serial dilutions of the sample were made in phosphate-buffered saline (pH 7.3). The number of viable organisms in the sample was assayed as described above.

(ii) Pellet system. The second method for measurement of phagocytic and bactericidal activity follows. For each sample, 1 ml of leptospiral suspension (10^6 leptospires per ml) was centrifuged at 2,000 × g for 20 min to a button in a polypropylene tube. Then, 1 ml of PMN suspension (10^6) was added, centrifuged at 4°C, at 260 × g for 7 min, and incubated at 37°C (for pathogenic leptospires, the sample contained 10% normal serum). After 0, 30, 60, and 120 min, samples were vortexed to resuspend leptospires and PMN. Measurement of phagocytic and bactericidal activity was made as above.

Observations of morphological interaction. Three techniques were used. (i) Light microscopy with phase-contrast and Nomarski optics including still and video tape observations were made of cover slip preparations of PMN and leptospires in the absence of serum for nonpathogenic leptospires or in the presence of 10% normal autologous serum for pathogenic organisms. The samples were prepared as previously described (15).

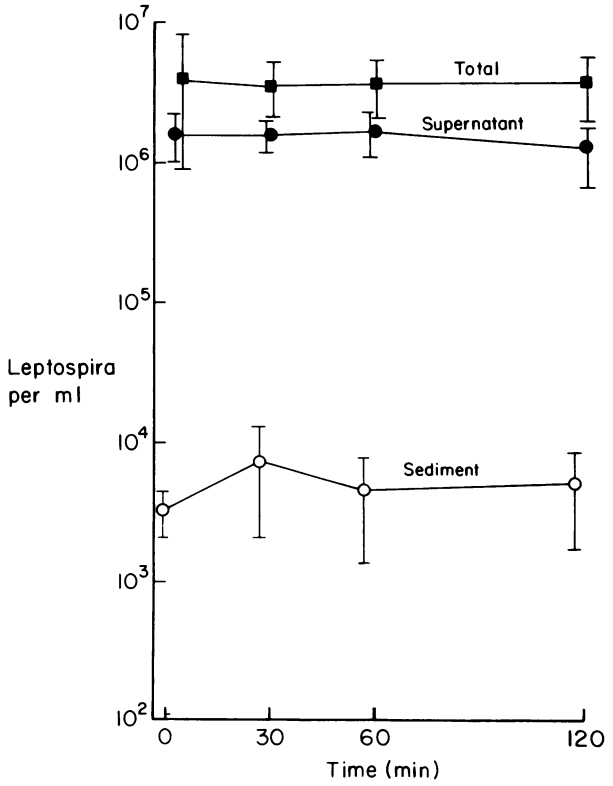

FIG. 1. Tumbling assay of phagocytic and bactericidal activity of PMN against nonpathogenic *L. biflexa*. Leptospires were incubated with PMN in the absence of serum at 37°C at 12 rpm. At intervals, total, supernatant, and sediment counts of viable leptospires were determined. Mean ± SEM for three to four determinations for each point are shown. Results indicate no ingestion or killing.

(ii) Scanning electron microscopic observation. Scanning electron microscope observations were made in monolayers of leptospires with PMN. Approximately 0.25 ml of whole venous blood was allowed to clot on a clean round cover slip (diameter, 2.2 cm) for 20 min in a moist chamber at 37°C. The clot was rinsed off with 0.9% NaCl at 37°C, and the cover slip with attached leukocytes was inverted on 0.5 ml of a suspension of 10^7 leptospires per ml (with 10% serum for pathogenic leptospires). The specimens were incubated at 37°C and at 15, 30, and 60 min, and preparations were fixed with 2% gluteraldehyde in HBSS for 30 min. Each specimen was washed in three baths of phosphate-buffered saline for 10 min each. Samples were dehydrated for 10 min in each of five baths in 40, 60, 80, and 100% (twice) ethanol. Specimens were immersed in a Samdri-780 Critical Point Drying Apparatus (Tousimis Research Corporation, Rockville, Md.) with liquid CO_2. Each cover slip was mounted on an aluminum stub and coated with gold palladium in a Hummer V sputter Coater (Technics, Alexandria, Va.). A JEOL-35 C scanning electron microscope (JEOL, Peabody, Mass.) was used, operating at 25 kV.

(iii) Transmission electron microscopic observations. Leptospires (10^8 suspended in HBSS with 10% serum for pathogenic leptospires or without serum for nonpathogenic organisms) and 10^6 PMN were centrifuged to a pellet in tubes and incubated as above. After 0, 15, 30, and 60 min, samples were fixed in 2% gluteraldehyde and osmium tetroxide, dehydrated in ethanol and propylene oxide, and embedded in Epon 812. Samples were cut with an ultramicrotome, stained with uranyl acetate and lead citrate, and examined and photographed with an electron microscope as described previously (25).

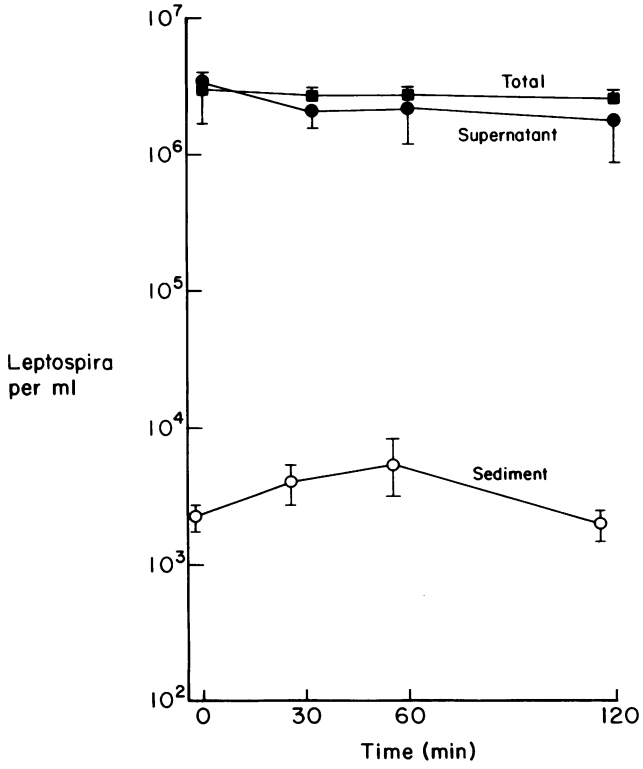

FIG. 2. Tumbling assay of phagocytic and bactericidal activity of PMN against pathogenic *L. interrogans*. Leptospires were incubated with PMN in 10% normal serum at 37°C in tumbling system. Mean ± SEM for two experiments are shown. Results indicate no ingestion or killing.

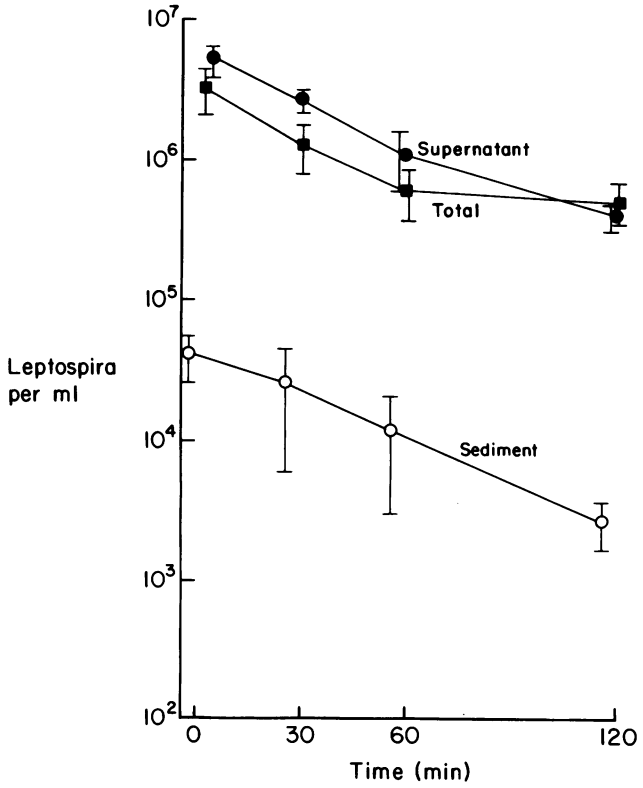

FIG. 3. Phagocytic and bactericidal activity of PMN agaist nonpathogenic *L. biflexa* in a pellet. Leptospires were incubated with PMN in pellets in the absence of serum at 37°C. Samples were resuspended after 0, 30, 60, and 120 min of incubation. The mean ± SEM for three to five experiments are shown. There was a significant decrease in the number of organisms in both supernatant and sediment, and the total number of organisms ($P < 0.01$), indicating effective ingestion and killing.

Statistical analysis. Data were analyzed with the Student *t* test. Results are expressed as the mean ± standard error of the mean (SEM).

RESULTS

Antileptospiral activity of normal human serum. More than 99% of nonpathogenic *L. biflexa* were killed when incubated with 1% normal serum for 1 h. Heated serum was also bactericidal. In contrast, no killing occurred for *L. interrogans* with 1 or 10% serum, even though 50% serum killed 56% of the organisms (Table 1).

Phagocytic and bactericidal activity. (i) Tumbling assay. (a) When incubated with PMN in the absence of serum, there was no increase in the number of CFU of nonpathogenic *L. biflexa* associated with PMN, as well as no decrease in total or supernatant organisms (Fig. 1), indicating neither ingestion nor killing.

(b) Similar results were obtained with the pathogenic leptospira strain. When PMN were tumbled with the organisms in the presence of 10% normal serum, there was neither ingestion nor killing (Fig. 2).

(ii) Pellet assay. (a) When the nonpathogenic leptospires were incubated with PMN in a pellet in the absence of serum, and then resuspended, 91 ± 6% of the organisms

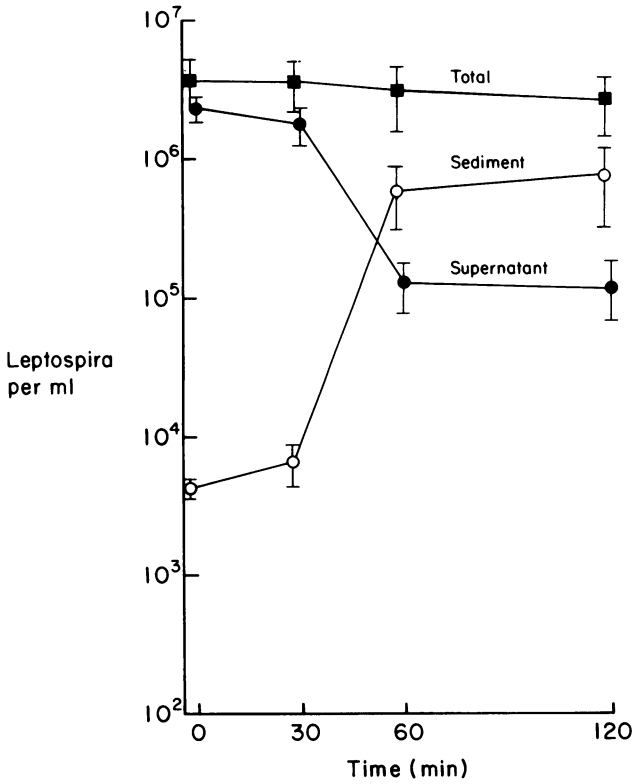

FIG. 4. Phagocytic and bactericidal activity of PMN against pathogenic *L. interrogans* in a pellet. Leptospires were incubated with PMN in 10% normal serum in pellets. Samples were resuspended after 0, 30, 60, and 120 min of incubation. There was significant increase in the number of leptospires in the sediment and a decrease in the number of organisms in the supernatant by 120 min ($P < 0.01$), but the total number of the organisms was unchanged. Mean and SEM for three experiments are shown. These results indicate cell association but no killing.

were removed from the supernatant by 120 min, and 93 ± 4% of the organisms that were associated with PMN were killed (Fig. 3). To test the comparability of the most-probable-number procedure, a determination by this method was made. Results were similar; 90.3% of the nonpathogenic leptospires were killed by PMN in the absence of serum.

(b) When the pathogenic leptospires were incubated with PMN in a pellet in the presence of 10% normal serum, there was a marked increase in the number of organisms in the sediment and a decrease in supernatant organisms. However, the total number of organisms was unchanged (Fig. 4), suggesting that the pathogenic leptospires attached to, or were ingested by, neutrophils, but were not killed.

Morphological observations. (i) Phase-contrast microscopic video sequences showed that PMN moved towards the nonpathogenic leptospires and attachment and ingestion took place. Pathogenic leptospires attached to PMN, but no ingestion was noted.

(ii) Scanning electron micrographs showed ingestion of nonpathogenic leptospires in the absence of serum. Specimens fixed after 15 min of incubation showed that as the nonpathogenic leptospires attached to the phagocyte membrane, the leptospires appeared to straighten out. The phagocyte membrane extended along the attached organisms, and engulfment of the whole leptospira took place (Fig. 5). As indicated by culture studies, pathogenic leptospires were associated with but were not killed by neutrophils even after 60 min of incubation. Figure 6 shows attachment of leptospires to the surface of a phagocyte.

(iii) Transmission electron micrographs showed that the nonpathogenic leptospires were internalized in large numbers by PMN in pellets (Fig. 7). After 30 min of incubation, most of the organisms were found within PMN vacuoles, and by 60 min, there were large phagocytic vacuoles containing numerous degenerated and lysed leptospires. Observations of neutrophils interacting with pathogenic leptospires revealed very few ingested organisms, but many organisms were attached to the surface of the neutrophils.

DISCUSSION

We found that nonpathogenic leptospires were killed by normal human serum and ingested and killed by human PMN in the absence of serum. Pathogenic leptospires were not sensitive to the antileptospiral action of either normal serum or neutrophils. In addition, as shown by scanning electron and phase-contrast microscopy, little phagocytosis of pathogenic leptospires took place. However, both microscopic and culture studies indicated that pathogenic leptospires became cell associated and survived in the PMN pellets.

In the 1960's, Johnson et al. (17–19) reported that normal mammalian sera possess antileptospiral activity primarily against nonpathogenic *L. biflexa* but not against pathogenic *L. interrogans*. Faine and Carter (13) have reported the presence of another bactericidal substance against nonpathogenic leptospires in addition to complement and lysozyme: a 2-mercaptoethanol-sensitive macroglobulin which was destroyed by being heated at 65 to 75°C for 20 min. Our data on the interaction of normal human serum with leptospires confirm that normal serum exerts bactericidal activity against nonpathogenic leptospires, but not against pathogenic leptospires.

PMN ingest certain bacteria efficiently only against a physical surface (21). This is the case for leptospires, since in the tumbling system, no phagocytosis of either nonpathogenic or pathogenic organisms occurred. Nonpathogenic leptospires are ingested and killed by PMN in a pellet, indicating that phagocytosis of unopsonized leptospires requires a physical surface (i.e., other PMN) against which the phagocytes can trap the organisms.

Phagocytosis of a strain of *L. interrogans* serovar *icterohemorrhagiae* by PMN was described by Faine et al. (14), whose conclusions were based on observations of silver-stained preparations by light microscopy. Data obtained from our experiments by the most-probable-number procedure for quantitating viable numbers of leptospires demonstrate that *L. interrogans* serovar *icterohemorrhagiae* become cell associated but survive in the PMN pellet. Our morphological observations suggest attachment with little ingestion.

Thus, in the nonimmune host, PMN are ineffective in ingesting and killing pathogenic leptospires. There is abundant evidence indicating that a specific antibody is an important host defense factor for leptospiral infection (1, 3, 12, 19, 26). The specific antibody, in the presence of complement, may be bactericidal for both pathogenic and nonpathogenic leptospires (2, 19). The interaction of human neutrophils with pathogenic leptospires opsonized with the specific antibody should be examined to determine the role of neutrophils in this situation.

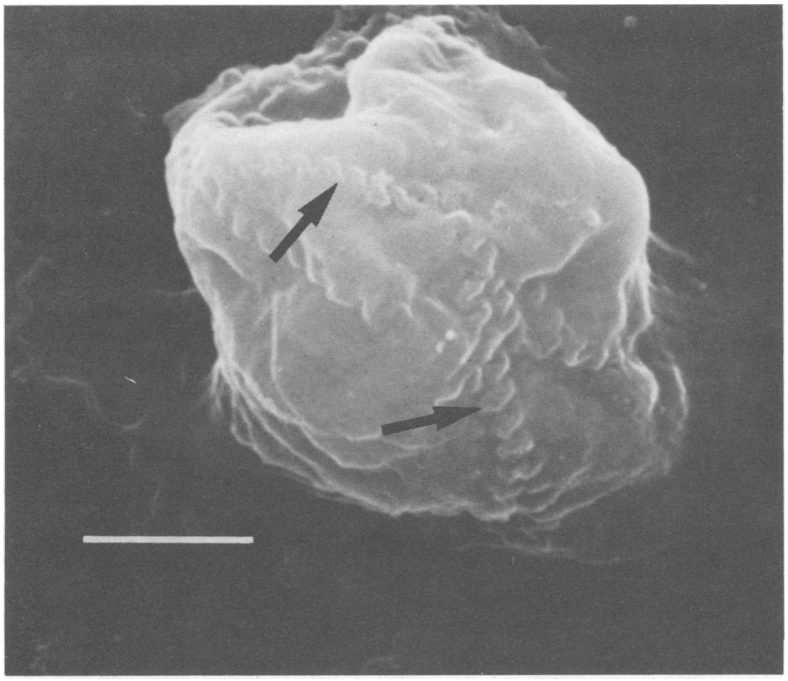

FIG. 5. Phagocytosis of nonpathogenic *L. biflexa* by phagocyte with no serum after 15 min of incubation demonstrated by scanning electron microscopy. Arrows indicate leptospires. Organisms can be seen inside phagocyte. Bar, 2 μm.

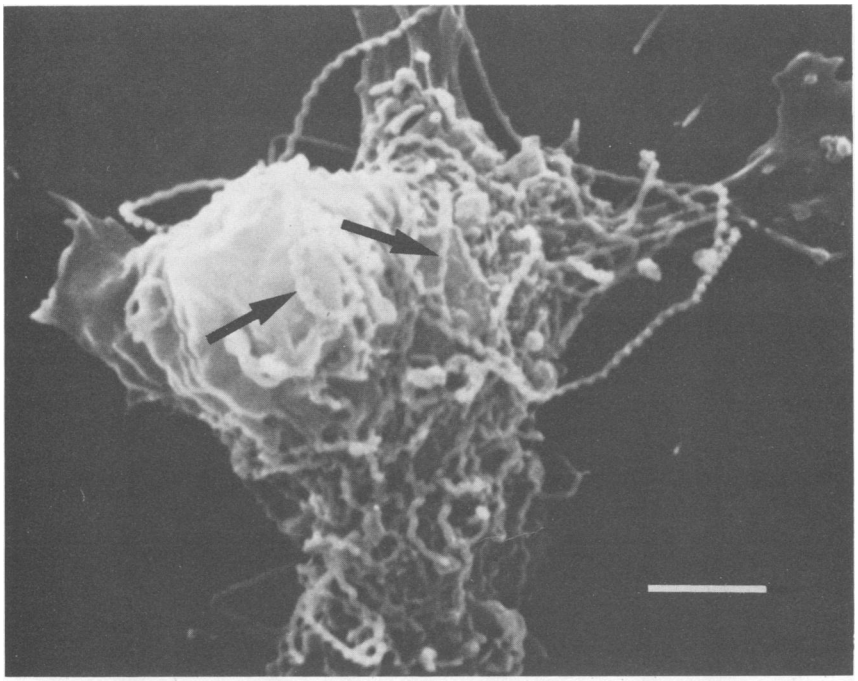

FIG. 6. Scanning electron micrograph showing the interaction between pathogenic *L. interrogans* and phagocytes with 10% normal serum. Arrows indicate leptospires. Numerous organisms attached to the tail and the body surface of phagocytes by 60 min of incubation. Bar, 2 μm.

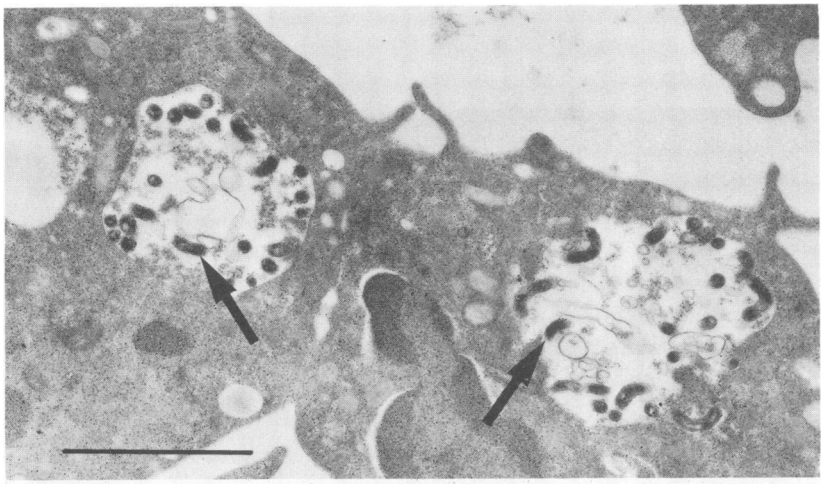

FIG. 7. Transmission electron micrograph showing phagocytosis of nonpathogenic *L. biflexa* by PMN without serum. Arrows indicate leptospires. After 60 min of incubation, large phagocytic vacuoles contained numerous degenerated leptospires. Bar, 2 μm.

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