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Phagocytosis of Opsonized *Treponema pallidum* subsp. *pallidum* Proceeds Slowly

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Macrophages were found to phagocytize *Treponema pallidum* subsp. *pallidum* attached to polycarbonate filters. This environment simulated the in vivo interaction of surface-adherent treponemes with macrophages. The phagocytosis of *T. pallidum* subsp. *pallidum* was found to proceed slowly. Heat-killed *T. pallidum* subsp. *pallidum* were susceptible to opsonization with 2% immune serum, whereas live treponemes were resistant to this concentration of antibody. High concentrations of immune serum were found to increase phagocytosis of the spirochetes. Live *T. pallidum* subsp. *pallidum* had bound limited quantities of immunoglobulin G in vivo, and only opsonization with 20% immune serum resulted in a detectable increase in surface-bound immunoglobulin in vitro. Kinetic studies suggested a steady rate of phagocytosis that is considerably slower than with other bacteria. Scanning electron microscopy studies of the phagocytizing macrophages showed that the treponemes were detached from the membrane filters and scooped onto the ruffled portion of the macrophage surface. This lengthy physical process, along with the lack of a dramatic increase in ingestion after opsonization, may account for the slow rate of phagocytosis.

Histologic studies have indicated that macrophages are involved in the immune response of the host during infection with *T. pallidum* subsp. *pallidum* (*T. pallidum*) (23, 37). Infiltration of macrophages into infected tissue correlated with a rapid decrease in the number of treponemes (32). In vitro assays confirming the phagocytosis of *T. pallidum* by macrophages have been difficult to perform because macrophages and motile treponemes do not interact well. Since 1978, only Lukehart and Miller (19) have been successful in demonstrating phagocytosis of *T. pallidum* by macrophages. They were unable, however, to demonstrate quantitative phagocytosis of treponemes.

Recently, we demonstrated quantitative phagocytosis of *T. pallidum* subsp. *pertenue*, the causative agent of yaws, in an in vitro system (1). This system involved phagocytosis of the yaws spirochete attached to polycarbonate filters. The number of treponemes phagocytized was enumerated by immunofluorescence. Similar systems have been proposed to be appropriate for in vitro simulation of phagocytosis of surface-adherent bacteria (9, 17).

In this investigation, we studied the interaction of *T. pallidum* in the presence or absence of immune serum with macrophages on polycarbonate filters. It has been reported that *T. pallidum* is resistant to antibody binding, as shown by fluorescent antibody (26, 28) and immunoelectron microscopy (15, 16) techniques. The kinetics of phagocytosis were also studied to investigate the proposed concept of treponema-mediated immunosuppression within the treponeme-macrophage system (4, 11, 13, 36). We demonstrated that *T. pallidum* organisms are difficult targets for phagocytosis and that immune serum in high concentrations can increase phagocytosis. Photographic and kinetic data suggested that the slow rate of phagocytosis was due to mechanical difficulty of detaching adherent treponemes from surfaces before phagocytosis.

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MATERIALS AND METHODS

Animals. Inbred LSH/Ss LAK hamsters were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.). Hamsters 6 to 8 weeks old were housed four per cage at an ambient temperature of 21°C. New Zealand White rabbits (Charles Rivers) were housed individually at 21°C.

Organisms. *T. pallidum* (Nichols strain) was maintained by passage in rabbits as reported previously (5, 20, 21). Rabbits were not treated with cortisone acetate. *T. pallidum* was harvested 3 weeks after intratesticular injection with 2×10^7 treponemes. *T. pallidum* subsp. *pertenue* was maintained by passage in hamsters (31). *T. pallidum* subsp. *pertenue* was harvested from hamsters 4 to 5 weeks after intradermal injection in the inguinal region with 10^6 treponemes. The treponemes were isolated from infected testicular or lymph node tissues by using Eagle minimum essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and 0.63 mM dithiothreitol (EMEM) as reported previously (1). After centrifugation at 1,000 × *g* for 10 min to remove cellular debris, the number of treponemes in the medium was determined by dark-field microscopy (3). When indicated, treponemes were killed with heat by incubation at 56°C for 2 h.

Preparation of macrophages. Peritoneal exudate cells were collected from LSH hamsters by peritoneal lavage. Briefly, 10 ml of sterile Hanks balanced salt solution was injected into the peritoneal cavity, which was massaged and aspirated aseptically. The exudate cells were centrifuged at 1,000 × *g* for 10 min and resuspended in Hanks balanced salt solution. Stimulated peritoneal exudate cells were harvested from hamsters injected 3 days previously with 5 μg of lipopolysaccharide (*Escherichia coli*; Sigma Chemical Co., St. Louis, Mo.).

Macrophages were separated from exudate cells by adherence (35). Briefly, the peritoneal exudate cells were adjusted to 5×10^6 cells per ml in RPMI 1640 medium supplemented with 10% fetal bovine serum, 5 mM L-glu-

tamine, 10 mM HEPES, 50 U of penicillin per ml, and 50 μg of streptomycin per ml. The cells were incubated in 60-mm-diameter polystyrene plates (Corning, Park Ridge, Ill.) for 24 h at 37°C in an atmosphere of 5% CO_2. The nonadherent cells were removed by three washes with warm Hanks balanced salt solution. The adherent macrophages were then recovered by addition of cold Hanks balanced salt solution on the plates. After incubation at 4°C for 20 min, the surfaces of the plates were scraped with a rubber policeman.

The macrophages were enumerated and found to be >95% viable by exclusion of 0.5% trypan blue and >95% phagocytic by ingestion of 0.007% neutral red. Macrophages recovered from hamsters injected with lipopolysaccharide had an enhanced ability to phagocytize opsonized sheep erythrocytes compared with the resident macrophages (35).

Inactivation of macrophages. Macrophages were inactivated by treatment with cytochalasin B. A stock solution of cytochalasin B (1 mg/ml in dimethyl sulfoxide) was added to macrophage preparations to yield 10 μg of cytochalasin B per ml.

Immune serum. Twenty hamsters were injected intradermally at two sites at weeks 0 and 12 with 10^6 treponemes. Two weeks after the last injection, the hamsters were bled by intracardiac puncture to obtain immune serum. Normal serum was collected from noninfected hamsters. The pooled immune and normal sera were sterilized by filtration (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.) and stored at −20°C until use. The pooled immune serum had a titer of 1,280 in the microhemagglutination *T. pallidum* test.

Opsonization of treponemes. Treponemes at 10^5 bacteria per ml of EMEM were incubated for 30 min with non-heat-inactivated normal or immune serum at a concentration of 2 or 20%.

Inoculation of filters with treponemes and macrophages. Treponemes and macrophages were inoculated onto polycarbonate filters as reported previously (1). Briefly, sterile polycarbonate filters (pore size, 0.2 μm; 25 mm; Nucleopore Corp., Pleasanton, Calif.) were loaded onto an alcohol-cleaned 12-sample filtration apparatus (Millipore). The filters were rinsed sequentially with 70% methanol and sterile saline under negative pressure. The filters were seeded under negative pressure with 10^5 opsonized or nonopsonized, viable or dead treponemes suspended in EMEM. After filtration, the treponemes on the filters were washed with sterile saline. The filters were then transferred to six-well tissue culture plates (Costar, Cambridge, Mass.) and covered with 4.0 ml of EMEM. Subsequently, the filters were overlaid with 2×10^5 macrophages in 1.0 ml of EMEM. The plates were incubated at 33°C with 3% O_2–5% CO_2–92% N_2 (14) in a humid environment for 1 to 24 h. After incubation, no spirochetes were detected in the supernatants, indicating that the treponemes remained attached to the filters. In addition, the number of treponemes on the control filters after incubation for 24 h did not differ significantly from the number of treponemes initially seeded onto the filters. The treponemes had a viability of 60 to 80% after incubation. Viability was determined by counting the number of motile and nonmotile treponemes scrapped from the filters with a rubber policeman.

Detection of treponemes on the filters. After incubation, treponemes were stained on the filters by using an indirect fluorescent antibody assay previously reported (1). Briefly, the filters were loaded onto a multisample filtration apparatus, washed with sterile saline, and fixed with 95% ethanol under vacuum. One milliliter of a 1/100 dilution of human reactive control serum (Clinical Sciences Inc., Whippany, N.J.) and 1 ml of a 1/50 dilution of fluorescent conjugated sheep anti-human immunoglobulin G (Sigma) were used to stain the treponemes. The filters were counterstained with 0.2% Evans blue and washed with distilled water under vacuum. The filters were then removed from the filtration apparatus, dried on glass slides, and immersed in 90% glycerin in phosphate-buffered saline (pH 7.2) for examination by epifluorescence.

Method of evaluation. Fluorescent labeled treponemes were enumerated in a blind count by determining the number of intact spirochetes in 25 microscopic fields per filter. Each assay included multiple replicate filters for each group, and the assays were repeated three to four times. The number of fluorescent labeled spirochetes was averaged, and the percent reduction in treponemal number was determined for the various macrophage preparations and treatments.

Electron microscopy. For electron microscopy, the treponeme and macrophage density was increased to 10^6 bacteria and cells per filter. Samples were fixed in 30% ethanol and mounted on poly-L-lysine-coated cover slips. The samples were dehydrated in increasing concentrations of ethanol (50-70-85-95-95-100-100-100%) for 10 min in each bath. These samples were then critical point dried in carbon dioxide (22). Dried samples were microsputtered with platinum and viewed in a Hitachi S-900 field emission scanning electron miscroscope modified for low-voltage use. The beam diameter obtained from the cold field emission source at 1 kV is 3 nm (24).

Immunoelectron microscopy. Live *T. pallidum* (10^6/ml) were opsonized with immune serum at a final concentration of 2 or 20% as stated previously. The treponemes were washed twice with phosphate-buffered saline and collected by centrifugation at 15,850 × *g*. Protein G-coated gold beads (20 nm; Sigma) were added at a 1/50 dilution by volume. The preparations were incubated for 12 h at room temperature and then washed twice as before and suspended in 1.2% formaldehyde in phosphate-buffered saline. The samples (5 μl) were affixed to poly-L-lysine-coated Formvar 200 mesh copper grids at room temperature for 1 h. The grids were then immersed in a modified Karnovsky fixative (pH 7.4) at room temperature for 1 h. After a buffer wash, the grids were postfixed in 0.1% osmium tetroxide for 20 min. Grids were negatively stained with 1% phosphotungstic acid (pH 6.2). High-voltage micrographs were obtained on the AEI 1-MeV transmission microscope at the Madison Integrated Microscopy Resource for Biomedical Research.

Statistics. The Student *t* test was used to examine pairs of means, and significance was set at $P \leq 0.05$ before the experiments were started.

RESULTS

Electron microscopy of phagocytosis of *T. pallidum*. Scanning electron microscopy showed the initial steps of phagocytosis of *T. pallidum*. The flat leading edge of macrophages appeared to move between treponemes and the filter (Fig. 1A). Treponemes were then bound by the ruffled portion of the macrophage and lifted off the filter (Fig. 1B). Finally, the treponemes were bound entirely within the ruffled portion of the macrophage membrane (Fig. 1C). In thin section, treponemes appeared inside membrane-bound vacuoles within macrophages (Fig. 1D).

Phagocytosis of nonviable treponemes. The ability of hamster macrophages to phagocytize killed *T. pallidum* and *T. pallidum* subsp. *pertenue* was examined. Hamster macrophages phagocytized killed *T. pallidum* and *T. pallidum*

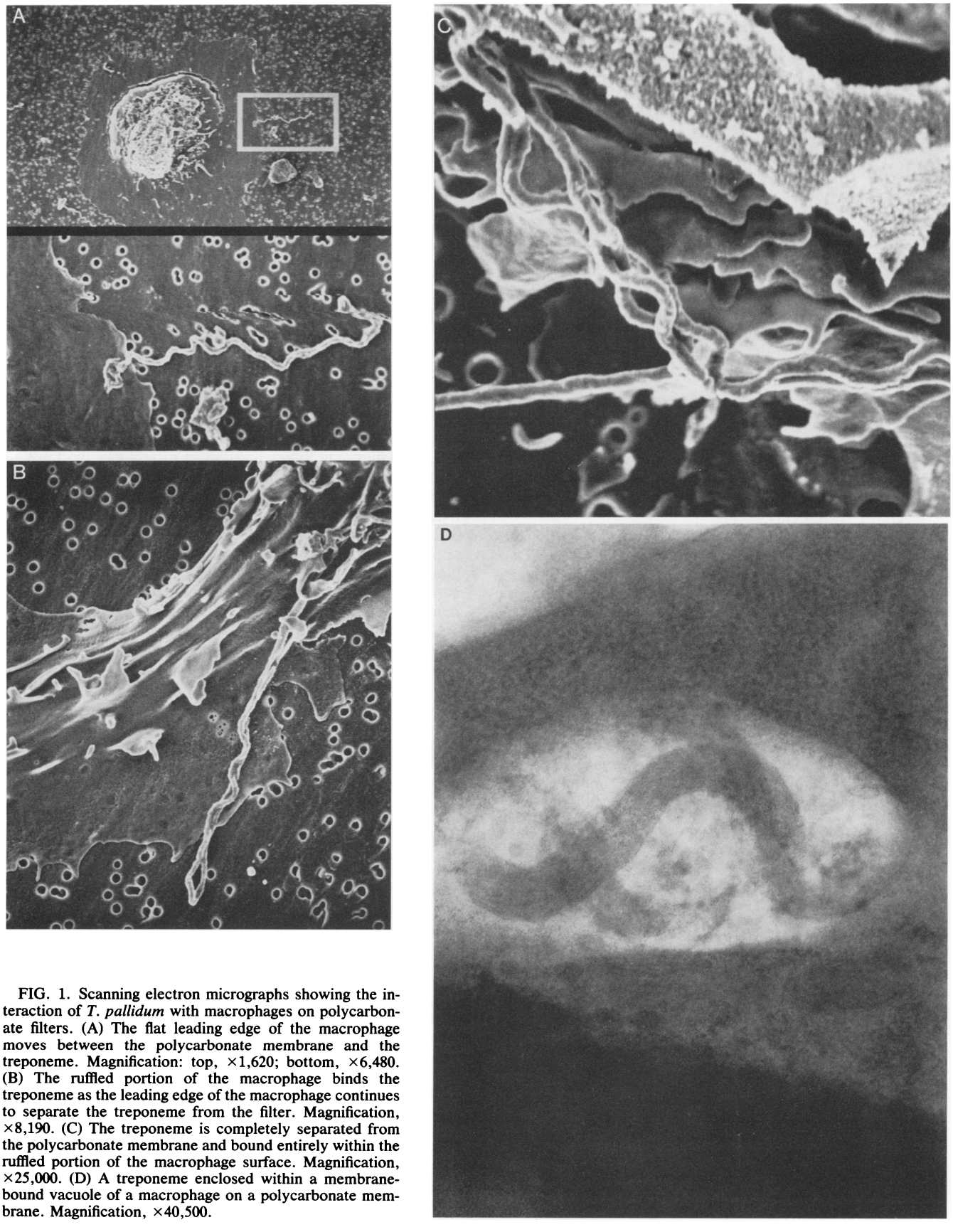

FIG. 1. Scanning electron micrographs showing the interaction of *T. pallidum* with macrophages on polycarbonate filters. (A) The flat leading edge of the macrophage moves between the polycarbonate membrane and the treponeme. Magnification: top, ×1,620; bottom, ×6,480. (B) The ruffled portion of the macrophage binds the treponeme as the leading edge of the macrophage continues to separate the treponeme from the filter. Magnification, ×8,190. (C) The treponeme is completely separated from the polycarbonate membrane and bound entirely within the ruffled portion of the macrophage surface. Magnification, ×25,000. (D) A treponeme enclosed within a membrane-bound vacuole of a macrophage on a polycarbonate membrane. Magnification, ×40,500.

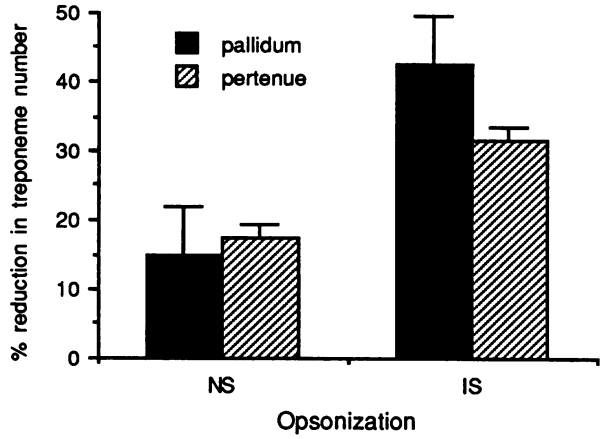

FIG. 2. Phagocytosis of killed *T. pallidum* and *T. pallidum* subsp. *pertenue* after opsonization with immune (IS) or normal (NS) serum. Macrophages and treponemes were incubated together for 24 h.

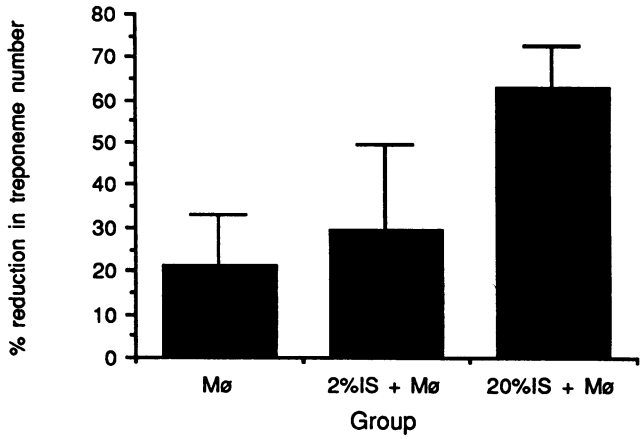

FIG. 4. Phagocytosis of live *T. pallidum* after opsonization with 2 or 20% immune serum (IS). Nonopsonized treponemes were also incubated with macrophages (Mø).

subsp. *pertenue* equally after opsonization with normal serum (Fig. 2). When *T. pallidum* and *T. pallidum* subsp. *pertenue* were opsonized with 2% immune serum, phagocytosis increased significantly ($P < 0.05$). No significant difference ($P > 0.05$) in the amount of phagocytosis was detected between the subspecies.

Resident and lipopolysaccharide-activated macrophages also did not differ in ability to phagocytize killed *T. pallidum* (Fig. 3). Opsonization of killed treponemes with 2% immune serum increased the amount of phagocytosis. However, no significant differences ($P > 0.05$) were detected between the resident and activated macrophages. Phagocytosis of treponemes was inhibited by treatment of the macrophage populations with cytochalasin B.

Effect of increased concentrations of immune serum on phagocytosis of *T. pallidum*. We next determined whether the concentration of immune serum affected the phagocytosis of live *T. pallidum*. Immune serum at a concentration of 2% failed to increase phagocytosis of *T. pallidum*. However, opsonization of live treponemes with 20% immune serum was effective in significantly ($P < 0.05$) increasing phagocytosis (Fig. 4). Opsonization with 20% normal serum did not increase phagocytosis of live treponemes (data not shown).

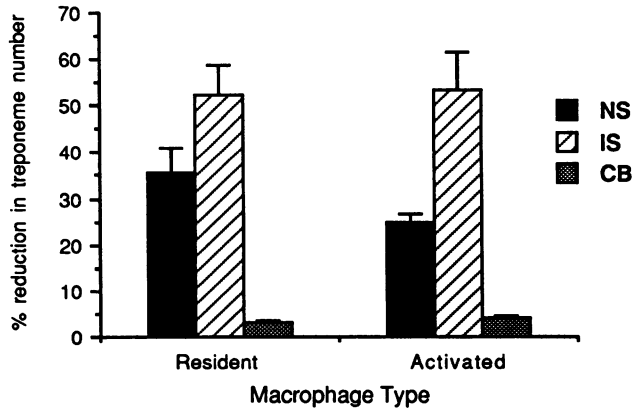

FIG. 3. Phagocytosis by resident and activated macrophages of killed *T. pallidum* after opsonization with immune (IS) or normal (NS) serum. Macrophages were treated with cytochalasin B (CB) as a control. Macrophages and treponemes were incubated together for 24 h.

Binding of immunoglobulin to the treponeme surface. Protein G-coated gold beads were used to monitor the binding of immunoglobulin to the surface of treponemes. Live treponemes recovered from rabbit testes were found to have a limited quantity of immunoglobulin G bound in vivo (Fig. 5A. Opsonization in vitro with 2% immune serum did not appreciably increase the amount of surface-bound immunoglobulin (Fig. 5B). A slight increase in the amount of surface-bound immunoglobulin was detected when treponemes were opsonized with 20% immune serum (Fig. 5C).

Kinetics of phagocytosis of viable *T. pallidum*. The kinetics of phagocytosis of live treponemes opsonized with 20% immune serum were monitored for 24 h. There was a steady decline in the number of treponemes over this interval (Fig. 6). At 1 h, there was no significant amount of phagocytosis. By 4 h, there was a 20% decrease in number of treponemes compared with the number in control cultures without macrophages. At 24 h, there was a 65% decrease in the number of spirochetes (Fig. 6).

DISCUSSION

The rate of phagocytosis of *T. pallidum* was slow, with only 65% of the treponemes phagocytized over 24 h. Activation of macrophages did not influence the rate of phagocytosis. Opsonization with low concentrations (2%) of immune serum did not increase phagocytosis of live treponemes. When the concentration of opsonizing immune serum was increased to 20%, phagocytosis of live treponemes increased. The data suggested that phagocytosis of pathogenic treponemes is a lengthy and involved process.

Microscopic evidence showed that *T. pallidum* was successfully phagocytized by hamster macrophages on polycarbonate filters. Treponemes attached to polycarbonate filters did not detach during incubation, since culture supernatants were free of spirochetes. Macrophages were observed with treponemes bound along the entire length of the spirochete. This type of binding is distinct from the "end on" and "horseshoe" binding of treponemes to monocytes reported by Brause and Roberts (7), which did not lead to phagocytosis. The flat leading edge of macrophages appeared to move between the treponeme and the filter as a prelude to scooping the spirochete onto the surface of the macrophage

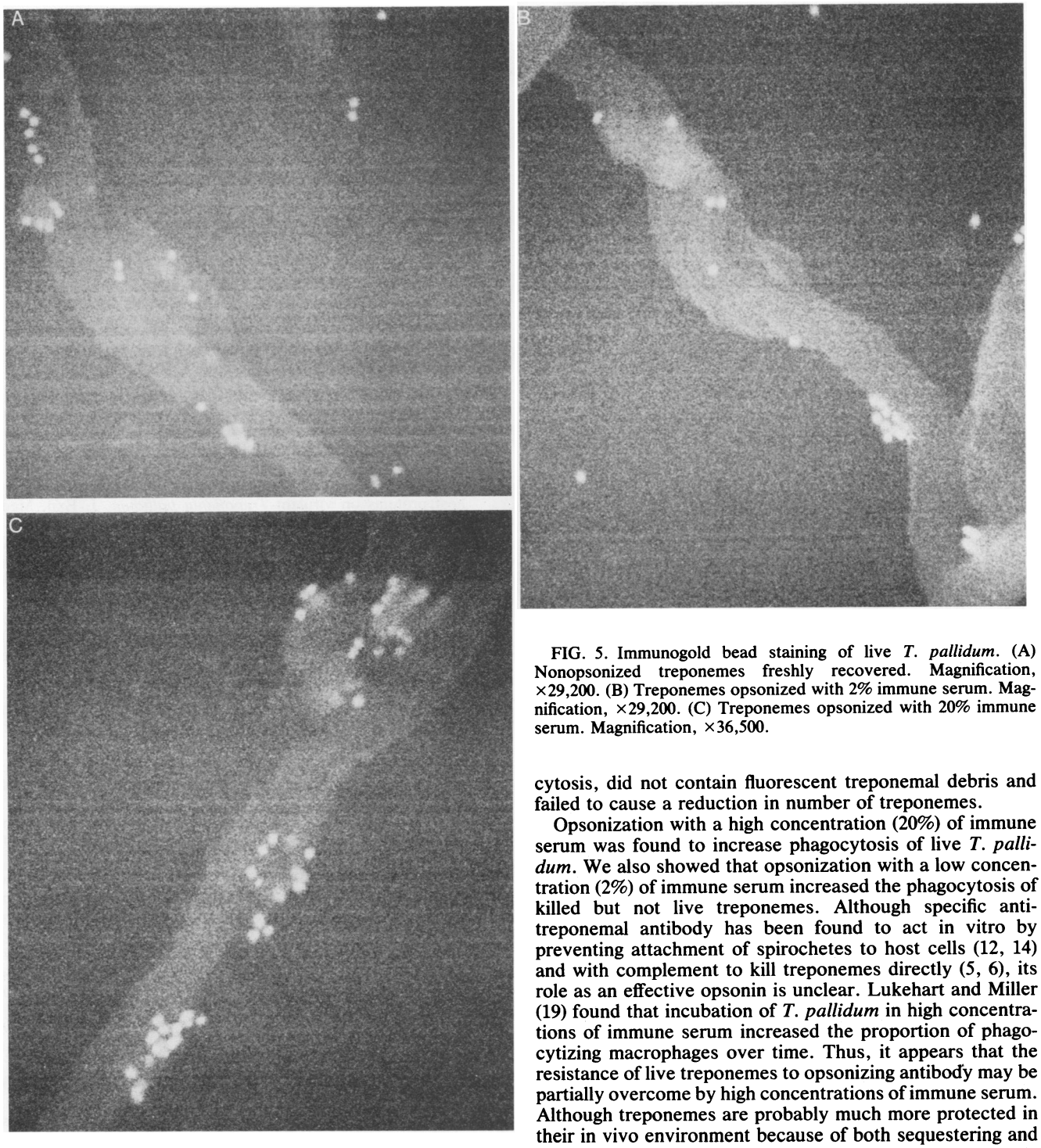

FIG. 5. Immunogold bead staining of live *T. pallidum*. (A) Nonopsonized treponemes freshly recovered. Magnification, ×29,200. (B) Treponemes opsonized with 2% immune serum. Magnification, ×29,200. (C) Treponemes opsonized with 20% immune serum. Magnification, ×36,500.

(Fig. 1A). Once the treponemes were scooped onto the ruffled portion of the macrophage membrane, projections appeared to envelope the spirochete (Fig. 1B and C). Treponemes were observed within membrane-bound vacuoles of macrophages (Fig. 1D). Also, after specific staining, fluorescent treponemal debris was observed primarily in macrophages that had incubated with treponemes. Macrophages incubated with cytochalasin B, an inhibitor of phagocytosis, did not contain fluorescent treponemal debris and failed to cause a reduction in number of treponemes.

Opsonization with a high concentration (20%) of immune serum was found to increase phagocytosis of live *T. pallidum*. We also showed that opsonization with a low concentration (2%) of immune serum increased the phagocytosis of killed but not live treponemes. Although specific antitreponemal antibody has been found to act in vitro by preventing attachment of spirochetes to host cells (12, 14) and with complement to kill treponemes directly (5, 6), its role as an effective opsonin is unclear. Lukehart and Miller (19) found that incubation of *T. pallidum* in high concentrations of immune serum increased the proportion of phagocytizing macrophages over time. Thus, it appears that the resistance of live treponemes to opsonizing antibody may be partially overcome by high concentrations of immune serum. Although treponemes are probably much more protected in their in vivo environment because of both sequestering and the binding of potentially shielding host factors (2, 10, 34), the continuous presence of high titers of antibody may influence their survival.

Staining with immunogold beads showed that live, freshly harvested treponemes contained immunoglobulin bound on their surface. These results are in agreement with those of Logan (18). Opsonization with immune serum at 2% did not greatly increase the amount of surface immunoglobulin on treponemes. However, opsonization with 20% immune serum resulted in increased binding of surface antibody. These staining results correlated well with the phagocytosis data,

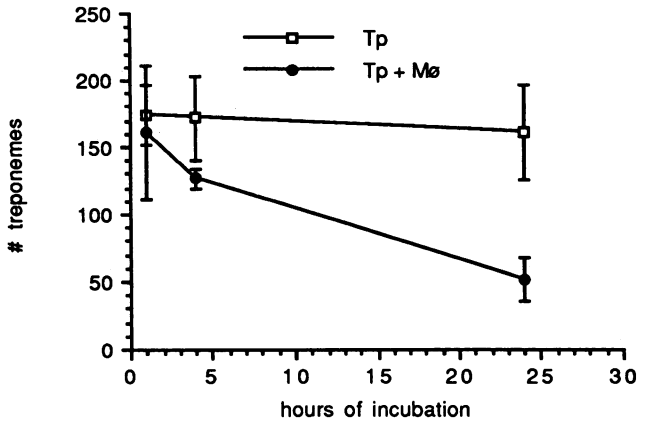

FIG. 6. Kinetics of the phagocytosis of live *T. pallidum* after opsonization with 20% immune serum. Treponemes were incubated either alone (Tp) or with an equal number of macrophages (Tp + Mø).

which showed an increase in phagocytosis of treponemes opsonized with 20% immune serum compared with that of nonopsonized treponemes or treponemes opsonized with 2% immune serum.

Recent work has suggested that the surface of *T. pallidum* possesses few protein antigens (27, 29). This antigenic inertness may contribute to the lack of a dramatic increase in surface-bound immunoglobulin or phagocytosis of treponemes after opsonization. The apparent limited mobility of treponemal surface proteins may also inhibit opsonization by limiting aggregation of surface bound immunoglobulin G (29). However, the demonstration that some treponemal proteins are proteolipids complicates interpretation, since these proteins may not be detected by freeze-fracture techniques (8).

Analysis of the rate of phagocytosis showed a relatively slow and steady decline of treponemal numbers over 24 h. Other bacteria are generally phagocytized at a much faster rate (25, 33). We showed that macrophages on polycarbonate filters can rapidly phagocytize *Staphylococcus aureus* (1). In other surface phagocytosis systems, bacteria are usually rapidly ingested at the flat leading edge of the macrophage (17). Occasionally, a few bacteria that escaped phagocytosis are observed on the ruffled portion of the macrophage membrane (17). Visual data obtained by scanning electron microscopy suggested that treponemes must be first detached from the surface of the membrane and scooped onto the ruffled portion of the macrophage membrane before phagocytosis. The outlines of ingested treponemes were not observed at the flat edge of the macrophage. Treponemes appeared to be engulfed by the ruffled portion of the macrophage membrane. The slow rate of phagocytosis could simply be due to difficulty in detaching and ingesting a relatively long, thin, and antigenically inert target attached to a membrane. This situation would also exist in vivo, where *T. pallidum* may initiate infection by first attaching to host tissue. This suggests that the slow rate of treponemal phagocytosis was not due to immunosuppression. The phagocytosis of surface-adherent bacteria may be best studied in vitro by using bacteria attached to a solid support rather than in solution (1, 9, 17).

An interesting finding of this and a previous (1) investigation was the similarity of the response of macrophages to *T. pallidum* and *T. pallidum* subsp. *pertenue*. The mechanisms by which these treponemes induce resistance may be closely related, and there is evidence that humoral and cellular mechanisms are cross-protective (30, 38).

In summary, surface-adherent *T. pallidum* were phagocytized by macrophages on membrane filters. When live treponemes were opsonized with high concentrations of immune serum, an increase in bound immunoglobulin and phagocytosis occurred. The rate of phagocytosis, however, was slow. Photographic evidence showed that phagocytosis of treponemes may be a lengthy physical process, involving detachment of the spirochetes from the membrane and scooping onto the ruffled portion of the macrophage surface before phagocytosis.

ACKNOWLEDGMENTS

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