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IN VITRO PHAGOCYTOSIS OF AVIRULENT *T. PALLIDUM* BY RABBIT MACROPHAGES

Daniel M. Musher, Nawfal N. Izzat, Kyung-Whan Min and Ferenc Györkey

From the Departments of Medicine (Infectious Diseases Section), Dermatology (Syphilology Section) and Pathology, Veterans Administration Hospital, Houston, Texas and Baylor College of Medicine, Houston, Texas, USA

Abstract. Uptake of avirulent *Treponema pallidum* by rabbit macrophages was shown to occur during incubation in vitro. Electron microscopic and metabolic studies suggested that this uptake reflected active phagocytosis by macrophages.

Electron microscopic examination of infected tissue has recently shown treponemes to be present within macrophages (2, 11). The present study is concerned with an in vitro demonstration of phagocytic activity of rabbit macrophages, when avirulent *T. pallidum* (Nichols) was added to these effector cells. The importance of such experimentation may shed light on the role of macrophages in syphilis immunity.

MATERIALS AND METHODS

Treponema pallidum. Nichols non-pathogenic strain of *T. pallidum* was used throughout the investigation. Organisms were grown in spirolate broth medium as adapted by Izzat et al. (4). After 7 days of incubation at 35°C the treponemes had normal motility and refractility when examined by darkfield microscopy. Motility was diminished only slightly by centrifugation (1 000 *g* for 30 min at room temperature), resuspension in Hanks' balanced salt solution (BSS) for brief periods of time and incubation up to 2 hours in tissue culture medium 199 enriched with 15% rabbit serum (TCM-RS). Nonmotile treponemes were obtained by refrigerating 7-day-old spirolate cultures at 4°C for 18 hours after which time darkfield examination showed intact organisms with abnormal refractility and absence of motility.

Macrophages. Six to eight ml proteose peptone solution (15% weight/volume) was injected intraperitoneally into 3–4 kg rabbits. Four days later, the animals were killed by exsanguination. The peritoneal cavity was opened and washed with 100 ml of BSS containing heparin 910 (units/ml). Leukocytes thus obtained, 85–90% of which were macrophages, were collected by centrifugation (800 *g* for 10 min at 4°C) and resuspended in TCM-RS.

Phagocytosis. Motile treponemes were distributed to Falcon tissue culture Petri dishes containing $0.6–1.4 \times 10^5$ macrophages in 2 ml TCM-RS so that a final treponeme/macrophage ratio of 1.5 : 1 was obtained. Identical aliquots of treponemes were added to 2 ml TCM-RS without macrophages. These suspensions were incubated at 37° in an atmosphere of air with 5% CO_2. After 1 hour, the cell layer was resuspended by gentle scraping with a rubber policeman and pipetting up and down with a Pasteur pipette. All treponemes that were free of cells or that could be seen attached to them were counted directly, using darkfield microscopy (4). For an experimental sample, three separate specimens were taken and 20 fields were counted for each.

Electron microscopy. Macrophages and treponemes in a ratio of five to one were incubated in TCM-RS for 1 hour, at the end of which period the cells were collected as described above, centrifuged for 10 min at 800 *g*, resuspended in 2.5% buffered glutaraldehyde, and post-fixed in osmium tetroxide (8, 10). After dehydration in a graded series of ethanol, the specimens were embedded in Epon-Araldite (6). Sections 1–2 μm in thickness were examined to select suitable areas. Ultrathin sections cut with an LKB 8800 Ultratome were stained with uranyl acetate, followed by lead citrate and studied with an RCA 3 G Electronmicroscope.

Metabolic studies. Decarboxylation of the first carbon of glucose was measured using methods described by Musher et al. (7). 1-^{14}C-glucose (0.25 μCi) was added to 1 ml TCM-RS containing 2×10^6 macrophages and 10^8 motile or nonmotile treponemes. Labelled glucose was added to other aliquots of TCM-RS which contained either 2×10^6 macrophages or 10^8 treponemes. After 30 min of incubation in a shaking water bath at 37°, the reaction was terminated by disrupting the cells with 1 ml of 2 N HCl. Radioactively labelled CO_2 released from cells was recovered in hydroxide of hyamine which was then dissolved in scintillation fluid (0.4% POP, 0.01% POPOP in toluene). Radioactive counting was done in a Packard liquid scintillation spectrometer. Data are re-

Table I. *Effect of incubation with rabbit macrophages on darkfield counts of T. pallidum*

Experiment	Darkfield counts ($\times 10^6$) following incubation of treponemes	
	With macrophages	Without macrophages
1	6.3[a]	11.9[a]
2	7.7	11.1
3	6.7	10.5
4	11.1	19.0
5	11.5	20.1
Average	8.6	15.3

[a] Counts represent an average of 20 darkfields from each of 3 separate specimens.

ported as counts per minute (cpm) $\pm$ 1 Standard Error of the Mean (S.E.M.). Statistical significance was computed using Student's *t* test.

RESULTS

Table I shows treponemal darkfield counts after incubation for 1 hour in TCM-RS with and without macrophages. In five consecutive experiments, exposure to macrophages reduced the number of treponemes from an average of 15.3×10^6 to 8.6×10^6 per ml. Electron microscopic examination of these exposed cells revealed macrophages with small irregular nuclei and abundant cytoplasm, having numerous cytoplasmic villi and intracytoplasmic phagocytic vacuoles (Fig. 1). Segments of treponeme(s) consisting of a dense treponemal body surrounded by an envelope (5) were seen within phagocytic vacuoles (Fig. 2).

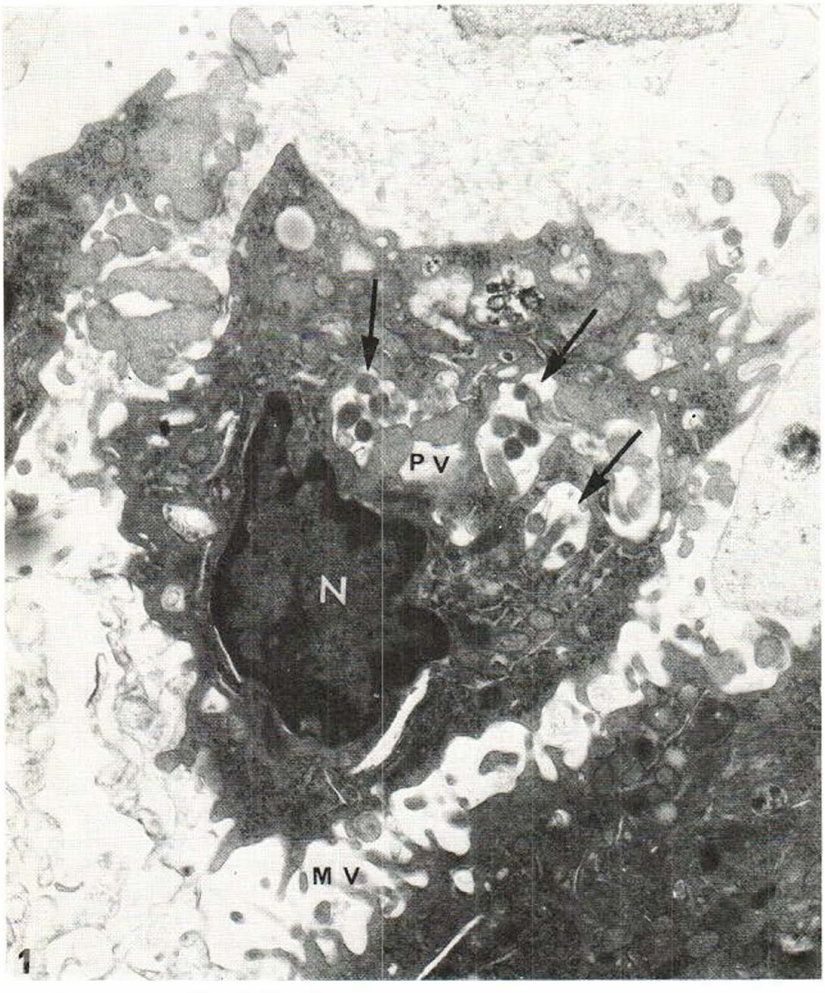

Fig. 1. Electron micrograph of a macrophage showing the nucleus (*N*), cytoplasmic microvilli (*MV*) and phagocytic vacuoles (*PV*) which contain treponeme(s) (*arrow*) cut in cross-section. × 11 000.

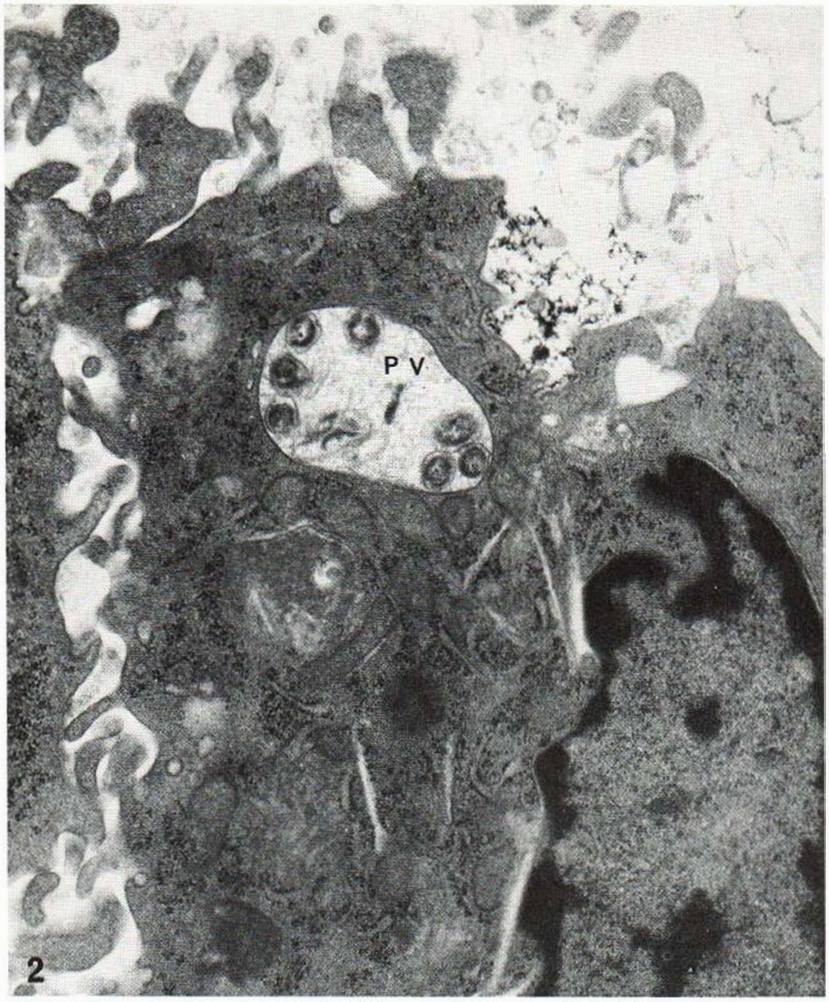

Fig. 2. A phagocytic vacuole (*PV*) at higher magnification containing treponemal structures characterized by a dense treponemal body and surrounded by an envelope. × 18 850.

Some vacuoles contained treponemes with broken membranes and loss of structural clarity, indicating disintegration. Treponemes were not detected within lymphocytes or polymorphonuclear leukocytes.

Table II shows that macrophages incubated in TCM-RS with 0.25 μCi of 1-^{14}C-glucose generated an average of $1\,242 \pm 69$ cpm of $^{14}CO_2$ per 10^6 macrophages. 10^8 motile or nonmotile treponemes produced 93 ± 6 and 79 ± 5 cpm respectively. When macrophages were incubated with motile or nonmotile treponemes, the amount of $^{14}CO_2$ which they generated increased to $2\,090 \pm 191$ (68.3 %) and $1\,730 \pm 135$ (40.9 %) respectively. In each case the increase was statistically significant ($p < 0.001$); there was no significant difference between the values achieved by macrophages phagocytizing motile or nonmotile treponemes ($p = 0.5$).

DISCUSSION

The data presented herein provide evidence that rabbit macrophages actively phagocytize avirulent *Treponema pallidum* in vitro. Before the recent demonstration of treponemes within macrophages in a chancre (2) or an infected rabbit testis (11), these organisms were either not seen at all in syphilitic lesions because light microscopy was used, or were thought, on the basis of electron microscopic studies, to be present only extracellularly (3, 5). Since treponemes were seen within cells other than macrophages, Azar et al. (2) and Sykes & Miller (11) felt that they could not

Table II. *Decarboxylation of glucose by macrophages during phagocytosis of treponemes*[a]

Macrophages alone		1 242 ± 69
Treponemes alone	motile	93 ± 6
	nonmotile	79 ± 5
Macrophages + motile treponemes		2 090 ± 191 (68.3 %)[b]
Macrophages + nonmotile treponemes		1 750 ± 135 (40.9 %)[b]

[a] Data represent the average of five separate studies, each carried out in triplicate, expressed as cpm/10^6 macrophages ± 1 S.E.M.

[b] Percentage increase in decarboxylation of glucose by phagocytizing compared with resting macrophages after subtracting the contribution by treponemes and background ($p < 0.001$). There is no statistically significant difference between 68.3 % and 40.9 % ($p = 0.5$).

exclude the possibility that macrophages might play a passive role (being penetrated by the spirochetes) rather than an actively phagocytic role.

In the present study, treponemal counts declined during incubation *in vitro* with rabbit macrophages. Electron microscopy provided evidence that the macrophages had actively phagocytized treponemes. Treponemes were seen within membrane-bound intracytoplasmic phagocytic vacuoles. In contrast, these organisms were not found in nonphagocytic cells, thus supporting the hypothesis that their presence in macrophages resulted from active phagocytosis rather than from an ability of treponemes to penetrate cells.

Further evidence was obtained by measuring decarboxylation of the first carbon of glucose by macrophages exposed to treponemes. This metabolic activity, which approximates metabolism of glucose via the hexose monophosphate shunt and is associated with phagocytosis by macrophages (9), increased by 68.3 % and 40.9 % when macrophages were exposed to motile or nonmotile treponemes, respectively. It is possible that some of the metabolic stimulation resulted from attachment of treponemes to the cell surface rather than by actual ingestion (1, 7). However, taken in conjunction with observations showing reduced treponemal counts following exposure to macrophages and the presence of organisms within phagocytic vacuoles, the metabolic data appear to reflect changes that have occurred during ingestion.

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D. M. Musher, M.D.
V. A. Hospital
Infectious Disease Lab.
Houston, Texas 77031
USA