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In Vitro Interaction of *Neisseria gonorrhoeae* Type 1 and Type 4 with Tissue Culture Cells

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As a basis for studies of gonococcal pathogenicity, tissue culture cells were infected with type 1 or type 4 *Neisseria gonorrhoeae* to determine intracellular viability. A simple and objective means of measurement was devised, based on the uptake of tritiated protein and deoxyribonucleic acid precursors by cycloheximide-inhibited cells infected with gonococci. Cycloheximide was found to inhibit protein synthesis by over 97% in tissue culture cells at a concentration of 100 μg/ml. In contrast, *N. gonorrhoeae* was found to be highly resistant to this antibiotic, and protein synthesis was unaffected by concentrations up to 1,000 μg/ml. Extracellular gonococci were eliminated by treatment with high concentrations of penicillin during cycloheximide inhibition and prior to the addition of radioisotope. Levels of protein and deoxyribonucleic acid synthesis by *N. gonorrhoeae* in the cycloheximide-treated cells were significantly higher in T1-infected cells (RE_2, HeLa, or HEp-2) than in the corresponding T4-infected cells. No differences were observed in tissue cell susceptibility to gonococcal infection. Intracytoplasmic localization of *N. gonorrhoeae* was confirmed by electron microscopy.

The importance of *Neisseria gonorrhoeae* in human infections has increased considerably over the past years. Both serum (1, 10) and cell-mediated (7, 11, 16) defense mechanisms have been implicated in the host resistance to infection. Although serum bactericidal factors have been demonstrated in vitro, they may not always operate in vivo. It would seem that gonococcal pathogenicity may involve other factors such as survival of gonococci within human polymorphonuclear leukocytes (4, 18, 24, 32, 38) or within normal eukaryotic cells (21, 25, 30, 31, 34, 35). Furthermore, electron microscopy of infected tissues have provided strong evidence of intracellular localization of gonococci in urethral exudate (8, 20) as well as in cultures of infected HeLa cells (34), human diploid cells (25), and monkey kidney cells (35). Correlation of adsorption to tissue cells and type variation of *N. gonorrhoeae* (15) has shown that virulent type 1 demonstrates enhanced attachment to various types of host cells (13, 26, 28, 33, 37) when compared with avirulent type 4.

It has been observed that attachment and passage into underlying tissues through mucosal cells is important in virulent *Shigella* infections (2). Tubercle and leprosy bacilli, brucellae, and *Listeria* spp. are the typical "intracellular" pathogens, and ability to grow within tissue cells is an important aspect of the pathogenicity of these bacteria (23). If gonococci are isolated within the cell and inaccessible to the action of drugs (30, 31) and antibodies, they may survive for long periods of time in the phagosome as a reservoir of infection and possible cause of asymptomatic gonorrhoea (20).

Attempts to quantitate association of gonococci to tissue cells have been difficult, and light microscope examination of infected cells fails to differentiate between intracellular and extracellular organisms (27, 35). To measure differences in intracellular metabolic activity between colonial variants type 1 and type 4 of *N. gonorrhoeae* in infected tissue cells, it was necessary to devise a more precise in vitro technique.

MATERIALS AND METHODS

Bacterial strain. *N. gonorrhoeae* strain 74123 was originally obtained from the Regional Public Health Laboratory, Ottawa, Ontario. Colony types T1 and T4 were maintained by daily passage and differentiated for colonial variation as described previously (15).

Growth conditions. *N. gonorrhoeae* strain 74123 was grown either on solid GC medium (GC base, Oxoid agar) containing chemically defined supple-

ment (15) or in Catlin liquid medium (Gibco Diagnostics, Chagrin Falls Ohio) (3). Incubation was conducted at 37°C under a controlled 10% CO_2 atmosphere.

Tissue culture cells. Human carcinoma of larynx (HEp-2) cells were obtained from R. Bather, Bureau of Biologics, Health and Welfare Canada, Ottawa, Ontario, and maintained in Eagle basal medium with Hanks salts. Rhesus monkey kidney cells (RE_2) and human cervical cancer cells (HeLa) were routinely cultured in medium 199 with Hanks salts. All tissue culture medium was antibiotic free and supplemented with 10% heat-inactivated fetal calf serum unless otherwise specified. The tissue culture media, supplements, and sera were obtained from Grand Island Biological Co., Grand Island, N.Y. Viable cell counts were performed by dilution of cell suspensions with 0.4% trypan blue in phosphate-buffered saline (PBS).

Bacterial suspensions for infection studies. *N. gonorrhoeae* was harvested after 18 h of growth in solid or liquid medium and suspended in PBS containing 0.1% gelatin. Only colonies of the desired type were removed from solid media for preparation of the bacterial suspension. *N. gonorrhoeae* cultured in Catlin medium were routinely plated to ensure that greater than 95% of the culture had maintained virulent-type morphology during growth according to the criteria of Kellogg et al. (15). Cell suspensions were adjusted to desired cell density and dilutions were prepared in PBS for determination of colony-forming units (CFU).

Antimetabolites. Cycloheximide (Upjohn, Kalamazoo, Mich.) was dissolved in PBS at a concentration of 1 mg/ml, filtered, and kept frozen at −70°C in small portions. Sodium benzylpenicillin (1.135×10^3 U/mg) and ampicillin trihydrate (835 μg/mg) (Bureau of Biologics, Ottawa, Ontario) were dissolved in PBS, filtered, and frozen in portions at −70°C.

Assay of radioisotope incorporation. Fifteen hours before the harvest of tissue culture cells, either 0.1 μCi of ^{14}C-labeled L-amino acid mixture, 1 μCi of [^{3}H]thymine (10 Ci/mmol), or 4 μCi of L-[^{3}H]leucine (60 Ci/mmol) was added to each 2 ml of culture medium, depending on the experiment. The cells were removed by trypsinization (0.25% for 15 min at 37°C) and centrifuged at 2,500 rpm for 20 min. The cell pellet was then suspended in 1 ml of distilled water, homogenized, and precipitated with 1 ml of ice-cold 10% trichloroacetic acid. Each suspension was poured onto a glass-fiber filter, washed twice with 5 ml of 95% ethanol, place in scintillation vials, and left to dry. A 5-ml portion of toluene–2,5-diphenyloxazole–1,4-bis-(5-phenyloxazolyl)benzene scintillation fluid was then added to each vial. Samples were counted in a (Beckman Instruments Inc., Fullerton, Calif.).

Electron microscopy. HeLa cells grown in 35-mm tissue culture dishes (Falcon Plastics, Oxnard, Calif.) and infected with *N. gonorrhoeae* for 8 h at 37°C were washed twice with warm PBS and fixed, in situ, with 2.5% glutaraldehyde in phosphate buffer for 1 h at pH 7. The fixed monolayer was then washed three times with 0.2 M sucrose in phosphate buffer, removed, centrifuged and postfixed in 2% unbuffered OsO_4 for 24 h at room temperature. After further washing and immersion in 0.5% buffered uranyl acetate for 1 h at room temperature, fixed specimens were dehydrated by passage through a series of increasing concentrations of acetone and finally embedded in Epon. Sections were cut on an LKB Huxley ultramicrotome, stained with uranyl acetate, and counterstained by Reynolds lead citrate (22). The preparations were examined and photographed with a Siemens Elmiskop 102 electron microscope.

RESULTS

Inhibition of protein synthesis by cycloheximide. To delineate subsequent experiments utilizing cycloheximide, levels of protein synthesis were established in control and treated cells under normal growth conditions. A total of 1×10^6 viable HeLa, RE_2, or HEp-2 cells, as determined by trypan blue exclusion, were cultured in 35-mm tissue culture dishes (Falcon Plastics) in a humidified 5% CO_2 incubator. After 18 h, the monolayers were rinsed twice with 2 ml of PBS, and then 2 ml of fresh tissue culture medium containing 2% fetal calf serum and 2×10^4 U of penicillin was added to each dish. Half of the dishes from each cell line received 200 μg of cycloheximide. After 2 h at 37°C, 4 μCi of [^{3}H]leucine was added, and incubation was continued for 15 h at 37°C under 5% CO_2. At the end of the incubation period, the cells were assayed for radioisotope incorporation. The results of Table 1 summarize the inhibition of protein synthesis by cycloheximide. Greater than 97% inhibition was observed in each cell line. The experiment demonstrates the consistently low levels of incorporation in uninfected inhibited tissue cells.

Effect of penicillin and cycloheximide on *N. gonorrhoeae*. To ensure adequate extracellular bactericidal concentrations of penicillin, protein synthesis as well as extracellular growth were monitored as a function of increasing penicillin concentration in a time-limited mock experiment. A 2-h infection experiment was devised since previous results with RE_2

TABLE 1. *Inhibition of protein synthesis in cycloheximide-treated tissue culture cells*

Cells	[^{3}H]leucine incorporation[a]		
	Control	Cycloheximide (100 μg/ml)	Inhibition (%)
RE_2	44,245 ± 2,151	1,151 ± 170	97.4
HeLa	70,192 ± 4,363	837 ± 30	98.9
HEp-2	49,141 ± 1,690	680 ± 69	98.7

[a] Mean counts per minute per culture ± standard deviation.

cells infected with *N. gonorrhoeae* (T1) have shown no phagocytosis of gonococci in ultrathin sections (19). In parallel, the sensitivity of T1 and T4 to varying cycloheximide concentrations was determined.

In the penicillin experiment, 1×10^6 RE_2 cells were grown in 35-mm dishes for 18 h. The monolayers were washed twice with PBS and inoculated with 2 ml of a suspension containing about 3×10^8 gonococci per ml in a mixture of 50% M199–50% Catlin medium. After 2 h at 37°C, the supernatant was discarded and the monolayer was rinsed thoroughly three times with 2-ml volumes of PBS. Fresh tissue culture medium containing 100 μg of cycloheximide per ml and varying penicillin concentrations was added to each dish. After another 2 h at 37°C, 1 μCi of ^{14}C-labeled L-amino acid mixture was added, and incubation was continued for 15 h at 37°C in a 5% CO_2 atmosphere. Prior to assay for radioisotope incorporation, portions of each infected culture were plated on GC agar and incubated for 48 h at 37°C in a candle extinction jar. Triplicate cultures were utilized for each concentration of penicillin. The penicillin section of Fig. 1 correlates the amino acid incorporation and extracellular gonococcal growth with penicillin concentration. For the purpose of this experimentation with cycloheximide-treated cells, a concentration of 10^4 U of penicillin per ml is sufficient to prevent growth and uptake of amino acid precursors by extracellular *N. gonorrhoeae*.

To measure the metabolism of intracellular gonococci in infected cycloheximide-treated cells, it was necessary to establish the sensitivity of T1 and T4 to cycloheximide. Suspensions of about 1×10^8 T1 and T4 in 50% M199–50% Catlin medium were incubated for 2 h at 37°C in the presence of increasing concentrations of cycloheximide before the addition of 0.25 μCi of ^{14}C-labeled L-amino acid mixture to each culture. After 15 h of incorporation, the gonococcal suspension was centrifuged at 3,500 rpm for 30 min and assayed for radioactivity. *N. gonorrhoeae* T1 and T4 were resistant to a minimum of 1,000 μg of cycloheximide per ml (Fig. 1). Protein synthesis as well as positive growth continued at all concentrations tested.

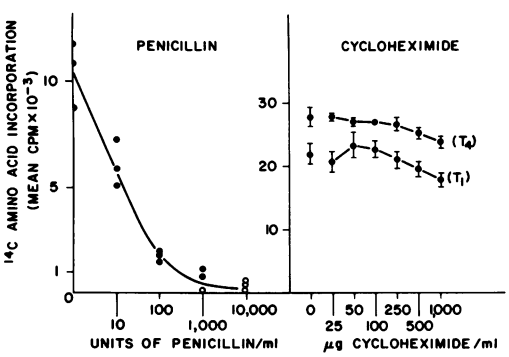

FIG. 1. *Dose effect of inhibitors on extracellular viability of N. gonorrhoeae. Symbols:* ●, *Positive growth;* ○, *negative growth. Penicillin dose effect was determined using RE_2 monolayers infected with T4 for 2 h at 37°C before the addition of the antibiotic. Cycloheximide dose effect was performed in the absence of tissue cells.*

Comparison of intracellular viability of *N. gonorrhoeae* types 1 and 4. Experimental conditions were defined to demonstrate differential infectivity of virulent and avirulent gonococci as well as tissue cell susceptibility. RE_2, HeLa, and HEp-2 cells were incubated for 18 h in 35-mm dishes at 1×10^6 cells per dish in antibiotic-free tissue culture medium supplemented with 10% fetal calf serum. Confluent monolayers were rinsed twice with PBS to remove excess serum and overlaid with 2 ml of a 50% M199–50% Catlin mixture containing 2% fetal calf serum and approximately 5×10^7 to 1×10^8 gonococci type 1 or type 4. Precise numbers of infecting CFU were determined by serial dilution and plate count. Replicate experiments were performed utilizing *N. gonorrhoeae* propagated on solid GC medium or in Catlin liquid medium. After 7 h of infection at 37°C under 5% CO_2, portions were removed from all dishes to test for maintenance of gonococcal type specificity and to confirm absence of bacterial contamination. The infecting medium was discarded and the monolayers were thoroughly washed twice with 2-ml volumes of PBS to remove unattached gonococci. To inactivate any remaining extracellular *N. gonorrhoeae* and inhibit tissue cell protein synthesis, dishes were treated with 2 ml of a mixture containing M199, 2% fetal calf serum, 200 μg of cycloheximide, and 2×10^4 U of penicillin. Cultures were returned to the incubator for 2 h before the addition of 0.1 μCi of [^{3}H]leucine, and incubation continued for 15 h at 37°C.

Control experiments were performed simultaneously to minimize variation and assess the significance of [^{3}H]leucine incorporation by intracellular gonococci. Uninfected tissue cells were maintained in the presence and absence of cycloheximide to measure the base level of incorporation in the inhibited system at 37°C. A separate set of controls, both infected and uninfected, were treated and maintained at 4°C during the 7-h infection time. Incubation at 4°C was carried out to inhibit phagocytosis but to include attachment of gonococci to tissue cells and thus establish the extracellular background for an infected experimental system.

The results were calculated as [(infected at 37°C − uninfected at 37° C) − (infected at 4°C)], and the mean counts per minute and standard deviation of the difference were recorded.

After 15 h of incorporation, portions from all dishes were plated for 48 h on GC agar to confirm the absence of viable extracellular gonococci. The assay of radioisotope incorporation was performed as described above.

Figures 2 and 3 represent the incorporation of [^{3}H]leucine by intracellular type 1 and type 4 gonococci in cycloheximide-treated cells. To determine variability caused by growth on liquid as opposed to solid media, the gonococci (T1 and T4) were propagated either in Catlin medium or on GC agar. Comparison of Fig. 2 and 3 indicates no significant difference with respect to varying growth conditions of the infecting gonococci. The results clearly demonstrate that the levels of protein synthesis by *N. gonorrhoeae* in the inhibited system are much higher in T1-infected cells (RE_2, HeLa, or HEp-2) than in the corresponding T4-infected cells. No significant differences were observed in susceptibility to infection of the cell lines. However, differences between the T1- and T4-infected cells were highly significant when analyzed statistically but the Student *t* test (all *P* values less than 0.0025).

Selective [^{3}H]thymine uptake in *N. gonorrhoeae*-infected HeLa cells. In an attempt to further elucidate the intracellular metabolism of *N. gonorrhoeae* types 1 and 4, deoxyribonucleic acid (DNA) synthesis was monitored by the uptake of [^{3}H]thymine in infected cycloheximide-treated cells. Cycloheximide inhibition was continued during this experimentation to maintain a low background, although the amount of thymine converted back to the corresponding nucleotide is negligible in mammalian cells (9) (Table 2). The infection, incubation procedure, and radioisotope extraction were performed as described in the preceding paragraphs. DNA synthesis by intracellular *N. gonorrhoeae* types 1 and 4 was measured by the addition of 1 μCi of [^{3}H]thymine to each infected HeLa cell monolayer. As previously reported in in vitro systems, the incorporation of thymine into cellular DNA is low and contrasts with the much greater incorporation of deoxythymidine (9, 12, 14). The results of control experiments in Table 2 suggest that thymine uptake is considerably higher in bacteria than in tissue culture cells and would provide a very sensitive index for the measurement of infection by gonococci. Analysis of the data in the lower portion of Table 2 indicates that DNA synthesis by *N. gonorrhoeae* type 1 is 10 times higher than in the corresponding cells infected with type 4. In the 4°C mock experiment where phagocytosis was inhibited, the [^{3}H]thymine uptake was consistently low.

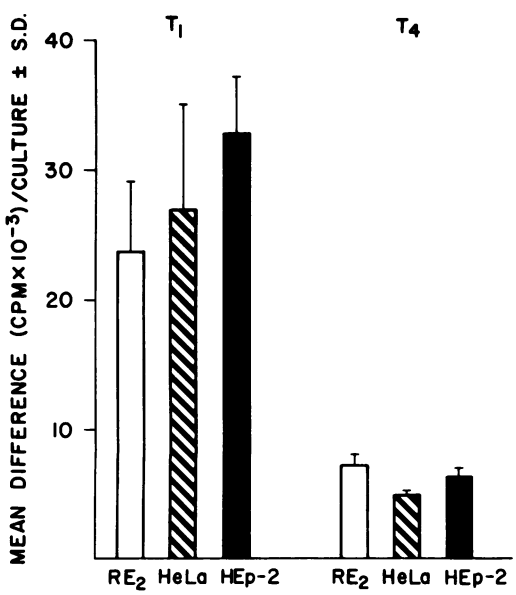

FIG. 2. *[^{3}H]leucine incorporation by intracellular N. gonorrhoeae T1 and T4 grown in liquid medium. Each column represents the mean difference counts per minute of infected cells at 37°C minus infected cells at 4°C.*

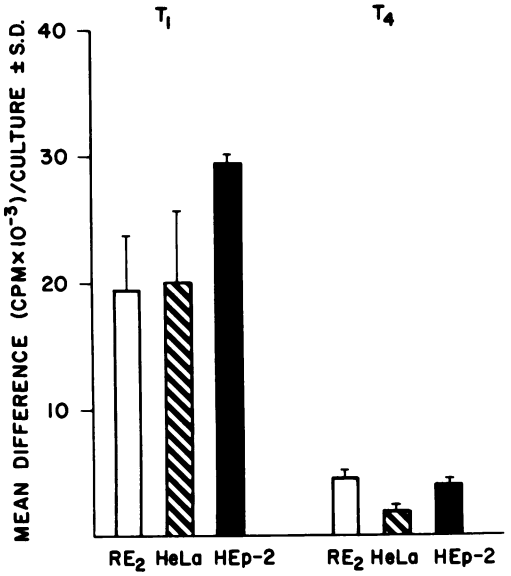

FIG. 3. *[^{3}H]leucine incorporation by intracellular N. gonorrhoeae T1 and T4 grown on solid medium. Each column represents the mean difference counts per minute of infected cells at 37°C minus infected cells at 4°C.*

Electron microscopy of infected HeLa cells.

TABLE 2. *DNA synthesis in cycloheximide-treated HeLa cells infected with N. gonorrhoeae*

N. gonorrhoeae (no. of GC/culture)[a]	Experimental conditions	^{3}H[thymine] uptake[b]
Type 1 (1×10^7)	Bacterial control	2,903 ± 301
Type 4 (1×10^7)	Bacterial control	7,705 ± 781
	HeLa cell control	
None	+ Cycloheximide	85 ± 16
None	− Cycloheximide	287 ± 46
Type 1 (1×10^7)	Cells infected at 4°C	654 ± 298
	Cells infected at 37°C	13,483 ± 4,144
Type 4 (1.25×10^7)	Cells infected at 4°C	482 ± 175
	Cells infected at 37°C	1,651 ± 236

[a] GC, Gonococci.
[b] Mean counts per minute ± standard deviation.

Adequate evaluation of the intracellular localization of gonococci requires electron microscope examination of ultrathin sections of infected tissue culture cells. HeLa cells were incubated for 18 h in 35-mm dishes at 1×10^6 cells per dish in antibiotic-free tissue culture medium supplemented with 10% fetal calf serum. The monolayers were rinsed twice with PBS and overlaid with 2 ml of a 50% M199–50% Catlin mixture containing 2% fetal calf serum and 5×10^7 type 1 *N. gonorrhoeae* 74123. After 8 h of incubation, infected monolayers were rinsed and fixed, in situ, as described above.

In cross sections of HeLa cells infected for 8 h, gonococci were observed in close association with the tissue cell membrane, and numerous morphologically intact organisms were found to be intracellular (Fig. 4). It is difficult to determine on the basis of these micrographs whether the gonococci situated in the cytoplasm of the HeLa cells are enclosed within membrane-limited vacuoles as observed by others (26, 34, 35). Organisms adhering to the tissue cell surface have portions of their outer membrane that are closely integrated with the cytoplasmic membranes of the tissue cell (Fig. 4b).

DISCUSSION

Studies of urethral exudate from acute cases of gonorrhea show a close association of the diplococci with polymorphonuclear cells in addition to numerous intracellular gonococci (17, 20). The present study provides evidence for the localization and metabolic activity of intracellular gonococci in tissue culture cells. Our results confirm previous observations by Swanson (26), Tyeryar et al. (34), and Waitkins and Flynn (35) that epithelial cells infected in vitro contain gonococci within membrane-limited structures but are contradictory to recent results (19) with RE_2-infected cells. Presently, the differences with the results of Ota et al. (19) cannot be explained. To date, association of gonococci with tissue cells has been evaluated qualitatively by light and electron microscopic examination (27). Hence, the relative association could only be measured by attachment plus ingestion, and the two phenomena could not be measured independently. Dilworth et al. (4) have devised a more sophisticated technique whereby interaction of gonococci with human neutrophils was studied. In this system, ingestion was separated from attachent using a combination of phase-contrast, fluorescent, and light microscopy. This study describes a simple, quantitative means of measuring the viability of intracellular *N. gonorrhoeae* in infected tissue culture cells. The technique is based on the uptake of tritiated protein and DNA precursors by cells infected with gonococci in a system where protein synthesis by the tissue cells was inhibited by cycloheximide, an antibiotic to which gonococci are highly resistant. The data clearly demonstrate that the levels of protein and DNA synthesis by *N. gonorrhoeae* in the cycloheximide-treated cells were significantly higher in T1-infected cells than in the corresponding T4-infected cells. These results suggest a correlation between virulence and intracellular location for *N. gonorrhoeae* analogous to systems infected with virulent *Shigella* sp., *Streptococcus* sp., and *Proteus* sp. (2, 5, 6). Ingestion of virulent gonococci by tissue culture cells could be mediated by a trypsin and heat-sensitive nonpilus membrane component described by Swanson et al. (29).

Furthermore, by using [^{3}H]thymine as a labeled precursor to monitor bacterial DNA synthesis in infected tissue culture cells, it was demonstrated that *N. gonorrhoeae* type 1 incorporates 10 times more thymine than avirulent type 4. It is well established in mammalian systems that the amount of thymine converted back to the corresponding nucleotide is negligi-

ble (9, 12), and this is verified by underdetectable uptake of [^{3}H]thymine in the HeLa cells (Table 2). Anabolism of [^{3}H]thymine into bacterial DNA is assured in the presence of deoxyribosyl donors and thymidine phosphorylase, generally localized near the surface of bacterial cells (14). The data in Table 2 show that exogenous [^{3}H]thymine is readily utilized for DNA synthesis by the type 1 gonococci in the infected HeLa cells, perhaps reflecting the potential differences in the number of virulent organisms capable of adhering and achieving an intracellular location or the possible inherent metabolic differences of intracellular type 1 as opposed to type 4 cells.

Attachment of gonococci to tissue cells in vitro is facilitated by the presence of pili (26), and other investigators (36) have suggested that firmly attached gonococci are capable of multiplying on the surface of epithelium-lined tissues. Failure of several antibiotics to kill phagocytized gonococci in tissue culture cells (30, 31) could be extended to explain recurrent infections in patients by the strong adherence

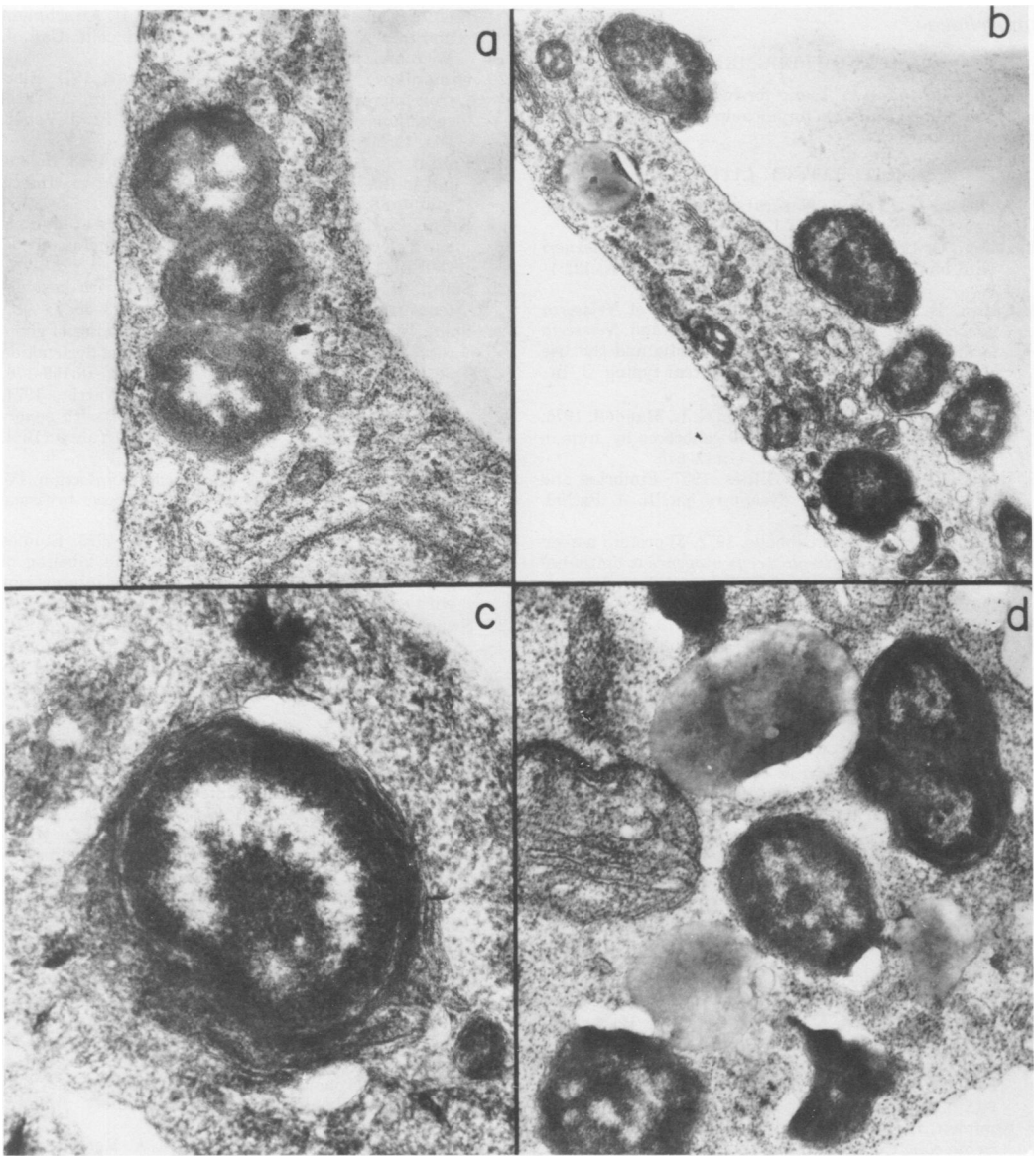

FIG. 4. *Intracytoplasmic localization of N. gonorrhoeae type 1 in ultrathin section of HeLa cells infected for 8 h. (a) ×30,000; (b) ×21,000; (c) ×60,000; (d) ×30,000.*

of virulent gonococci to cellular cytoplasmic membranes or by the intracytoplasmic persistence of viable gonococci.

This investigation supports the hypothesis that epithelial cell penetration is a major factor in determining the pathogenicity of *N. gonorrhoeae* (36). The results presented in this paper describe an in vitro model of infection whereby the intracellular viability of virulent and avirulent gonococci may be measured differentially. This model will enable us to study the effect of specific gonococcal antisera and/or metabolic inhibitors on the infection parameters of *N. gonorrhoeae*.

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