

Growth of *Legionella pneumophila* in a human macrophage-like (U937) cell line

Eric Pearlman,¹ Asmina H. Jiwa,¹ N. Cary Engleberg^{1,2,3} and Barry I. Eisenstein^{1,2*}

¹Departments of Microbiology and Immunology and ²Internal Medicine, University of Michigan, Ann Arbor, MI 48109-0620, U.S.A. and ³Ann Arbor Veterans Administration Hospital

(Received March 16, 1988; accepted April 18, 1988)

Pearlman E. (Dept. of Microbiology and Immunology, University of Michigan, Ann Arbor, MI 48109-0620, U.S.A.), A. H. Jiwa, N. C. Engleberg and B. I. Eisenstein. Growth of *Legionella pneumophila* in a human macrophage-like (U937) cell line. *Microbial Pathogenesis* 1988; 5: 87–95.

We established a model of the bacteria-macrophage interaction to study the cellular basis of *Legionella pneumophila* pathogenesis and to characterize avirulent *L. pneumophila*. We found that U937 cells, which are derived from a human histiocytic lymphoma cell line, support intracellular growth of *L. pneumophila* with a doubling time of 6 h, and that sustained intracellular growth is associated with a cytopathic effect (CPE) that can be detected by microscopic examination and quantified with the vital stain 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). An *L. pneumophila* isolate obtained directly from infected guinea-pig spleens can grow and produce CPE in these cells, destroying most of the cell layer after 72 h of growth. Only 10⁶ organisms of this strain are required to kill 50% of guinea-pigs inoculated by the intraperitoneal route. In contrast, an avirulent isolate derived by 203 successive plate passages of the same strain can neither kill guinea-pigs at an intraperitoneal inoculum of 10⁷ nor grow or produce CPE in U937 cells. Since the cells were able to differentiate between a virulent and an avirulent strain of *L. pneumophila*, we conclude that U937 cells are an appropriate model system for study of the bacteria-macrophage interaction.

Key words: *L. pneumophila*; U937 cells; intracellular growth.

Introduction

During the course of Legionnaires' disease, *Legionella pneumophila* replicates primarily intracellularly within alveolar macrophages.^{1,2} To characterize the intracellular life cycle of this organism, investigators have employed a variety of host cells capable of supporting growth, including human monocytes,³ free-living protozoa,^{4–6} human and animal epithelial cell lines,^{7,8} and primary explanted macrophages from various origins.^{9–14} To our knowledge, there have been no prior attempts to demonstrate intracellular growth of *L. pneumophila* in a host cell having both the properties of a human alveolar macrophage and the properties of a continuous cell line.

The purpose of this study was to determine if a cell line with these combined properties would permit the intracellular growth of *L. pneumophila* and whether this cell line could discriminate between guinea-pig virulent and avirulent derivatives of *L.*

* Author to whom correspondence should be addressed.

pneumophila. Such a cell line would have the obvious advantages of ease of use, reproducibility, and relevance to native human infection. As a candidate cell model system, we chose the U937 cell line, which was derived from a human histiocytic lymphoma.¹⁵ This cell line is maintained as replicative non-adherent cells having many of the biochemical and morphological characteristics of blood monocytes.¹⁶ When treated with phorbol myristate acetate (PMA), U937 cells differentiate to become adherent, non-replicative cells with characteristics of tissue macrophages, including isoenzyme patterns,¹⁷ CR3 expression,¹⁸ and other phenotypic markers.¹⁶ Because of these properties, this cell line has been used by investigators to support the growth of other intracellular pathogens.¹⁹⁻²¹

We validated the U937 cell line as a model for *L. pneumophila*/macrophage interaction by demonstrating that PMA-transformed U937 cells support the intracellular growth of *L. pneumophila* bacteria. In addition, we showed that this macrophage model can distinguish guinea-pig-virulent from avirulent strains of *L. pneumophila*.

Results

Kinetics of intracellular growth

In preliminary experiments we confirmed that PMA-transformed U937 cells would adhere to the base of wells on polystyrene tissue culture plates and that the cells resisted displacement by vigorous washing. Using this monolayer system we next determined whether *L. pneumophila* would grow intracellularly.

We incubated *L. pneumophila* with U937 cell monolayers for 2 h, at which time we removed the extracellular bacteria by washing the monolayer three times. During the logarithmic phase of growth, the bacteria doubled in number every 6 h (Fig. 1), and the infection continued until the macrophage layer was destroyed. To show that growth is dependent on the presence of intact cells, we incubated the bacteria with 5×10^5 U937 cells that had been disrupted by three cycles of freeze/thaw. No growth was noted under these conditions (Fig. 1), or when the bacteria were incubated

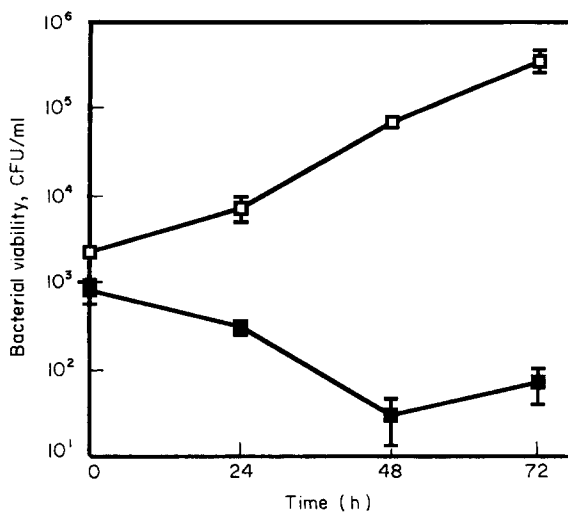


Fig. 1. Kinetics of growth of *L. pneumophila* in U937 cells. *L. pneumophila* were incubated either with live U937 cells (□) or with U937 cells that had been disrupted by freeze/thawing (■). At each time point, the number of viable bacteria was determined by lysing intact cells and culturing the samples on BCYE agar.

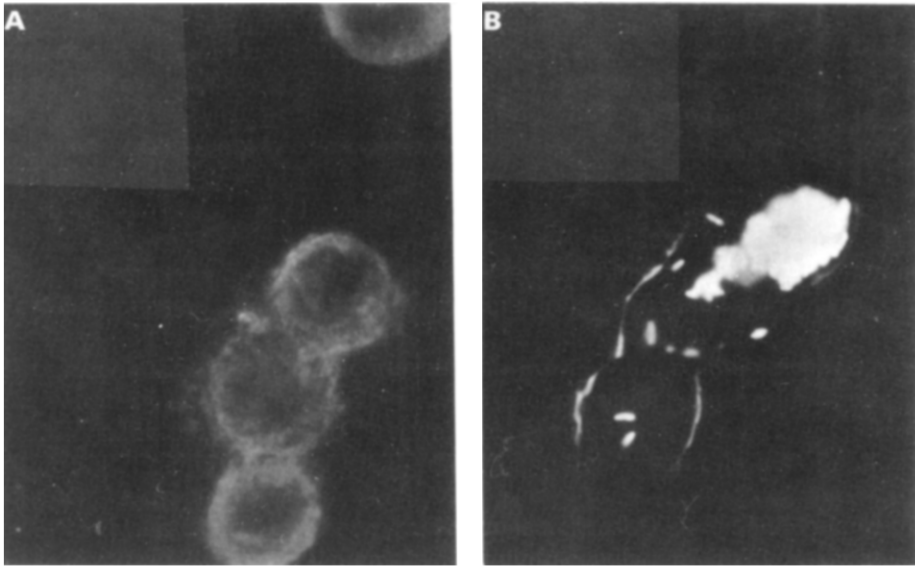


Fig. 2. Fluorescence microscopy of U937 cells infected with *L. pneumophila*. Infected cultures were stained with an FITC labeled anti-*L. pneumophila* monoclonal antibody 48 h after infection. The cultures were stained when live (to visualize only extracellular bacteria) (A), or after methanol fixation (to visualize both intracellular and extracellular bacteria) (B). Note that although no bacteria are visualized in the live, intact cells (A), there are numerous individual bacteria associated with the four adjacent cells shown in panel B, and a large mass of fluorescent bacteria in the uppermost cell. The fluorescence of the cells themselves in panel A was red in color and therefore not due to fluorescein staining. This non-specific background was diminished after methanol fixation, as can be seen in panel B.

with Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum (DMEM/FCS) alone (data not shown).

To corroborate the ability of U937 cells to support the intracellular growth of *L. pneumophila*, we examined the 72 h-infected monolayer by fluorescence microscopy after staining with an FITC-labeled anti-*L. pneumophila* monoclonal antibody. When we examined the living cells, which do not permit antibody penetration, we saw no bacteria associated with the cells [Fig. 2(A)]. In contrast, when the cells were first fixed with methanol to permit intracellular penetration of the antibody, we found that bacteria were clearly visible in association with the cells [Fig. 2(B)]. Thus, the organisms were present and growing within the cells.

Loss of U937 cell viability during bacterial growth

During the course of the intracellular growth experiments, we noted that the U937 cells were destroyed as the infection proceeded. Using the vital stain 3-(4,5-dimethyl thiazol-2-yl)-2,5,-diphenyl tetrazolium bromide (MTT) to quantitate the number of living cells present during the infection, we were able to measure the amount of monolayer destruction. MTT, a colorogenic substrate for mitochondrial dehydrogenase, is reduced in living cells to a purple formazan which can be quantitated spectrophotometrically.²² As described below (Materials and Methods), there is a linear correlation between MTT staining, measured at OD₅₅₀, and U937 cell viability.

To quantitate the monolayer destruction by the growing bacteria, we incubated the organisms with the U937 cells at a ratio of 10:1 (bacteria: cells) for two hours and then killed the extracellular organisms with gentamicin sulfate, an antibiotic that does not penetrate phagocytic cells.²³ The only surviving bacteria would be those sequestered within the U937 cells. After removing the antibiotic, we determined the

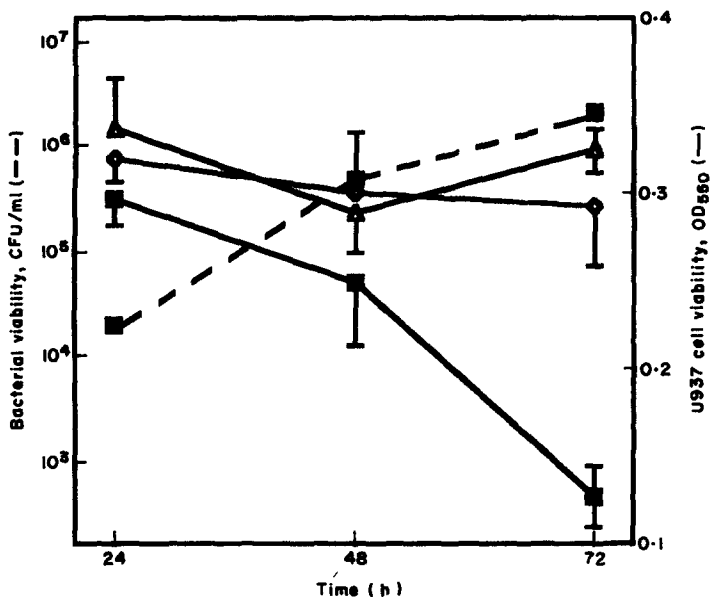


Fig. 3. Effect of intracellular growth of *L. pneumophila* on U937 cell viability. U937 cells were infected either with *L. pneumophila* (followed by killing of the extracellular organisms with gentamicin sulfate), with organisms that had been previously killed by 2 h preincubation with the antibiotic, or with no bacteria. At each time point we determined the number of viable bacteria (■) by plate counts, and the number of viable U937 cells remaining by measuring the OD₅₅₀ following infection with either live bacteria (■), killed bacteria (△), or no bacteria (◇).

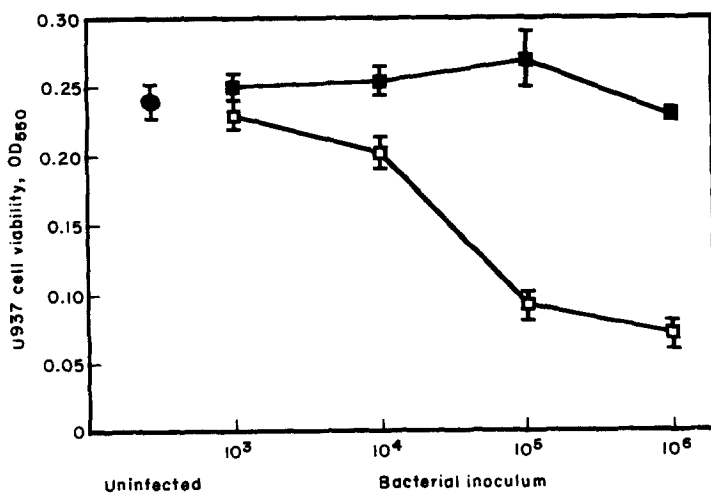


Fig. 4. Effect of *L. pneumophila* infection on U937 cell viability. The number of viable U937 cells remaining was determined by staining with MTT and measuring OD₅₅₀ 48 h after infection with *L. pneumophila* that either were guinea-pig-passaged (□) or were passaged serially on agar 203 times (■). Uninfected cells (●).

number of viable (i.e., intracellular) organisms by mild detergent lysis of the cells, and the number of viable U937 cells by vital staining. We found that the number of viable U937 cells associated with the cultures decreased over time as the bacteria increased in number (Fig. 3). In contrast, U937 cells incubated with dead *L. pneumophila* showed no loss in viability over this time demonstrating that host cell death is

Table 1 Comparison of the parent *L. pneumophila* strain with the multiply passaged derivative.

	<i>L. pneumophila</i> strain	
	130b	130b:203
Guinea pig virulence	2/3 ^a	0/3 ^a
Intracellular growth in U937 cells ^b	5 × 10 ⁵	< 10 ²
Extracellular growth in yeast extract broth ^c	1.7 × 10 ⁹	1.8 × 10 ⁹

^a Number of guinea-pigs killed 3 d after inoculation by the intraperitoneal route with 10⁶ bacteria.

^b CFU's after 72 h of infection were determined by detergent lysis of the cells followed by plate counts.

^c Obtained after 16 h growth in yeast extract broth. During this time the cultures increased in optical density from OD₆₆₀ = 0.15 to OD₆₆₀ = 0.85 and 0.88 in the parent and 130b:203, respectively.

dependent upon intracellular growth of *L. pneumophila*, and not due to the effects of a preformed bacterial toxin.

Characterization of an avirulent L. pneumophila derivative

To demonstrate that U937 cells are a relevant model of *L. pneumophila* pathogenicity, we investigated the capacity of the U937 cell model system to distinguish virulent from avirulent derivatives of an *L. pneumophila* strain. Several studies have shown that passaging *L. pneumophila* on artificial media reduces both the virulence of the organisms for guinea-pigs^{24,25} and their ability to grow in human monocytes.²⁶ To attenuate a virulent strain, we passaged *L. pneumophila* strain 130b on BCYE agar twice weekly for over two years (203 passages) to give rise to strain 130b:203. This agar-passaged strain was compared with the strain utilized throughout this study (strain 130b recently recovered from guinea pigs) by the following parameters: (a) guinea pig virulence; (b) growth in U937 cells, and (c) growth in yeast extract broth.

We found that, in contrast to the parent strain, *L. pneumophila* strain 130b:203 was incapable of killing guinea-pigs after intraperitoneal inoculation (Table 1). Whereas there was no difference in the ability of the strains to grow in artificial media, the parent strain was much more capable of growing in U937 cells (Table 1) and producing CPE during intracellular growth (Fig. 4) than the long-term passaged derivative. Thus, loss of guinea-pig virulence was paralleled by loss of U937 cell virulence but not by change in growth ability in artificial media.

Discussion and conclusions

The purpose of the present study was to establish a reproducible and relevant *in vitro* system of *L. pneumophila*/macrophage interaction so that we can proceed to investigate the interaction between the parasite and the host at the cellular level. We have previously identified and cloned several protein antigens of *L. pneumophila*,²⁷⁻²⁹ and we plan to determine the role of these proteins in the interaction of these bacteria both with U937 cells and with human alveolar macrophages. To perform these studies, we require a model of cellular infection that reflects the *in vivo* interaction and can be used to screen mutants that are defective in intracellular growth. For this purpose, the system of choice must: (a) support intracellular growth of virulent *L. pneumophila*; (b) fail to support the growth of avirulent *L. pneumophila*; and (c) reliably reproduce significant findings in subsequent experiments.

L. pneumophila has been grown intracellularly in a variety of other systems.³⁻¹⁴

Although explanted human alveolar macrophages would seem to be the most physiologic model available, it presents two serious problems as a screening assay: (1) the difficulty in obtaining a continuous supply of fresh cells, and (2) the variability of the cells in successive experiments (since both the donor and the state of macrophage activation would not be controllable).

The macrophage-like cell line employed in our experiments overcome these disadvantages. U937 cells are monoblastic in continuous culture, but their neoplastic nature can be reversed by various inducers such that they simultaneously lose their proliferative capacity and develop features of tissue macrophages.¹⁵⁻¹⁸ The advantages of such a cell line are several: (i) they are of human origin; (ii) they are available in a continuous and renewable supply; (iii) their state of activation is experimentally controlled and can therefore be standardized; and (iv) they are phenotypically similar, if not identical to macrophages, being actively phagocytic and having receptors for IgG and C3. In addition, the U937 cells have been reported to respond to lymphokines by inhibiting the growth of *Toxoplasma gondii*,²⁰ and by increased levels of antibody dependent cellular cytotoxicity.³⁰

Since we have shown that *L. pneumophila* replicates in U937 cell culture and that the increase in bacterial number over time is due to intracellular growth of the organism, the U937 cell model fulfills the most basic requirements as a model for the study of *L. pneumophila*/macrophage interaction. The model also shows that growth is coincident with a loss of viability of the host cells. Moreover, we found that a derivative of strain 130b that had been passaged on BCYE plates more than 200 times not only lost its ability to infect guinea-pigs, but also became unable to grow in U937 cells and cause the associated CPE. By these criteria, the U937 cells are a suitable model for the study of *L. pneumophila*/macrophage interaction.

Materials and methods

Bacteria. Maintenance of *L. pneumophila* serogroup 1, strain 130b has been described.²⁹ The bacteria (10^7) were inoculated into the peritoneal cavities of guinea-pigs and recovered from the spleen three days later. The organisms were stored in 0.01 M phosphate buffered saline, pH 7.4 (PBS) at -70°C and passaged minimally on buffered charcoal yeast extract agar (BCYE). The strain was again passaged through guinea-pigs immediately prior to this study.

For each experiment several colonies were scraped off the surface of the agar and inoculated into filtered yeast extract broth as described.²⁹ After 24 h on a shaking incubator at 37°C , the culture had reached a cell density of $1-2 \times 10^{10}$ CFU/ml. The bacteria were then harvested, washed in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, U.S.A.), and used to inoculate the U937 cells.

U937 cells. U937 cells were kindly provided by Dr J. J. Marr (University of Colorado, Denver, CO, U.S.A.) and maintained as the replicative non-adherent monocyte-like cells in DMEM supplemented with 10% fetal calf serum (FCS) and 50 $\mu\text{g/ml}$ gentamicin sulfate. Under these conditions, the doubling time of the U937 cells was typically 12 h (data not shown).

When the culture was at late logarithmic phase of growth (around 10^6 cells/ml), the cells were diluted to $2-3 \times 10^6$ cells/ml and 12-O-tetra decanoylphorbol-13-acetate (TPA, Sigma, St Louis, MO, U.S.A.) was added at a final concentration of 10^{-8} M for 48-72 h. The cells were removed from the flask with 0.2% EDTA, washed with DMEM and distributed into wells of either a 24-well or a 96-well plate (Costar, Cambridge, MA, U.S.A.) at cell densities of 5×10^5 /well and 2×10^5 /well, respectively. By examining the plates with an inverted microscope, we estimated that these densities resulted in a 50-75% covering of the surface of the plate.

Coincubation of *L. pneumophila* with U937 cells. After the macrophages had settled onto the plates (1-2 h), the bacteria were added in DMEM at a ratio of 1:1 (bacteria:cells) and incubated for 2 h. After three washes with 1 ml DMEM, the cultures were incubated in DMEM/FCS. At each time point, the cells were lysed by the addition of 50 μl of 2% Triton X-

100 (final concentration of 0.1%, which did not affect the viability of the bacteria). The bacterial count was determined by diluting the lysate in saline and spotting aliquots (10 μ l) onto BCYE plates.

An equivalent number of macrophages (5×10^5 /ml) were disrupted by three cycles of freezing and thawing. When stained with the vital stain Trypan blue we found that all the cells were dead. The disrupted macrophages were then incubated with 10^3 organisms (without washing or incubating with antibiotics), and the number of viable bacteria was determined every 24 h.

Cytopathic effect. The cytopathic effect (CPE) of the organisms was determined by assaying the number of viable U937 cells remaining in the well after infection with *L. pneumophila*. To distinguish between living and dead U937 cells, we used a vital stain, the tetrazolium salt 3-(4,5-dimethyl thiazol-2-yl)-2,5,-diphenyl tetrazolium bromide (MTT, Sigma, St. Louis, MO, U.S.A.), as previously described.²²

To standardize the relationship between color reaction and the number of remaining viable cells, we plated serial dilutions of transformed U937 cells into 24-well plates. We added 50 μ l of a stock solution of MTT (5 mg/ml dissolved in PBS and filter sterilized) to the wells of the assay and incubated for 4 h at 37°C. The supernatants were expelled and 1 ml acid-isopropanol (0.04 N HCl in isopropanol) was added to each well. After thorough mixing, the optical density of each well was read at several wavelengths using acid-isopropanol as a blank. The correlation between the optical density and number of viable cells was determined by staining various numbers of live cells. A regression analysis using the method of least squares showed a high degree of correlation between staining and cell number ($r > 0.99$).

As living aerobic bacteria would also be expected to incorporate the dye, we determined the minimum concentration of *L. pneumophila* required to give absorbance under these conditions. This concentration (10^8 /ml) was never attained during the growth of these organisms in the U937 cells (data not shown).

To demonstrate a cytopathic effect on the cells, we plated U937 cells onto a 96-well plate and infected them with *L. pneumophila* at various bacteria: cell ratios. We then incubated the cultures with gentamicin sulfate (50 μ g/ml) to kill the extracellular organisms. (By performing bacterial assays with different concentrations of gentamicin, we found that >99% of the inoculum had been killed after 2 h with a concentration of 50 μ g/ml.) We then washed the cultures, incubated them in 0.1 ml DMEM/FCS and at each time point we added 5 μ l of the stock solution of MTT as described above, reading the plates on an EAR 400 AT automatic ELISA reader (SLT Labinstruments USA, Ronkonkoma, NY) at OD₅₅₀. In some experiments the organisms were killed with gentamicin prior to adding the bacteria to the monolayers.

Production and characterization of a serogroup 1 specific monoclonal antibody. *L. pneumophila* were harvested from colonies grown on BCYE agar, washed in PBS, and adjusted to a concentration of 10^{10} bacteria/ml. The bacteria were then either boiled for 30 min or incubated for 12 h in 2% formaldehyde at 4°C. The bacteria were then stored at -70°C. For each inoculum, the bacteria were pooled and inoculated intraperitoneally into two Balb/c mice (Jackson Laboratories, Bar Harbor, ME, U.S.A.). The first inoculation contained a total of 1×10^9 organisms and the subsequent inoculations, given 2 and 4 weeks after the original, contained 5×10^8 organisms. Four days after the last injection, the mice were sacrificed and splenic cells were fused with SP2 cells as described by Oi *et al.*³¹ Monoclonal antibody (mAb) 1C5 (IgG3) was found to be specific for *L. pneumophila* serogroup 1 and was purified on a Protein A sepharose column (Biorad, Richmond, CA, U.S.A.) according to the manufacturer's directions.

Direct fluorescence assay of intracellular bacteria. Fluorescent labeling was performed by previously described methods.³² Briefly, 1 mg of mAb 1C5 was dialysed against a 0.1 M carbonate buffer, pH 9.5, 10 μ g fluorescein isothiocyanate (FITC) was added and the mixture was stirred in the dark for 2 h. The unbound FITC was removed by filter dialysis on a mini-Amicon system (Danvers, MA, U.S.A.). The conjugated 1C5 (FITC-1C5) was stored at -70°C and diluted 1:100 in PBS for routine use.

TPA-treated U937 cells were plated onto 12 mm diameter glass coverslips, and inoculated as described in the kinetics studies. All subsequent stops were performed at room temperature. The cultures were fixed in methanol for at least 5 min to allow the mAb to penetrate the macrophages. The samples were then washed in PBS, and 50 μ l of FITC-1C5 was added for 30 min. The samples were then washed in distilled water, air dried, and mounted onto a slide with Bacto FA mounting fluid (Difco, Detroit, MI, U.S.A.). The slides were examined with a Zeiss fluorescent microscope (Carl Zeiss Inc., New York, NY, U.S.A.) using vertical illumination.

and photographs were taken with a Zeiss Ikon camera using T-MAX 100 film (Eastman Kodak Co, Rochester, NY, U.S.A.).

Living cultures were stained by inverting the coverslip on a drop of FITC-1C5 and incubating 15 min at 37°C in the presence of 7% CO₂. The cultures were examined as before and photographs were taken within 15 min.

We thank Dr J. J. Marr of the University of Colorado for providing the U937 cells, Dr Lathe Clafflin of this department for the use of the ELISA reader, and Drs Garth Jones and Rolf Freter, also of this department, for the use of the fluorescent microscope. This work was supported by Public Health Service grant AI24731 and by the Veterans Administration. N.C.E. is supported by a Veterans Administration Career Development Award.

References

1. Blackmon JA, Hicklin MD, Chandler FW, *et al.* Legionnaires' disease: pathological and historical aspects of a 'new' disease. *Arch Path Lab Med* 1978; 102: 337-43.
2. Davis GD, Winn WC, Jr, Gump DW, Beaty HN. The kinetics of early inflammatory events during experimental pneumonia due to *Legionella pneumophila* in guinea pigs. *J Infect Dis* 1983; 148: 823-35.
3. Horwitz MA, Silverstein SC. Legionnaires' disease bacterium (*Legionella pneumophila*) multiplies intracellularly in human monocytes. *J Clin Invest* 1980; 66: 441-50.
4. Holden EP, Winkler HH, Wood DO, Leinbach ED. Intracellular growth of *Legionella pneumophila* within *Acanthamoeba castellanii* neff. *Infect Immun* 1984; 45: 18-22.
5. Newsome AL, Baker RL, Miller RD, Arnold RR. Interactions between *Naegleria fowleri* and *Legionella pneumophila*. *Infect Immun* 1985; 50: 449-52.
6. Fields BS, Barbaree EB, Shotts EB Jr, *et al.* Comparison of guinea pig and protozoan models for determining virulence of *Legionella* species. *Infect Immun* 1986; 53: 553-9.
7. Daisy JA, Benson CE, McKittrick J, Friedman HM. Intracellular replication of *Legionella pneumophila*. *J Infect Dis* 1981; 143: 460-4.
8. Oldham LJ, Rodgers FG. Adhesion, penetration and intracellular replication of *Legionella pneumophila*: an *in vitro* model of pathogenesis. *J Gen Microbiol* 1985; 131: 697-706.
9. Jacobs RF, Locksley RM, Wilson CB, Haas JE, Klebanoff SJ. Interaction of primate alveolar macrophages and *Legionella pneumophila*. *J Clin Invest* 1984; 73: 1515-23.
10. Kishimoto RA, Kastello MD, White JD, *et al.* In vitro interaction between normal cynomolgus monkey alveolar macrophages and Legionnaires' disease bacteria. *Infect Immun* 1979; 25: 761-3.
11. Kishimoto RA, White JD, Shirey FG, *et al.* In vitro response of guinea pig peritoneal macrophages to *Legionella pneumophila*. *Infect Immun* 1981; 31: 1209-13.
12. Nash TW, Libby DM, Horwitz MA. Interaction between the Legionnaires' disease bacterium (*Legionella pneumophila*) and human alveolar macrophages. *J Clin Invest* 1984; 74: 771-82.
13. Yamamoto Y, Klein TW, Newton CA, Widen R, Friedman H. Growth of *Legionella pneumophila* in thioglycolate-elicited peritoneal macrophages from A/J mice. *Infect Immun* 1988; 56: 370-5.
14. Yoshida S, Mizuguchi Y. Multiplication of *Legionella pneumophila* Philadelphia-1 in cultured peritoneal macrophages and its correlation to susceptibility of animals. *Can J Microbiol* 1986; 32: 438-42.
15. Sundstrom C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U937). *Int J Cancer* 1976; 17: 565-77.
16. Harris P, Ralph P. Human leukemic models of myelomonocytic development: a review of the HL-60 and U937 cell lines. *J Leukocyte Biol* 1985; 37: 407-22.
17. Radzun HJ, Parwaresch MR, Sundstrom C, Nilsson K, Eissner M. Monocytic origin of the human hematopoietic cell line U937 and its convertibility to macrophages evidenced by isoenzyme mapping. *Int J Cancer* 1983; 31: 181-6.
18. Gilbert D, Peulve P, Daveau M, Ripoche J, Fontaine M. Modulation of complement receptors of a human monocyte cell line, U937, during incubation with phorbol myristate acetate: expression of an iC3b-specific receptor (CR#). *Eur J Immunol* 1985; 15: 986-91.
19. Looker DL, Martinez S, Horton JM, Marr JJ. Growth of *Leishmania donovani* amastigotes in the continuous human macrophage cell line U937: studies of drug efficacy and metabolism. *J Infect Dis* 1986; 154: 323-7.
20. Wing EJ, Koren HS, Fisher DG, Kelley V. Stimulation of a human macrophage-like cell line (U937) to inhibit replication of an intracellular pathogen. *J Reticuloendoth Soc* 1981; 29: 321-9.
21. Folks TM, Justement J, Kinter A, Dinarello CA, Fauci AS. Cytokine-induced expression of HIV-1 in a chronically infected promonocyte line. *Science* 1987; 238: 800-2.
22. Green LM, Reade JL, Ware CF. Rapid colorimetric assay for cell viability: application to the quantitation of cytotoxic and growth inhibitory lymphokines. *J Immunol Met* 1984; 70: 257-68.
23. Mandell GL. Interaction of intraleukocytic bacteria and antibiotics. *J Clin Invest* 1972; 52: 1673-9.

24. Elliott JA, Johnson W. Virulence conversion of *Legionella pneumophila* serogroup 1 by passage in guinea pigs and embryonated eggs. *Infect Immun* 1982; 35: 943–6.
25. McDade JE, Shepard CC. Virulent to avirulent conversion of Legionnaires' disease bacterium (*Legionella pneumophila*)—its effect on isolation techniques. *J Infect Dis* 1979; 139: 707–11.
26. Horwitz MA. Characterization of avirulent mutant *Legionella pneumophila* that survive but do not multiply within human monocytes. *J Exp Med* 1987; 166: 1310–28.
27. Engleberg, NC, Pearlman E and Eisenstein BI. *Legionella pneumophila* surface antigens cloned and expressed in *Escherichia coli* are translocated to the host cell surface and interact with specific anti-*Legionella* antibodies. *J Bacteriol* 1984; 160: 199–203.
28. Engleberg NC, Pearlman E, Dixon D and Eisenstein BI. Antibodies isolated using cloned surface antigens recognize antigenically related components of *Legionella pneumophila* and other *Legionella* species. *J Immunol* 1986; 136: 1415–17.
29. Pearlman E, Engleberg NC, Eisenstein BI. Identification of protein antigens of *Legionella pneumophila* serogroup 1. *Infect Immun* 1985, 47: 74–9.
30. Larrick JW, Fischer DG, Anderson SJ, Koren H. Characterization of a human macrophagelike cell line stimulated *in vitro*: a model of macrophage functions. *J Immunol* 1980; 125: 6–12.
31. Oi VT, Herzenberg LA. Immunoglobulin-producing hybrid cell lines. In: BB Mishell and SM Shiggi, eds. *Selected methods in cellular immunology*. San Francisco. WH Freeman and company, 1982; 351–72.
32. Hurn BAL, Chantler SM. Production of reagent antibodies. In: Van Vunakis H, Langone JJ, eds. *Methods in enzymology* vol. 30: Immunochemical techniques. Academic Press Ltd, 1980. 105–6.