ATTACHMENT OF *Legionella pneumophila* TO CELLS *IN VITRO*

THESIS

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The attachment and/or penetration of animal cells by two strains of Legionella pneumophila was studied in three vertebrate cell lines in vitro. The study focused on (1) differences in attachment and penetration between the two bacterial strains (an environmental isolate, Johannesburg-2, and a clinical isolate, Chicago-8) and between the cell lines (Hep-2, WI-38 and a murine line); (2) effects of L. pneumophila on cell morphology and growth; and (3) the effects of pyruvate and six sugars or sugar derivatives (D-mannose, D-galactose, D-glucose, L-glucose, D-fructose, and 2-deoxy-D-glucose).

The Chicago 8 strain of L. pneumophila was less able to attach than was the Johannesburg 2 strain. Some additives (2-deoxy-D-glucose, L-glucose and pyruvate) enhanced, while some (D-galactose and D-mannose) inhibited attachment. Cytopathic effects of the legionellae were minimal , microvilli being the principal aberration noted by scanning electron microscopy. Both rate of growth and maximal numbers of animal cells in culture were suppressed by the two strains of Legionella.

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CHAPTER I

INTRODUCTION

Legionnaires' disease gained its name from an explosive outbreak of pneumonia which followed an American Legion convention in Philadelphia in July, 1976 (46). Twenty-nine deaths occurred among 182 cases. The disease was characterized by either fever and X-ray evidence of pneumonia or a temperature of at least 102°F and a cough. Since then, sporadic cases have been reported from many of the states (23) as well as from many other countries (7, 14, 15, 42, 58, 77, 85). The original etiologic agent, after being isolated from lung tissue from victims of the Philadelphia outbreak, was named Legionella pneumophila by McDade (20).

Studies of several epidemics indicate that legionellosis is spread by the airborne route (46, 47, 56, 122). Between July 27 and August 27, 1965, 81 cases of acute respiratory illness ocurred among patients at St. Elizabeth Hospital in Washington, D.C. Soon after identification in 1977 of the Legionnaires' disease bacterium (LDB) as the cause of the Philadelphia outbreak, immunofluorescence procedures were developed enabling indirect fluorescent-antibody tests on stored sera from the St. Elizabeth outbreak. All sera from patients involved in the latter episode were positive,

indicating that the illness was due to LDB (122). Soil excavation for the purpose of installing a new sprinkler system was temporally and geographically linked to risk of the disease. Attack rates for residents within 20 feet of the excavation site were significantly higher than the rate for residents housed further away from the site. Moreover, the number of cases dropped markedly upon completion of the excavation. It was speculated that excavation, plus heavy rains followed by hot weather, resulted in airborne spread from that site. Evidence that those patients sleeping close to open windows near the excavation site as well as those having ground privileges were at a higher risk of acquiring disease supports this hypothesis.

A relationship between legionellosis and air-conditioning, humidifying, or ventilation systems has been well documented. In an outbreak in Memphis no new cases occurred nine days after the cooling tower was shut down (34). It has been found that potable water can serve as a reservoir and as a vehicle of transmission of legionellae (5). Many workers have shown air-conditioning systems (29, 34) and tap water (117, 124) as sources of infection.

Nature of Legionella pneumophila

The etiologic agent of Legionnaires' disease, L. pneumophila (20), is a Gram-negative bacillus, the morphology of which varies considerably with the conditions employed for

its cultivation (79, 83). In human lung tissue and the chick yolk sac, LDB are predominantly bacillary with a width of 0.3 - 0.7 μm and a length of 1 - 3 μm, although coccobacillary and filamentous forms also have been described. On bacteriological media, early forms are also bacillary, but with age they become highly filamentous and reach lengths of over 50 μm.

Although Gram-negative, LDB stains poorly by the conventional Gram stain (24), particularly in tissue section, because after decolorization it does not adsorb the counterstain. However, the use of a "half-a-Gram" stain was found to improve visualization (33). Upon completion of the second step of Gram's procedure (application of the iodine solution), the organisms show up under the light microscope as intensely-stained purple cells.

Electron microscopy of LDB has revealed an ultrastructure much like other Gram-negative bacteria. Studies of the Philadelphia 1 strain (serogroup 1) (45) have shown the cell to be surrounded by two complete trilamellar membranes, each composed of two electron-dense layers separated by an electron-transluscent layer. Upon isolation of these membranes, the denser fraction was found to contain a high concentration of 2-keto-3-deoxyoctonate, a monomer characteristically found in the lipopolysaccharide of the outer membrane of Gram-negative organisms. On the other hand, the lighter fraction of Legionella cell membranes was found to

contain most of the succinic dehydrogenase activity; this is characteristic of the cytoplasmic membrane of Gram-negative bacteria.

Association of Legionella pneumophila With Protozoa

Rowbotham (104, 105, 106) demonstrated that L. pneumophila is pathogenic for freshwater and soil amoeba and presented an hypothesis that may be relevant to the epidemiology of Legionnaires' disease. He showed that L. pneumophila infects free-living amoeba of the genera Naegleria and Acanthamoeba. Free-living amoebae are ubiquitous in nature and have been isolated in large numbers from soil, humidifiers, and evaporator condensors. Evidence has increasingly indicated that L. pneumophila is also widely distributed in the natural and artificial aquatic environment, being found in some of the same ecologic reservoirs as amoeba. It is conceivable that, in ecologic systems harboring both organisms, L. pneumophila parasitize amoeba. If this is true, persons exposed to aerosols of contaminated humidifier systems, air-handling units, water, or dust may be exposed simultaneously to L. pneumophila and amoeba.

Inhalation of amoeba containing L. pneumophila may help to explain the pathogenesis of primary infection in humans. Legionella pneumophila has been shown to retain its virulence in this association; upon adaptation to extracellular growth on bacteriologic media, it loses its ability to cause

infection of experimental animals (84, 93). Rowbotham (104, 105, 106) suggested that Legionnaires' disease may be acquired by inhaling amoebae that are infected with L. pneumophila rather than by inhaling free-living bacteria. Moreover, he suggested that intracellular L. pneumophila in amoebic vesicles may be protected from rapid dehydration; this may help explain Broad Street pneumonia (46) and other cases that have occurred outside of buildings during epidemics of Legionnaires' disease.

The study of Anad et al. (4) suggested that legionellae infect amoeba in nature and multiply in them at a rate influenced by temperature. Nagington and Smith (87) subsequently found that A. polyphaga could use L. pneumophila as a food source. Tyndall and Domingue (127) extended these studies and showed that when L. pneumophila was cocultured with A. royreba or N. lovaniensis, the number of viable bacteria increased over an extended period, although the bacteria did not survive long in the amoeba culture medium alone. They also suggested that amoeba may produce a soluble factor which enhances the growth of L. pneumophila. This possibility was tested by Holden et al. (60). When L. pneumophila was added to a compartment containing amoeba-conditioned medium, but separated from the amoeba (A. castellanii) by a 0.4-um membrane filter, the counts of viable bacteria declined to undectable levels within 48 hours. In contrast, when L. pneumophila was inoculated into the same compartment

as the amoeba, the viable bacterial counts associated with the amoebae increased by three orders of magnitude over a 72-hour period. This result makes it extremely unlikely that A. castellanii produces a diffusible factor which promotes L. pneumophila growth. They also demonstrated that L. pneumophila are capable not only of surviving but of actively growing within acanthamoebae.

Besides amoeba, Fields et al. (44) found L. pneumophila to infect and multiply intracellularly in the ciliate, Tetrahymena pyriformis. They found that the bacterium could not multiply by itself in sterile tap water but did so in co-culture with the protozoa. Tests revealed that L. pneumophila could not multiply in a suspension of lysed T. pyriformis cells suspended in sterile tap water nor in a cell-free extract of a spent T. pyriformis culture.

Pathology and Pathophysiology of Legionellosis

While active immunity to LDB occurs, delayed hypersensitivity (DH), or cell-mediated immunity, to Legionella antigens would appear to play an important role in legionellosis. Wong et al. (135) showed that the intradermal injection of LDB cross-reacting antigens into sensitized guinea pigs evoked skin hypersensitivity. Histological examination of biopsies taken from the test sites 48 hours later revealed infiltration of mononuclear cells in and around the small subcutaneous blood vessels and throughout the dermis;

this finding is compatible with DH. Thus, it may be possible to detect presence of past LDB infection by a skin test. Such a skin test, not yet developed, would permit rapid screening of populations for epidemiological evidence of prior exposure, as well as facilitating clinical assessment. Demonstration of DH by means of a skin test has not yet been reported in human legionellosis; however, evidence from studies on peripheral blood leukocytes strongly indicates that DH plays a role in the infection. For example, Plouffe and Baird (98) showed that peripheral blood leukocytes from patients who had recovered from Legionnaires' disease evinced significantly increased incorporation of (^{3}H) thymidine upon in vitro stimulation with a L. pneumophila sonic extract as compared to control blood leukocytes from individuals with no clinical history of Legionnaires' disease. In follow-up studies, some controls showed significant incorporation of (^{3}H) thymidine after stimulation with L. pneumophila antigens in vitro. Additional studies (99, 100) revealed that the level of responsiveness of peripheral blood leukocytes from younger uninfected individuals to the LDB sonicates was lower than that of leukocytes from older individuals. Peripheral blood leukocytes contain both B- and T-cells. Since about 80% of the lymphocytes in humans are T cells, these investigators believe that the responding cells are very likely T cells. These studies have been interpreted tentatively as supporting DH as one of the immune-responses to Legionella infection.

In a related series of studies, Horwitz and Silverstein (62, 63, 64, 65) showed that human monocytes from peripheral blood inhibited the growth of L. pneumophila, if derived from patients who had recovered from the disease. Control monocytes behaved similarly only when activated with supernatants from concanavalin A-stimulated normal lymphoid cells, which produce activating factors (lymphokines) for macrophages. Human polymorphonuclear leukocytes (PMN) readily ingested and killed Legionella cells, especially after opsonization by antibody and complement. Fresh, normal human serum exhibited in vitro no bactericidal activity toward the legionellae.

Many studies of Legionnaires' disease in man and in animal models have led to an understanding of the acquisition and histopathology of this disease. Inhalation of aerosols containing L. pneumophila is thought to be the mode of infection in humans (78), and is the best method of producing pneumonic Legionnaires' disease in animals. Small particles (<5 μm) seem to be necessary, presumably because they more readily penetrate the respiratory bronchioles and alveoli than larger particles. Large particles, which lodge in the upper respiratory tract, may not indicate infection, or they may produce a different clinical disease. The possibility of an oral route of infection with L. pneumophila is occasionally mentioned, particularly since the organism has been found in potable water (8). The experiments of Katz

and co-workers (74), using oral dosing of guinea pigs with L. pneumophila, are intriguing. After gastric intubation with drinking water containing legionellae, the animals developed fever; macrophages and PMNs were found in alveolar walls, and the agent could be isolated from the lungs and spleen. A possible pathogenetic pathway here might be penetration of the gastrointestinal tract and entry into the lymphatic system, followed by dissemination of the bacteria to the lungs and spleen.

Fever and bacteremia occur in both humans and animals. Mortality has been reported to vary from 10 to 20% in untreated human cases (46). In guinea pigs, mortality is dose dependent and thus can be manipulated to result in from 0 to 100% mortality. Macroscopic lung lesions are similar in humans and in aerosol-infected animals. They are largely in the bronchi and become confluent and, frequently, lobar in extent, accompanied by a variable pleural effusion. In over two-thirds of human cases, there is multilobar involvement, with no preferential distribution (134). In aerosol-infected animals, all lobes show consolidation to a similar degree. The quantitative analysis carried by Winn et al. (132) showed that the extent of the area of the lungs affected in guinea pigs is proportionate to the size of the infecting dose. The greater the extent of the lesions, the poorer the animal's chance of survival. This is probably because much of the effect of the consolidation is to cause hypoxemia by

interfering with the ventilation, diffusion, and perfusion function of the lung, in addition to toxemia.

In humans and animals the lesions are very similar, although the greater variation is seen in humans, probably because of such variables as concurrent disease, age, general health and immunosuppression. As noted in the original work by the Centers for Disease Control (CDC) (17), legionellosis is an acute fibrinopurulent pneumonia affecting the acinus, with no upper respiratory tract lesions and relatively little damage to the epithelium of bronchioles. In contrast, small-particle aerosol infection with other bacteria, such as Francisella tularensis and salmonellae, does cause nasal and upper respiratory tract lesions (9); thus, this particular histopathology seems to be a property of legionellosis and not merely a function of the size of the infecting particles. The alveolar cellular exudate in all animals consists of PMNs and macrophages in varying proportions, with PMNs often predominating. The most significant lesion in the lungs in human and animal Legionnaires' disease is exudation into alveoli of edema fluid and fibrin (10, 16,133). There is some focal alveolar epithelial necrosis, though not on the scale of some other bacterial or viral pneumonias. The necrosis seems to be more extensive in humans than in experimental animals.

Bacterial Adherence

Studies on the role of adherence in the pathogenesis of of Legionnaires' disease are few. Thus far it seems that L. pneumophila has little or no affinity for ciliated columnar epithelium (11). This is perhaps in accordance with its being an opportunistic, rather than an obligatory pathogen. Although some strains of legionellae possess fimbriae, organelles known in other bacteria to aid in attachment (22, 36, 49), they have little affinity for surface ligands on airway epithelial cells (102).

The phenomenon of adherence of bacteria to tissue surfaces has gained increasing attention as an important initial event in the pathogenesis of some bacterial infections (115). The infectious process in animals and man can be envisioned as a sequence of events in which the bacteria must first adhere to a tissue surface. The failure of adherence would result in the bacteria being swept away by the fluids constantly bathing the tissue surfaces and being ultimately phagocytosed. Adherence of pathogens to particular tissues is followed by colonization, during which either cytotoxicity from toxins produced by the colonizing organisms and/or invasion by the bacteria themselves would occur. In the deeper tissues, attachment of the bacteria to phagocytic cells generally results in their ingestion and destruction. Organisms whose surfaces are not recognized by antibody and complement or by phagocytic cells can multiply

to produce systemic infections. The surfaces of both prokaryotic and eukaryotic cells have a negative potential that results from the ionization of various chemical groups of the cells' surfaces (71). Strong adhesion of microbial cells to a surface, including animal cells, requires intimate contact. Since both cells carry a net negative charge, close proximity of the two surfaces is not favored because of this electrical repulsion barrier. Balanced against repulsion are attractive forces such as ionic bonding, van der Waals forces, and hydrophobic and hydrogen bonding, each of which functions at a very short range. Although individually weak, collectively these forces can overcome the electrical barrier, especially if one or more of them are intensified.

There is much evidence to suggest that bacteria possess molecules (ligands or adhesins) on their surfaces capable of binding, in a stereospecific fashion, with complementary molecules (receptors) on the surfaces of cells of the host (71, 88). The interaction between bacterial ligands and host cell receptors has been compared to antigen-antibody interactions (111). Thus, the specificity of the interaction can be demonstrated by (1) inhibiting the interaction with excess of haptens, either identical to or resembling the native ligand or the native receptor; (2) abolishing or altering, by chemical treatment of the bacteria or cells, the specific molecule(s) involved in adherence; and (3)

blocking the ligand or receptor with specific antibodies directed against antigens comprising these structures.

Many different adhesive properties are found amongst the majority of bacteria in the family Enterobacteriaceae. Fimbriae of the organisms in this family have been classified into four main types by Duguid (37). Type 1, or the common fimbriae, are produced by most strains of Escherichia coli and by Enterobacter cloacae, Shigella flexneri, Klebsiella spp., Serratia marcescens and by many serotypes of Salmonella. They average 70 Å in diameter and 2 μm in length (38). The most unifying characteristic of type 1 fimbriae is that D-mannose reverses their adhesive property (92). Also, D-mannose suppresses slime production by bacteria with type 1 fimbriae (90). Type 2 fimbriae, produced by some salmonellae, are thought to be non-adhesive forms of type 1 (91). Type 3 fimbriae, produced by K. aerogenes and S. marcescens, are 48 Å in diameter (38). They mediate bacterial adhesion to fungal cells, plant cells, cellulose fibers and glass, but not to animal cells unless they are first modified by chemical means (36). Type 4 fimbriae of Proteus spp. are about 40 Å in diameter, agglutinate selected erythrocytes and are unaffected by D-mannose (38).

A colonization factor (CF) discovered in a strain of E. coli pathogenic for man is associated with fimbriae measuring 80-90 Å in diameter (41). The CF promotes successful bacterial colonization of the intestine of laboratory

animals; the isolated fimbriae cause mannose-resistant hemagglutination of guinea-pig erythrocytes. Studies of strains of E. coli infecting calves (K-99) and piglets (K-88) have revealed them to possess a fimbriate surface structure (a K antigen) that specifically adheres to the epithelial cells lining the small intestine. These antigens are pilus-like structures that provide intimate contact between the toxin producing organisms and the intestinal cells. The significance of this association is supported by the fact that antibodies directed against these antigens are protective. The K-88 antigen is produced by a limited number of serotypes of E. coli of porcine origin. It is known to be produced in vivo and to function as an adhesin which allows the bacteria to colonize the intestinal mucosa of the host (69). Bacteria that do not produce K-88 fail to adhere to the mucosa and hence fail to colonize and cause disease (69). The K-99 antigen, present in some bovine strains of E. coli, plays a role in colonization similar to that of K-88 (69).

Neisseria gonorrhoeae colonial types designated 1 and 2 produce fimbriae (119), approximately 85 Å in diameter (118), whereas those gonococci that grow as colonial types 3 and 4 do not produce fimbriae and are far less virulent (118). Studies with a variety of animal cells have established that fimbriate gonococci are significantly more adhesive than nonfimbriated gonococci (120, 121) and that fimbriate gonococci cause hemagglutination and enhance adherence to the

mucosal surface of fallopian tubes (131).

Four species of oral streptococci have been shown to colonize preferentially particular surfaces of the buccal cavity (53, 54). Streptococcus salivarius preferentially colonizes the dorsum of the tongue, S. sanguis and S. mutans predominate on the surface of teeth and S. mitis inhabits the nonkeratinized oral mucosa. Thus, different species, and perhaps strains, of oral streptococci possess different adhesive substances. The specificity of these adhesins dictates the specific niche of these bacteria.

Adhesins can be classified as proteinaceous or non-proteinaceous (71). Included in the proteinaceous adhesins are the type 1 fimbriae and K-88 antigens of E. coli, the fimbriae of N. gonorrhoeae, Bordetella pertussis and of several other species. The type 1 fimbriae of E. coli are composed of protein, with less than 0.6% nucleic acid, phosphorus and carbohydrate. Protein accounts for over 90% of the dry weight of the K-88 of a strain of E. coli (71). Some streptococci and lactobacilli attach to cell surfaces by means that do not appear to include proteinaceous adhesins. A study by Beachey and Ofek (12) demonstrated that adhesion of S. pyogenes was inhibited if the target epithelial cells were pretreated with lipoteichoic acids (LTA) or with the lipid component of LTA, or if the bacteria were treated with LTA antibody. Consequently, the LTAs of cell walls of group A streptococci appear to play a major role in

adhesion. The adhesive activity of some lactobacilli is slightly impaired by treatment with trypsin, but markedly impaired by pepsinization (48).

Mammalian cell surface receptors have been identified as either carbohydrate (including glycoproteins) or lipid (72). Adhesins that appear to interact with carbohydrate receptors are the type 1 fimbriae of E. coli and a hemagglutinin of Pseudomonas aeruginosa -- also, fimbriae of mycoplasmas, Vibrio cholerae, avian lactobacilli and some streptococci (72). The several carbohydrates found to be involved in the adhesin-receptor interactions include D-mannose, L-fucose, D-galactose, neuraminic acid and glycoproteins (50, 70, 92, 94). Streptococcus salivarius and S. pyogenes adhesins also interact with lipids of the animal cell membranes (39). Adhesion of S. salivarius to epithelial cells was found to be supressed in the presence of sphingomyelin, lecithin, phosphatidyl-L-serine and phosphatidylethanolamine (52).

Fibronectin (Fn), a fiber-forming glycoprotein (about 5% carbohydrate by weight), is an extracellular-matrix glycoprotein that promotes cell adhesion (68, 107, 137). It exists as large aggregates in the extracellular space. Fibronectin first attracted attention when it was discovered to be present in greatly reduced amounts on the surface of fibroblasts derived from tumors compared to normal fibroblasts. In general, there is a good inverse correlation

between cell-surface Fn and the ability of cultured transformed cells to cause tumors, invade tissues, and to metastasize when reinjected into animals. Transformed cells behave differently from normal cells in culture: they adhere poorly to the substrate and fail to flatten out and develop organized intracellular actin filament bundles called stress fibers; and they grow to a much higher density than do normal cells (1). If larger amounts of Fn are added to cultures of transformed cells that themselves make relatively little Fn, the cells rapidly adhere, flatten out, and generate well-organized intracellular actin filament bundles. While they look like normal fibroblasts, they still grow to abnormally high density. This suggests that Fn promotes cell adhesion but does not directly influence the control of cell proliferation. Purified Fn has now been shown to promote the adhesion of a variety of cell types to other cells. In addition, recent evidence suggests that Fn functions as a receptor for certain bacterial cells. Stanislawski *et al.* (116) found plasma Fn adsorbed to human buccal epithelial cells to enhance binding of *S. pyogenes* and to decrease binding of *E. coli*. Their results are consistent with the idea that Fn on the surface of host cells modulates bacterial adherence by providing receptors for certain bacteria and blocking receptors for others.

Fibronectin has also been shown to bind to lipoteichoic acid (LTA) on the surface of group A streptococci (13, 30).

Poirier and Dale (101) found that, in addition to LTA, group A streptococci contain a common polypeptide that acts as a binding site for Fn. In another study, LTAs from group A streptococci were found to inhibit the binding of Fn to group A streptococci but not to pneumococci (31); thus, the Fn-binding site for pneumococci would appear to be different from the binding sites on staphylococci and group A streptococci. Christensen and Courtney (26) have shown that pretreatment of plastic microtiter plates with LTA isolated from S. pyogenes or Staphylococcus epidermidis inhibited the adherence of 3 strains of coagulase-negative staphylococci in a dose-dependent manner. Adherence was not affected by pretreatment of the microtiter plates with lipopolysaccharide, cardiolipin, or various detergents. These data indicate that LTA is one of several adhesins which bind coagulase-negative staphylococci to smooth surfaces. Toy and his co-workers (126) demonstrated that the adherence of five of seven S. aureus strains decreased significantly when fibrin thrombi had been depleted of Fn; adding Fn back reversed the effect in a dose-dependent manner. Bacteria known not to bind to Fn (E. coli and S. epidermidis) adhered 100-fold less well than S. aureus; their adherence was unaffected by the adherence of most S. aureus strains to solid fibrin thrombi, and thus is important in wound infections by staphylococci.

Cultivation of Legionella pneumophila in Cell Cultures

In vitro studies employing cell cultures infected with L. pneumophila support histopathologic observations made of human alveolar tissues that these bacteria are intracellular pathogens (136). Kishimoto et al. (75) reported approximately five percent of cynomolgus monkey alveolar macrophages to contain L. pneumophila within three hours after exposure. The organisms grew intracellularly in membrane-bound cytoplasmic vesicles. Although not avidly phagocytosed, once ingested, the bacteria resisted lysosomal enzymes and replicated rapidly, killing the macrophages. Wong and associates (136) demonstrated that cultured human embryonic lung fibroblasts, cells not specialized for phagocytosis, supported rapid intracellular growth of L. pneumophila, resulting in their death. They also found that bactericidal concentrations of penicillin and streptomycin were highly effective in killing extracellular but not intracellular legionellae. This observation may explain the discordance between results of in vitro and in vivo antimicrobial agents (82, 123). Horwitz and Silverstein (62, 63, 64) examined in vitro the interaction of L. pneumophila with monocytes, PMNs, and complement and specific antibody. In tissue cultures, L. pneumophila grew within intact monocytes, while neither intra- nor extracellular growth occurred in lymphocyte cultures (62). Monocytes bound and phagocytosed, but did not kill, L. pneumophila in the absence of antibody and

complement; three times as many organisms were bound when specific opsonins were present. They also demonstrated that PMNs require complement and specific antibody to phagocytose and kill L. pneumophila (63). Like PMNs, monocytes required complement and specific antibody to kill L. pneumophila. However, neither mononuclear cells nor PMNs killed legionellae efficiently, even in the presence of opsonins.

In a study on guinea pig peritoneal macrophages, Kishimoto et al. (76) found virulent strains of L. pneumophila to survive and proliferate within phagocytes after ingestion, whereas an avirulent strain of the bacterium was killed. Daisy et al. (32) noted L. pneumophila to grow intracellularly in several human cell lines (MRC-5, Hep-2, Hela) and a mouse cell line (McCoy) in culture. In the presence of the antibiotic gentamicin, the bacteria grew within the cells but not in the supernatant fluid.

The legionellae can grow within a variety of cells in addition to those mentioned above; these include monkey alveolar macrophages (75), guinea pig macrophages (76), the MRC-5 line of human embryonic lung fibroblasts (136), and human monocytes and macrophages (62, 65). Elliott and Winn (40) have shown that virulent L. pneumophila can grow within guinea pig alveolar but not peritoneal macrophages. Avirulent legionellae either did not grow in, or were killed by all, macrophage populations studied. Levi et al.(81) also demonstrated the ability of legionellae to multiply within

alveolar macrophages of guinea pigs. Vilde et al. (129) showed that a virulent strain of L. pneumophila multiplied in human monocyte-derived macrophages; an avirulent strain multiplied less well. Their study confirmed a previous study (64) showing the enhancement of binding of L. pneumophila to mononuclear phagocytes by antibodies and complement. They found intracellular multiplication of a non-virulent strain within human mononuclear phagocytes to be slower and of lower magnitude than that of a virulent strain; this has been shown for other facultative intracellular bacteria such as salmonellae (128). No growth was observed in the control media without cells in any of the studies mentioned above. If human mononuclear phagocytes respond in a similar fashion in vivo, the known efficacy of erythromycin in the treatment of legionellosis becomes clear since this antibiotic is readily absorbed by eukaryotic cells, although it is of relatively low potency in cultures of L. pneumophila.

Rodgers and Oldham (103) were the first to describe adherence of L. pneumophila in cell cultures. They found that the Legionella organisms would adhere reversibly to three cell lines (MRC-5, Hep-2 and Vero) within one hour of inoculation. Adherence was firm enough to resist washing. No cell receptors or bacterial adhesins such as pili or flagella could be visualized by scanning electron microscopy (SEM). They also noticed that adherence was followed by intracellular

multiplication and cytopathology. Although nuclei were often disrupted, no organisms were detected within them. While some studies have been made of the effect of antibiotics on the intracellular multiplication of L. pneumophila (32, 64, 66), none have dealt with the effect of antibiotics on attachment.

It would appear that L. pneumophila is no exception to the rule that cellular attachment or adherence must occur before bacteria can colonize or invade cells of the host. However, the nature of cellular adherence by the legionellae is poorly understood. Accordingly, this project was designed so as (a) to visualize microscopically attachment and penetration of tissue culture cells by two strains of L. pneumophila; (b) to determine whether the ligand-receptor bond could be weakened by certain carbohydrates or metabolites; and (c) to observe the influence of attachment/penetration upon growth and histology of the host cells.

CHAPTER II

MATERIALS AND METHODS

Preparation of Legionella pneumophila

The Johannesburg 2 (Jo 2) (serogroup 1) and Chicago 8 (Chi 8) (serogroup 7) strains of L. pneumophila were obtain- from Dr. William Bibb, The Centers for Disease Control (CDC), Atlanta, Georgia. They were maintained by monthly transfer on charcoal yeast extract (CYE) agar (80) (Table I). Suspensions of the bacteria were prepared in sterile phosphate-buffered saline (PBS) from growth on 3-4-day streak plates of CYE agar incubated in air at 37°C. The cells were washed once by centrifugation and resuspended in PBS to a density that would provide approximately 125 bacteria/tissue culture cell upon the addition of 0.05 ml (50 µl) to the tissue culture medium.

Tissue Culture Cells

All cell lines were incubated in an atmosphere of humidified air and carbon dioxide (5%) in a CO_2 incubator (Hotpack Coorporation, Philadelphia, PA). Three cell lines were used in this project. The WI-38 human diploid cell line was purchased from Dr. Leonard Hayflick, Center for Gerontological Studies, University of Florida, Gainesvelle, Florida. This cell line was derived by Dr. Hayflick from normal

TABLE I

CHARCOAL YEAST EXTRACT AGAR

1. Difco yeast extract	10.0 g
2. Activated charcoal (Norit SG, Sigma Chemical Co., St. Louis, Mo, Catalogue #C5510)	2.0 g
3. Difco agar	17.0 g
4. Distilled water	980 ml
5. L-cysteine HCl	0.4 g
6. Soluble ferric pyrophosphate	0.25 g

Ingredients (1), (2), (3) and (4) were autoclaved at 121°C for 15 minutes and cooled to 50°C. Separate filter-sterilized solutions (0.2-µm membrane filter) of L-cysteine HCl (10 ml of a 4% aqueous solution) and ferric pyrophosphate (10 ml of 2.5% aqueous solution) were added just before use to the cooled sterile base and the pH was adjusted to 6.9.

embryonic (3rd month of gestation) lung tissue of a Caucasian female (3). The growth medium used was Eagle's basal medium (BME) in Earle's balanced salt solution (GIBCO Laboratories, Grand Island, New York), supplemented with 10% fetal bovine serum (GIBCO Laboratories).

The Hep-2 cell line was obtained from the American Type Culture Collection, Rockville, Maryland. This cell, derived from a human carcinoma of the larynx, was established by Moore et al. (86) from tumors that had appeared in irradiated-cortisonized weanling rats injected with epidermoid carcinoma tissue from the larynx of a 56-year-old Caucasian male (125). These cells, an epithelial type, were grown in Eagle's minimum essential medium (MEM) with Earle's balanced salt solution supplemented with 10% fetal bovine serum.

The third tissue culture, a line of rat embryo fibroblasts (REF), was isolated in our laboratory from minced embryos recovered from a pregnant Sprague Dawley rat. Four embryos (13th day of gestation) were excised aseptically, washed several times with 10 ml of sterile 0.25% trypsin to remove blood fibrin and tissue debris, chopped by means of a sterile scalpel in a 50 ml sterile centrifuge tube. The minced tissues were suspended in 10 ml of 0.25% trypsin for 15 minutes, and then the supernatant fluid containing cells was transferred into the second centrifuge tube. This step was repeated once again and 5 ml of growth medium was added which stopped the action of trypsin. The cell suspension was

centrifuged in a bench top centrifuge (International Equipment Co., Needham HTS., Mass), resuspended in 10 ml of growth medium, and inoculated into a tissue culture plate (100 X 20 mm, Falcon, Oxnard, CA). The REFs were maintained in MEM as was employed for growing Hep-2 cells.

Attachment Assay

Figure 1 summarizes the procedure used in the attachment assay. Cultures obtained from 100 X 20 mm tissue culture plates with confluent growth were trypsinized (0.25 % trypsin in PBS with 0.02 M ethylene-diamine-tetra-acetic acid, EDTA, GIBCO); the cells were evenly suspended in the growth medium with a pipette. Cells in suspension were counted with a hemocytometer (American Optical Scientific Instrument Division, Buffalo, New York). Each plate (60 X 15 mm) was seeded with 5 ml medium containing 2 X 10^4 cells. Also, each plate contained four sterile glass cover slips (18 X 18, No. 1. Clay Adams, Parsippany, New Jersey) on which monolayers could be examined. Cultures were incubated at 37°C in an atmosphere of humidified air containing 5% CO_2 for 24 hours, during which time the monolayers formed.

Bacteria (Jo 2 and Chi 8 strains) from CYE agar plates were suspended in PBS and 2 µl of the suspension were spread evenly upon two 15-mm circles of Fluoro Slides (Curtin Matheson Scientific Inc., Houston, TX). The slides were air-dried, heat-fixed and the bacteria were stained with

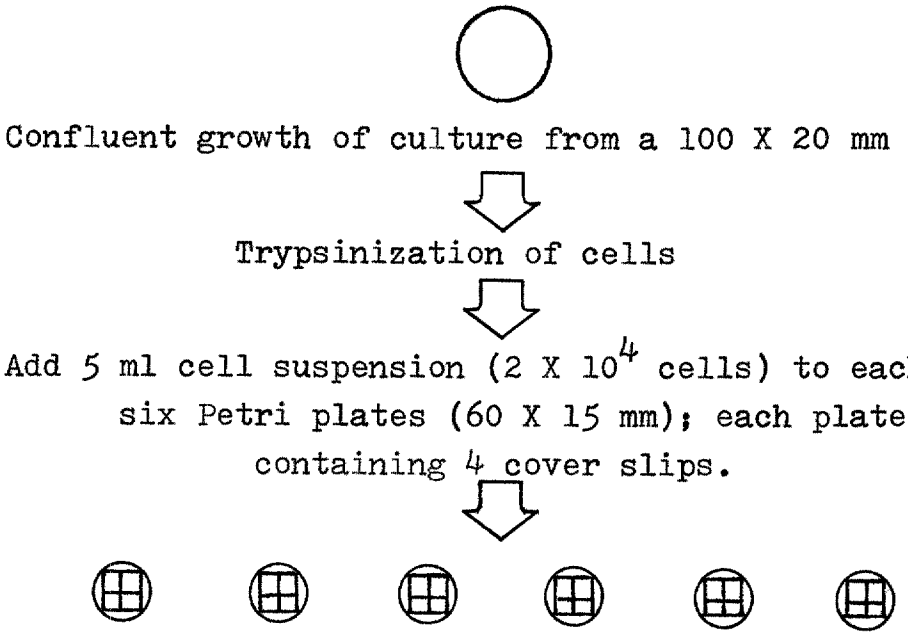


Figure 1 -- Procedure for Attachment Assay

serogroup-specific immunofluorescent conjugate, provided by CDC, and counted with the aid of a fluorescence microscope (Carl Zeiss, Germany) at 400X. The average area of the circles (176.625 mm^2) on the slides was 1,218 times that of a microscopic field (0.145 mm^2) at 400X. Let X be the average number of bacteria counted in a field; from the equation: $X \cdot 1218/2 \cdot 10^{-3} = Y$, where Y was the total number of bacteria per ml of suspension. Upon adjusting the density of the suspension, the culture dishes were inoculated with 50 µl of suspension containing 2.5 X 10^6 bacterial cells and the cultures were immediately returned to the 37°C incubator.

The bacteria were counted as follows: cover slips for examination of attachment and penetration were sampled at intervals during incubation, namely, 0.5, 1, 12, 24, 48, 72 and 168 hours. At each time interval, three cover slips with the animal cells and bacteria were removed from the culture dishes aseptically, washed three times in PBS, and then fixed immediately with methanol/acetic acid (3:1) for 15 minutes. This was followed by application of methanol for five minutes. The slides were then air-dried.

To detect the legionellae the immunofluorescent conjugate was applied directly to the cover slip preparations (25). The CDC conjugates were serogroup specific (group 1 for the Knoxville strain and group 7 for the Chi 8 strain of L. pneumophila). The gamma-globulins had been conjugated with fluorescein isothiocyanate (FITC) to yield fluorescein

to protein (F/P) ratios of approximately 20 g FITC/mg protein, and had been diluted with tetramethylrhodamine isothiocyanate (TMRITC)-labeled normal rabbit serum to their optimum working dilutions, lyophilized and packaged in 2-ml volumes. When needed, the dehydrated conjugates were rehydrated in 2 ml of special PBS (Table II).

After application of the immune conjugate, the preparations were incubated in a moist chamber (Petri dish with water-saturated filter paper) at room temperature for 30 minutes. The cover slips were washed twice with the special PBS, immersed in PBS in separate chambers for 10 minutes, and rinsed with distilled water. Air-dried cover slips were mounted onto precleaned Fluoro Slides with the specimen side down. Buffered glycerol was employed as the mounting medium (Table III). Immunofluorescence was detected using a Zeiss fluorescence microscope with an HBO 200 w/4 super-pressure mecury lamp, and with the exciter filter and barrier filter set at BG 12 and 50, respectively. The number of organisms attached to the cell surface and in the cytoplasm or the nucleus was quantitated by counting three fields of cells at 400X magnification in each of the three samples. Examples of fluoromicrographs are shown in Figures 2, 3 and 4.

Later, attachment was examined in greater detail by means of scanning electron microscopy (Figure 5). Cultures on cover slips, 48 hours post-infection, were washed three

TABLE II

PHOSPHATE BUFFERED SALINE FOR IMMUNO FLUORESCENCE
pH 7.6 (0.01 M Buffer; 0.85% NaCl)

A. Concentrated Stock Solution:	
Na_2HPO_4 (anhydrous reagent grade)	12.36 g
NaH_2PO_4 (reagent grade)	1.80 g
NaCl (reagent grade)	85.00 g
Distilled water to make final volume	1,000 ml
B. Working Solution (pH 7.6: 0.01 M Buffer; 0.85% NaCl):	
Concentrated stock solution	100 ml
Distilled water to make	1,000 ml

TABLE III

GLYCEROL MOUNTING MEDIUM

Preparation:

Buffer, pH 9.0*	1 part
Glycerol (glycerin), neutral	9 parts

*Carbonate-Bicarbonate Buffer, pH 9.0 (0.5 M)
4.4 ml of 0.5 M Na_2CO_3 was mixed with 100 ml 0.5 M $NaHCO_3$
If necessary, adjust pH to 9.0 by the addition of 0.5 M Na_2CO_3.

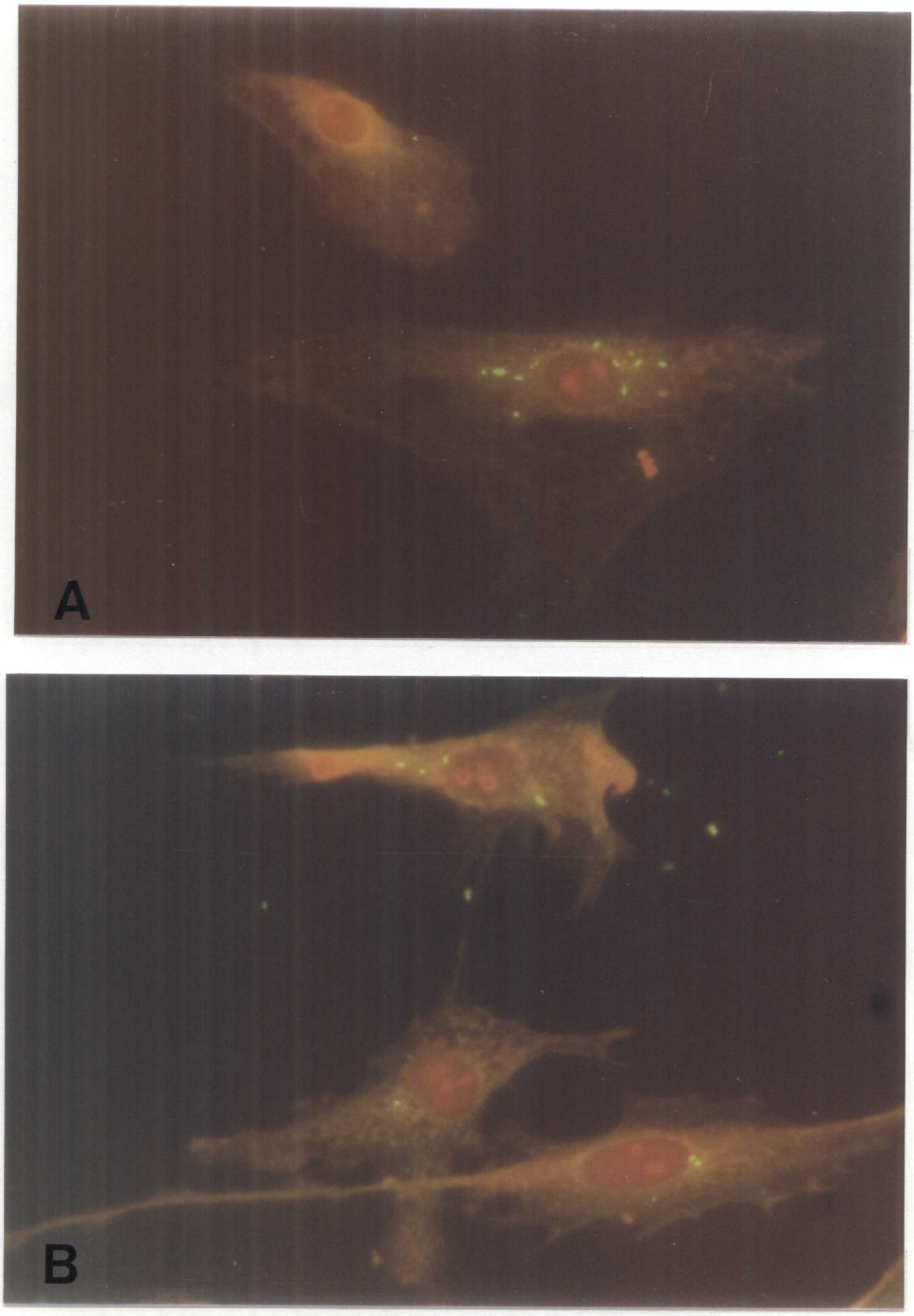


Figure 2 -- Fluoromicrograph of Legionella and tissue culture cells. (A) Rat embryo fibroblast cells infected by Jo 2 strain, 48 hours of incubation, 400 X. (B) Rat embryo fibroblast cells infected by Chi 8 strain, 48 hours of incubation, 400 X.

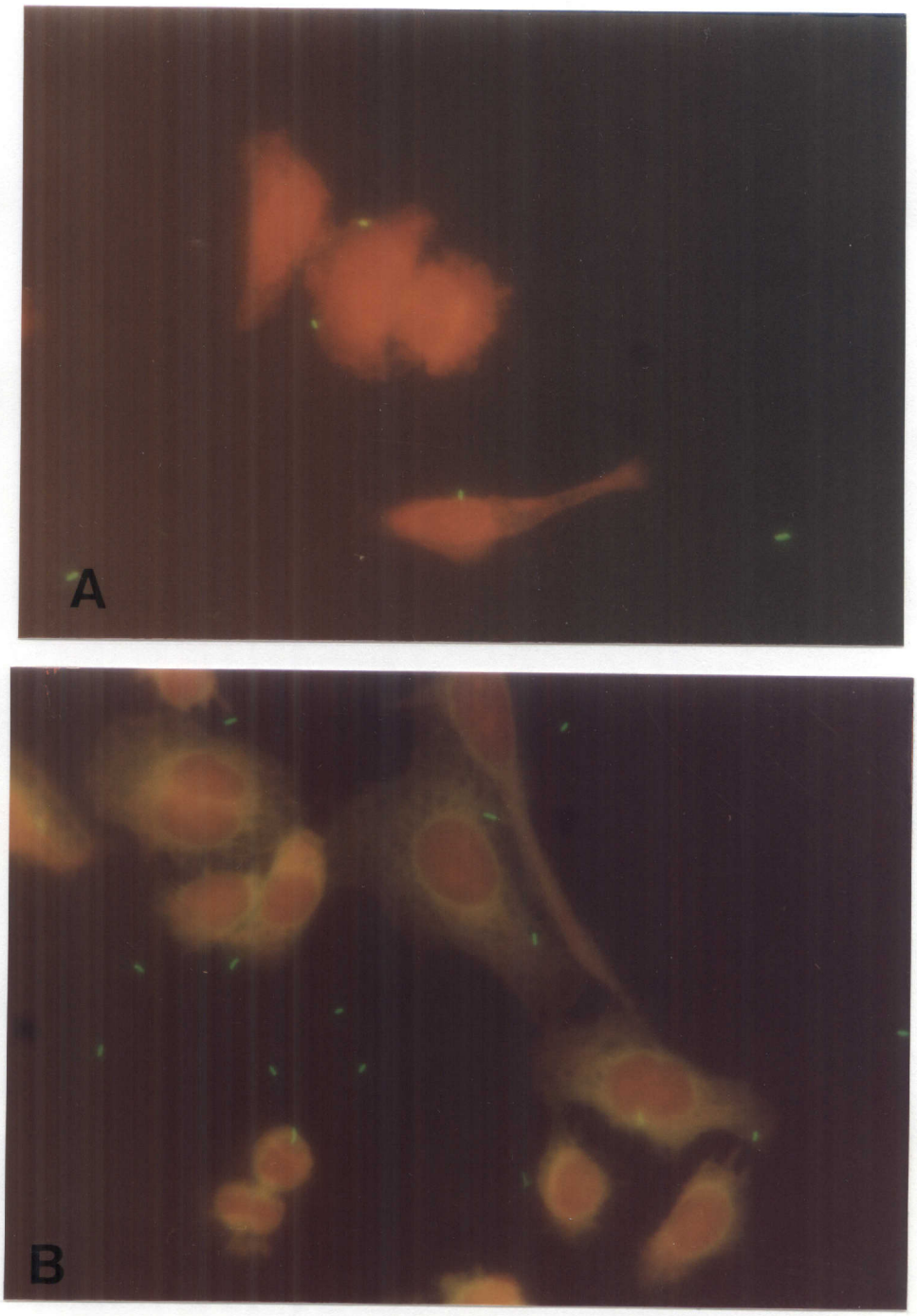


Figure 3 -- Fluoromicrograph of Legionella and tissue culture cells. (A) Hep-2 cells infected by Jo 2 strain, 48 hours of incubation, 400 X. (B) Hep-2 cells infected by Chi 8 strain, 48 hours of incubation, 400 X.

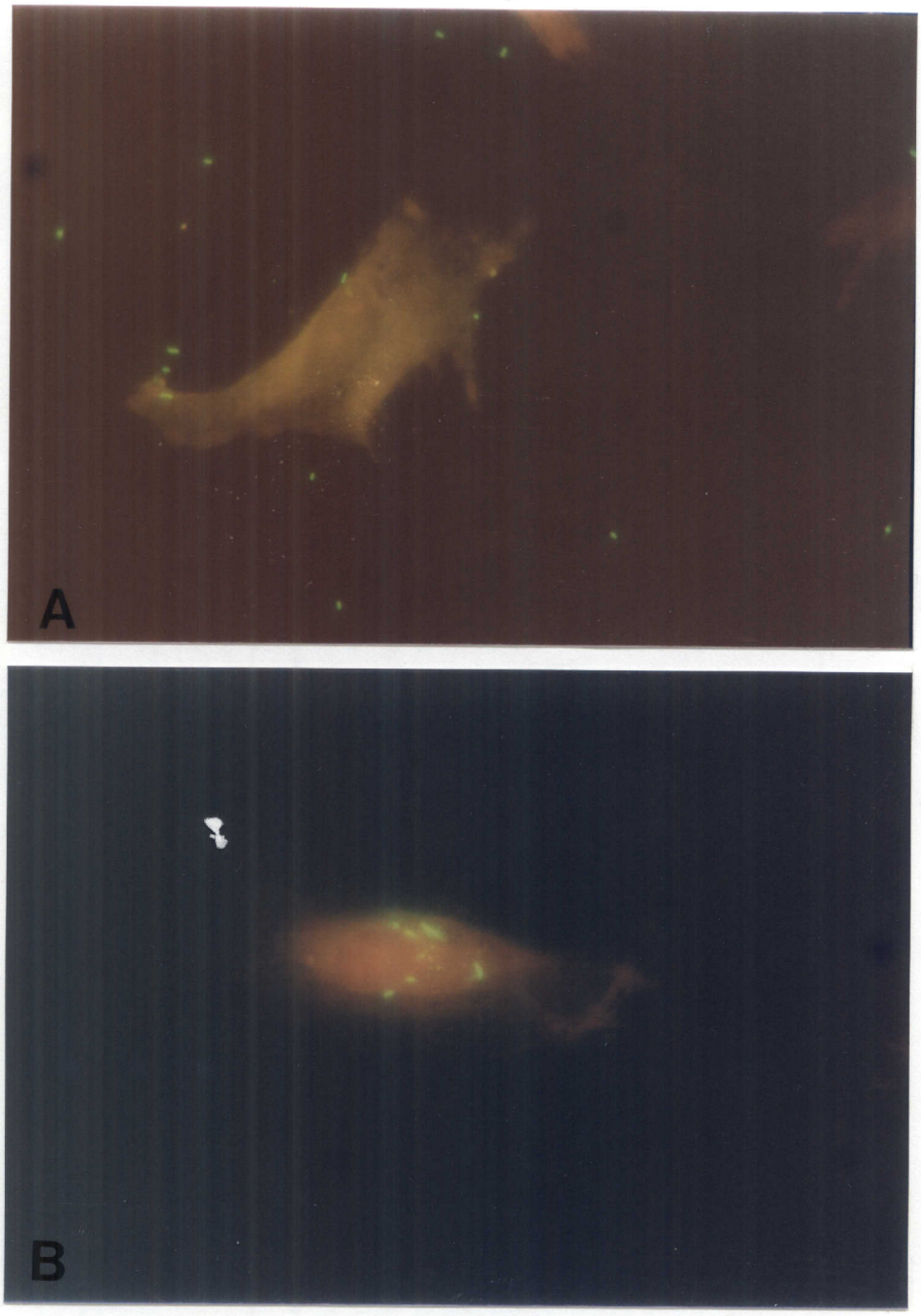


Figure 4 -- Fluoromicrograph of Legionella and tissue culture cells. (A) WI-38 cells infected by Jo 2 strain, 48 hours of incubation, 400 X. (B) WI-38 cell infected by Chi 8 strain, 48 hours of incubation, 400 X.

Wash cover slips three times in phosphate buffered saline and fix with 3% glutaraldehyde (overnight) in phosphate buffer*.

⇩

Wash off glutaraldehyde with phosphate buffer

⇩

Dehydrate the culture in a series of alcohol solutions:

(1) 25% Alcohol : 75% H_2O	15 - 20 mins.
(2) 50% Alcohol : 50% H_2O	15 - 20 mins.
(3) 75% Alcohol : 25% H_2O	15 - 20 mins.
(4) 95% Alcohol : 5% H_2O	15 - 20 mins.
(5)100% Alcohol	15 - 20 mins.
(6)100% Alcohol	15 - 20 mins.

⇩

Critical-point dry with liquid CO_2

⇩

Glue cover slips to stubs with silver paint; coat in a sputtercoater with gold-palladium

⇩

Examine coated samples with a scanning electron microscope (25 KV)

*Phosphate Buffer (0.2 m):

NaH_2PO_4	6.41 g
Na_2HPO_4	41.30 g
H_2O	1,000 ml

Figure 5 -- Preparation of tissue culture cells growing on cover slips for scanning electron microscopy.

times in PBS, fixed in 3% glutaraldehyde and dehydrated through the alcohol series. After dehydration the preparations were critical-point dried with liquid CO_2 (Critical Point Dryer, Omar, SPC-900/EX, Tacoma, WA). The cover slips were glued to stubs with silver paint and coated in a sputtercoater (SEM coating unit E5100, Polaron Instruments Inc., West Chester, PA) with gold-palladium and then examined with a scanning electron microscope (JEOL JSM-T300 Scanning Microscope, Dearbon Road Peabody, Mass.) at 25 KV.

Effect of Carbohydrates and Pyruvate on the Attachment

The interaction between L. pneumophila and three cell lines was studied in the presence of five sugars, a metabolic intermediate, and a glucose analogue, namely: D-mannose (Difco); D-galactose and D-glucose (Fisher); L-glucose and β-D(-)fructose (Sigma); pyruvic acid (Sigma); and 2-deoxy-D-glucose (Sigma).

All compounds were employed at concentrations of 1 mg and 5 mg/ml in the tissue culture media. The media were filter-sterilized through a 0.2 um membrane filter (Micro Filtration System, Dublin, CA) and kept at 4°C until use. The effects of the additives were tested in two ways: (a) cells were treated for 24 hours, the conditioned medium removed, the cells washed twice with 2 ml of fresh medium and 5 ml fresh medium added; or (b) the test compounds were continuously present in the medium. Cultures without any treatment served as

controls for comparing the effect of the additives on attachment/penetration.

After the initial 24-hour period, the cells were challenged with 2.5 X 10^6 L. pneumophila. Cultures were replenished once with fresh medium, with or without the specific additive, soon after the sampling at 48 hours post-infection. Figure 6 outlines the procedure employed.

The Influence of Legionella pneumophila on Cell Morphology and Growth

Cytopathic effect (CPE) is any form of macroscopic or microscopic, localized or generalized degenerative change or abnormality in the cells of a monolayer tissue culture (or membranes of an embryonated egg) due to infection by microorganisms (59). In virus-cell systems, CPE may include cell death, cytoplasmic vacuolation, the formation of syncytia, or the formation of discrete foci of hyperplasia (microtumors) (97).

In a study on tissue culture monolayers of MRC-5 (human fetal lung fibroblasts), HeLa (human cervical carcinoma), McCoy (mouse synovial cells), and Hep-2 cells, Daisy et al.(32) found the Philadelphia 2 strain (serogroup 1) of L. pneumophila to cause CPE, beginning as localized granularity and progressing to lysis of most of the cells. Rodgers and Oldham (103) showed the development of microvilli on MRC-5 and Hep-2 cells infected by strain Nottingham N 7 (serogroup 1) of L. pneumophila as well as the same kind

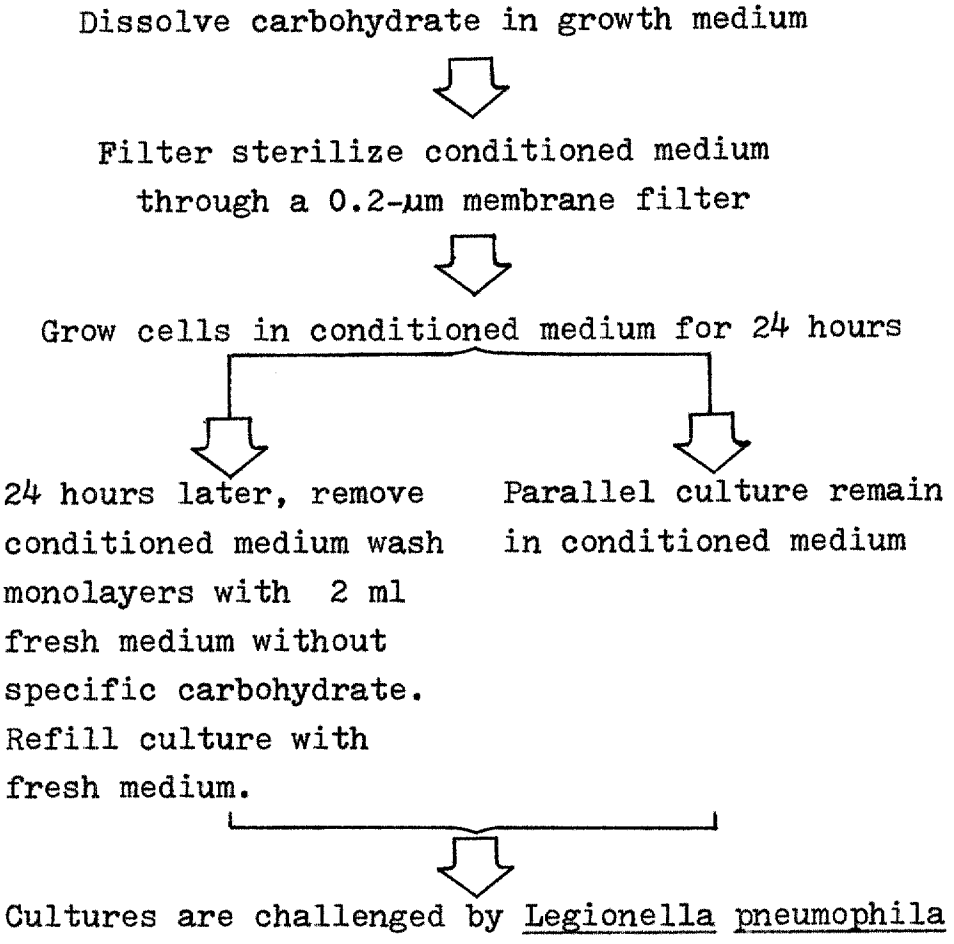


Figure 6 -- Procedure for testing the effect of carbohydrates on the attachment of Legionella pneumophila to cells in culture.

of CPE observed by Daisy et al..

Animal cells in culture display very much the same type of growth pattern as microorganisms. When cells are taken from a stationary culture there is at first a "lag phase" of some hours to some days before growth commences. Growth then proceeds steadily with the population doubling every 15 to 20 hours in the case of fast growing cells. This is known as "logarithmic phase". Once the maximal population is reached, the cells enter the "stationary phase". During the logarithmic phase the cell population increases according to the formula $N = N_0 2^n$

$$\text{i.e., } \log N = \log N_0 + n \log 2$$

(where N_0 = initial inoculum, N = total population, and n = generation time). From which $n = (\log N - \log N_0)/\log 2$. Since $1/\log 2 = 3.32$ this can be written in the form

$$n = 3.32(\log N - \log N_0) \quad (96).$$

In preparing to observe cytopathic effects, cells infected with L. pneumophila for attachment assay were maintained in tissue culture plates for another two weeks and compared microscopically with uninfected controls. Signs of CPE were checked daily using an inverted microscope (Olympus, Model CPK) at a magnification of 400X. During this period the cells were maintained in the customary manner, i.e., periodic medium change and subculture upon reaching confluency.

Cells were than transferred into three 24-well tissue culture plates (Falcon, Oxnard, CA) at a concentration of 10^4 cells/ml medium/well. The cultures were incubated in a humidified CO_2 incubator at 37°C. The growth curve of each culture was determined every 24 hours by triplicate hemocytometer counts of trypsinized (1 ml 0.25% trypsin) cells recovered from the wells.

Survival of Legionella pneumophila in Tissue Cultures and in the Tissue Culture Medium

Legionella spp. contamination has been repeatedly reported to occur in hot water systems and in associated plumbing fixtures (21, 28, 130). Recent outbreaks of legionellosis have been attributed to water aerosols created by shower heads and heat exchange systems, such as cooling towers or air-conditioning units (6, 29, 34). Legionella pneumophila has been reported also to be able to survive and even multiply in hot water storage tanks and in tap water (18, 138). Surprisingly, it was also found to grow and multiply intracellularly but not in tissue culture media (32, 66, 103).

In my study, 5 ml of tissue culture medium in 60 X 15 mm plates, with (2×10^4) or without mammalian cells, were inoculated with 2.5×10^6 L. pneumophila cells. Viable counts were performed in duplicate on harvested culture fluids daily for 8 days and the colony forming unit (CFU) per milliter was estimated by spread plates on CYE agar.

CHAPTER III

RESULTS

At various time intervals (0.5, 1, 12, 24, 48, 72 and 168 hours) after adding legionellae to the cell culture, the cover slips were collected and washed three times with PBS to remove unattached bacteria. Cells on the cover slips were then fixed and immunofluorescently stained. The number of bacteria attached to the surfaces or penetrating into the cytoplasm or the nuclei of cells and the number of cell in each microscopic field (400X) were recorded, as shown in Table IV for example. These data enabled the calculation of the attachment/penetration indices (AI or PI) which simply express the average number of bacteria (attached/penetrating) /cell (number of bacteria/ cell X 100), as in Table V (as calculated from raw data in Table IV). Because of the large number of parameters employed in these experiments, it is necessary to utilize a graphical method of presentation of the data to facilitate interpretation of the results. For this purpose, data from each group of experiments were plotted by means of the least squares fit according to the quadratic function, $Y = aX^2 + bX + c$ (61). Where the abscissa stands for the time interval and the ordinate stands for AI or PI; a, b, and c are constants. In brief, the

TABLE IV

INTERACTION BETWEEN LEGIONELLA (JOHANNESBURG 2) AND TISSUE CULTURE CELLS

Cell		Time of Incubation																					
		30 mins			1 hr			12 hrs			24 hrs			48 hrs			72 hrs			168 hrs			
REF	Cells Counted	6,	8,	12	9,	8,	10	16,	15,	15	14,	12,	15	16,	17,	20	20,	14,	17	50,	60,	60	
	No. of Bacteria on/in																						
	Surface	0	0	0	1	1	0	1	1	1	2	1	1	1	0	1	0	2	0	15	6	3	
	Cytoplasm	0	0	0	0	0	0	5	3	3	1	2	1	0	0	0	0	1	0	6	12	7	
	Nucleus	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	3	0	1	
Hep-2	Cells Counted	21,	18,	17	13,	21,	26	55,	40,	32	27,	23,	32	35,	40,	40	55,	60,	60	80,	60,	60	
	No. of Bacteria on/in																						
	Surface	0	0	0	1	0	0	4	0	0	0	1	3	4	0	0	1	1	1	4	4	3	
	Cytoplasm	0	0	0	0	0	0	1	2	0	0	0	0	0	0	2	2	0	1	7	6	1	
	Nucleus	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	3	2	1	
WI-38	Cells Counted	9,	8,	9	11,	7,	9	15,	14,	16	13,	12,	18	16,	15,	22	15,	12,	24	60,	35,	40	
	No. of Bacteria on/in																						
	Surface	0	0	0	0	0	0	2	0	0	1	1	1	0	1	1	2	1	2	2	1	2	
	Cytoplasm	0	0	0	0	0	0	1	1	0	0	2	0	0	1	1	1	3	0	1	0	0	
	Nucleus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

About 2×10^4 cells were incubated with 2.5×10^6 Legionella in tissue culture plates (60 x 15 mm). Each plate contained four sterile glass cover slips. At each time interval three cover slips were aseptically removed, washed with PBS and fixed. The bacteria were stained with serogroup-specific immunofluorescent conjugate and counted with the aid of a fluorescence microscope.

TABLE V

ATTACHMENT/PENETRATION INDICES* OF Legionella pneumophila (JOHANNESBURG 2) WITH TISSUE CULTURE CELLS

Cell	Index	Time of Incubation						
		30 mins	1 hr	12 hrs	24 hrs	48 hrs	72 hrs	168 hrs
REF	AI	0	7.40	6.52	9.76	3.77	3.92	14.12
	PI (Cytoplasmic)	0	0	23.91	9.76	0	1.96	14.71
	PI (Intranuclear)	0	0	0	2.44	0	0	2.35
Hep-2	AI	0	2.00	3.15	4.88	3.48	1.71	5.50
	PI (Cytoplasmic)	0	0	2.36	0	1.74	1.71	7.00
	PI (Intranuclear)	0	0	0.79	1.22	0	0	3.00
WI-38	AI	0	0	4.44	6.98	3.77	9.80	3.70
	PI (Cytoplasmic)	0	0	4.44	4.65	3.77	7.84	0.74
	PI (Intranuclear)	0	0	0	0	0	0	0

* Data in TABLE IV were calculated an attachment/penetration index (AI or PI) which expresses the ratio of bacteria (attached/penetrating) to animal cells (No. of bacteria/No. of cells X 100).

quantity

$$S = \sum_{i=1}^{n} \left(Y_i - (aX_i^2 + bX_i + c)\right)^2$$

would be minimized by setting the following derivatives equal to zero:

$$\frac{\partial S}{\partial a} = 0 = \sum 2\left(Y_i - (aX_i^2 + bX_i + c)\right)(-X_i^2)$$

$$\frac{\partial S}{\partial b} = 0 = \sum 2\left(Y_i - (aX_i^2 + bX_i + c)\right)(-X_i)$$

$$\frac{\partial S}{\partial c} = 0 = \sum 2\left(Y_i - (aX_i^2 + bX_i + c)\right)(-1)$$

Expanding and collecting terms:

$$a\sum X_i^4 + b\sum X_i^3 + c\sum X_i^2 = \sum X_i^2 Y_i$$

$$a\sum X_i^3 + b\sum X_i^2 + c\sum X_i = \sum X_i Y_i$$

$$a\sum X_i^2 + b\sum X_i + cn = \sum Y_i$$

These equations may then be solved for the constants a, b, and c. The parabola derived from this quadratic function then shows the trend of interaction between Legionella and animal cells.

Attachment/Penetration of Animal Cells by Chicago 8 and Johannesburg 2 Strain of Legionella pneumophila (Figures 7-9)

In these control experiments in the unmodified medium, strain Jo 2 showed a much higher affinity for cell surfaces than Chi 8. In Figure 7 it can be seen that the Chi 8 strain no longer remained attached to any of the three animal cell

lines after one week's incubation. However, Jo 2 at that time gave an AI of approximately 4 (WI-38) to 15 (REF). The AI of Jo 2 with Hep-2 was about one-third that of REF. In Figure 8, REF is seen to support the highest cytoplasmic PI (PI = 14) with strain Jo 2 after one week; with WI-38 the PI peaked on the fourth day (PI = 10), dropping to 4 after one week. Neither Legionella strain showed much penetration of nuclei (Figure 9). In fact, Chi 8 was not seen intranuclearly in any of the three cell lines.

Effect of Selected Carbohydrates and Pyruvate on Attachment/Penetration of Animal Cells by *Legionella pneumophila*

1. D-mannose (Figures 10-15)

The attachment of both Chi 8 and Jo 2 to cell surfaces was inhibited by D-mannose (Figures 10, 13) except for the Hep-2 cell line to which the affinity of Jo 2 was enhanced by treatments of 5 mg D-mannose/ml (24 hours) and 1 mg/ml (continuous). After 48 hours, the AI of 12 for Jo 2 with Hep-2 was six times that of the control, while its cytoplasmic PI ranged from approximately two to four times that of the control in all three cell lines (Figure 11). The cytoplasmic PI of Chi 8 was relatively unaffected by D-mannose (Figure 14). The intranuclear PI of both *Legionella* strains was virtually unaffected by D-mannose (Figures 12, 15).

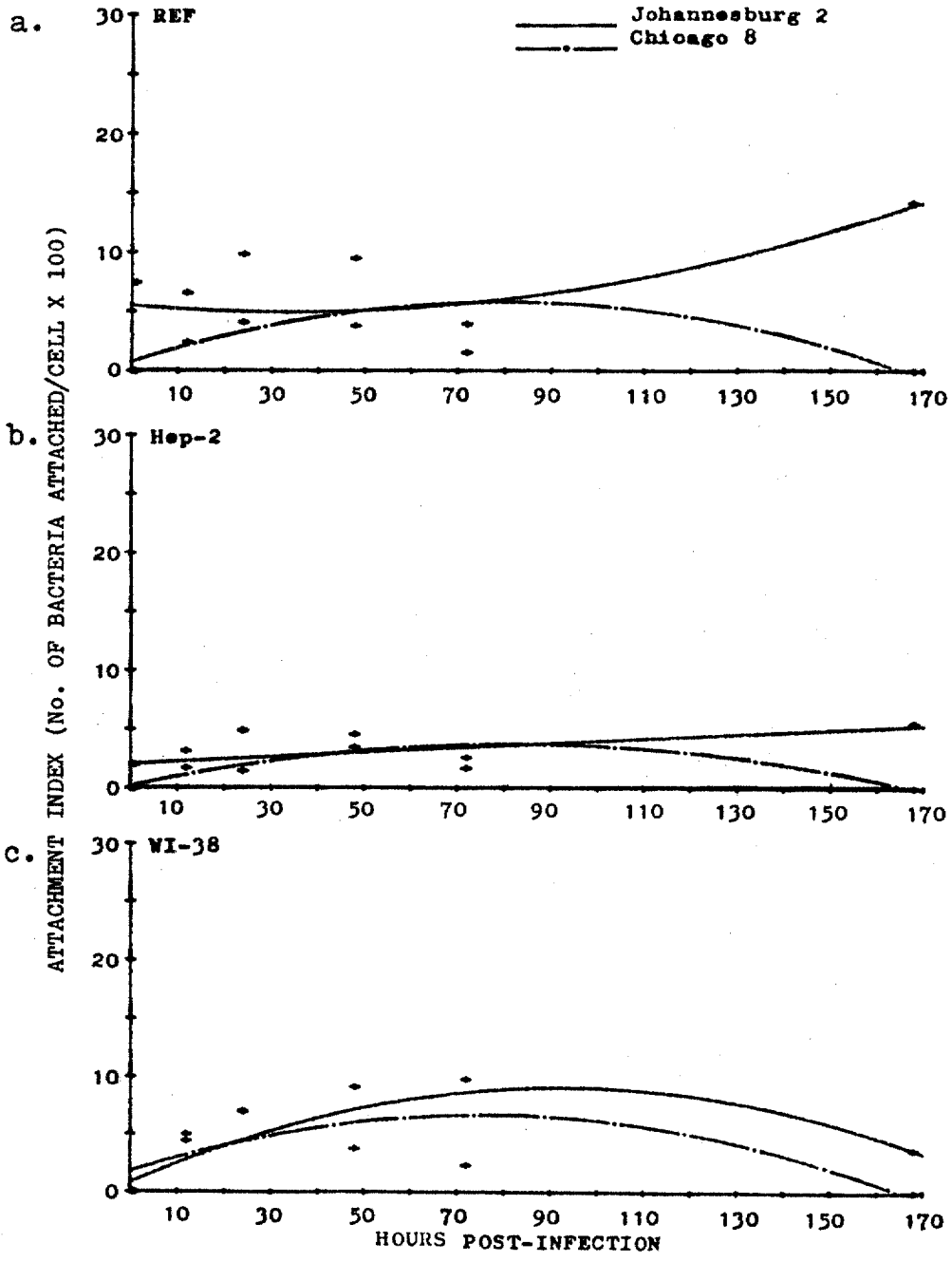


Figure 7 -- Attachment of Legionella pneumophila to surfaces of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

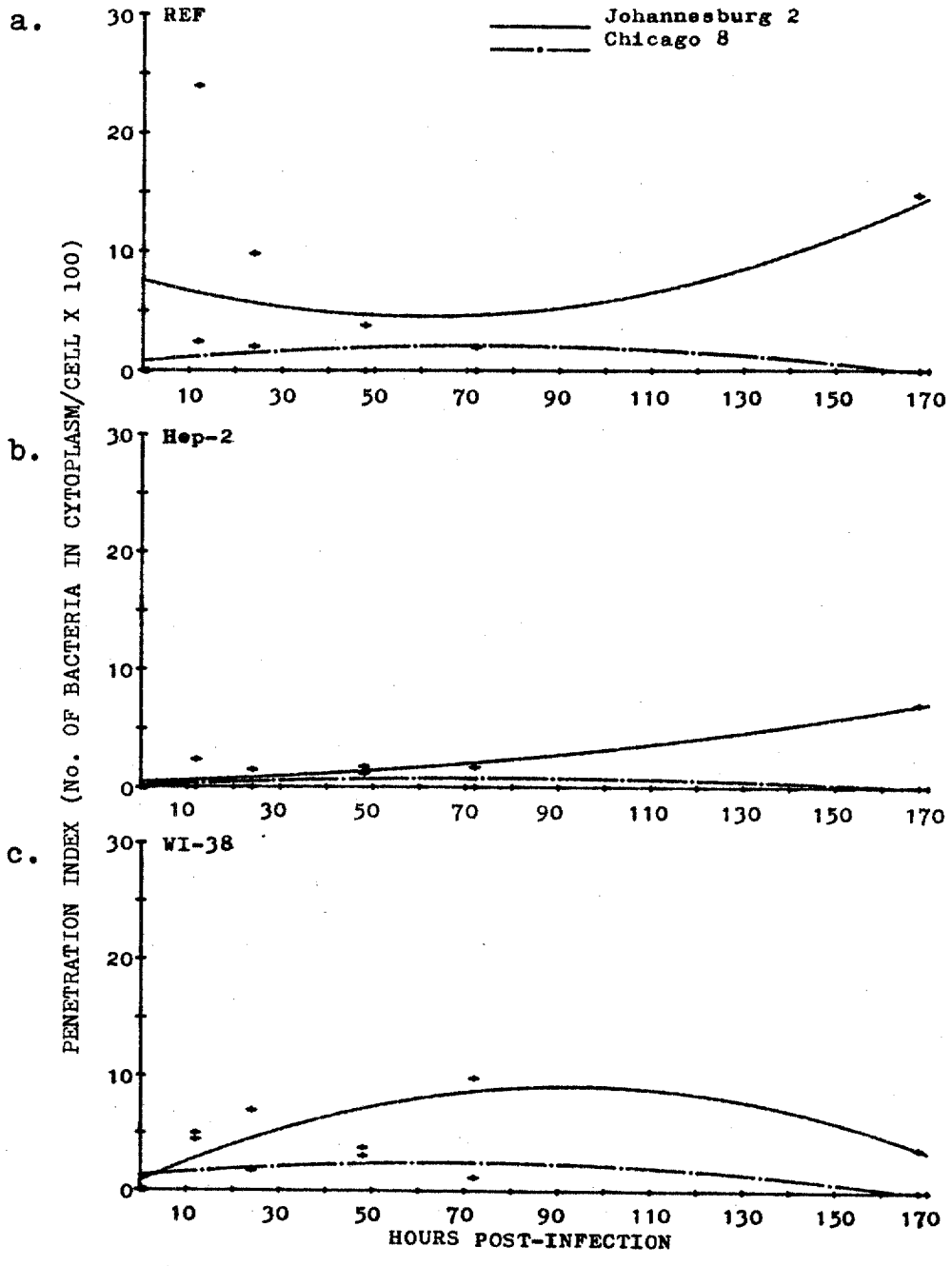


Figure 8 -- Penetration of Legionella pneumophila into cytoplasm of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

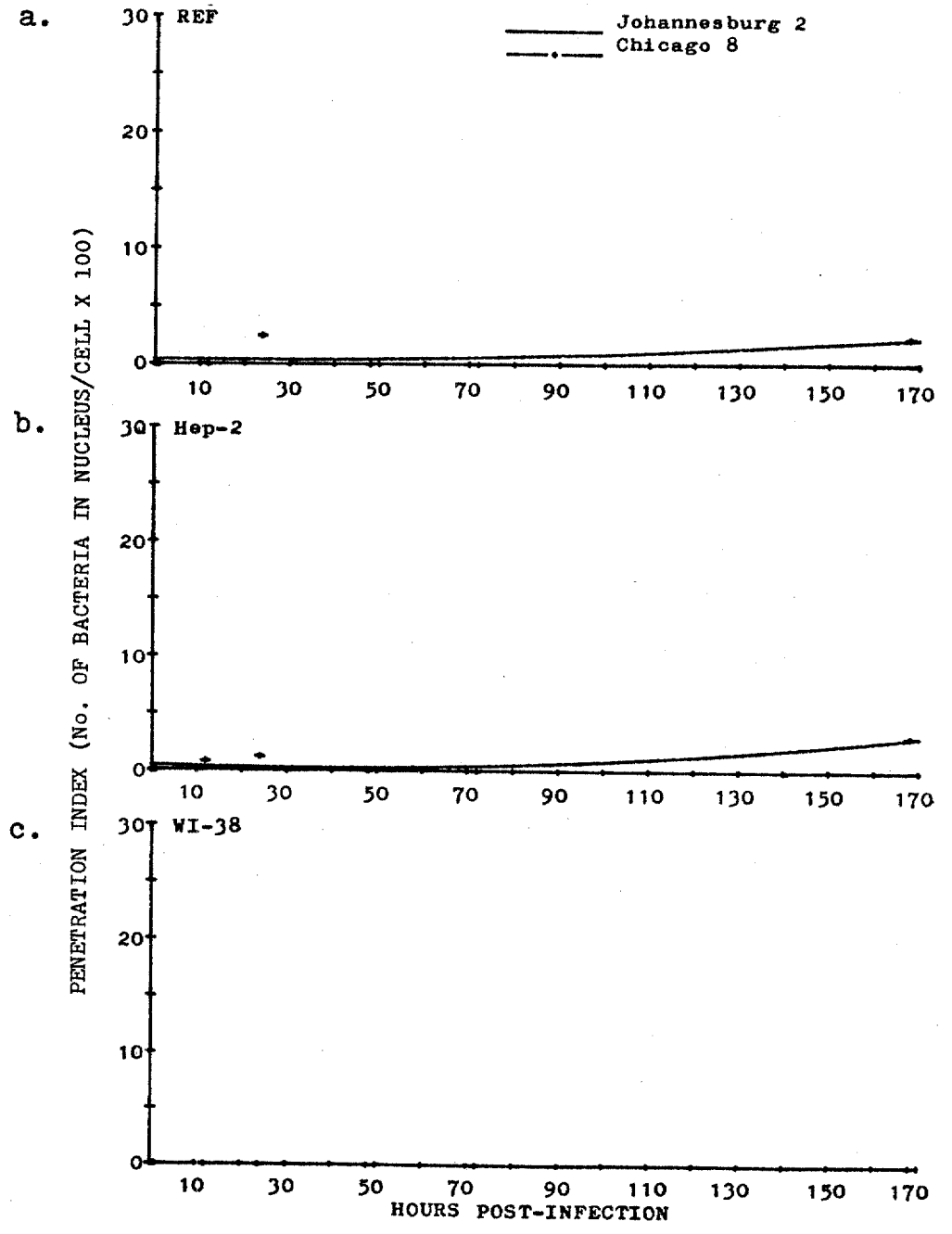


Figure 9 -- Penetration of Legionella pneumophila into nuclei of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

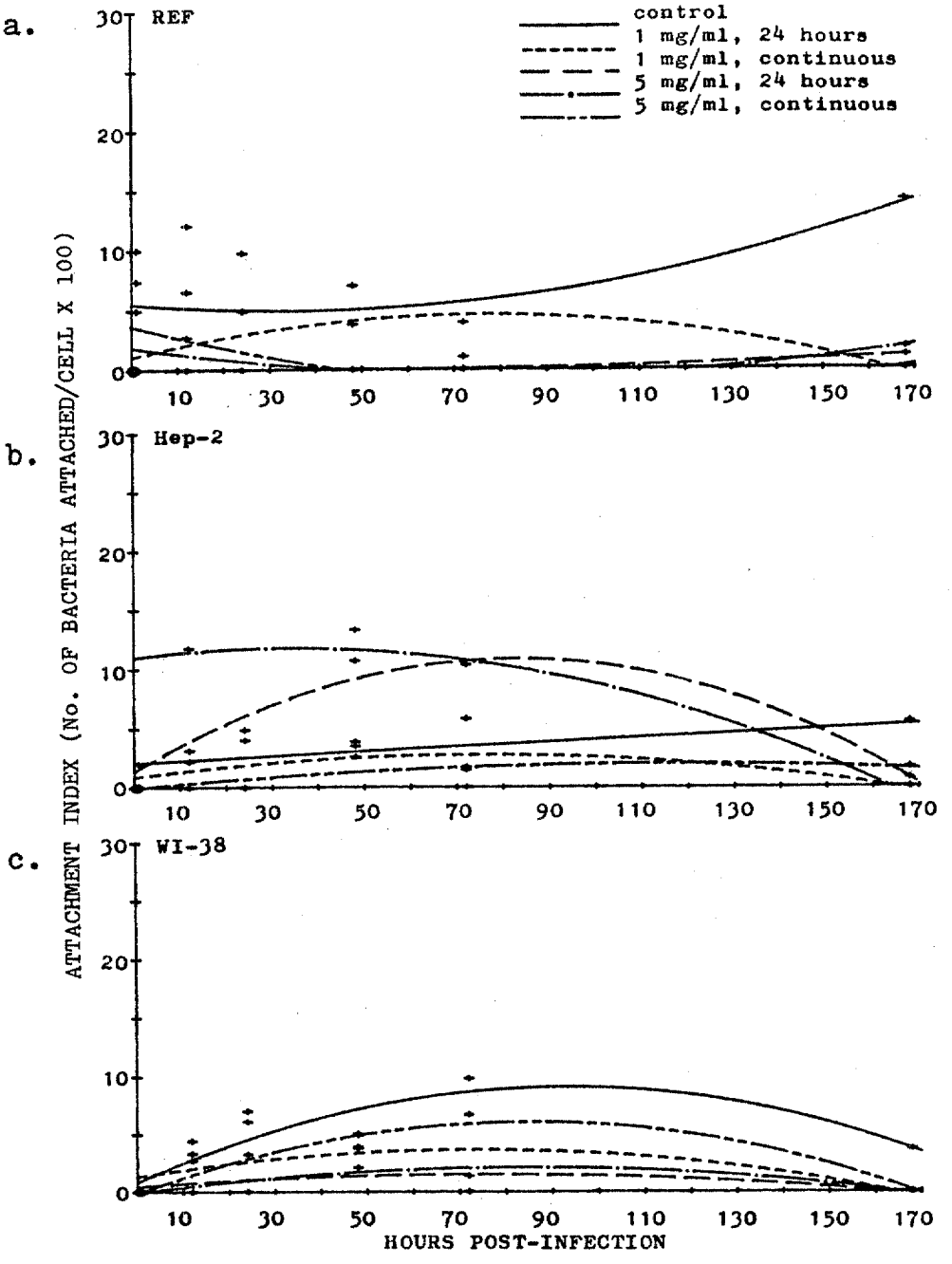


Figure 10 -- Effect of D-mannose on attachment of Johannesburg 2 strain of Legionella pneumophila to surfaces of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

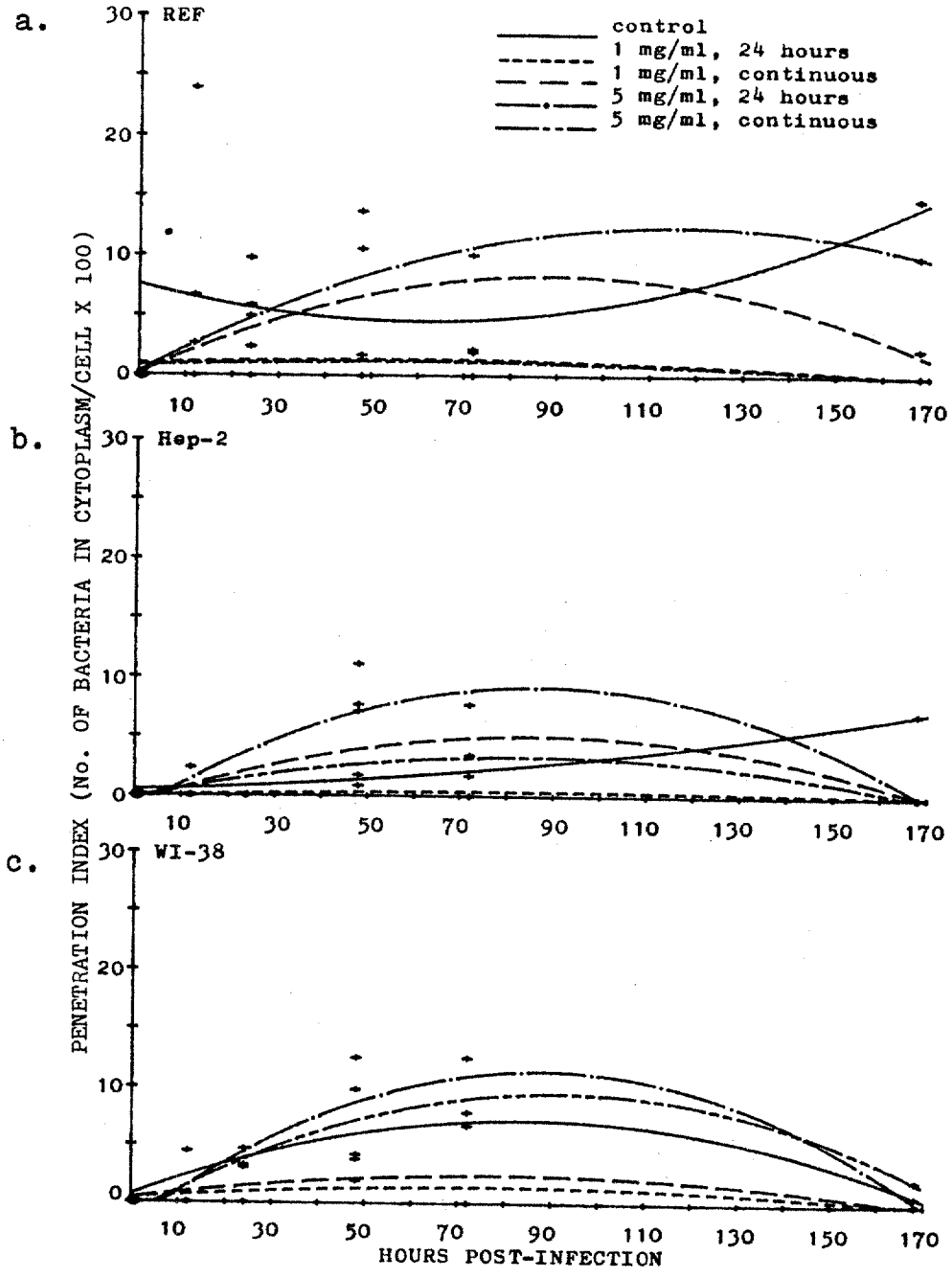


Figure 11 -- Effect of D-mannose on penetration of Johannesburg 2 strain of Legionella pneumophila into cytoplasm of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

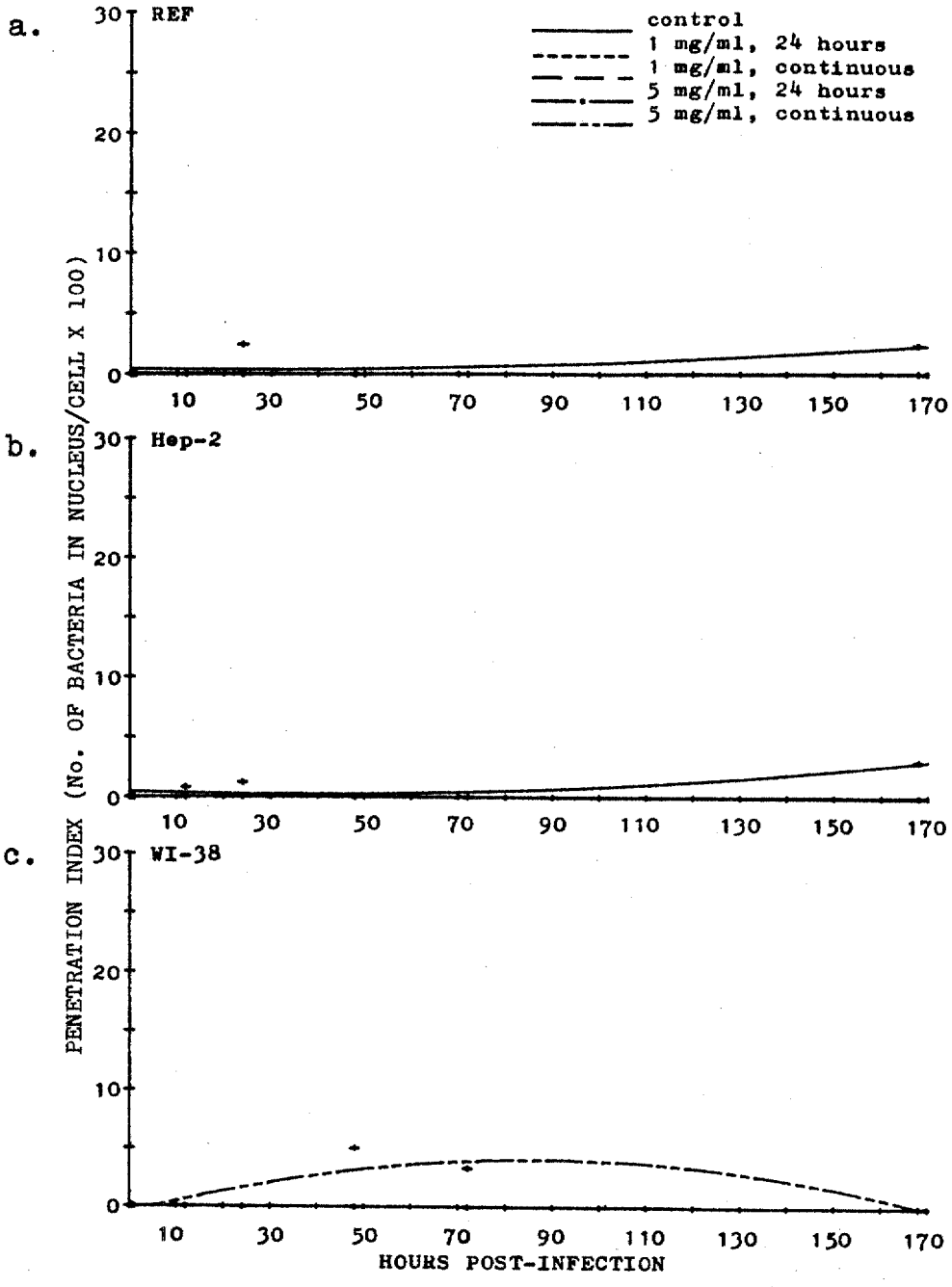


Figure 12 -- Effect of D-mannose on penetration of Johannesburg 2 strain of Legionella pneumophila into nuclei of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

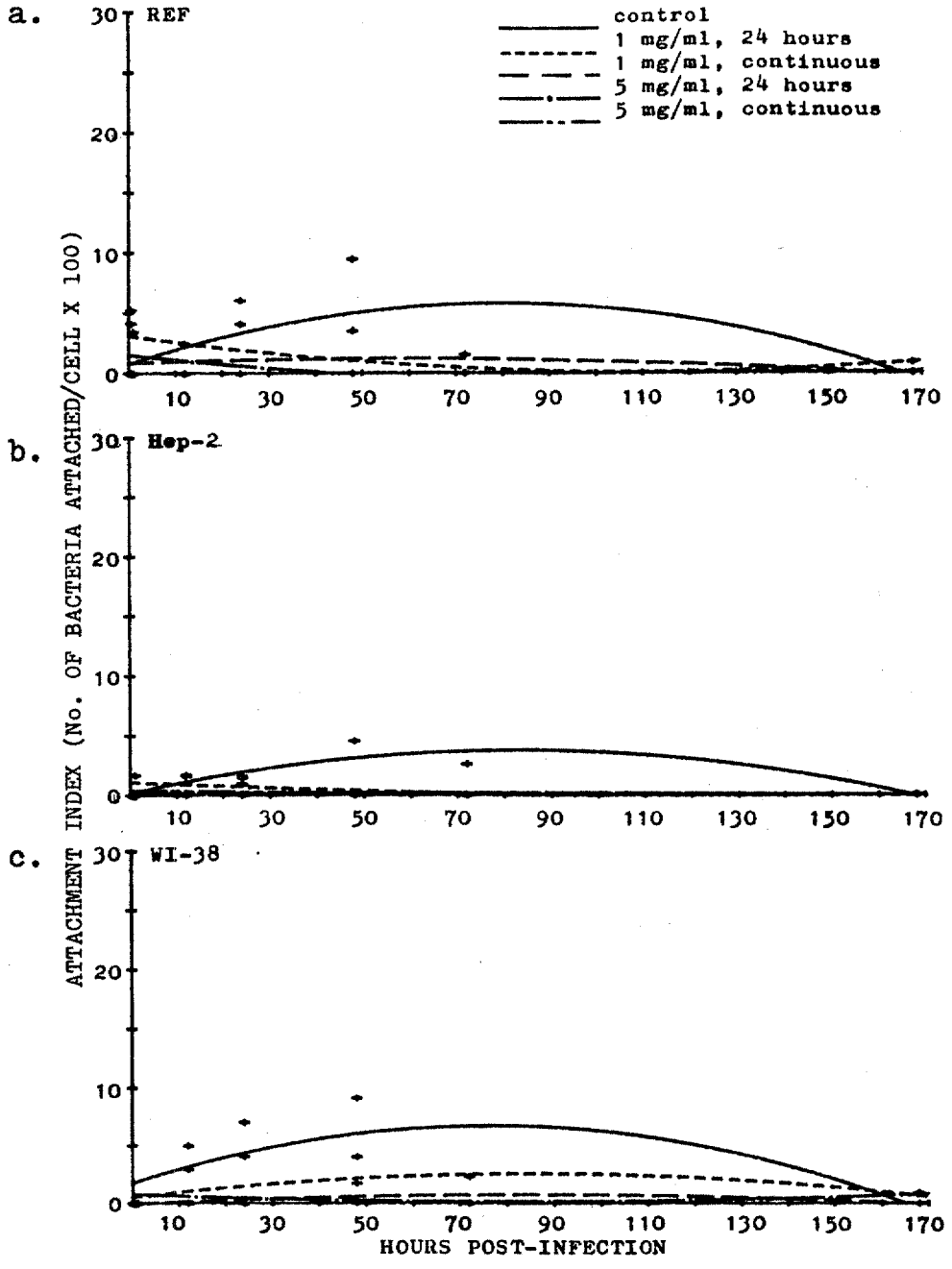


Figure 13 -- Effect of D-mannose on attachment of Chicago 8 strain of Legionella pneumophila to surfaces of tissue culture cells. a: Rat embryo fibroblast cells ; b: Hep-2 cells; c: WI-38 cells.

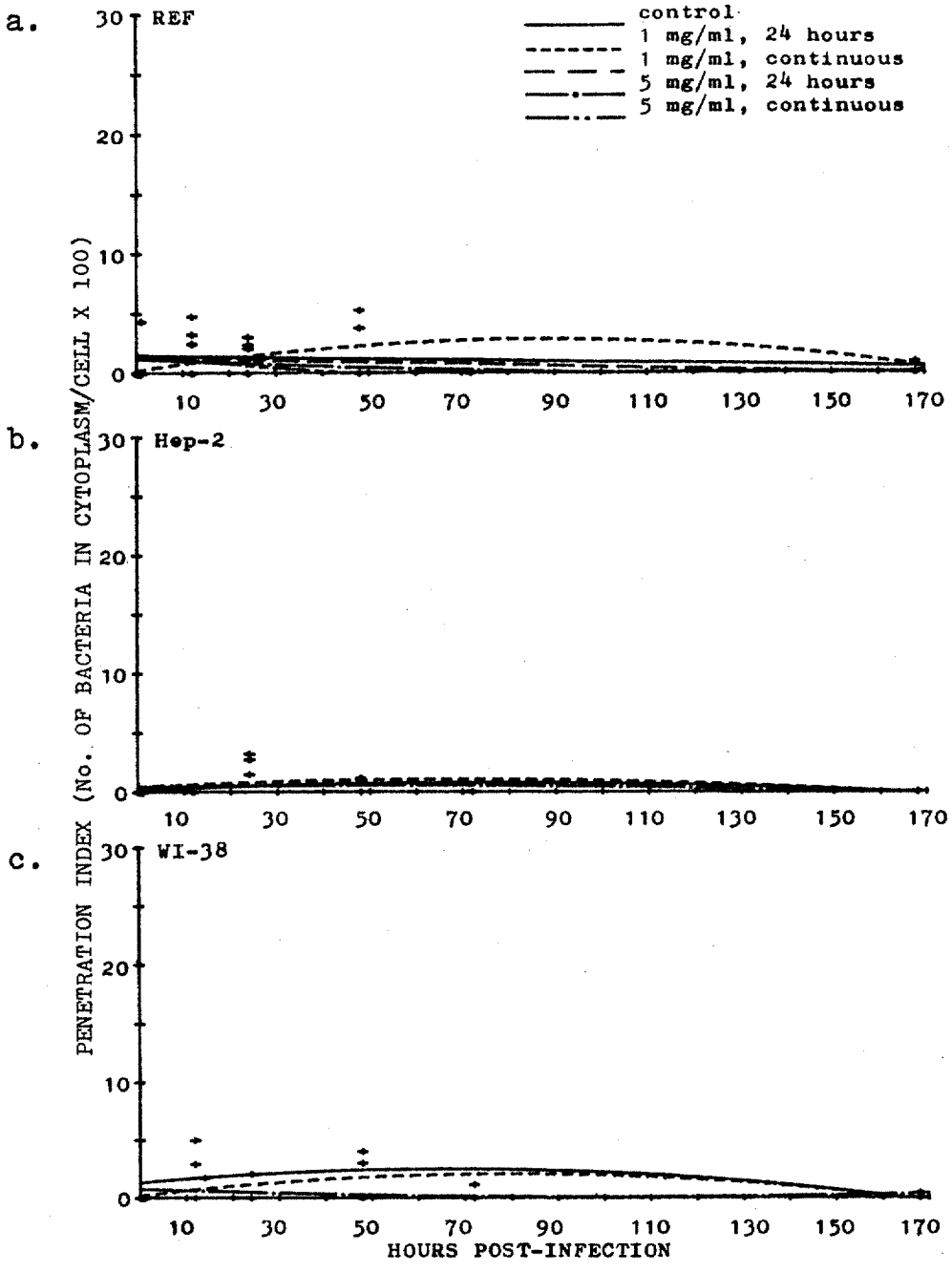


Figure 14 -- Effect of D-mannose on penetration of Chicago 8 strain of Legionella pneumophila into cytoplasm of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

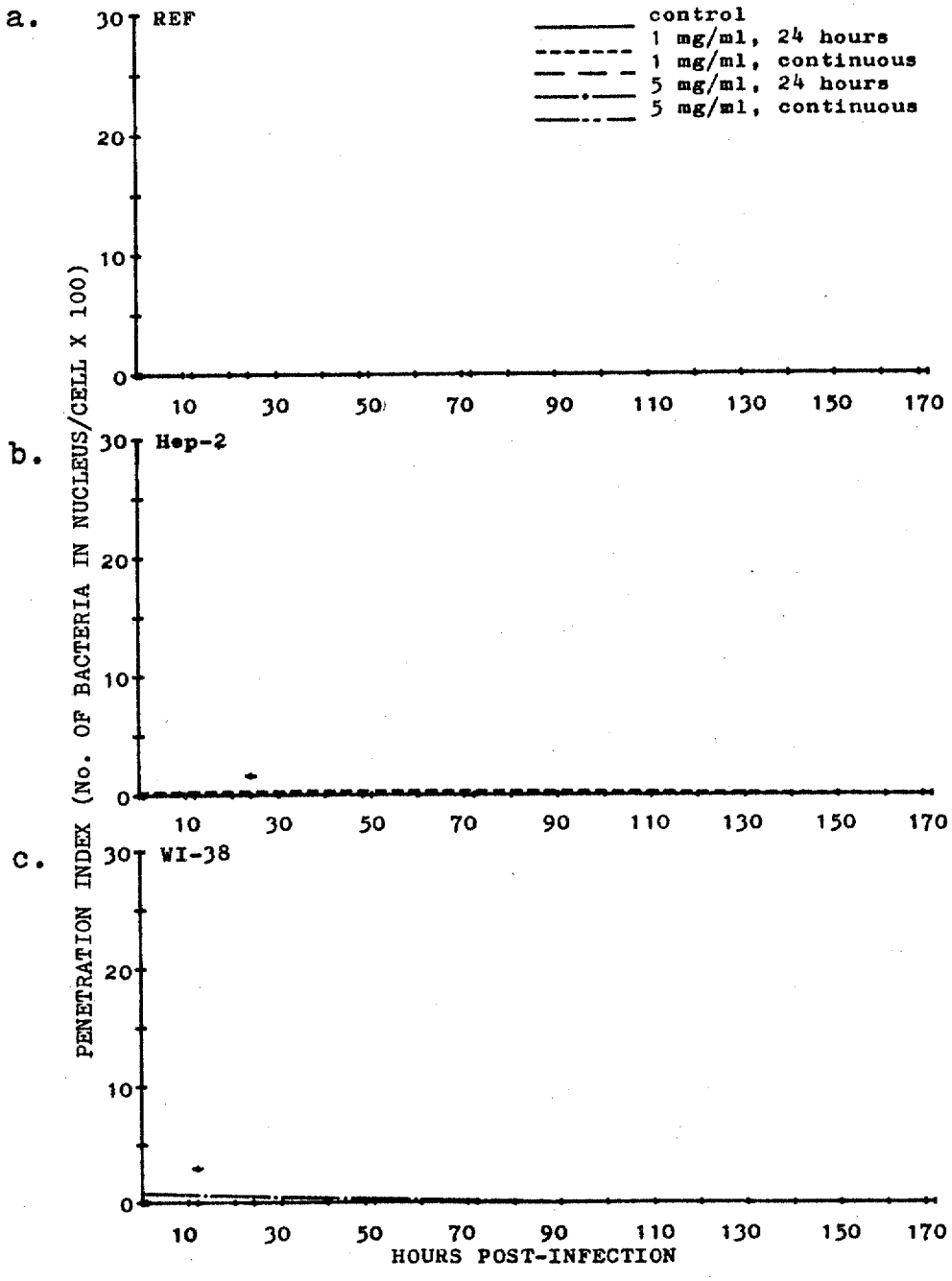


Figure 15 -- Effect of D-mannose on penetration of Chicago 8 strain of Legionella pneumophila into nuclei of tissue culture cells. a: Rat embryo fibroblast cells ; b: Hep-2 cells; c: WI-38 cells.

2. D-galactose (Figures 16-21)

The attachment of Jo 2 to WI-38 was enhanced somewhat by treatment with both levels of D-galactose for 24 hours; Chi 8 was unaffected (Figures 16, 19). The ability of both Legionella strains to penetrate the cytoplasm of Hep-2 and WI-38 was enhanced by D-galactose, especially with Jo 2 which showed cytoplasmic peak PIs of 8 and 17, respectively (versus controls of 2 and 7 at the same time); by the same token, those of Chi 8 were 2 and 7 (vs. <1 and 2) (Figures 17, 20). Cytoplasmic penetration of REF by both strains was either unaffected (Chi 8) or inhibited (Jo 2) by D-galactose (Figures 17, 20); nucleolization was very slightly enhanced, except for Chi 8 cultivated with REF (Figures 18, 21).

3. D-glucose (Figures 22-27)

D-glucose (5 mg/ml, continuously) increased some three-fold the attachment (AI $=17$) to an cytoplasmic penetration (PI $=18$) of REF by Jo 2 after three days' incubation (Figures 22, 23). Also, attachment (early) and cytoplasmic penetration by Jo 2 of WI-38 was greatly promoted by D-glucose. With Chi 8, both adherence and cytoplasmic penetration were inhibited except for the WI-38 line in which 5 mg D-glucose/ml (24-hour exposure) elevated somewhat both the AI (9 vs. 7) and PI (6 vs. 3) (Figures 25, 26). Nucleolization by both strains was only slightly affected by D-glucose (Figures 24, 27).

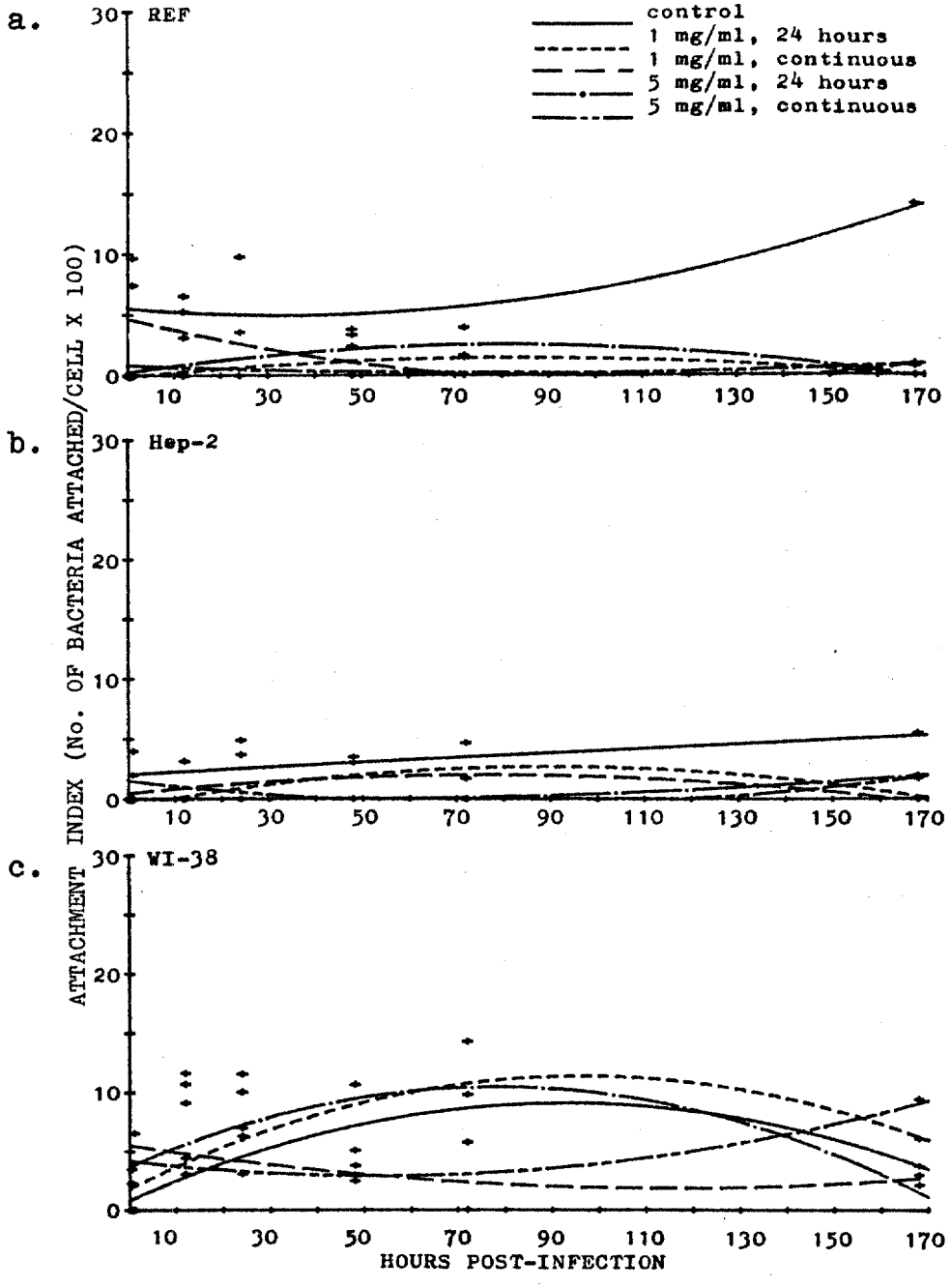


Figure 16 -- Effect of D-galactose on attachment of Johannesburg 2 strain of Legionella pneumophila to surfaces of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

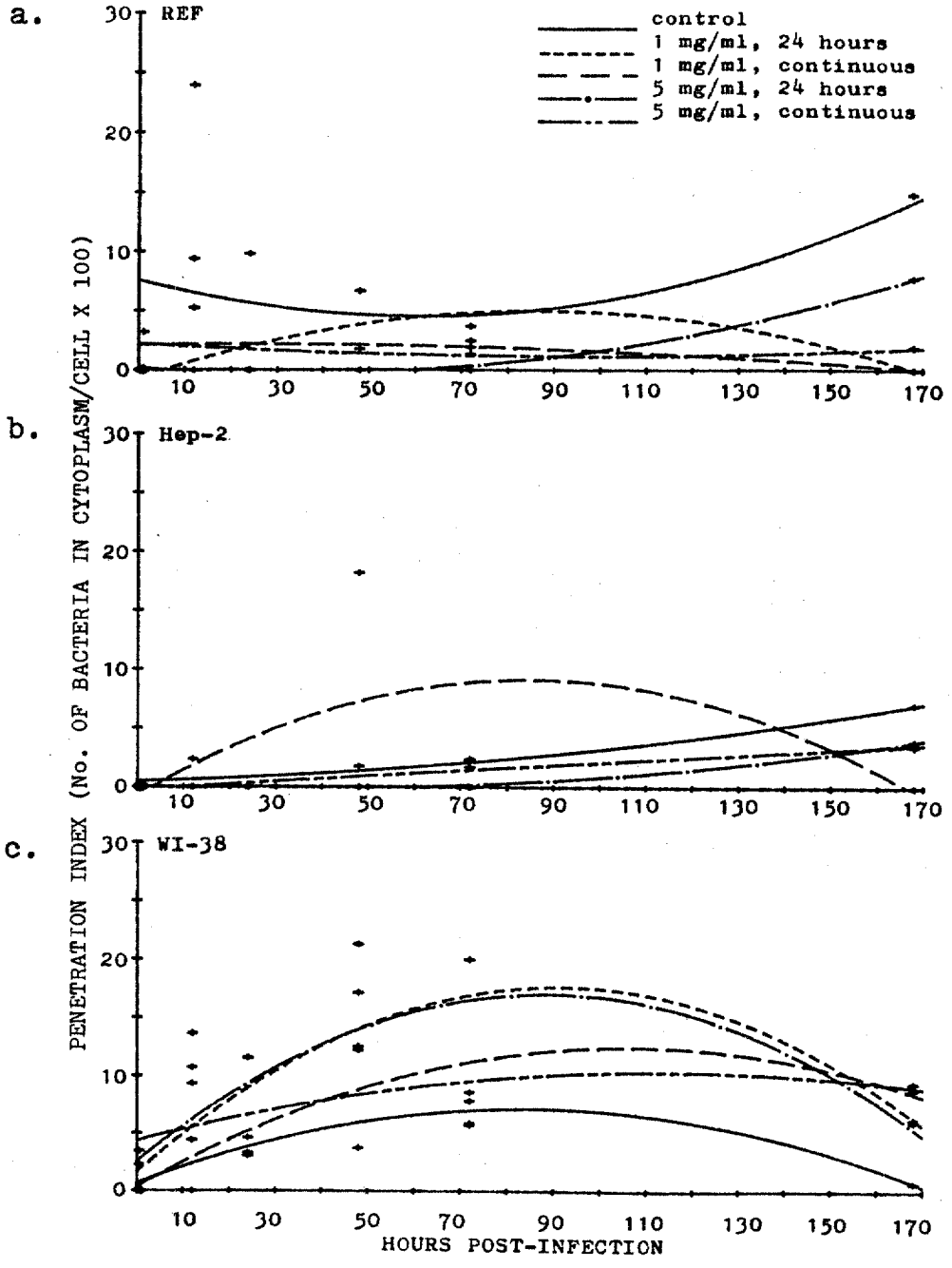


Figure 17 -- Effect of D-galactose on penetration of Johannesburg 2 strain of Legionella pneumophila into cytoplasm of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

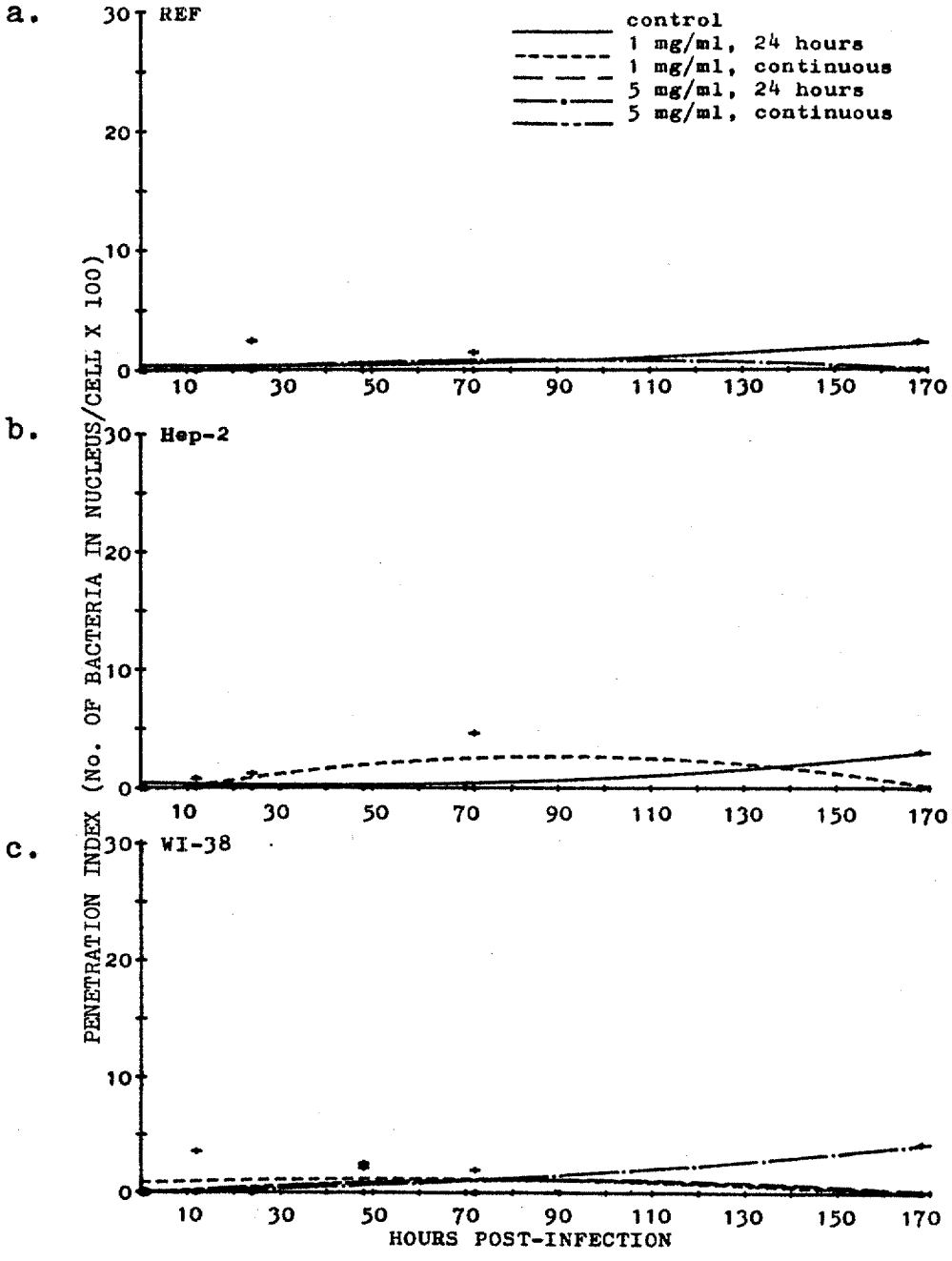


Figure 18 -- Effect of D-galactose on penetration of Johannesburg 2 strain of Legionella pneumophila into nuclei of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

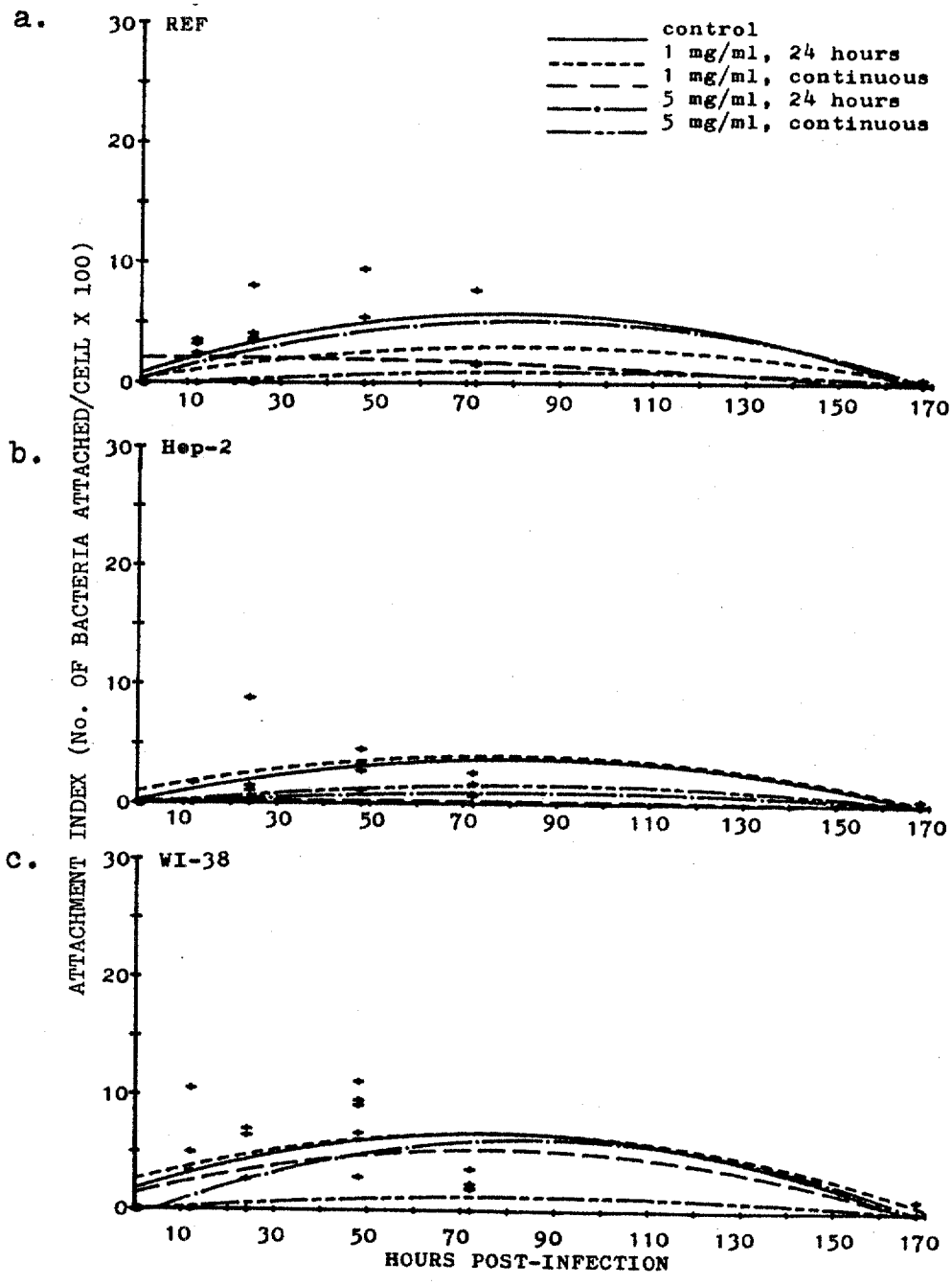


Figure 19 -- Effect of D-galactose on attachment of Chicago 8 strain of Legionella pneumophila to surfaces of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

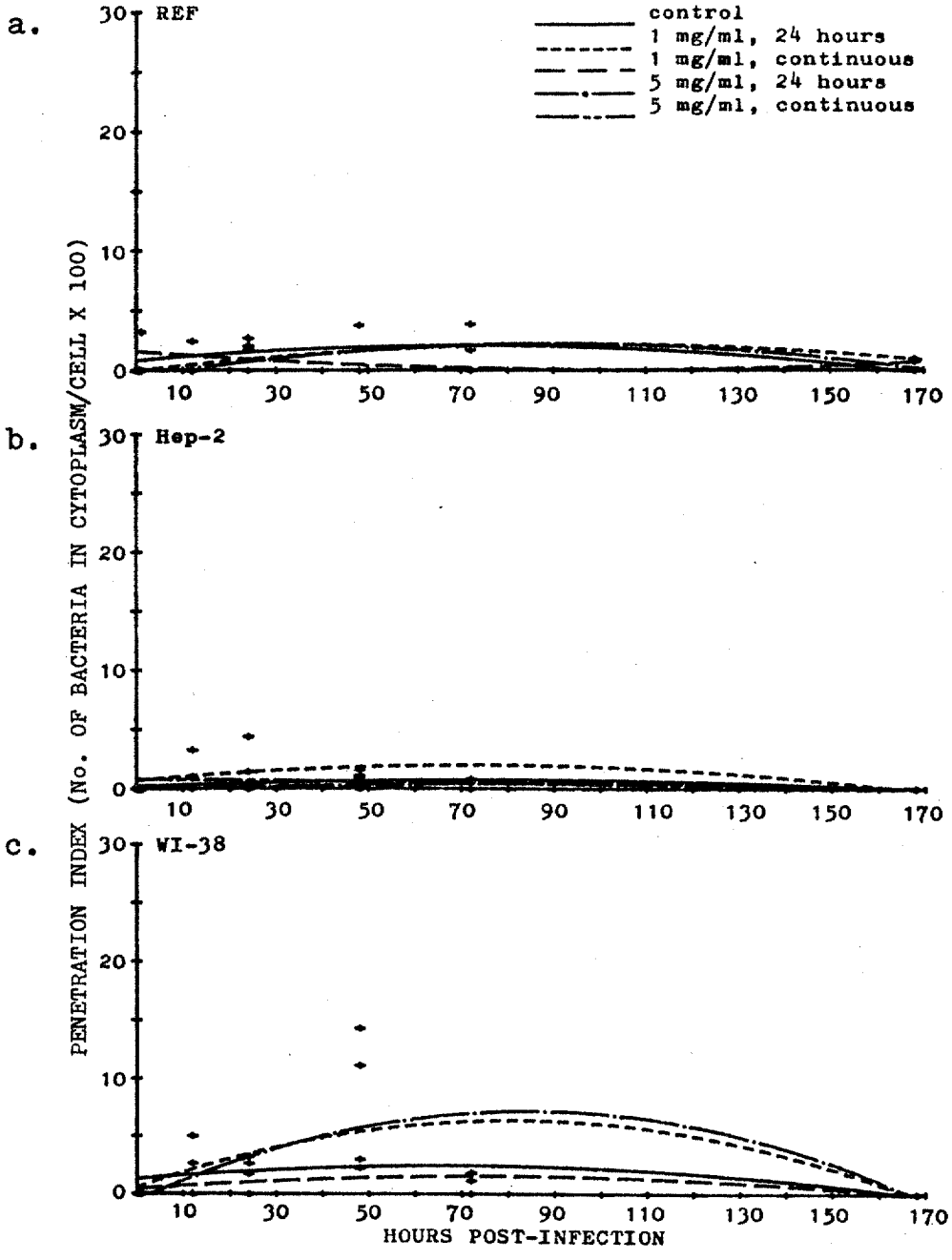


Figure 20 -- Effect of D-galactose on penetration of Chicago 8 strain of Legionella pneumophila into cytoplasm of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

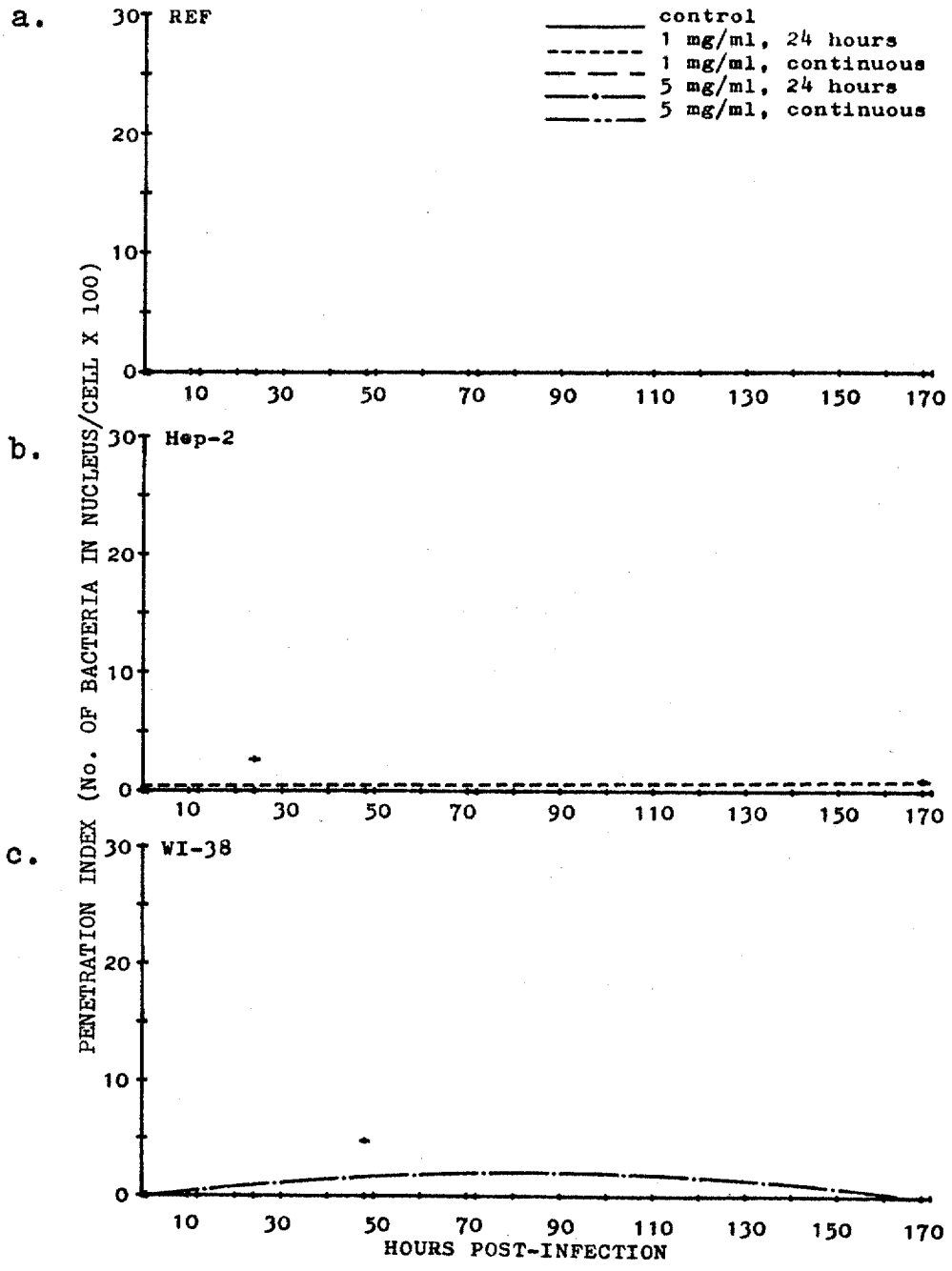


Figure 21 -- Effect of D-galactose on penetration of Chicago 8 strain of Legionella pneumophila into nuclei of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

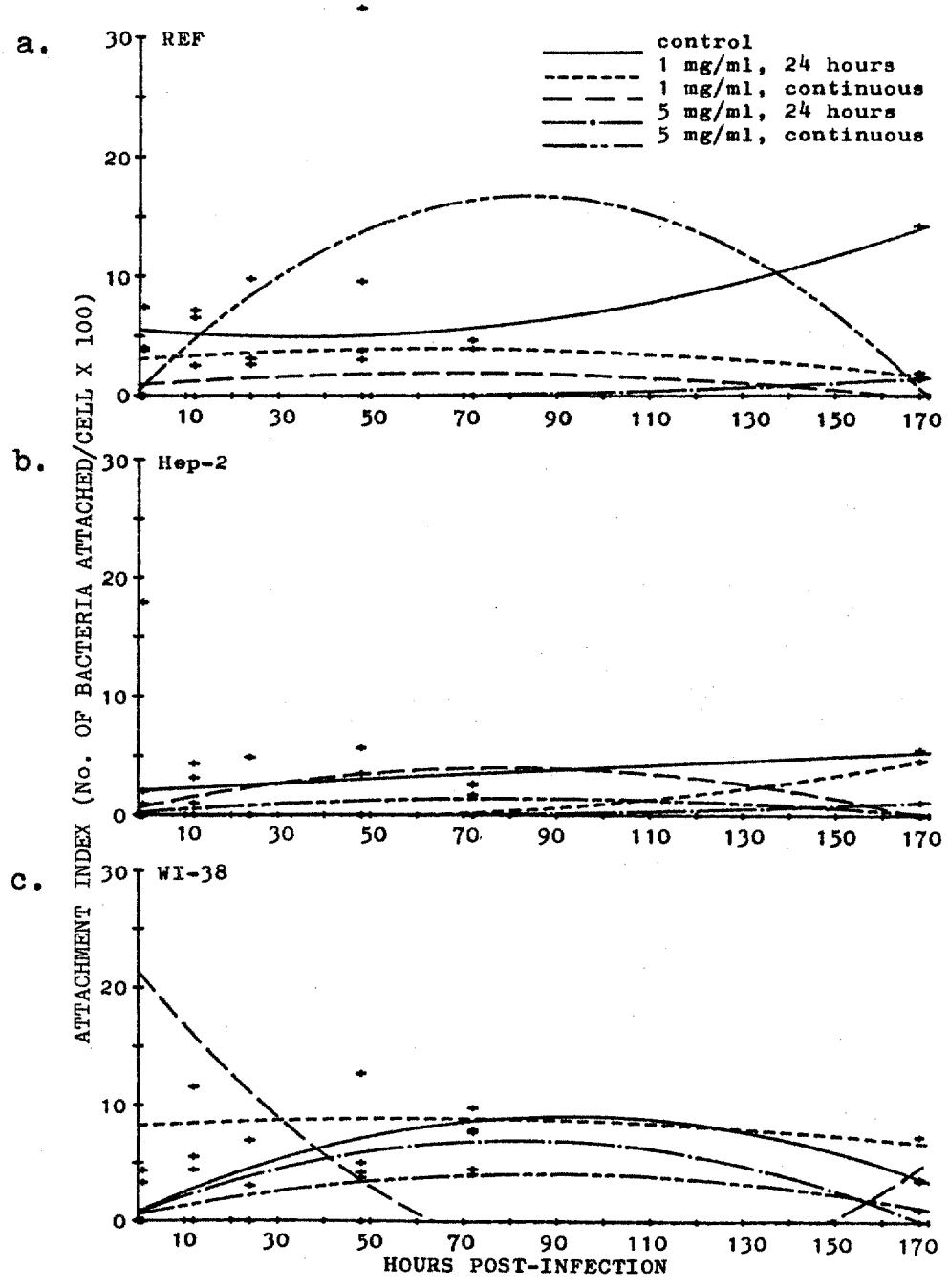


Figure 22 -- Effect of D-glucose on attachment of Johannesburg 2 strain of Legionella pneumophila to surfaces of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

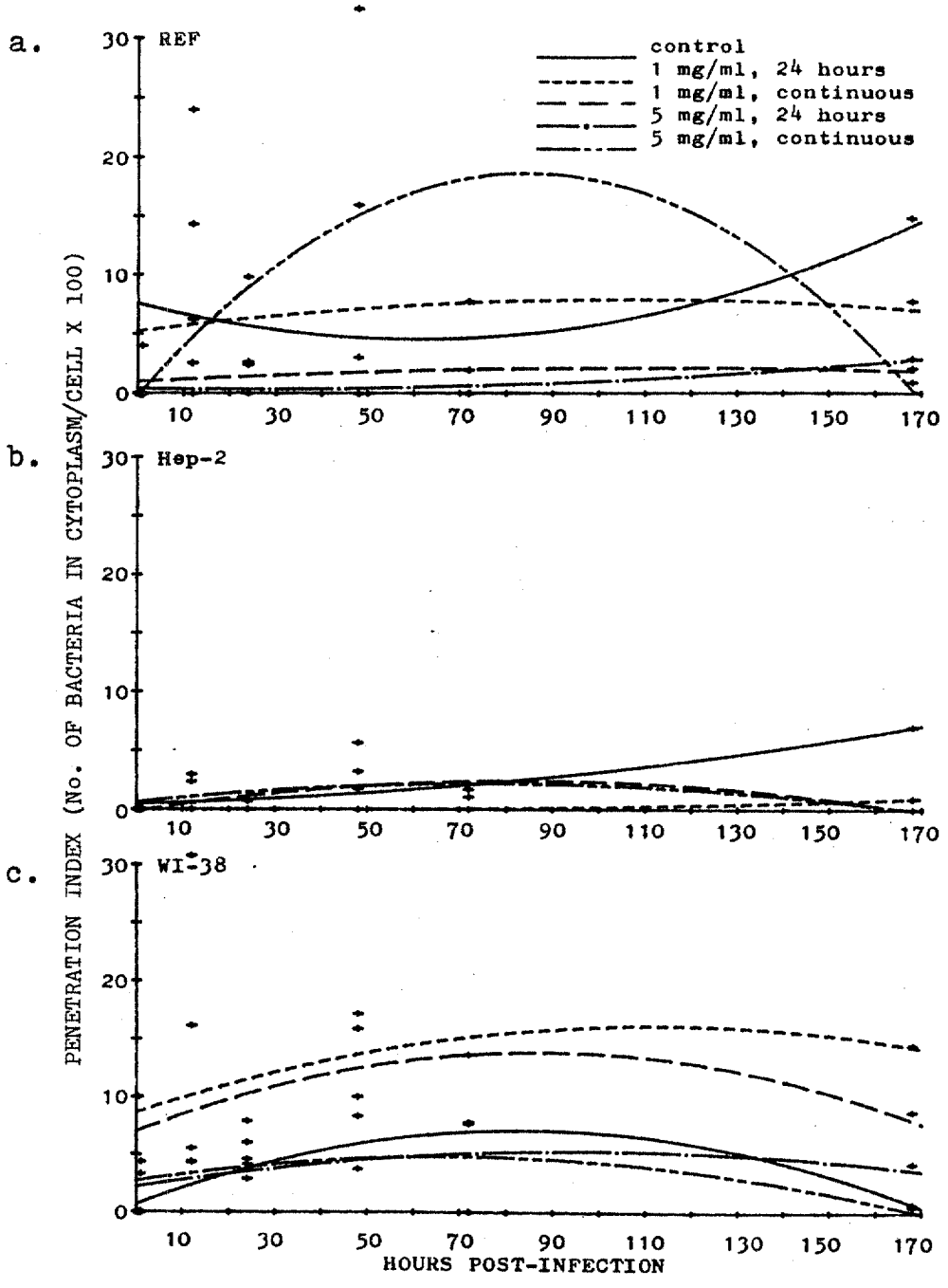


Figure 23 -- Effect of D-glucose on penetration of Johannesburg 2 strain of Legionella pneumophila into cytoplasm of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

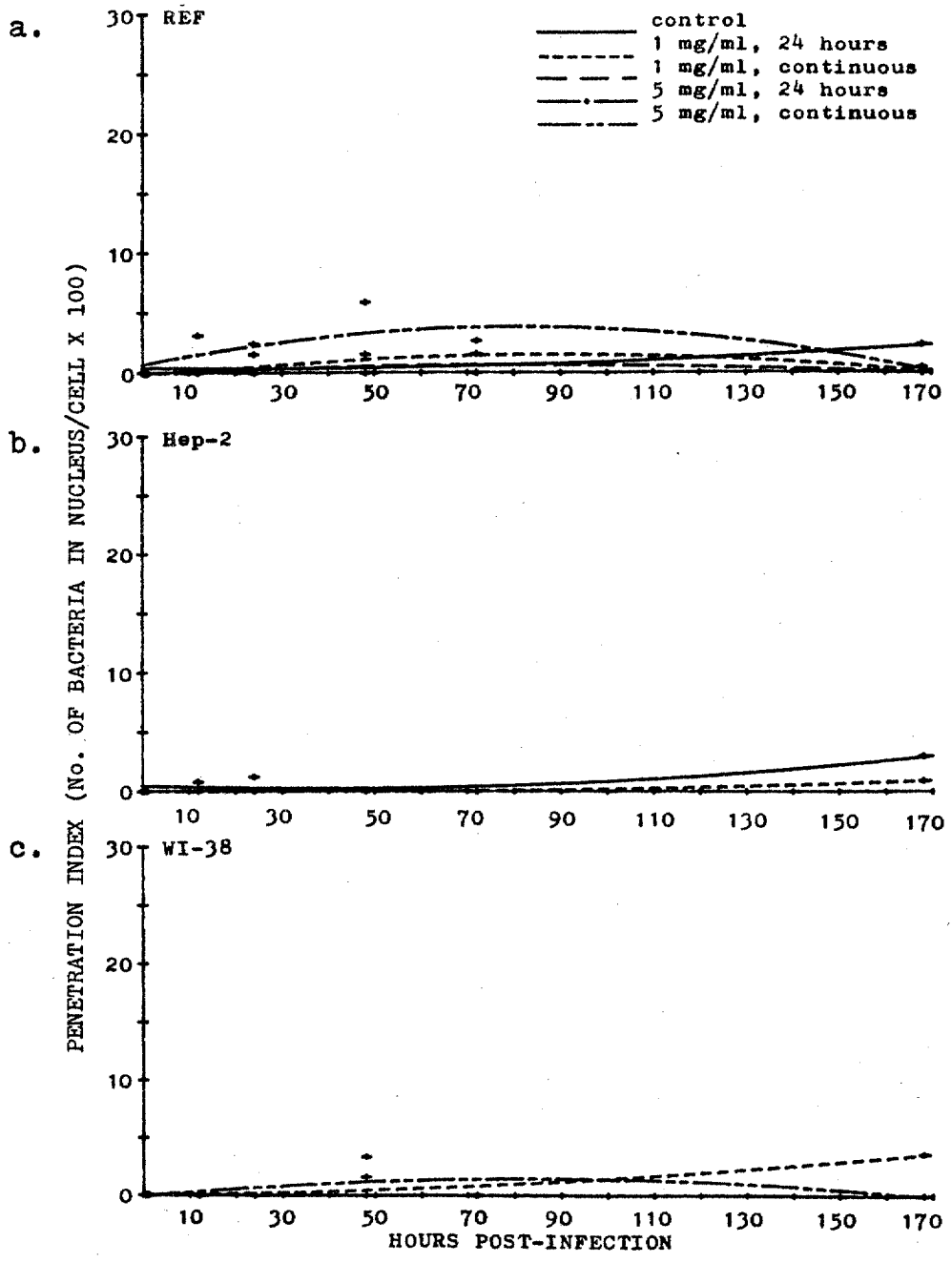


Figure 24 -- Effect of D-glucose on penetration of Johannesburg 2 strain of Legionella pneumophila into nuclei of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

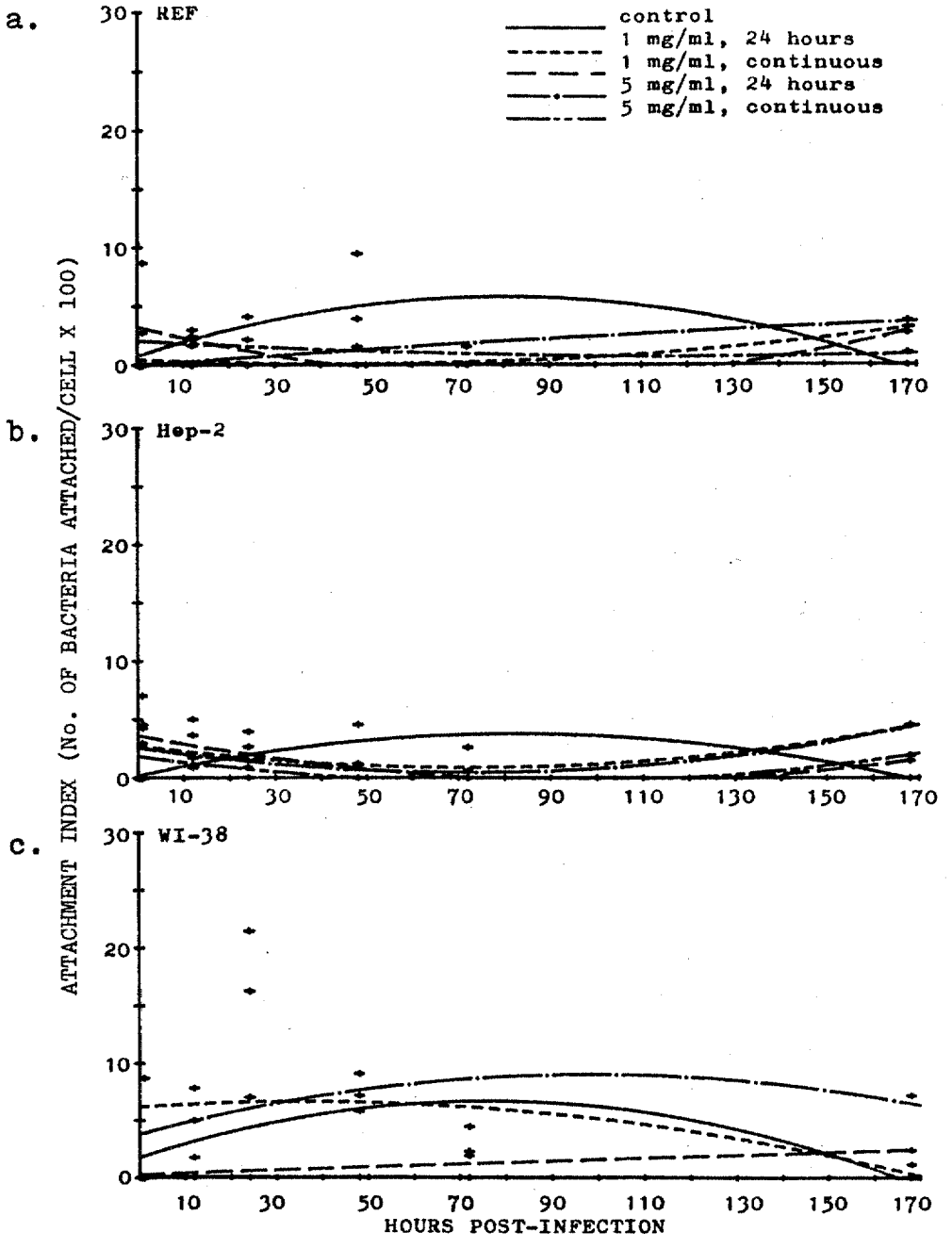


Figure 25 -- Effect of D-glucose on attachment of Chicago 8 strain of Legionella pneumophila to surfaces of tissue culture cells. a: Rat embryo fibroblast cells ; b: Hep-2 cells; c: WI-38 cells.

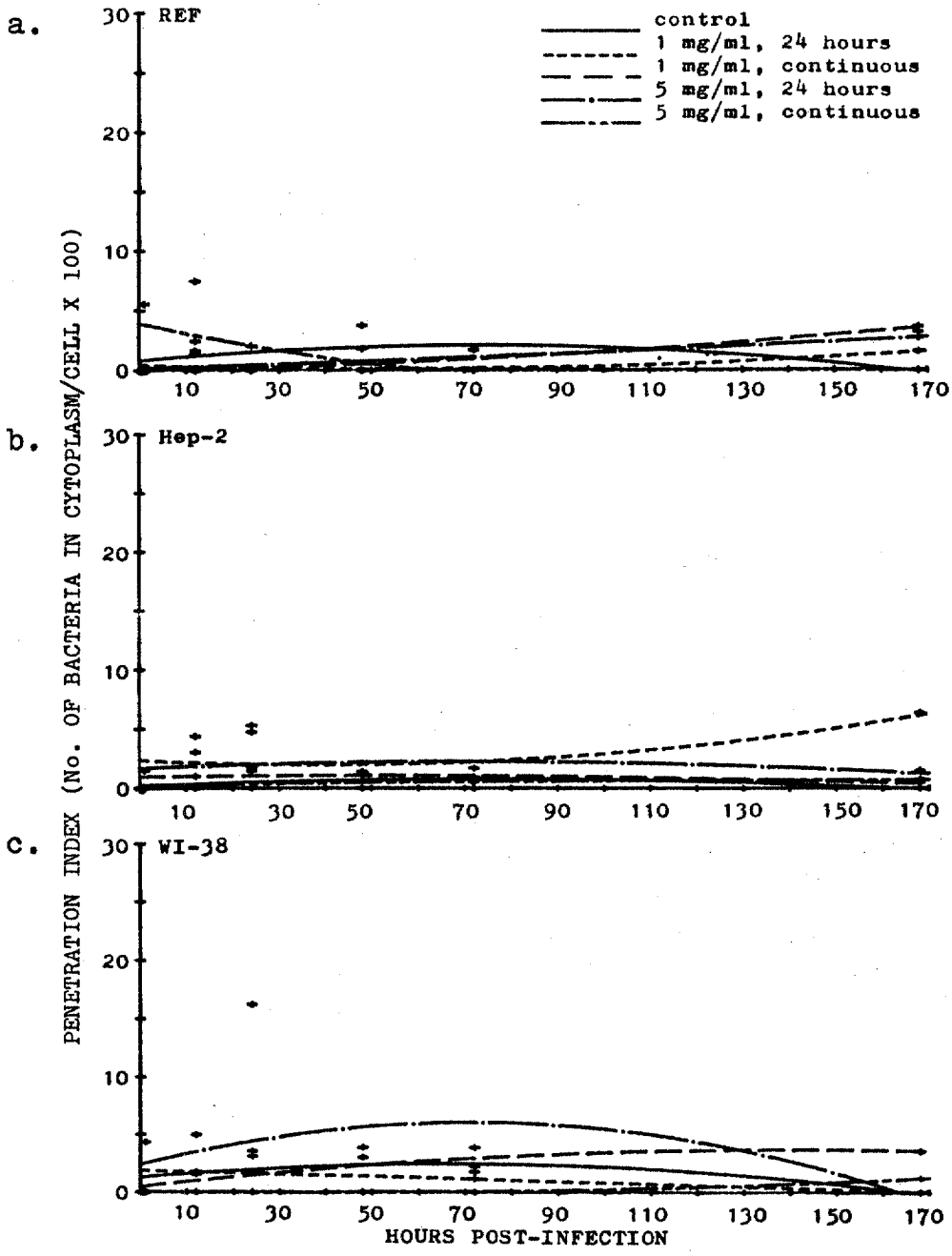


Figure 26 -- Effect of D-glucose on penetration of Chicago 8 strain of Legionella pneumophila into cytoplasm of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

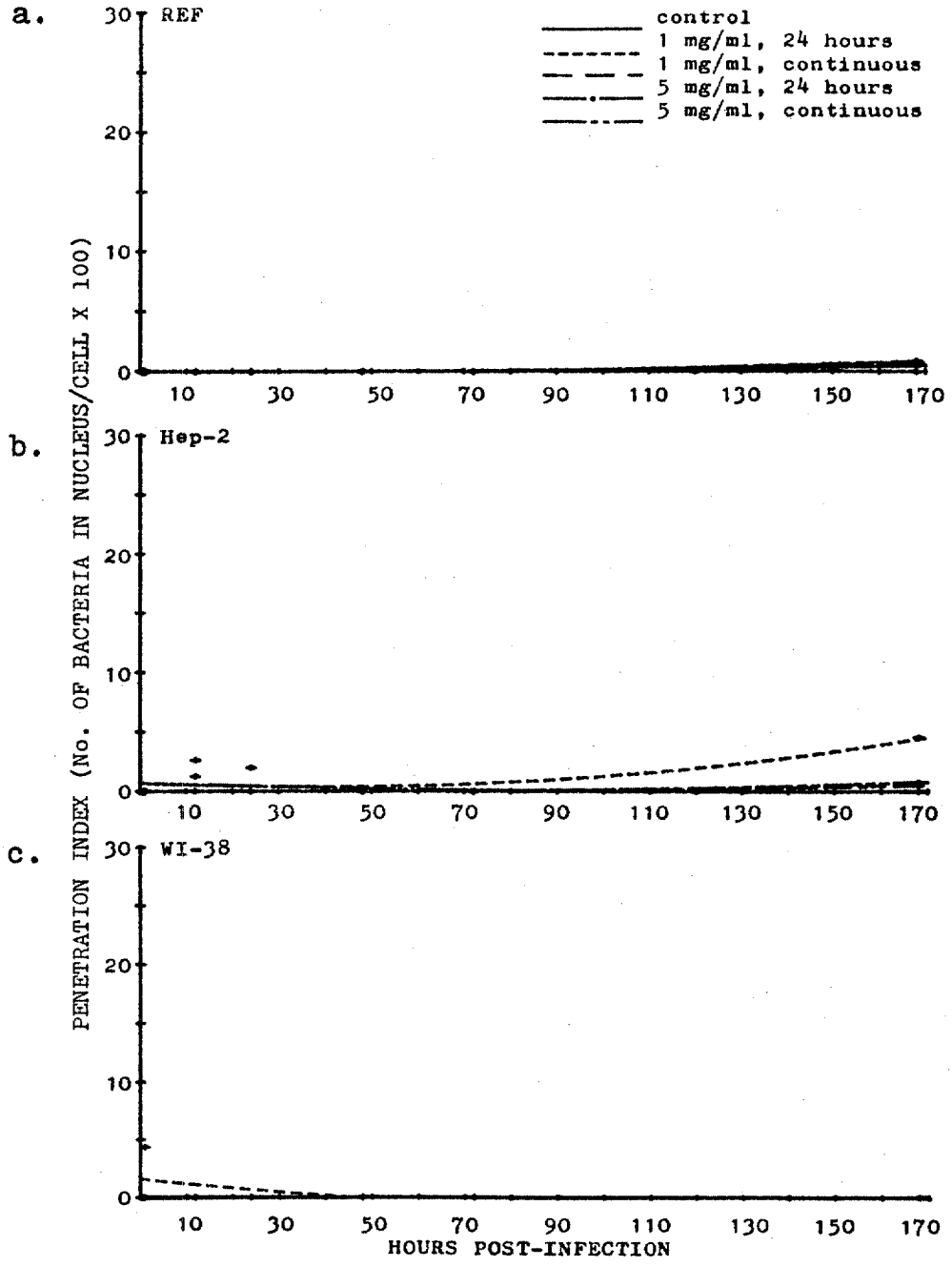


Figure 27 -- Effect of D-glucose on penetration of Chicago 8 strain of Legionella pneumophila into nuclei of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

4. L-glucose (Figures 28-33)

The effects of L-glucose on attachment and penetration of the cells by the legionellae were striking. Thus, after 3 days' incubation, L-glucose (1 mg/ml, 24 hours) had raised by about three-fold the AI (17.5) and the cytoplasmic PI (20) of Jo 2 with REF -- 5 mg/ml was less effective (Figures 28, 29). Attachment and cytoplasmic penetration by Jo 2 of the other two cell lines were likewise promoted by L-glucose. Thus, after 7 and 5 days, Jo 2 showed AIs of 15 with Hep-2 (control = 16) and 25 with WI-38 (control = 9), respectively (1 mg L-glucose/ml, 24 hours). Again, the higher concentration of L-glucose had less effect. Exposure to 1 mg L-glucose/ml for 24 hours also raised the cytoplasmic PIs of Jo 2 in Hep-2 and WI-38 (two- to three-fold) while increasing the AIs some three-fold.

The AIs and cytoplasmic PIs of Chi 8 were increased greatly by L-glucose in REF and WI-38; with Hep-2, L-glucose reduced the AI while increasing the PI (Figures 31, 32). The maximal AIs of Chi 8 with REF and WI-38 were 17 and 16 (controls = 5 and 7), while the maximal cytoplasmic PIs with REF, Hep-2 and WI-38 were 25, 16 and 15, respectively (controls = 2, 1, 2). Intranuclear PIs of both *Legionella* strains with three cell lines were increased by L-glucose approximately two- (Jo 2, WI-38) to four-fold (Chi 8, REF) (Figures 30, 33).

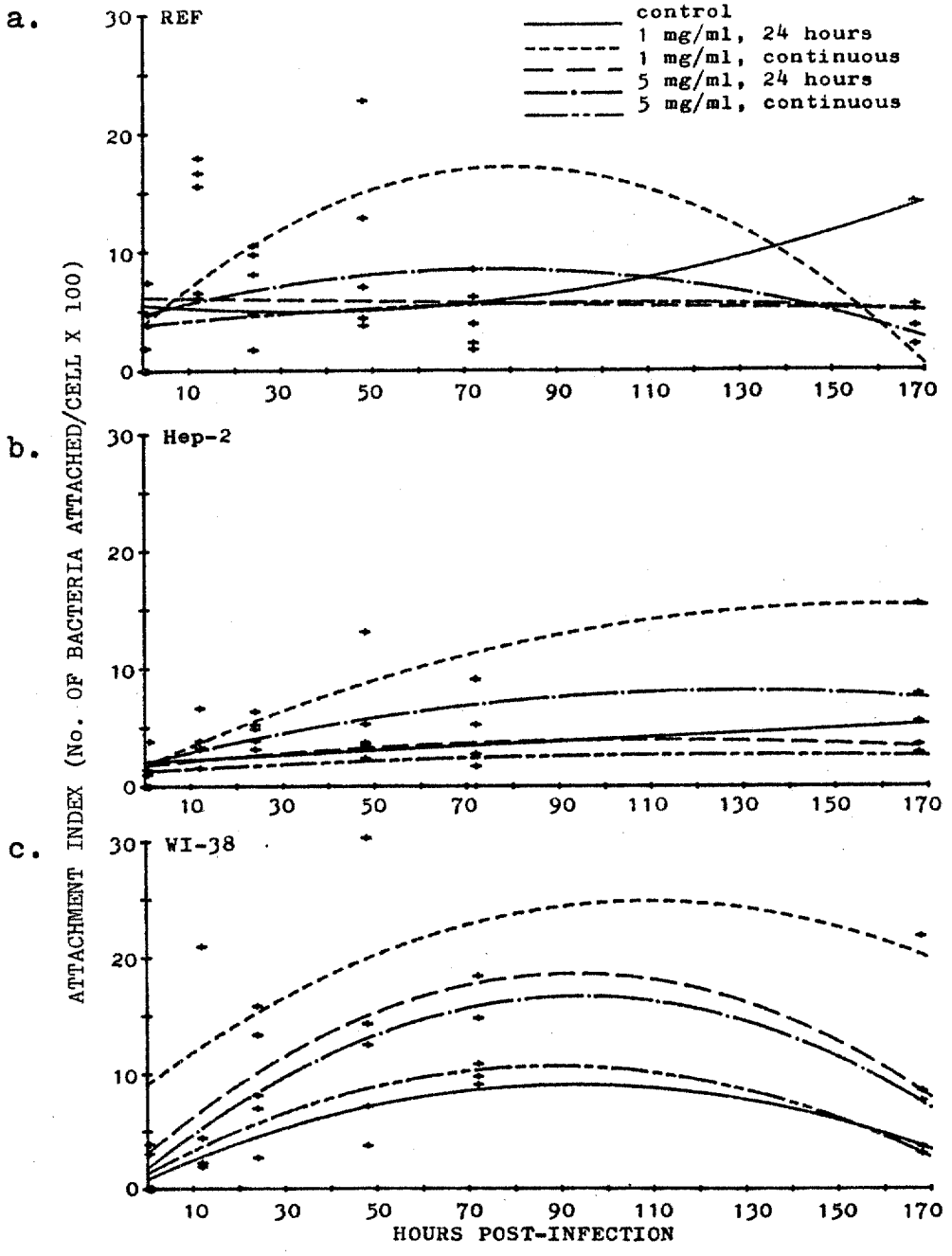


Figure 28 -- Effect of L-glucose on attachment of Johannesburg 2 strain of Legionella pneumophila to surfaces of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

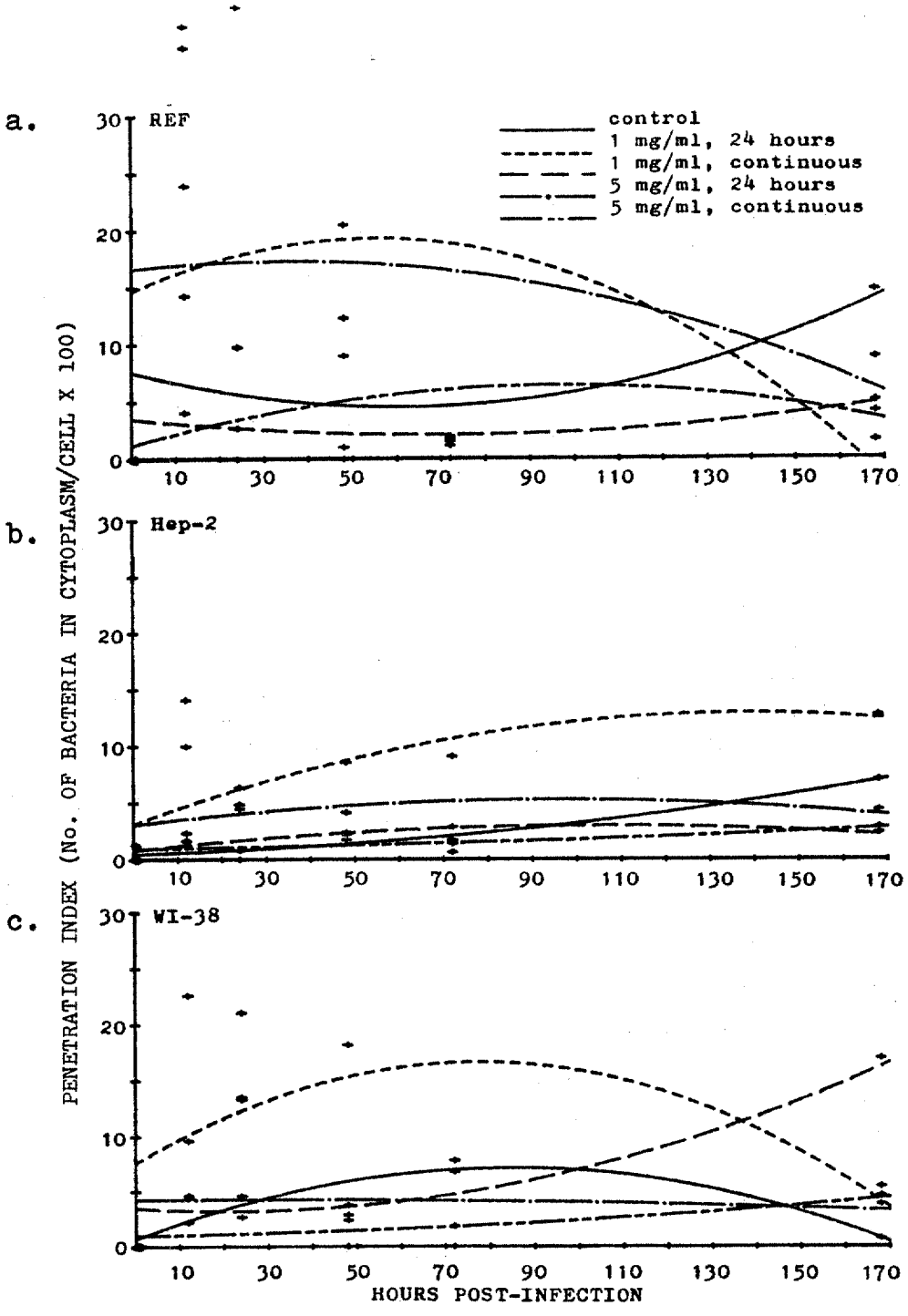


Figure 29 -- Effect of L-glucose on penetration of Johannesburg 2 strain of Legionella pneumophila into cytoplasm of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

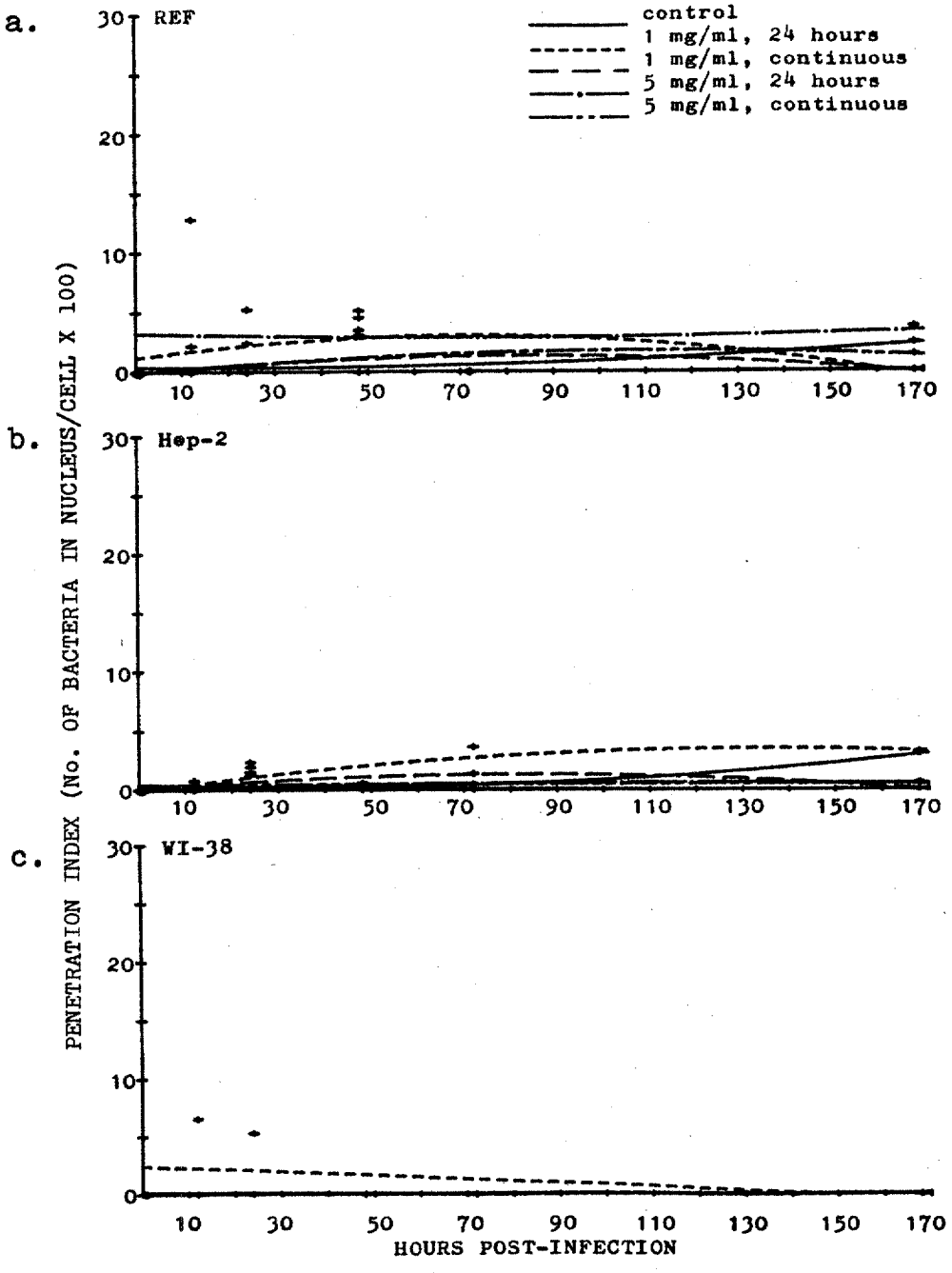


Figure 30 -- Effect of L-glucose on penetration of Johannesburg 2 strain of Legionella pneumophila into nuclei of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

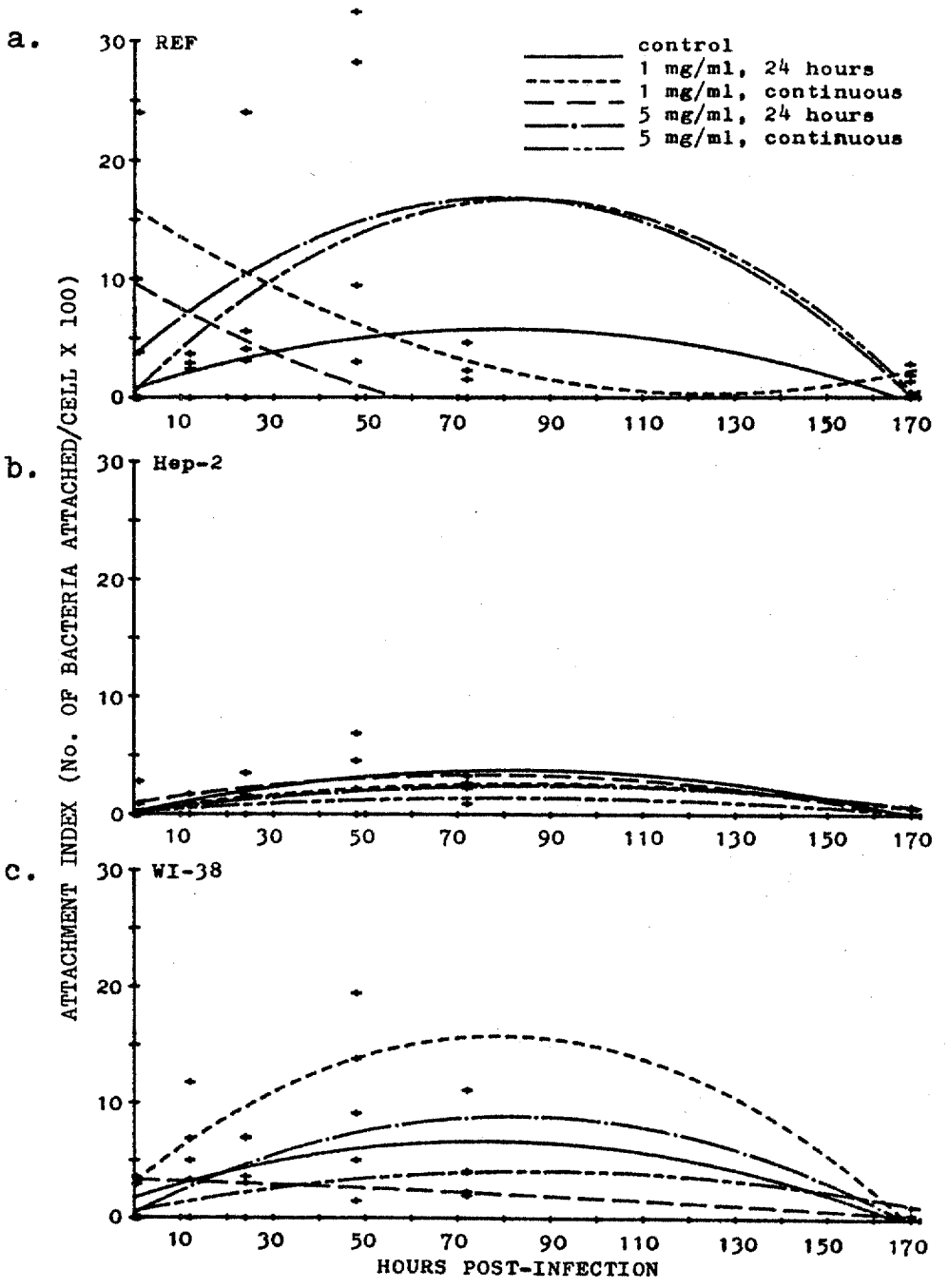


Figure 31 -- Effect of L-glucose on attachment of Chicago 8 strain of Legionella pneumophila to surfaces of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

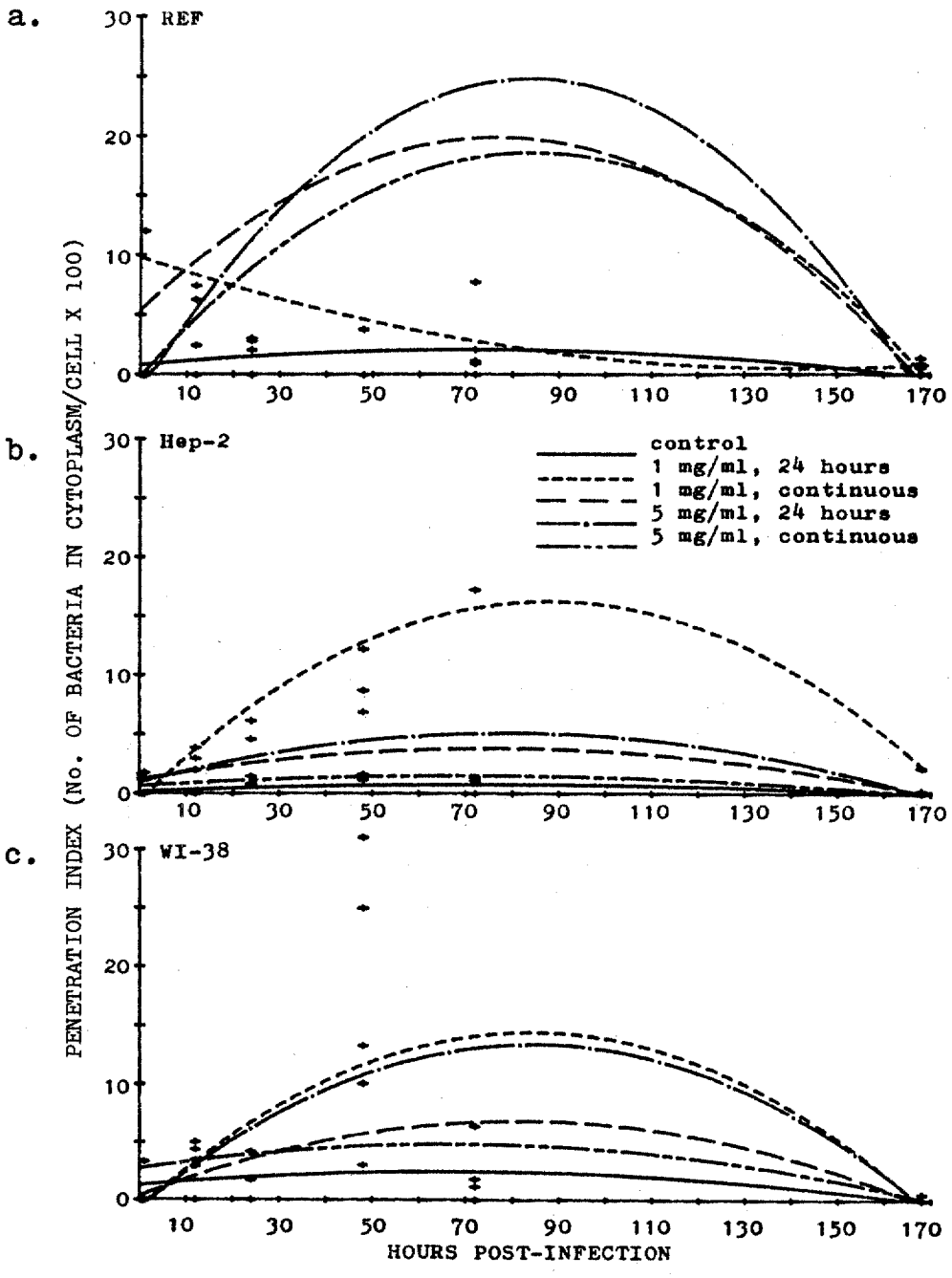


Figure 32 -- Effect of L-glucose on penetration of Chicago 8 strain of Legionella pneumophila into cytoplasm of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

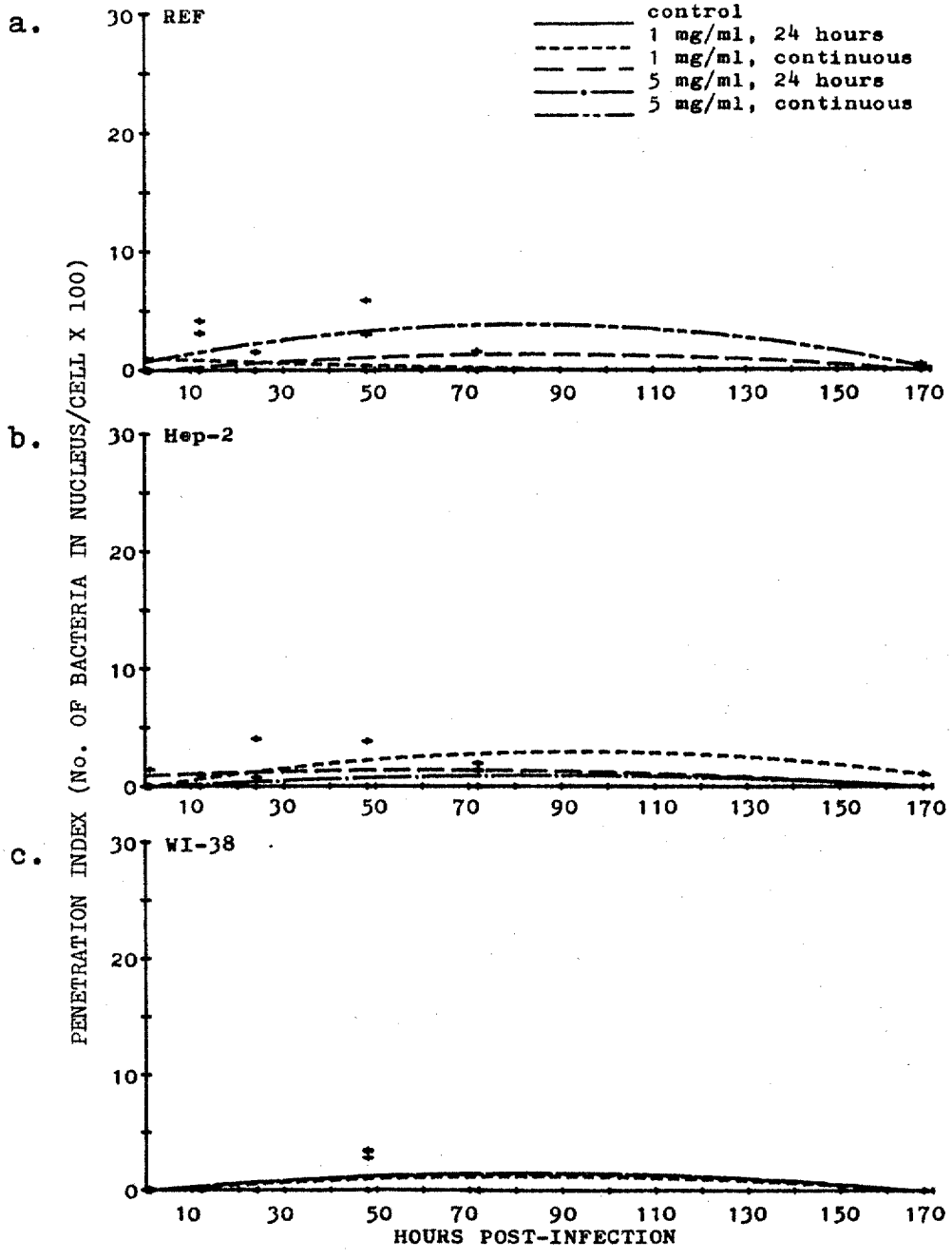


Figure 33 -- Effect of L-glucose on penetration of Chicago 8 strain of Legionella pneumophila into nuclei of tissue cells. a: Rat embryo fibroblast cells ; b: Hep-2 cells; c: WI-38 cells.

5. 2-deoxy-D-glucose (Figures 34-39)

This glucose derivative (2DG) rendered positive effects on attachment, cytoplasmic and intranuclear penetration of both Legionella strains with all three cell lines. Attachment to WI-38 was affected the most, with the AI of Jo 2 reaching 25 (3 days) and that of Chi 8 reaching 29 (4 days) upon treatment with 5 mg/ml (24 hours). Controls at those times were 7 and 9, respectively (Figures 34, 37). Cytoplasmic penetration was also greatly enhanced by 2DG, especially with Chi 8 which gave peak PIs of 28 (REF), 24 (Hep-2) and 26 (WI-38) on the third day of treatment with 1 mg 2DG (continuous) (REF) or with 5 mg/ml (24 hours) (Hep-2 and WI-38). Control cytoplasmic PIs on the third day were 2, 1, and 2, respectively. Effects of 2DG on cytoplasmic penetration were far less pronounced with Jo 2 than with Chi 8 (Figure 35). Enhancement of intranuclear penetration by 2DG was less with Jo 2 than with Chi 8 (compare Figure 36 with 39). Intranuclear PIs with the latter ranged from 4 (REF) to 6 (Hep-2 and WI-38), at the fourth day following treatments of 5 mg 2DG/ml (24 hours and continuous) while controls showed no nuclear penetration at any stage of incubation.

6. Pyruvate (Figures 40-45)

Pyruvic acid promoted adherence, cytoplasmic and intranuclear penetration of both Legionella strains with each

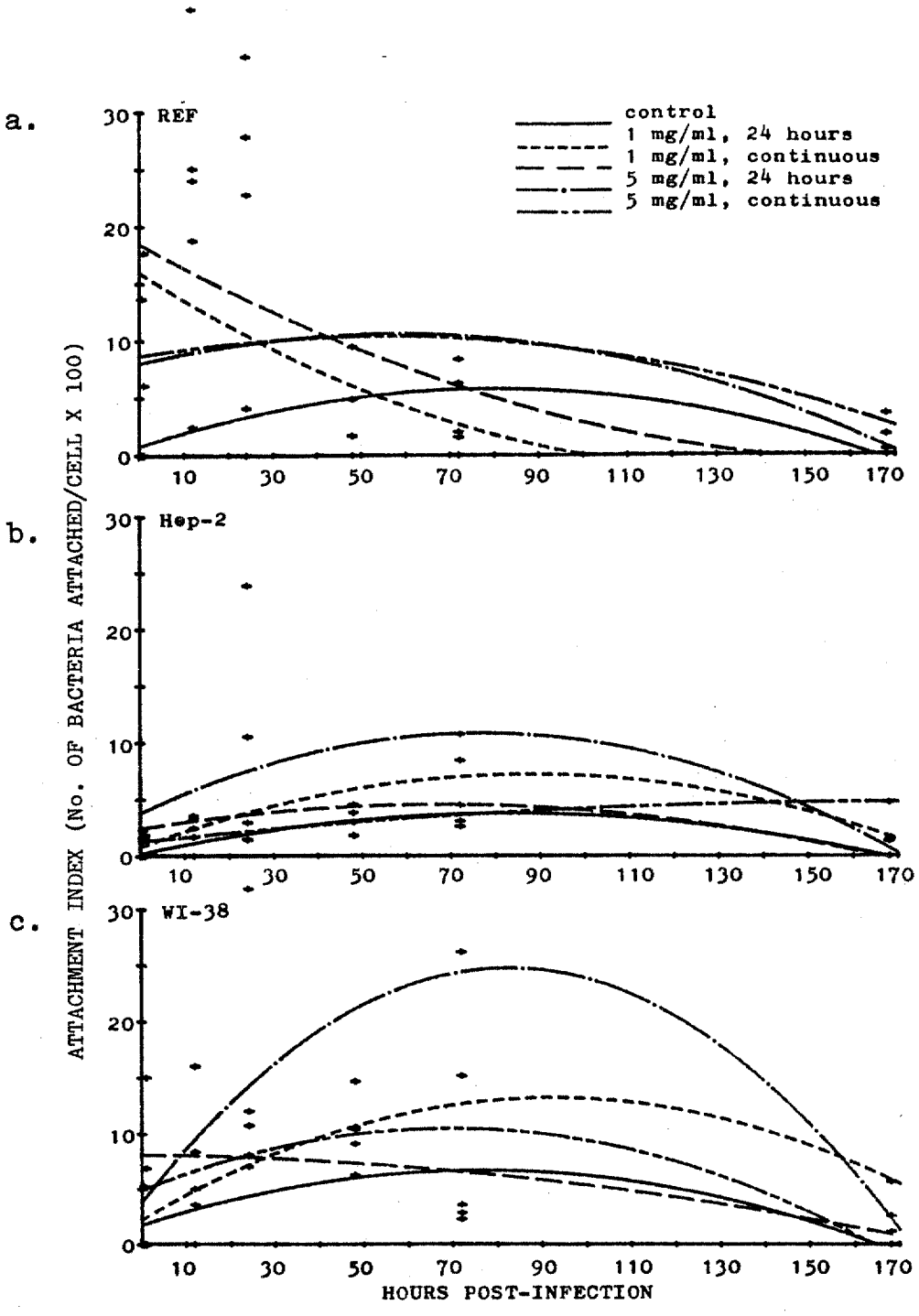


Figure 34 -- Effect of 2-deoxy-D-glucose on attachment of Johannesburg 2 strain of Legionella pneumophila to surfaces of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

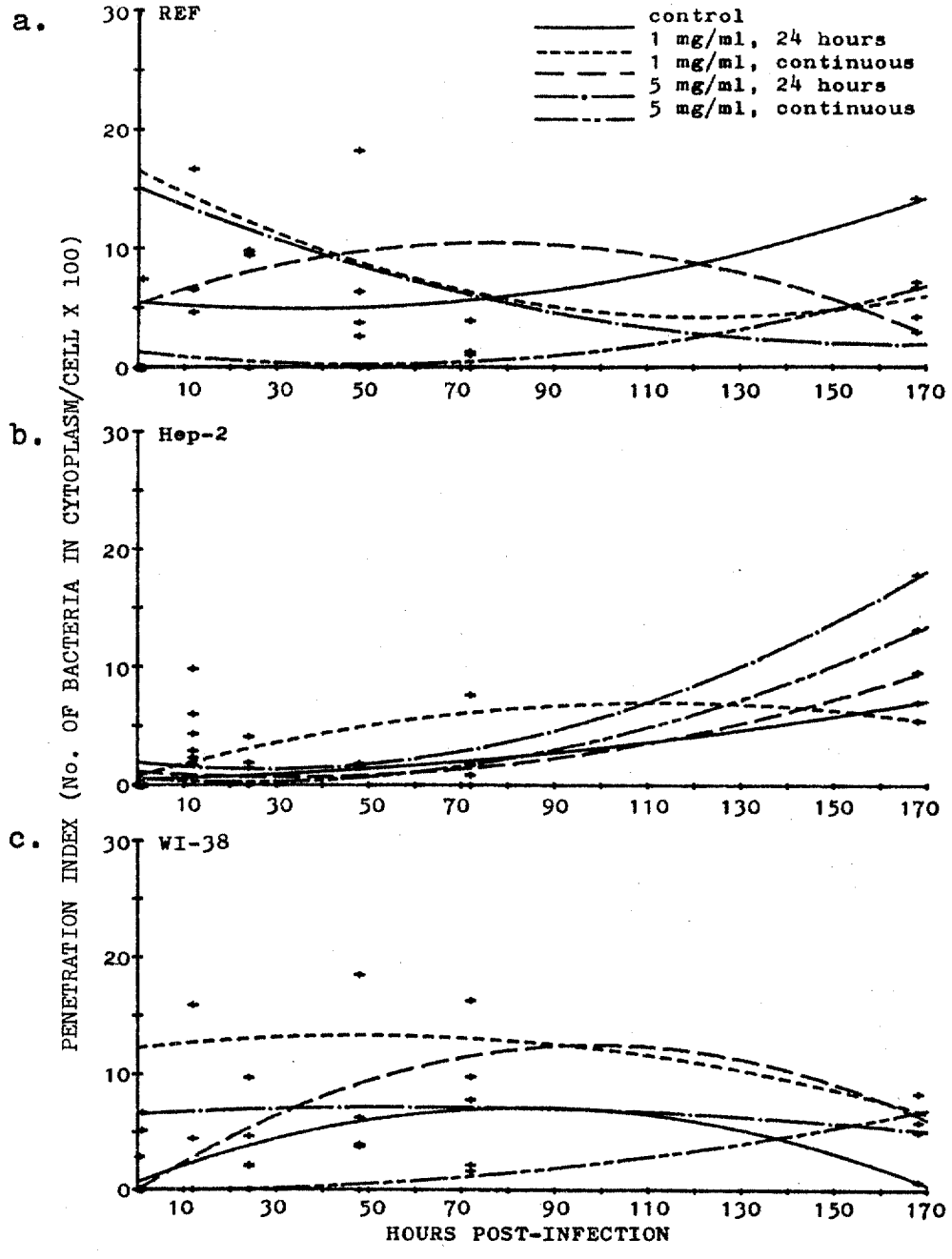


Figure 35 -- Effect of 2-deoxy-D-glucose on penetration of Johannesburg 2 strain of Legionella pneumophila into cytoplasm of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

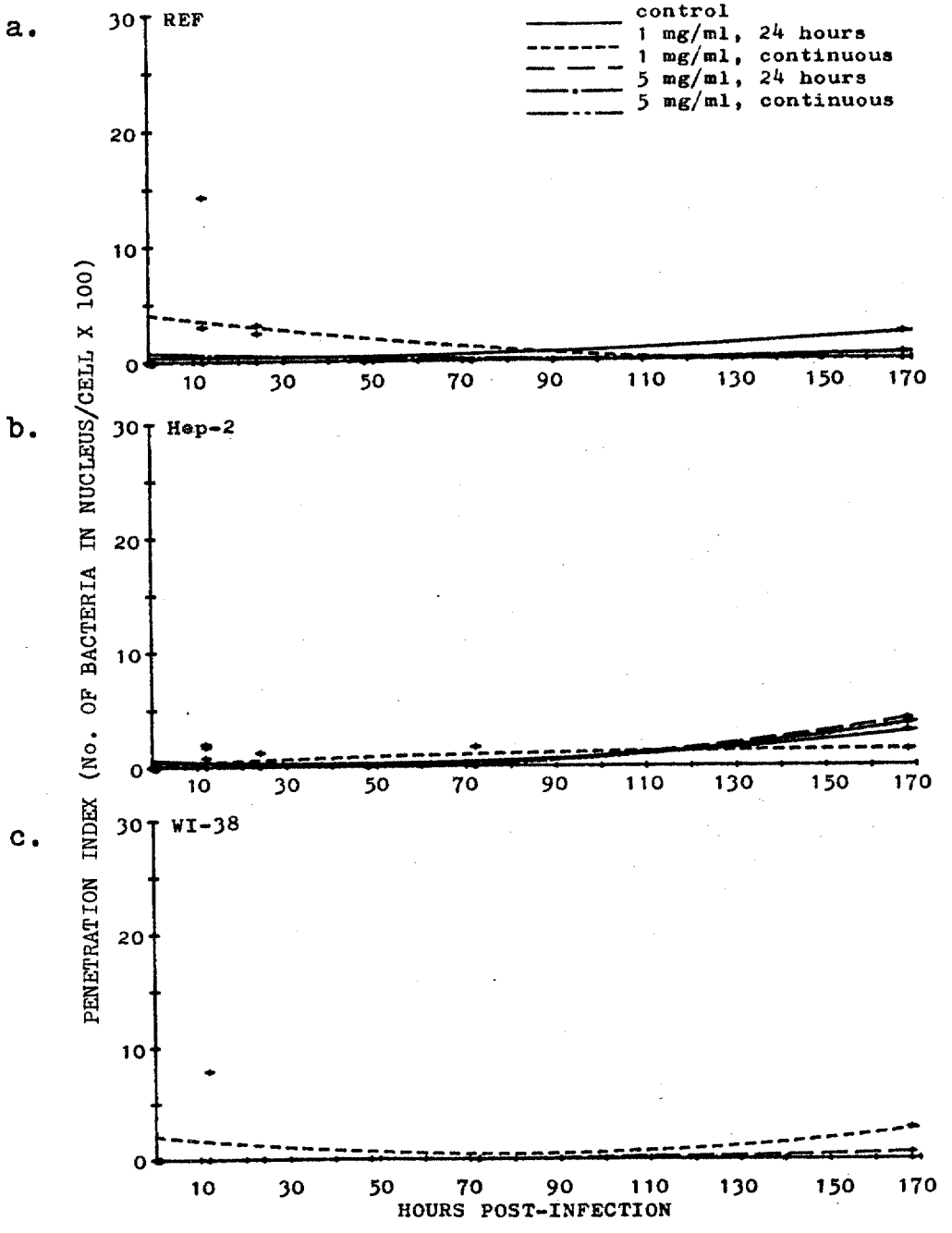


Figure 36 -- Effect of 2-deoxy-D-glucose on penetration of Johannesburg 2 strain of Legionella pneumophila into nuclei of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

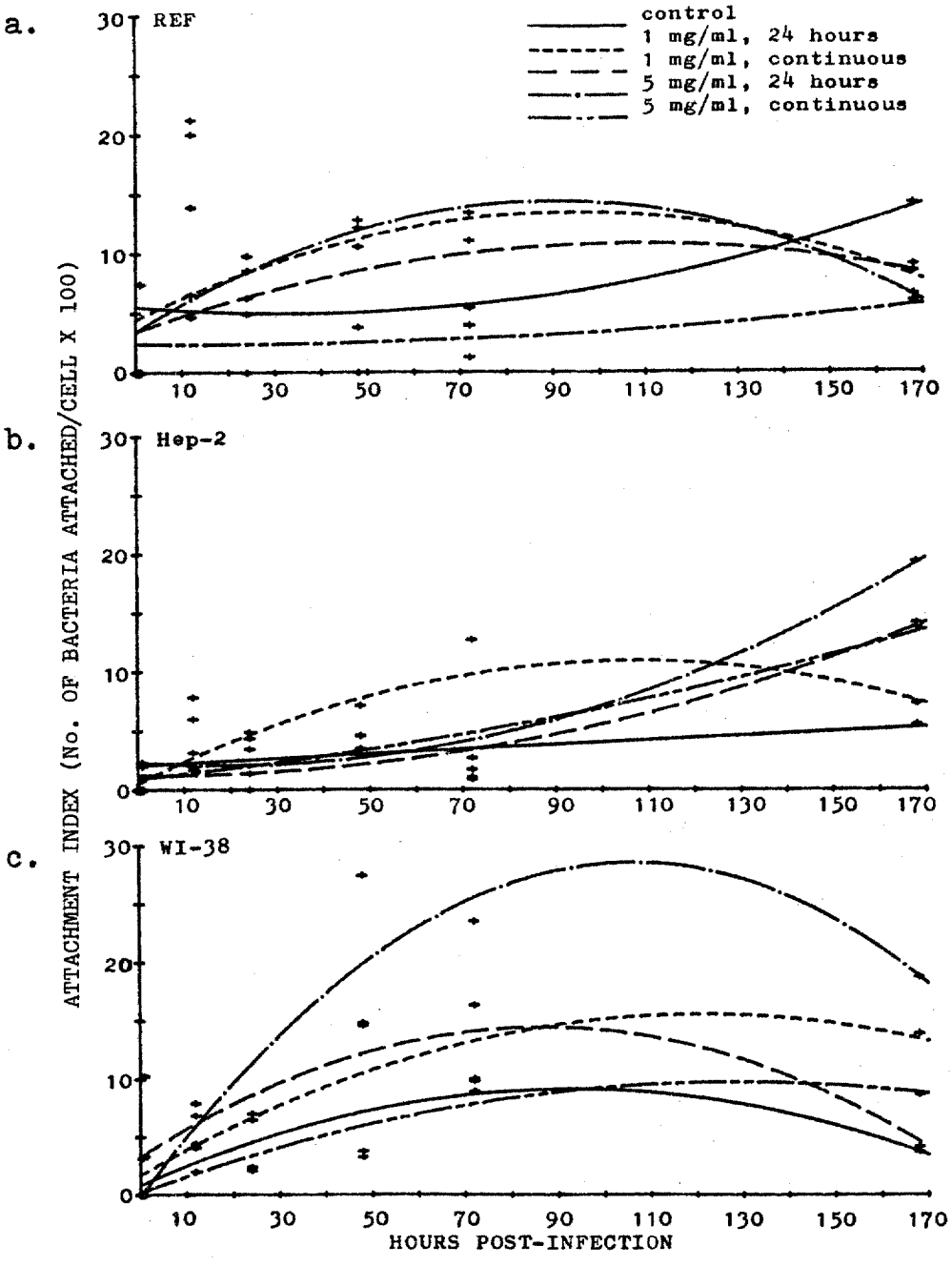


Figure 37 -- Effect of 2-deoxy-D-glucose on attachment of Chicago 8 strain of Legionella pneumophila to surfaces of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

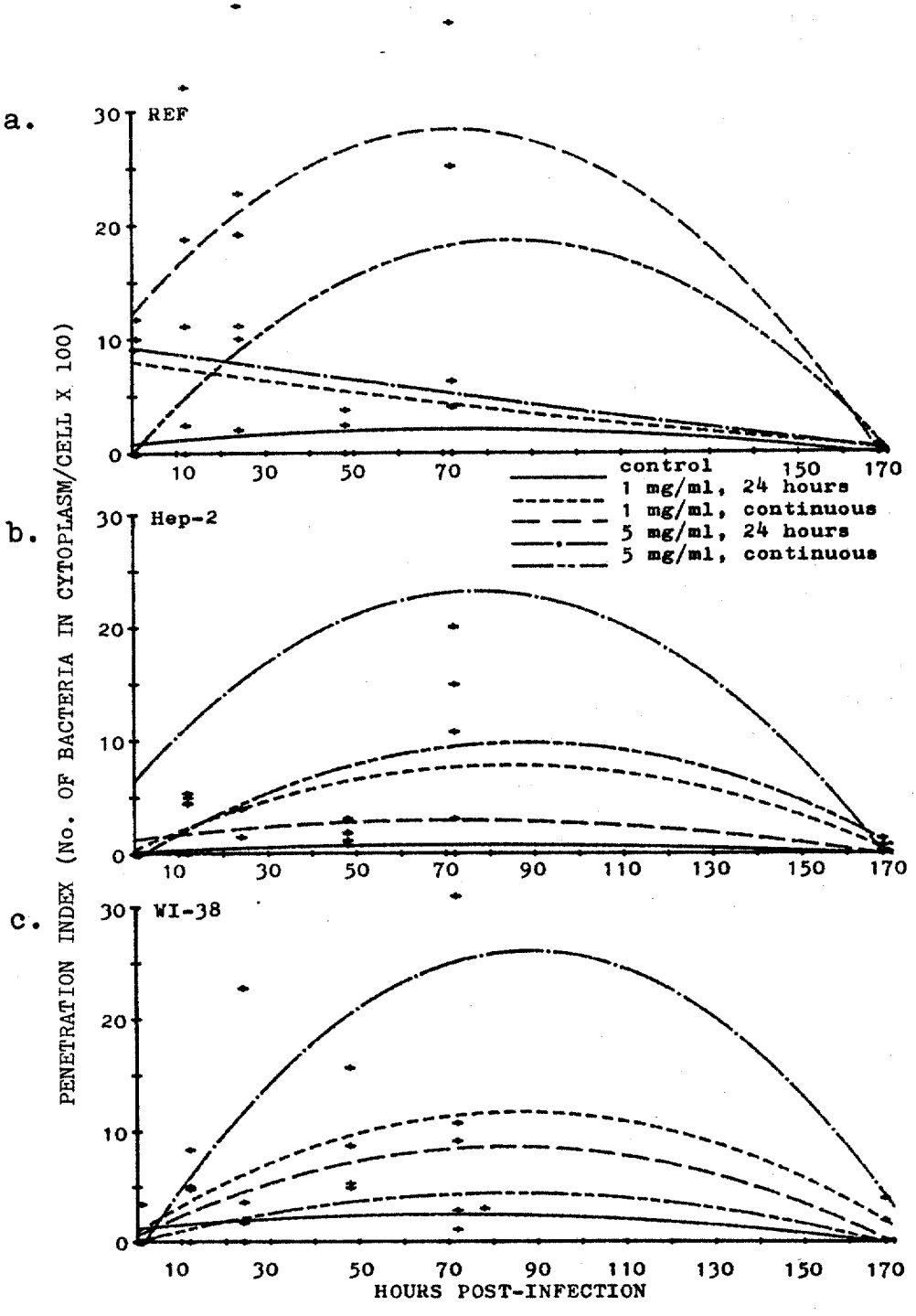


Figure 38 -- Effect of 2-deoxy-D-glucose on penetration of Chicago 8 strain of Legionella pneumophila into cytoplasm of tissue culture cells. a: Rat embryo fibroblast; b: Hep-2 cells; c: WI-38 cells.

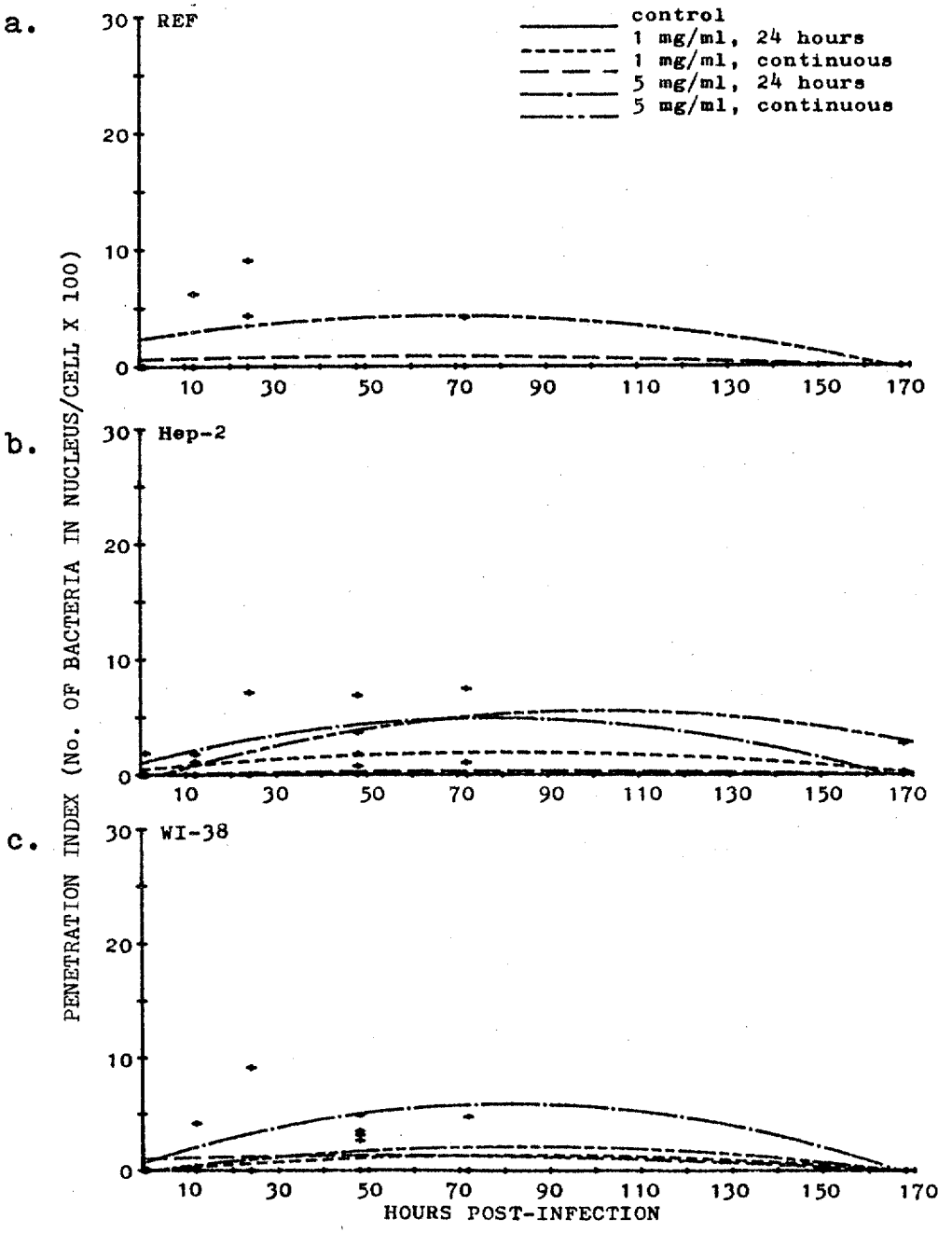


Figure 39 -- Effect of 2-deoxy-D-glucose on penetration of Chicago 8 strain of Legionella pneumophila into nuclei of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

cell line. Peak AIs of Jo 2 were 15 (REF), 8 (Hep-2) and 22 (WI-38) at the fourth day upon treatment with 5 mg pyruvate /ml (Figure 40), while controls gave AIs of 6, 4 and 8, respectively. At the third day, the peak AIs of Chi 8 were 13 (REF), 5 (Hep-2) and 12 (WI-38) with 5 mg (continuous), 5 mg (24 hours) and 1 mg (continuous) of pyruvate/ml, respectively (Figure 43). Comparable controls were 6, 3 and 7, respectively. It can be seen in Figure 41 and 44 that all cytoplasmic PIs were elevated by pyruvate. With REF and WI-38 exposed for 24 hours to 5 mg pyruvate/ml the PI of Jo 2 peaked between the third and fourth day (PI = 26 and 13, respectively), dropping to 0 after one week (controls 5, 7 and 0, respectively); with Hep-2 the peak AI of 10 was formed at the third day in cells exposed to 1 mg pyruvate for 24 hours (control = 2.5). The latter dropped to zero after one week, at which time the control AI had reached its maximum (PI = 7). In the case of Chi 8, cytoplasmic PIs were less affected by pyruvate, with all three cell lines showing no cytoplasmic penetration after one week. Intranuclear PIs of both strains of bacteria were increased slightly by pyruvate, the greatest enhancement being seen with Jo 2/REF on days 3 and 4 with 5 mg pyruvate/ml (24 hours); the PI in this case dropped to zero after one week. With 1 mg/ml (continuous), the peak nuclear PI of 7.5 occurred after one week, at which time the control was 3 (Figures 42 and 45).

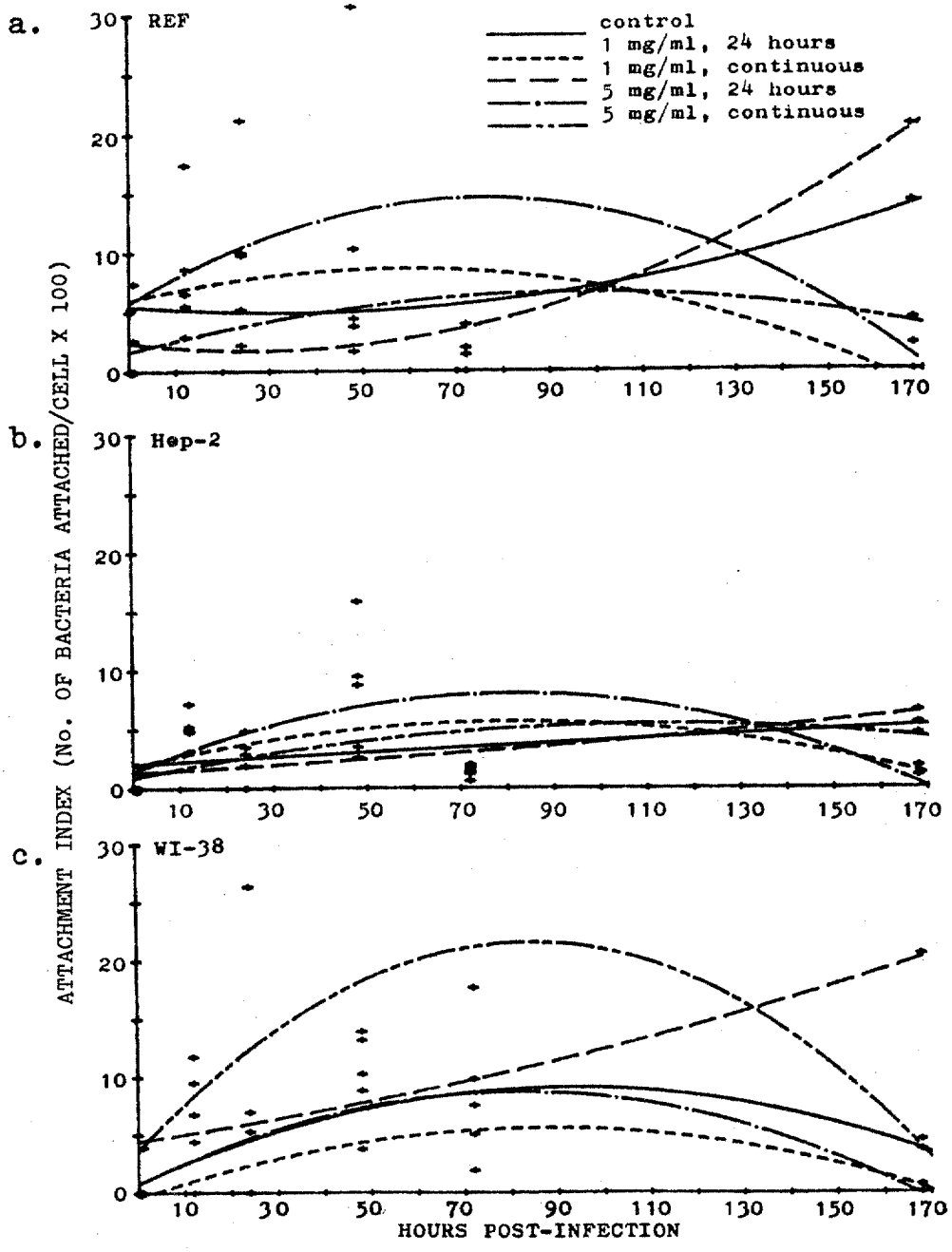


Figure 40 -- Effect of pyruvate on attachment of Johannesburg 2 strain of Legionella pneumophila to surfaces of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

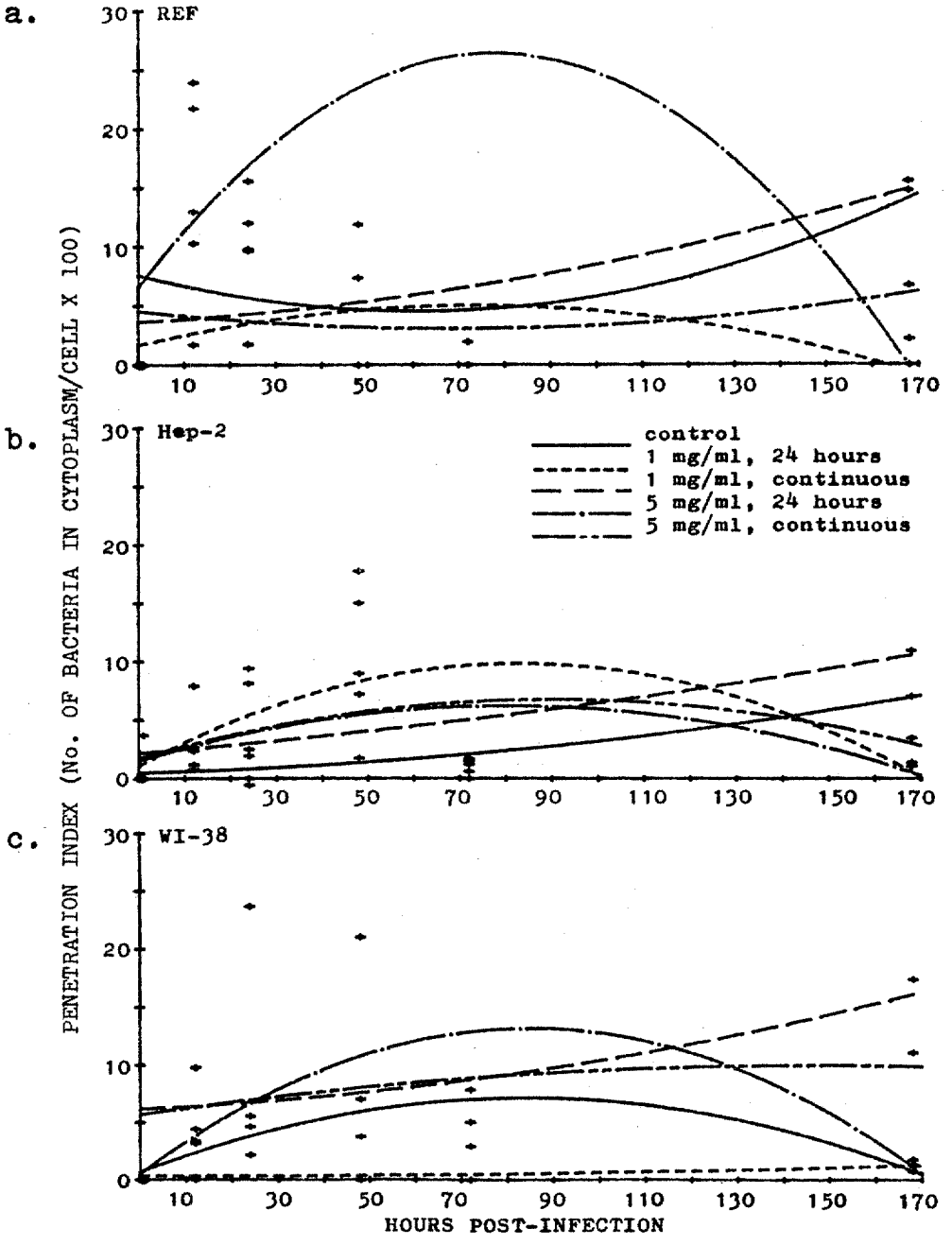


Figure 41 -- Effect of pyruvate on penetration of Johannesburg 2 strain of Legionella pneumophila into cytoplasm of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

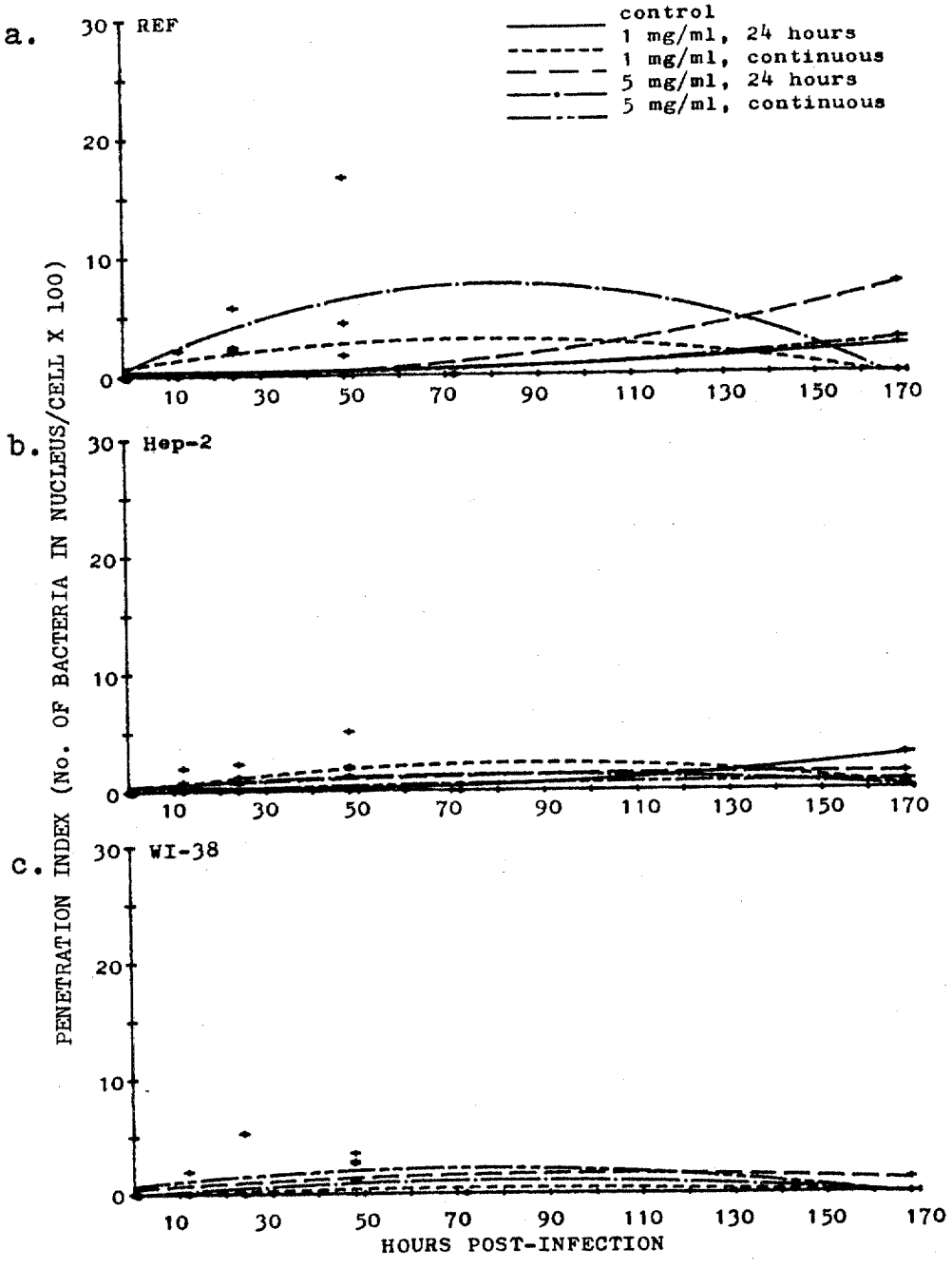


Figure 42 -- Effect of pyruvate on penetration of Johannesburg 2 strain of Legionella pneumophila into nuclei of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

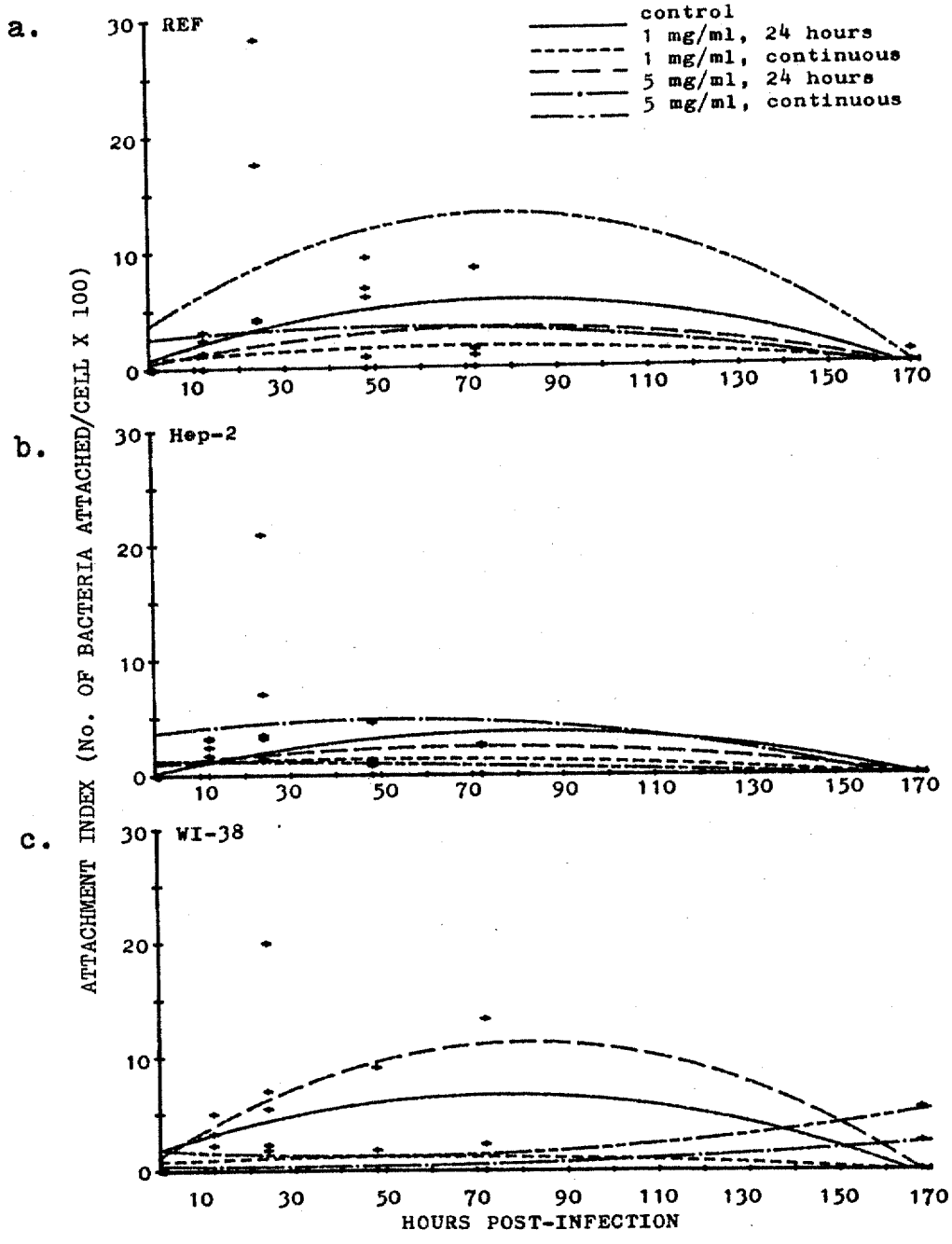


Figure 43 -- Effect of pyruvate on attachment of Chicago 8 strain of Legionella pneumophila to surfaces of tissue culture cells. a: Rat embryo fibroblast cells ; b: Hep-2 cells; c: WI-38 cells.

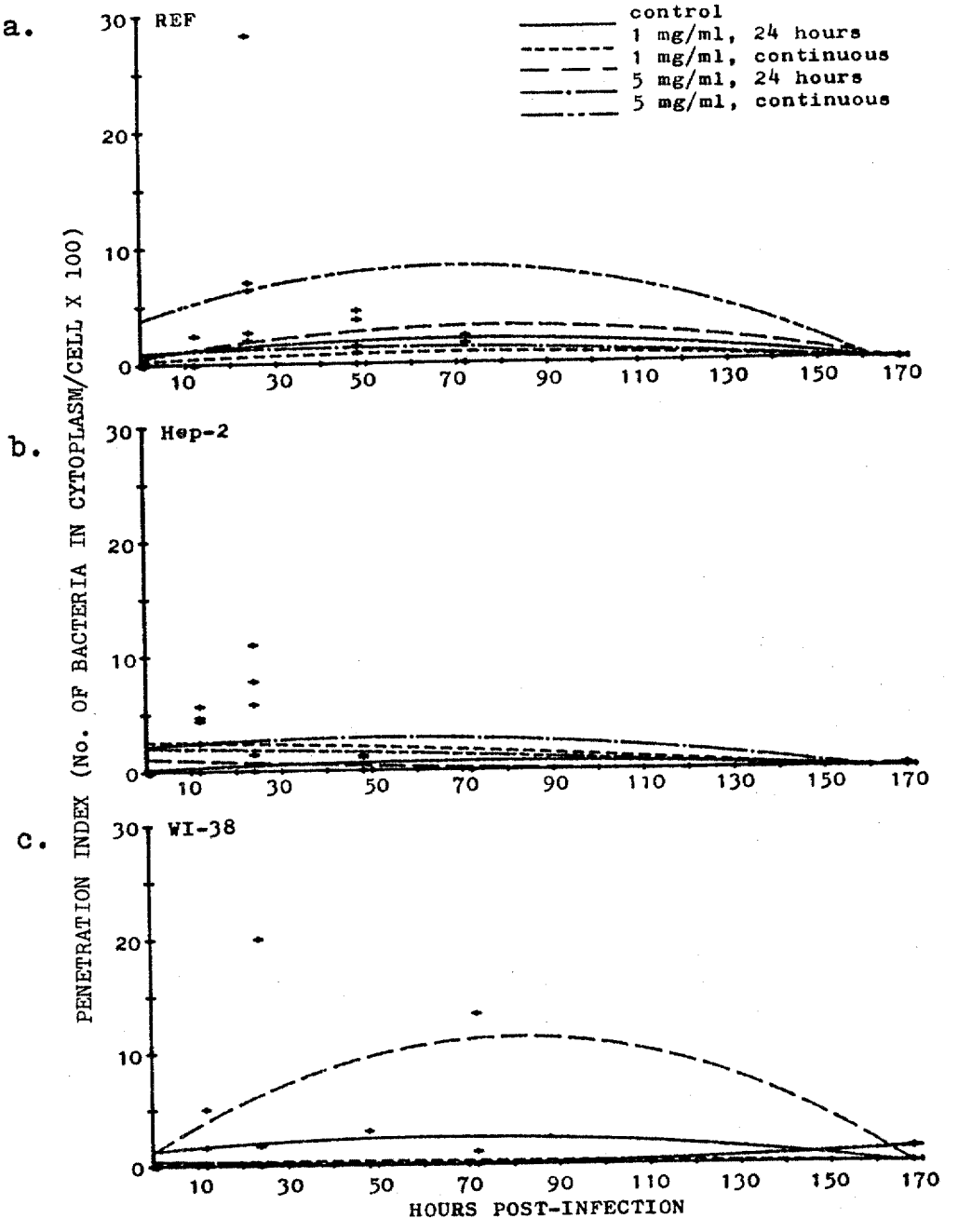


Figure 44 -- Effect of pyruvate on penetration of Chicago 8 strain of Legionella pneumophila into cytoplasm of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

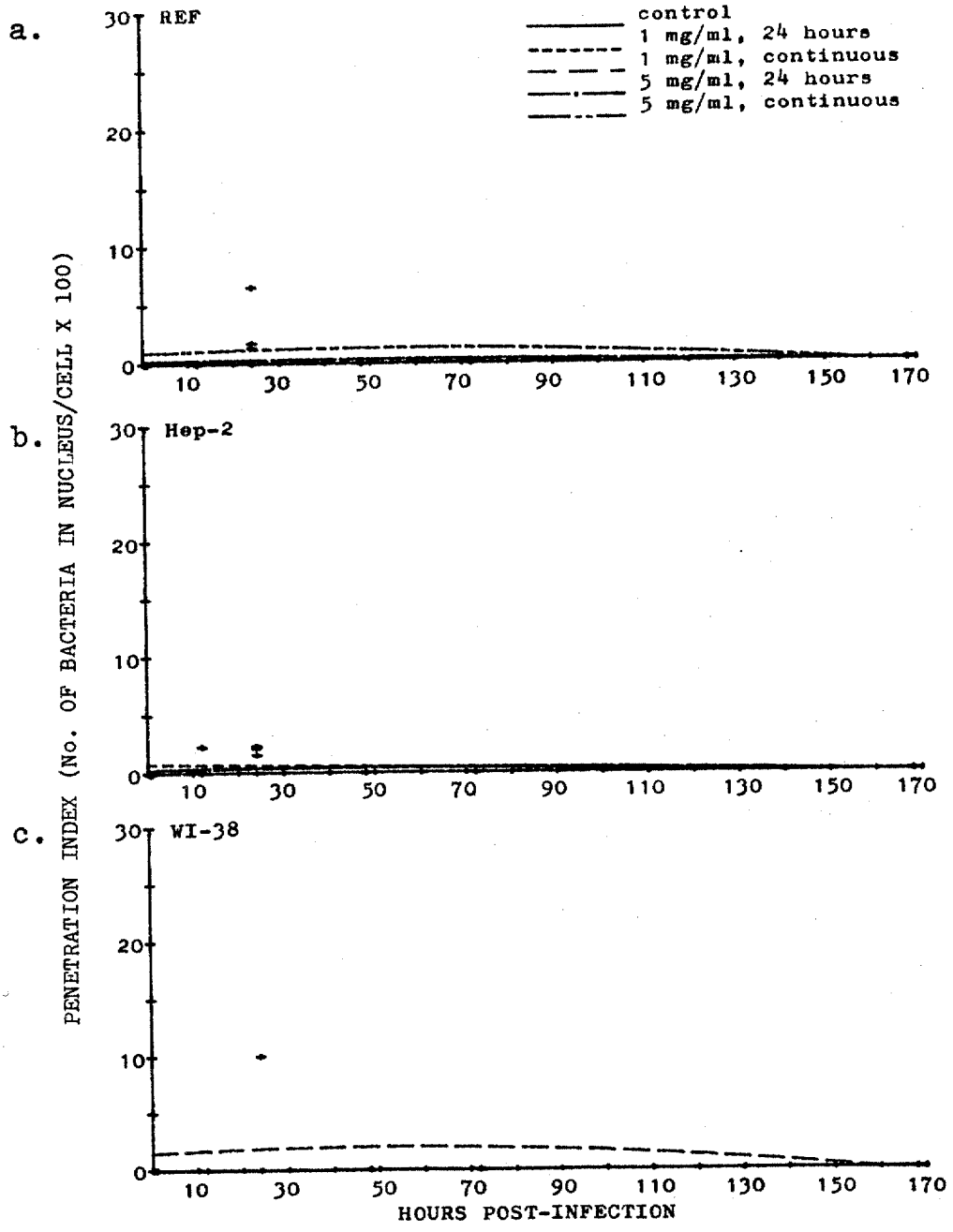


Figure 45 -- Effect of pyruvate on penetration of Chicago 8 strain of Legionella pneumophila into nuclei of tissue culture cells. a: Rat embryo fibroblast cells ; b: Hep-2 cells; c: WI-38 cells.

7. β-D-(-)fructose (Figures 46-51)

Attachment of Jo 2 was either enhanced or inhibited slightly by fructose, depending on the dose and exposure. For example, the AI of Jo 2 with REF was increased during the first four days slightly above the controls by exposure to 1 or 5 mg fructose/ml for 24 hours; however, it dropped to zero after one week, at which time the control AI had risen to 14 (Figure 46). However, continuous exposure to 1 mg fructose/ml resulted in a marked increase in the REF/AI during the last three days, reaching a peak of 20 on the seventh day (AI = 20), a bit higher than the control. Continuous exposure to 5 mg fructose/ml, however, lowered the AI of Jo 2 to REF cells. With WI-38 and Jo 2, a similar picture is seen, although the dose responses were different. The peak AI of Jo 2 with WI-38 was 19 at one week following a 24-hour exposure to 5 mg fructose/ml. The control AI at that time was 3. Results of effects of fructose on Jo 2/ Hep-2 were less clear than with REF and WI-38. The AIs of Chi 8 with all three cell lines was inhibited slightly by fructose (Figure 49).

The cytoplasmic PIs of Jo 2 with all cells were elevated by fructose. For example, with REF the cytoplasmic PIs peaked on the third day (PI = 9-10, control: 5) with an initial 24-hour exposure to 1 or 5 mg fructose/ml, while peaking after a week by continuous treatment with 1 mg/ml (Figure 47). With Hep-2, the cytoplasmic PI of Jo 2 peaked

at 10 (control = 3) on the fourth day following a 24-hour exposure to 5 mg fructose/ml. The latter exposure of Jo 2/ WI-38, however, reached a peak of 15 after one week (control = 1). The cytoplasmic PI of Chi 8 with REF and Hep-2 was both increased (5 mg/ml, 24 hours) or slightly suppressed by fructose; no enhancement occurred with WI-38 (Figure 50).

Intranuclear penetration by either strain of Legionella was virtually unaffected in any cell by the fructose regimen (Figures 48, 51).

The Influence of Legionella pneumophila on Cell Morphology and Growth

1. Growth Curves of Tissue Culture Cells

Cells infected with L. pneumophila were grown in 24-well tissue culture plates with an initial concentration of 10^4 cells/ml/well. Counts of trypsinized cells were made in triplicate every 24 hours with the aid of a hemocytometer. Plots of these quantitations, as seen in Figure 49 to 51, reveal some differences between the three lines. Growth of REF plateaued after 11 days, reaching a maximal population of 7.0×10^5 cells/ml: their numbers declined slowly after the fourteenth day (Figure 52). Cytologically, the only aberration seen in the REF cultures infected by Legionella was an elongation of the cells during the stationary phase. Infected REF cultures achieved lower densities which declined more rapidly than the controls, especially in the Chi 8-

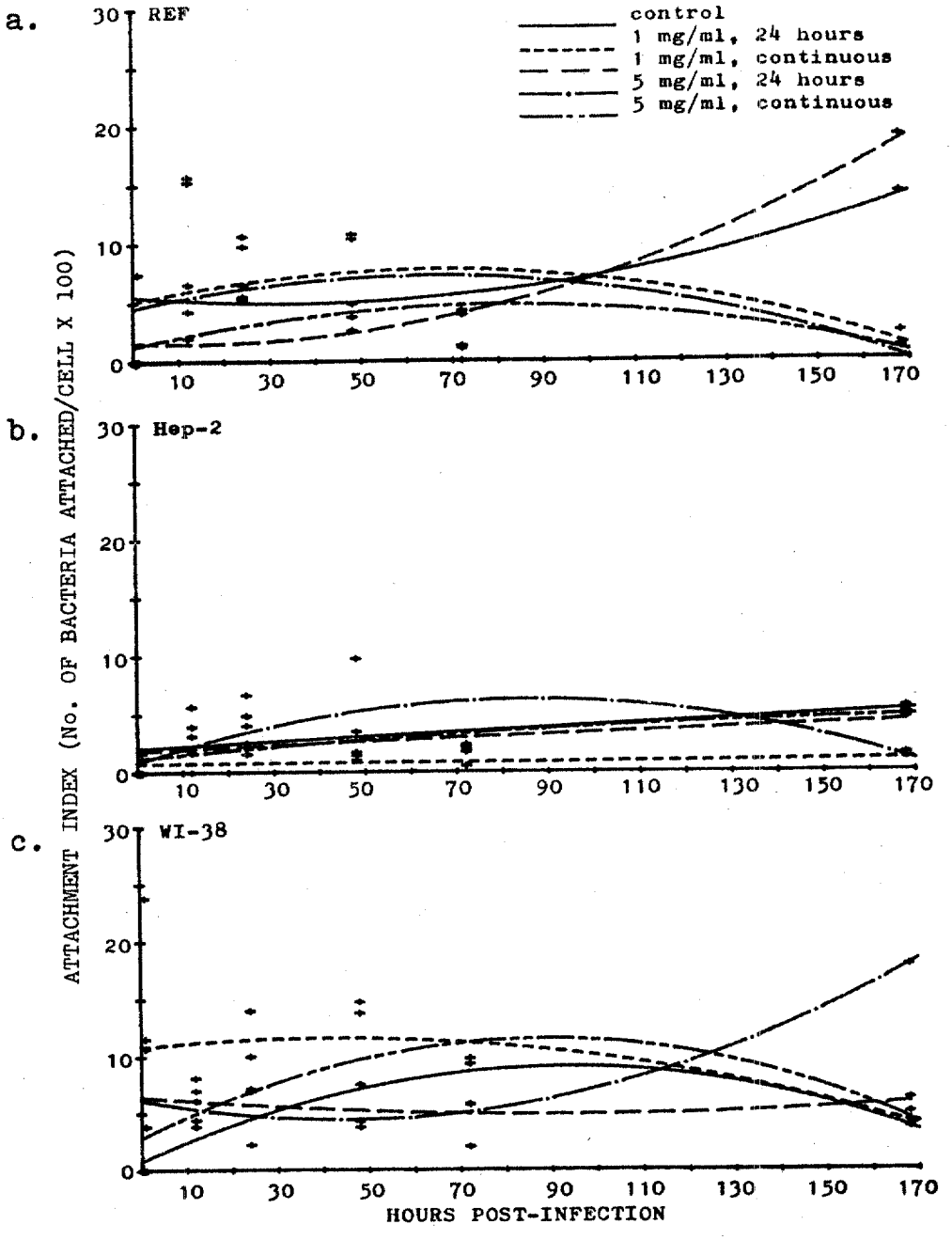


Figure 46 -- Effect of β-D-fructose on attachment of Johannesburg 2 strain of Legionella pneumophila to surfaces of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

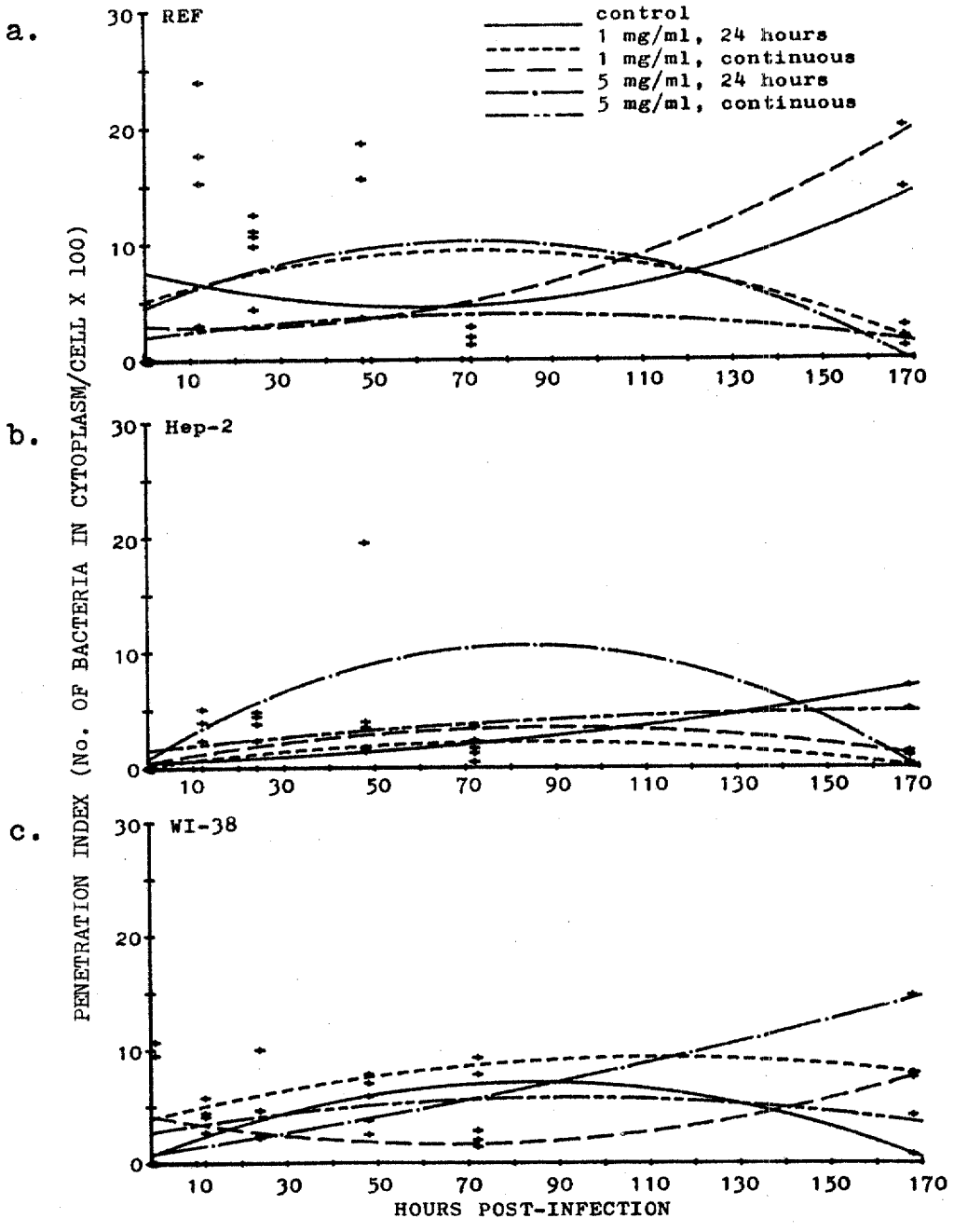


Figure 47 -- Effect of β-D-fructose on penetration of Johannesburg 2 strain of Legionella pneumophila into cytoplasm of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c:WI-38 cells.

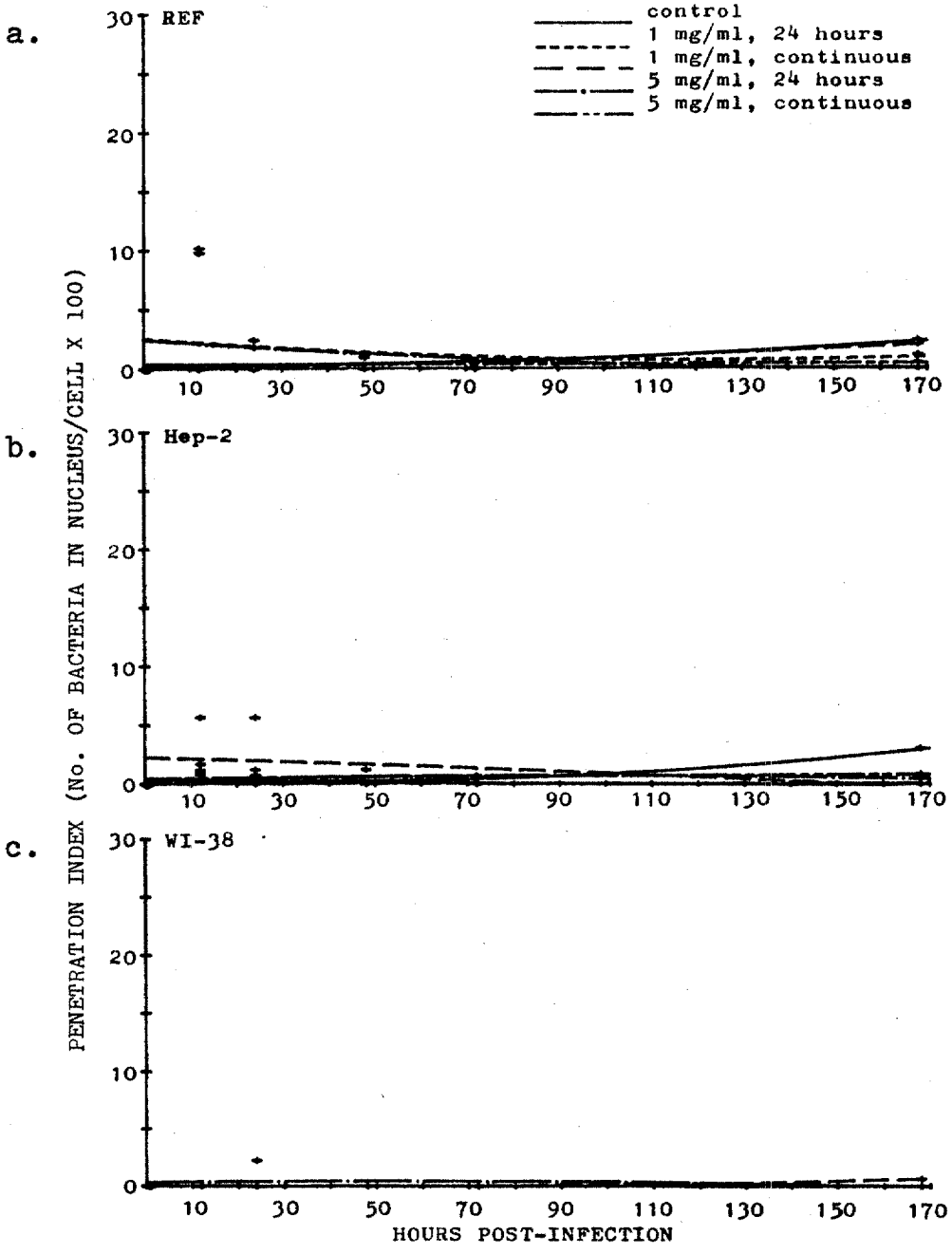


Figure 48 -- Effect of β-D-fructose on penetration of Johannesburg 2 strain of Legionella pneumophila into nuclei of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

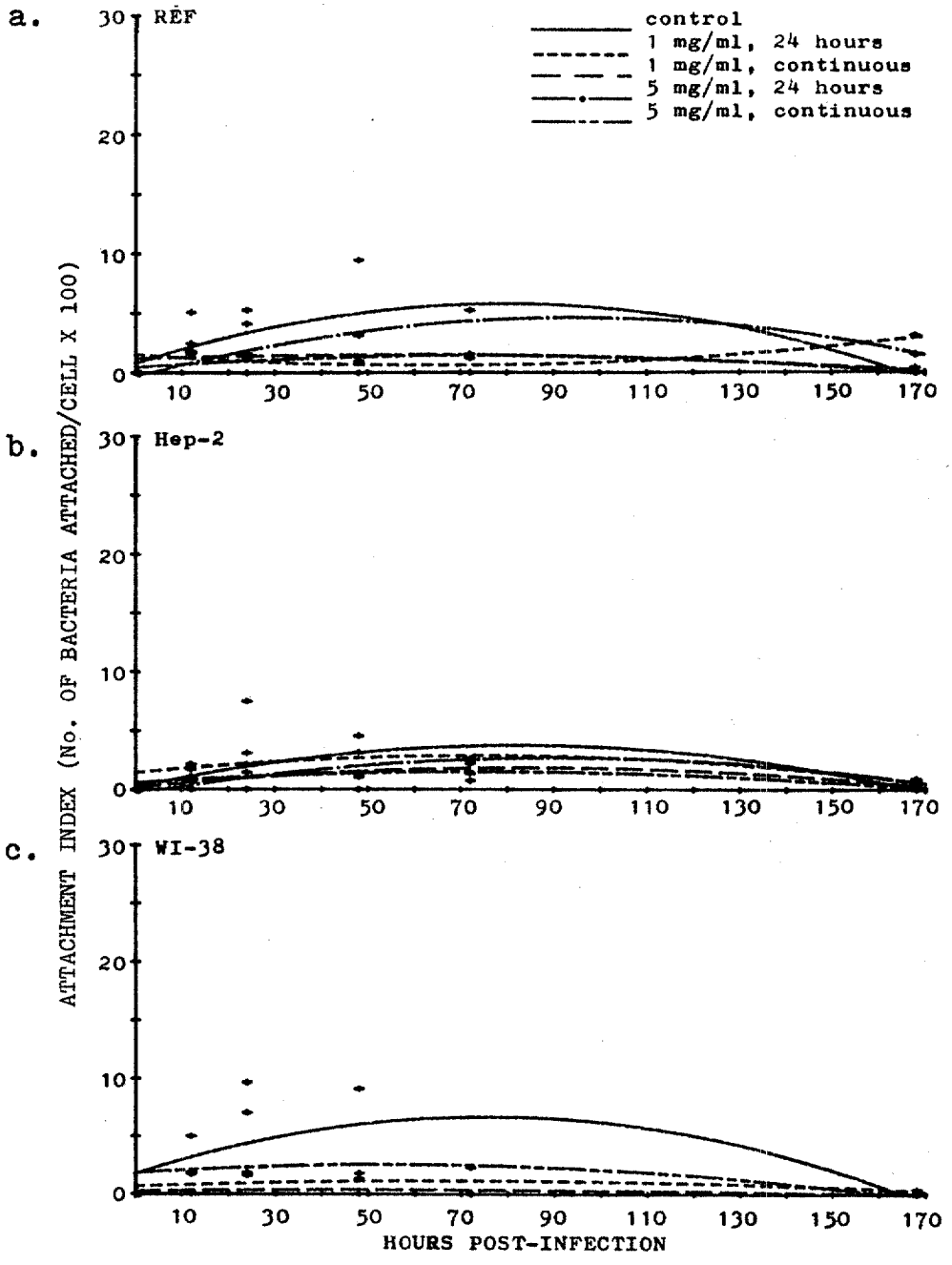


Figure 49 -- Effect of β-D-fructose on attachment of Chicago 8 strain of Legionella pneumophila to surfaces of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

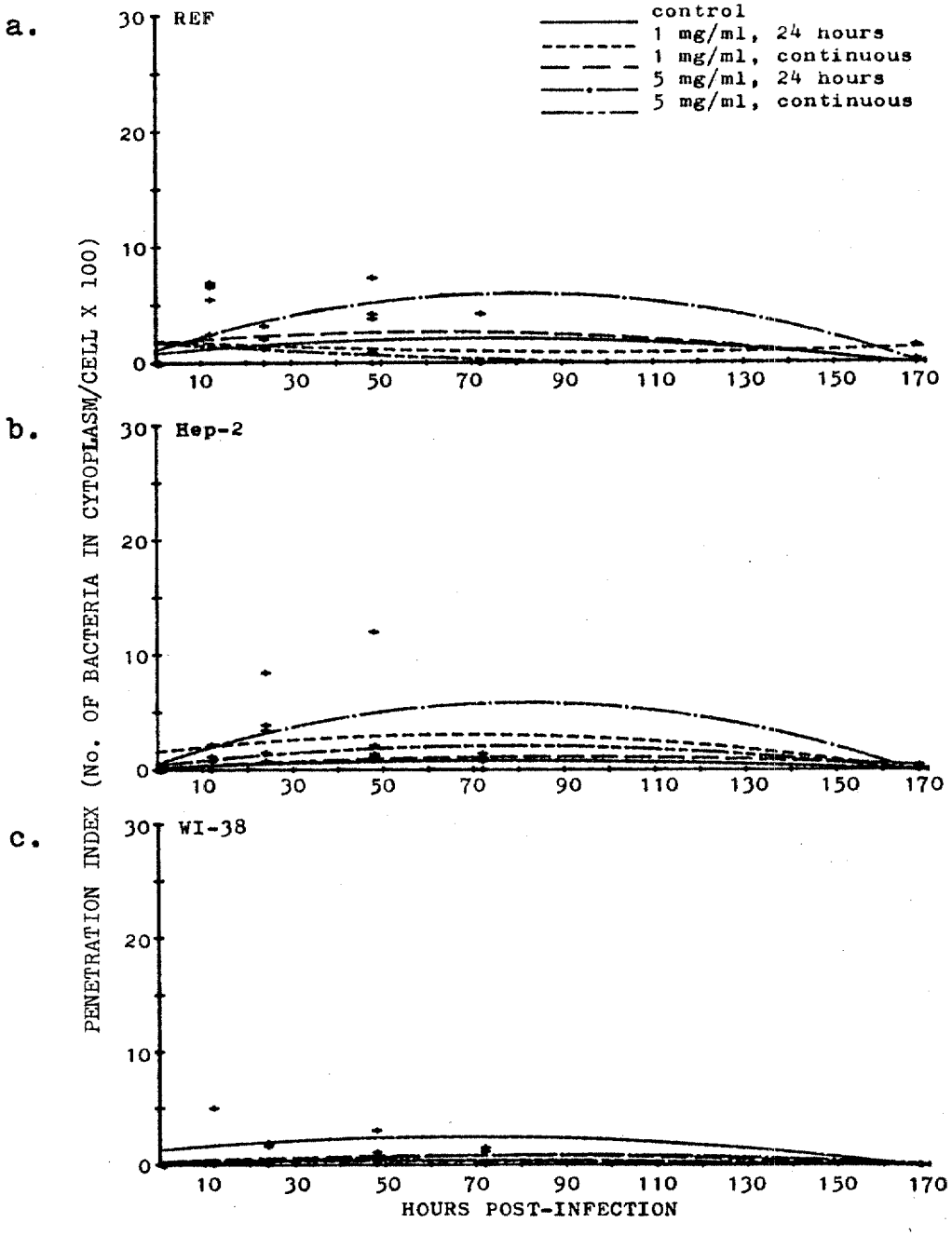


Figure 50 -- Effect of β-D-fructose on penetration of Chicago 8 strain of Legionella pneumophila into cytoplasm of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

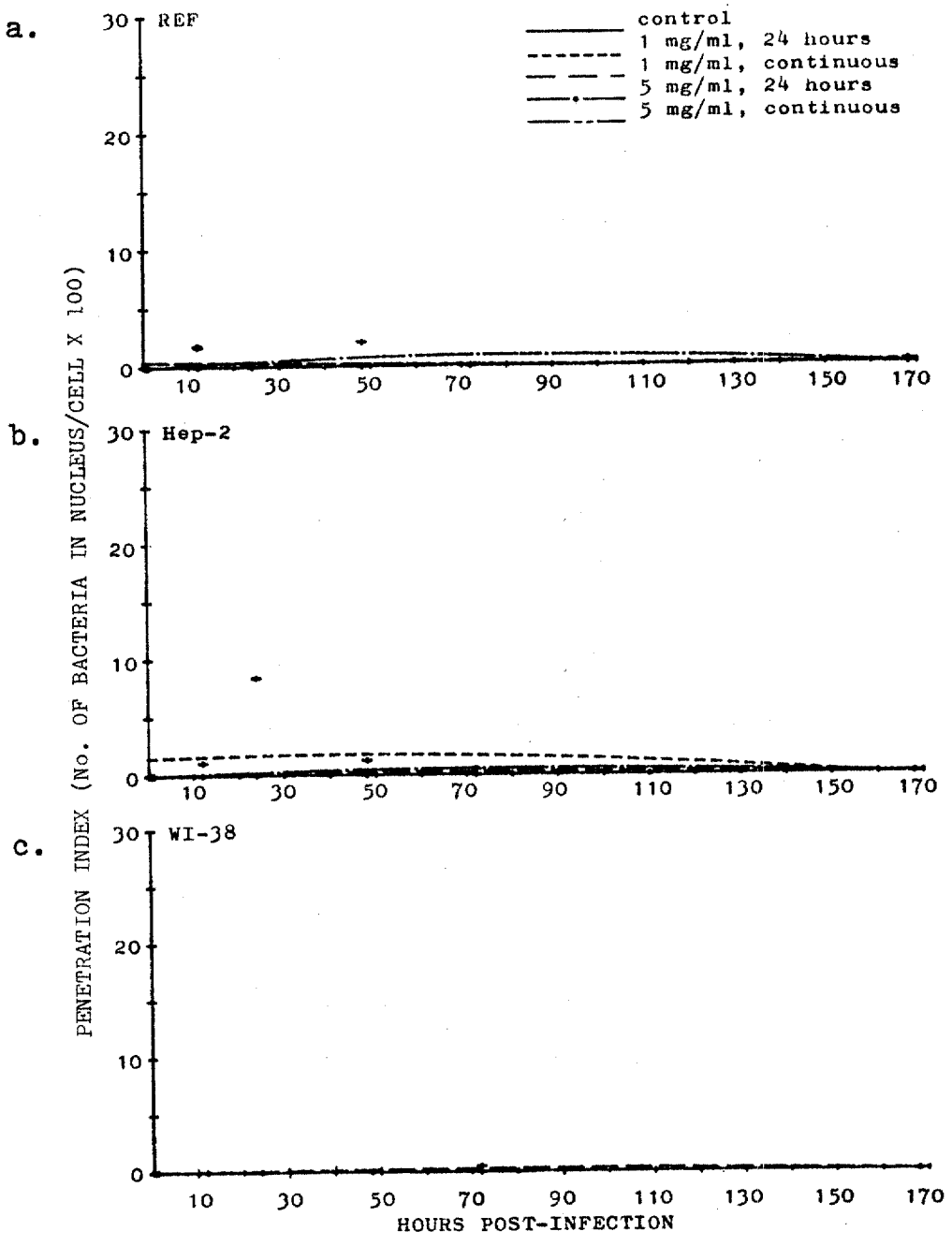


Figure 51 -- Effect of β-D-fructose on penetration of Chicago 8 strain of Legionella pneumophila into nuclei of tissue culture cells. a: Rat embryo fibroblast cells; b: Hep-2 cells; c: WI-38 cells.

inoculated culture.

Hep-2 cells by the thirteenth day reached the highest density (1.2 X 10^6 cells/ml) among the three cell lines tested (Figure 53), maintaining a plateau through the twentieth and final day. Infected Hep-2 cells did not maintain their maximal density; instead, their numbers dropped immediately after the peak seen on the twelfth day, especially under the influence of Chi 8. No morphological changes could be discerned by light microscopy.

WI-38 culture took 16 days to reach the stationary phase, the longest among the three lines; they also had the longest lag phase (about 3 days) (Figure 54). Growth of infected WI-38 cells was retarded at all stages. No cytopathology was noted in this line.

2. Electron Microscopy of Cells Infected by Legionellae

Except for elongation noted in REFs, the Legionella bacteria did not appear to affect cellular morphology as observed under the light microscope. However, scanning electron microscopy (SEM) revealed not only various patterns of attachment, but also morphological variations (Figures 55-57). The principal morphological variations seen were in microvillus formation and roughness of the animal cell surfaces.

The surfaces of REF incubated with Legionella was not smooth as seen in control cultures (Figure 55-A). In Figure

55-B a Jo 2 bacterium can be seen attached along its length to the rat embryo fibroblast cell; note the microvilli in the vicinity of bacterial cell. In another example, the adherence between Jo 2 and a Hep-2 cell would seem to be supported by two protrusions which appear to originate at the pole of the bacterium, extending to the cell membrane (Figure 56-A). A good example of microvilli formation can be seen in Figure 56-B which shows a portion of a Hep-2 cell from a culture infected with Jo 2. Although the nucleus can not be seen in this photography, it would be noted that the greatest concentration of villi was generally noted to occur in close proximity to the nucleus. The cell surfaces of Hep-2 infected by Chi 8 were especially rough (Figures 56-C, 56-D) compared with normal Hep-2 cells (Figure 56-E). No microvilli were apparent. Both polar and "side-on" types of bacterial attachment were noticed in WI-38 cells with both strains of Legionella (Figures 57-A, 57-B). As with REF, the attachment of Legionella to WI-38 cells was accompanied by the production of cellular microvilli, especially in response to strain Jo 2. WI-38 cells growing under axenic conditions had relatively smooth surfaces without microvilli (Figure 57-C). The cytoplasmic extrusions (filopodia) seen in the latter photograph were less commonly encountered in infected cultures.

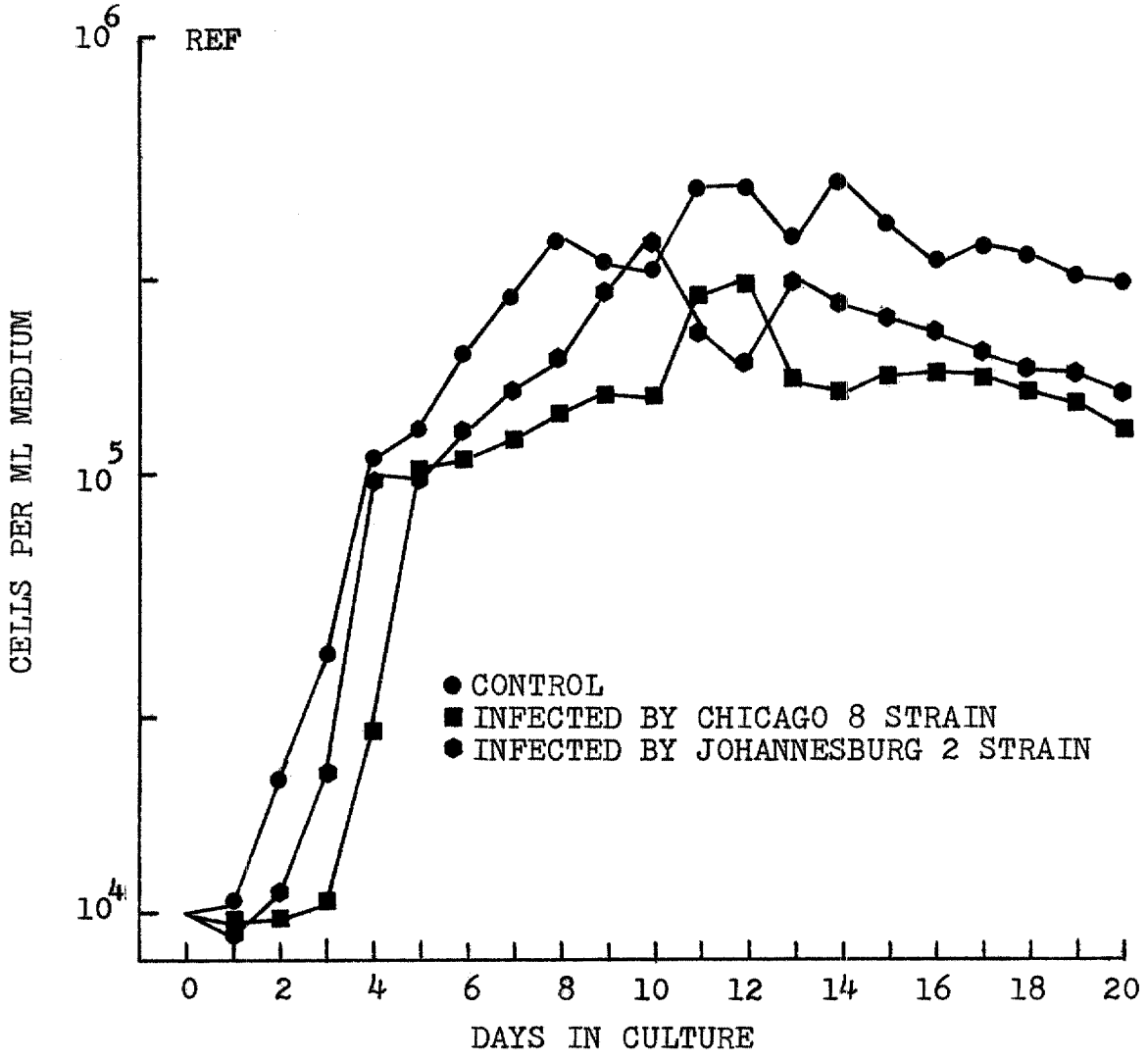


Figure 52 -- Growth curve of REF cell line

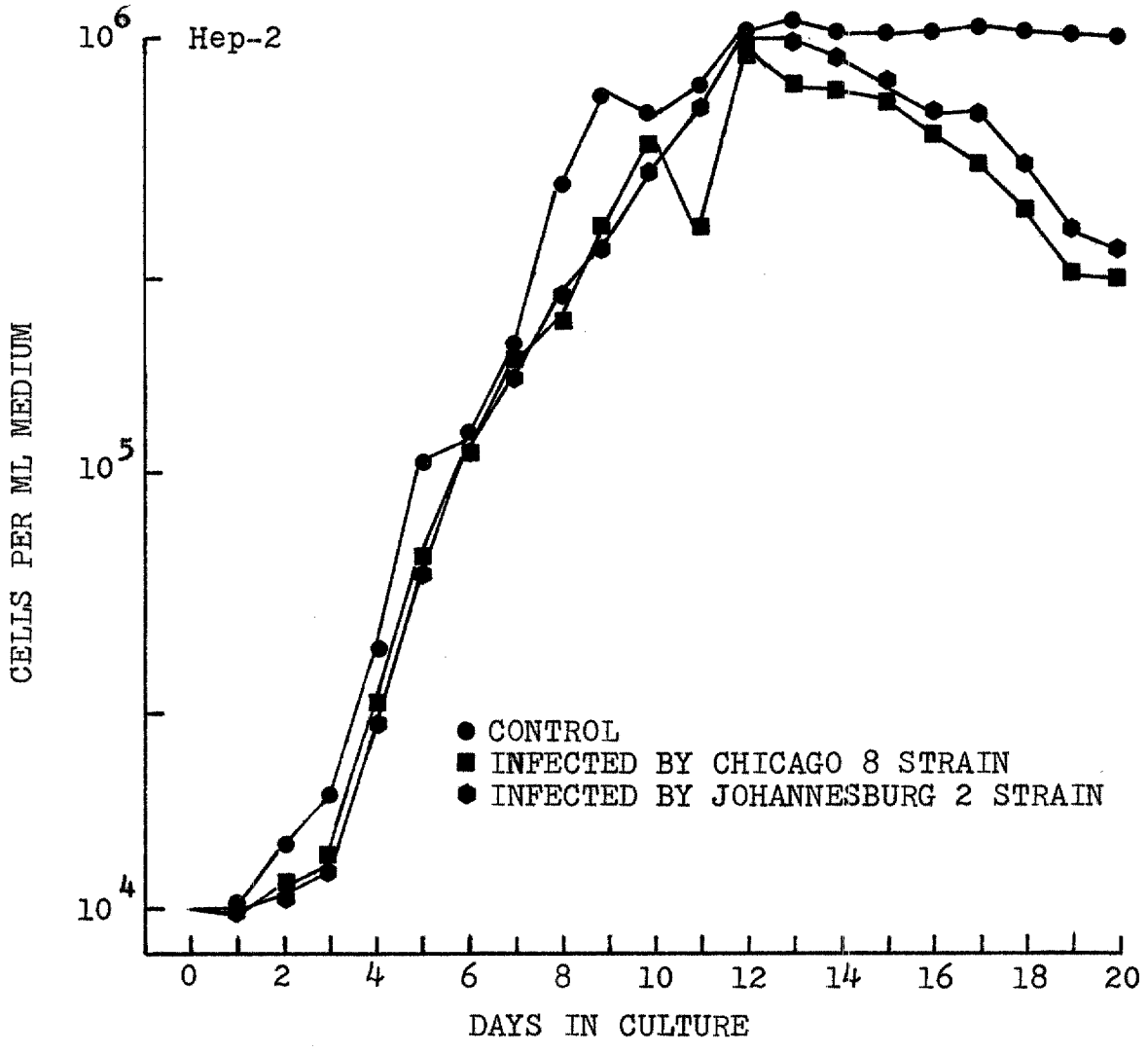


Figure 53 -- Growth curve of Hep-2 cell line

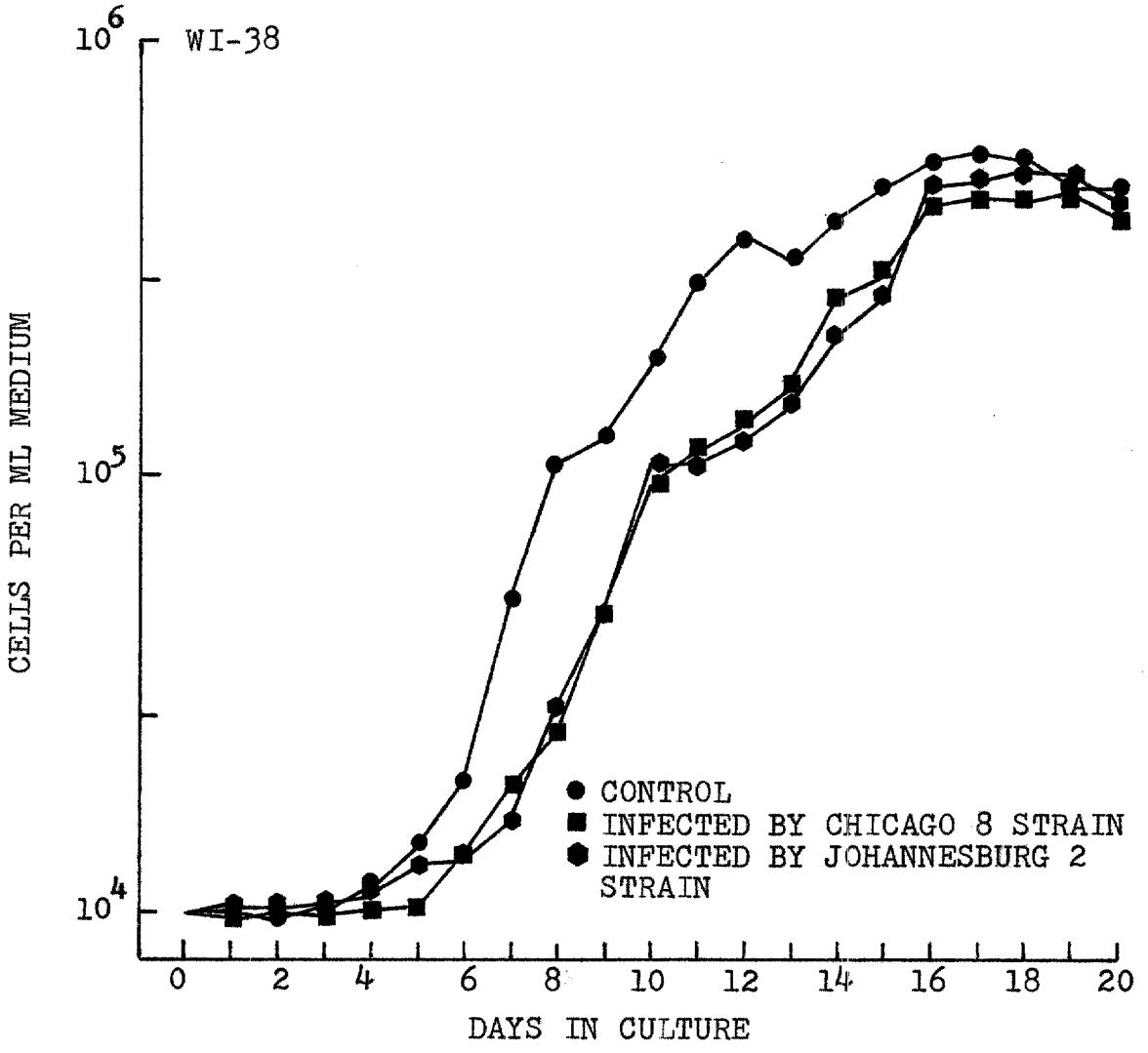


Figure 54 -- Growth curve of WI-38 cell line

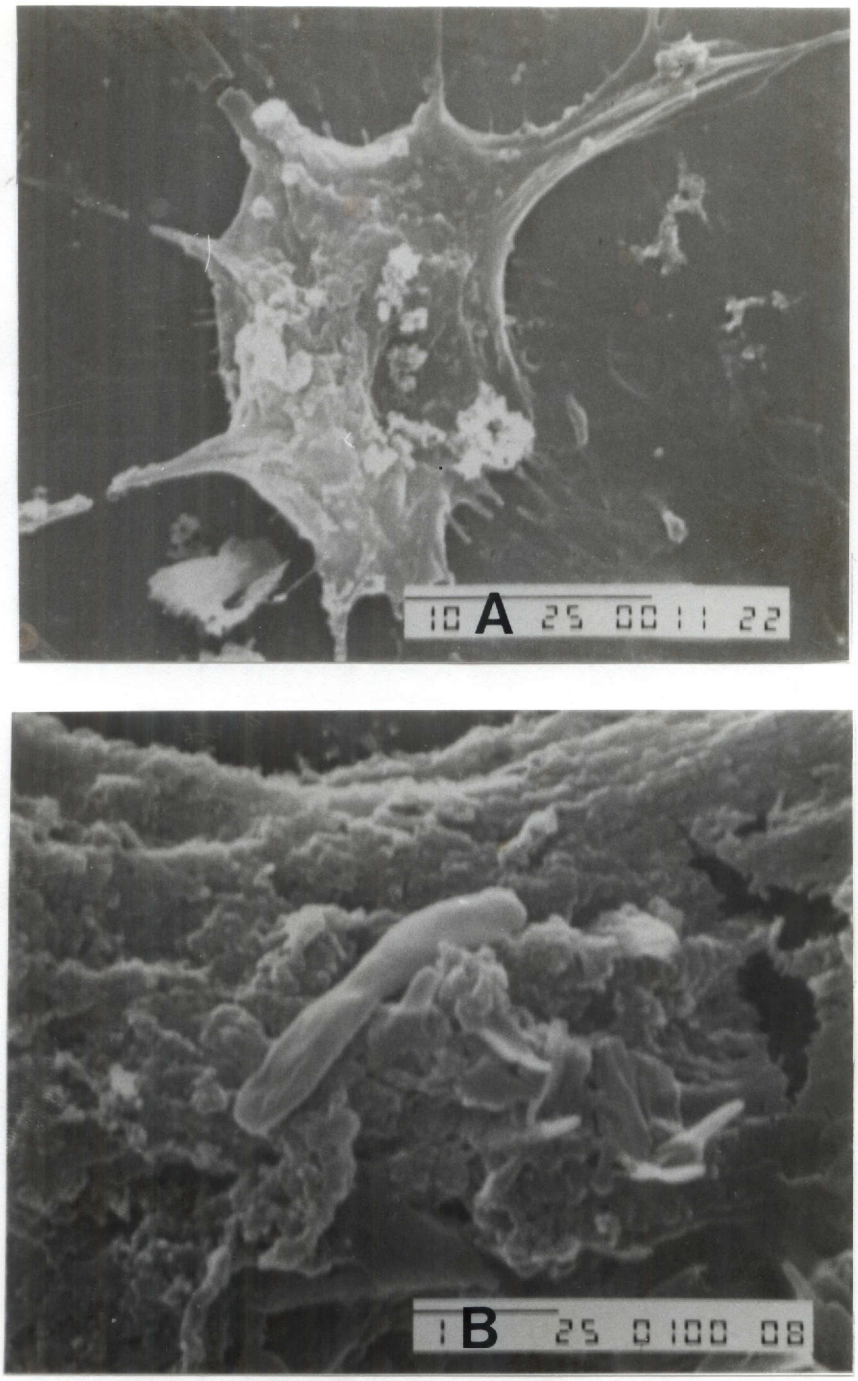


Figure 55 -- SEM of REF cell monolayers inoculated with Legionella pneumophila, 48 hours post-infection. (A) A REF cell from culture challenged with Chi 8 strain. Note the surface is rough. Bar, 10 μm. (B) A single Jo 2 is seen attached to the surface of REF cell. Cellular microvilli are seen in the vicinity of the bacterial cell. Bar, 1 μm.

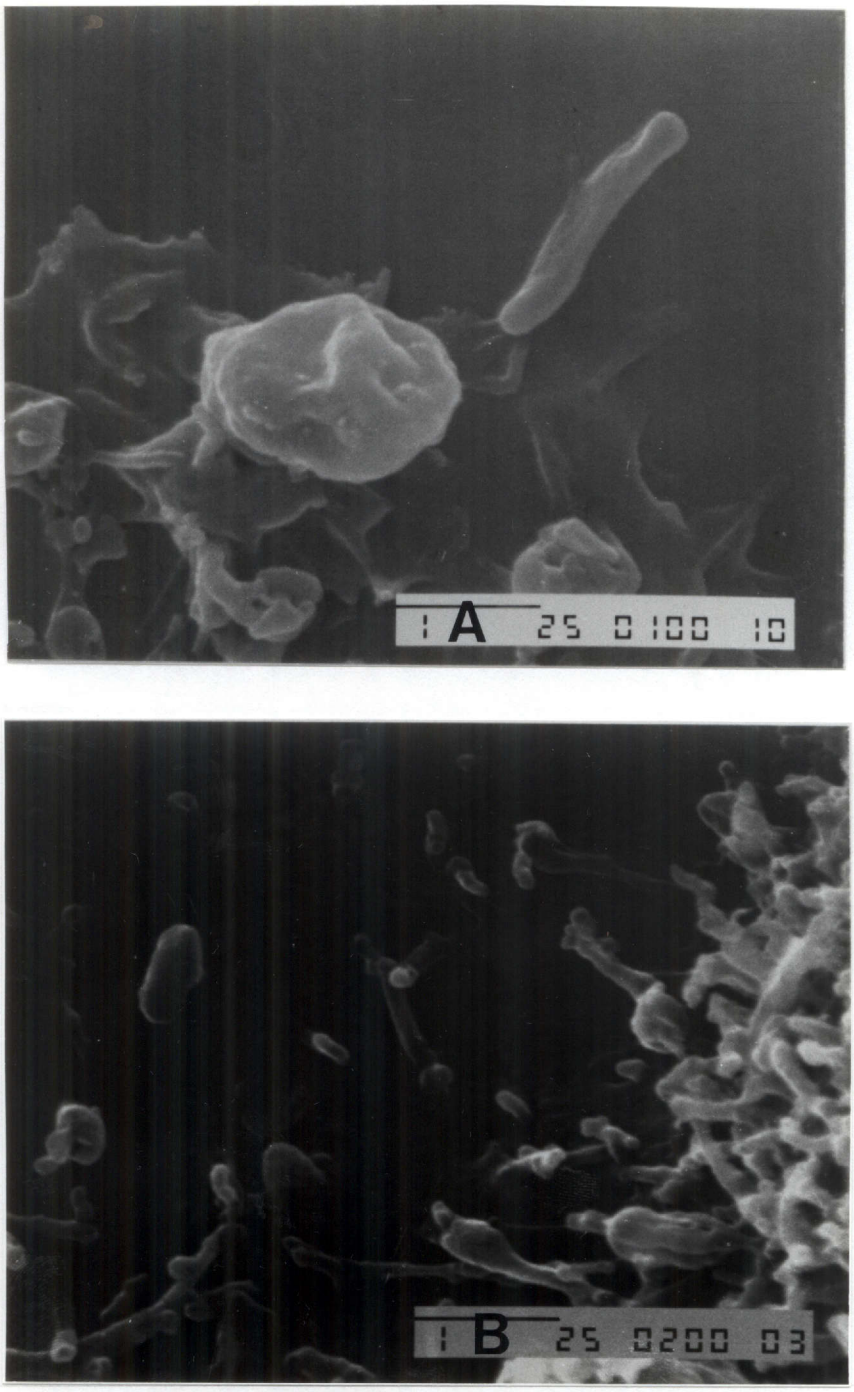
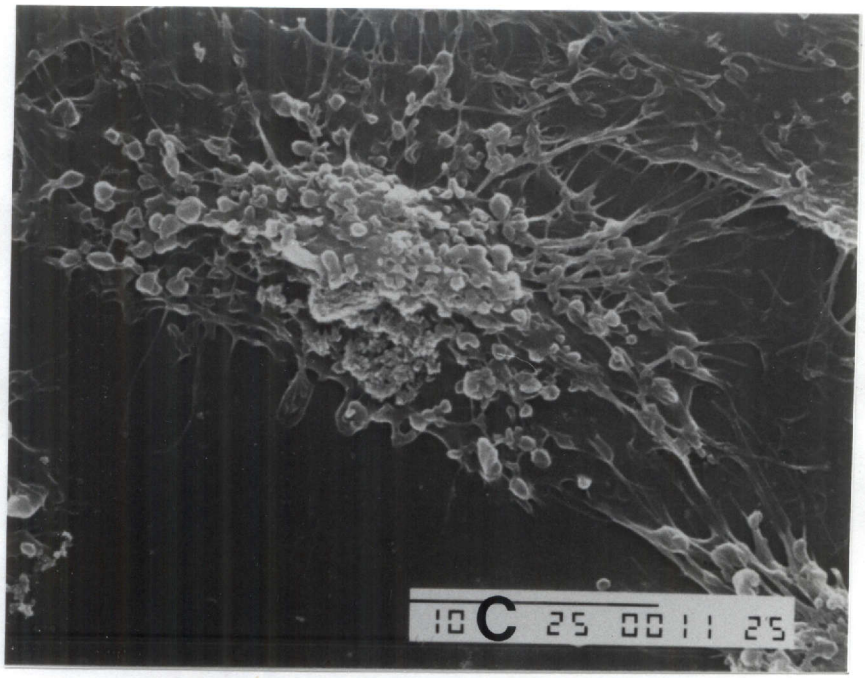


Figure 56 -- SEM of Hep-2 cell monolayers inoculated with Legionella pneumophila, 48 hours post-infection. (A) Two protrusions are seen originating at the pole of the bacterium (Jo 2). Bar, 1 µm. (B) Microvillus formation is induced by Jo 2, 48 hours post-infection. Bar, 1 µm. (C) Hep-2 cell infected by Chi 8. Bar, 10 µm. (D) Higher magnification of (C). Bar, 1 µm. (E) Surface of uninfected Hep-2 cell. Bar, 1 µm.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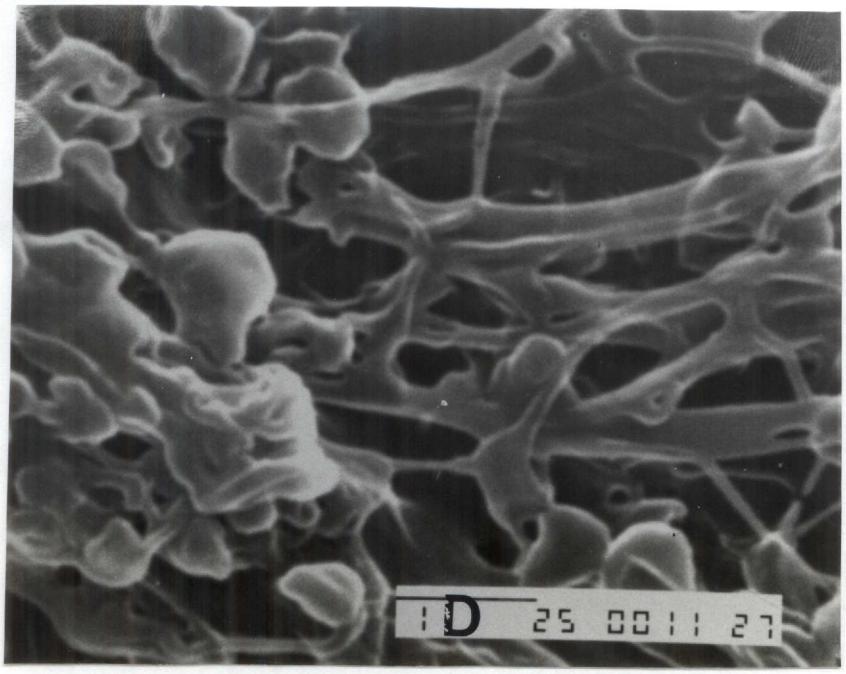
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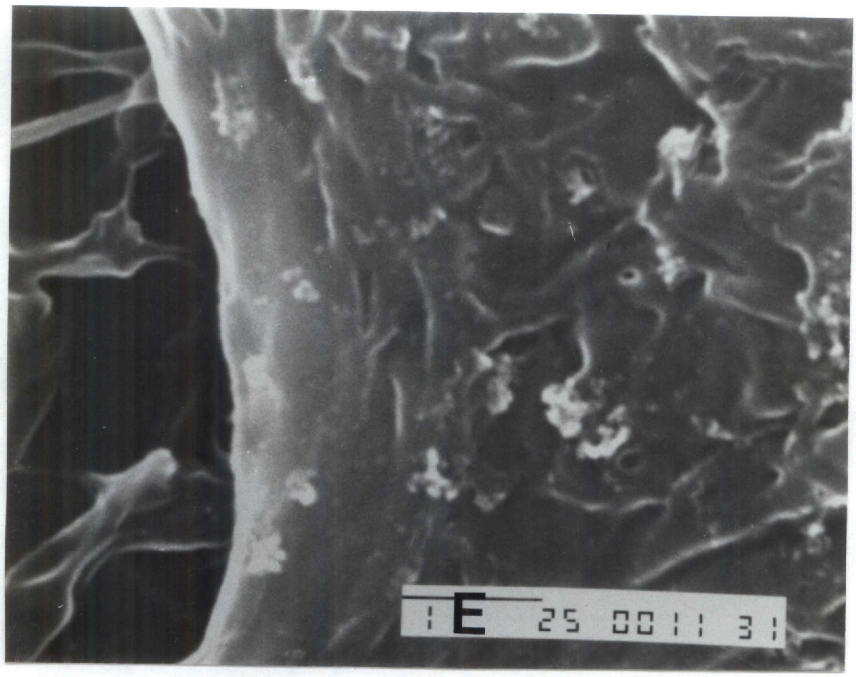
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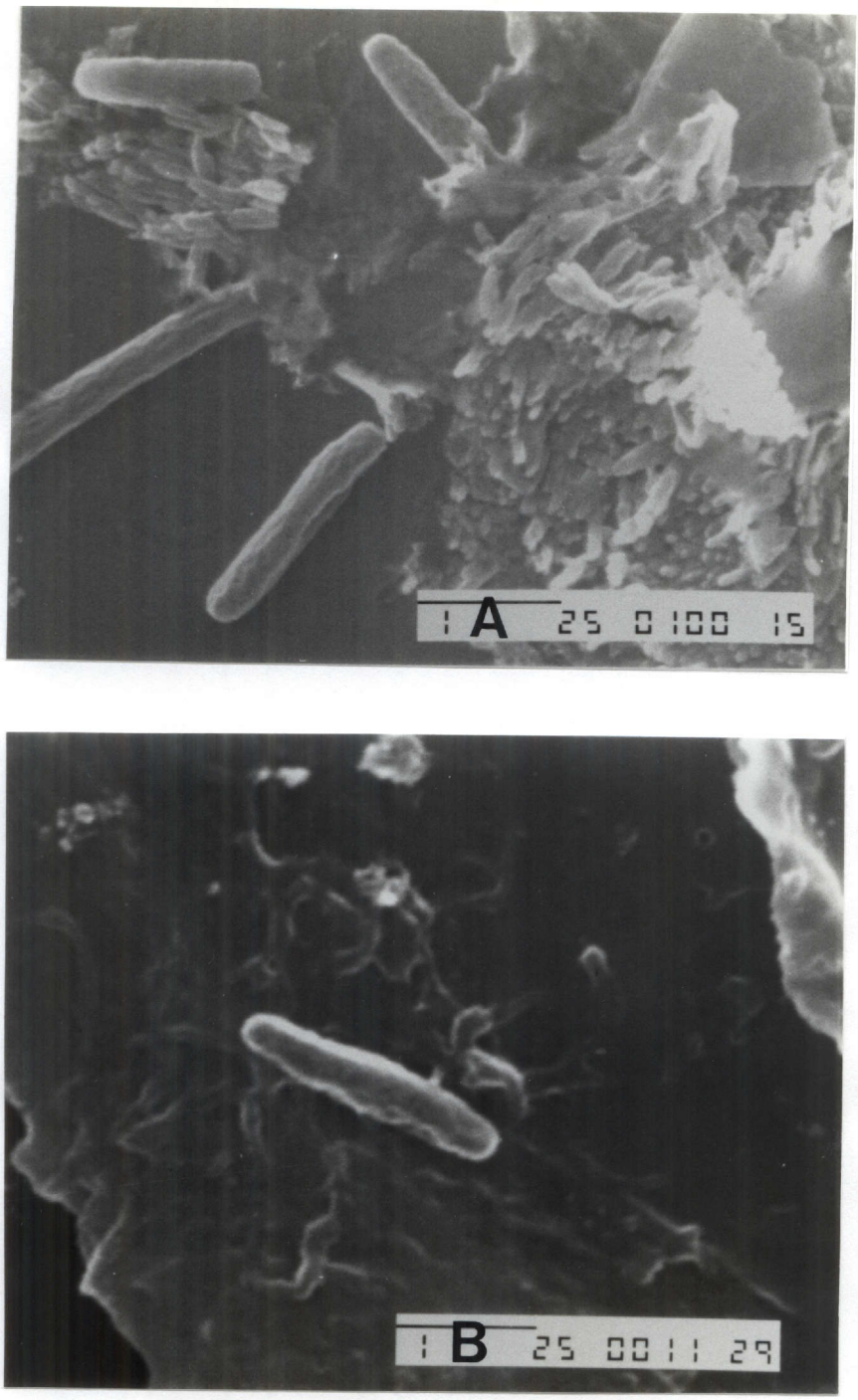
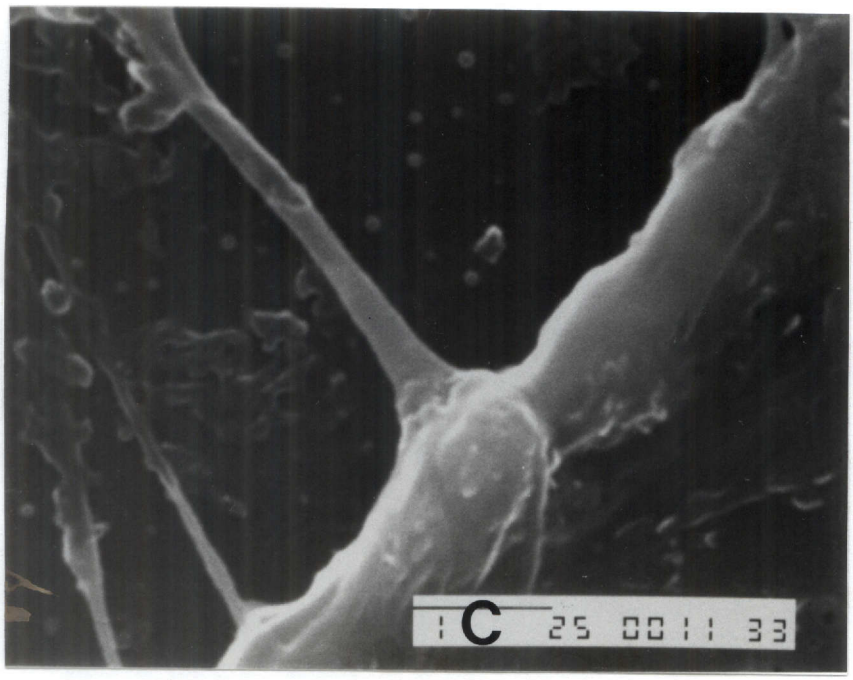


Figure 57 -- SEM of WI-38 cell monolayers inoculated with Legionella pneumophila, 48 hours post-infection. (A) Cell infected by Jo 2. Penetrating and microvilli are clearly seen. Bar, 1 µm. (B) Single bacterium (Chi 8) on the surface of WI-38 cell. (C) Smooth surface and filopodia of uninfected WI-38 cell. Bar, 1 µm.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3. Survival of Legionella pneumophila in Tissue Cultures

Viable counts of Legionella were performed in duplicate on harvested culture fluids daily for 8 days. At each time interval, duplicate 5 µl quantities of culture fluids were spread on CYE agar plates and the colony forming units (CFU) per milliliter was estimated.

Neither the tissue culture medium nor the cell cultures were able to sustain the legionellae. As can be seen in Table VI and VII, the numbers of both strains of L. pneumophila declined steadily, whether eucaryotic cells were present or not. Occasional bursts of growth were followed by a prompt decline. These data, however, do not rule out surface-associated or intracellular growth of the bacteria.

TABLE VI

VIABLE COUNTS OF CHICAGO 8 STRAIN OF *Legionella pneumophila* IN TISSUE CULTURE AND MEDIA (No. OF BACTERIA/ML MEDIUM) (X 10^5)

Culture Conditions	Time of Incubation									
	1 hr	12 hrs	1 day	2 days	3 days	4 days	5 days	6 days	7 days	8 days
BME[a]	4.80	4.00	2.88	2.44	1.45	1.00	1.28	1.13	0.28	0.22
MEM[b]	0.16	1.12	1.42	0.76	0.88	0.21	0.10	0.08	0.018	0.022
REF[c]	0.026	1.12	0.95	0.39	3.00	0.10	0.26	1.80	0.13	0.15
Hep-2[d]	3.28	3.00	0.16	0.60	0.25	0.36	0.10	0.07	0.06	0.42
WI-38[e]	2.78	2.40	4.58	1.60	3.00	1.45	1.38	0.67	0.58	0.50

[a] Eagle's basal medium only

[b] Eagle's minimun essential medium only

[c] Rat embryo fibroblast cell culture

[d] Hep-2 cell culture

[e] WI-38 cell culture

TABLE VII

VIABLE COUNTS OF JOHANNESBURG 2 STRAIN OF *Legionella pneumophila* IN TISSUE CULTURE AND MEDIA (No. OF BACTERIA/ML MEDIUM) (X 10^5)

Culture Conditions	Time of Incubation									
	1 hr	12 hrs	1 day	2 days	3 days	4 days	5 days	6 days	7 days	8 days
BME[a]	5.12	5.00	4.40	2.10	2.65	1.25	1.30	0.99	0.81	0.78
MEM[b]	4.10	2.42	0.15	2.32	0.58	0.062	0.052	0.076	0.072	0.05
REF[c]	0.40	0.40	0.036	0.30	0.046	0.008	0.006	0.012	0.008	0.006
Hep-2[d]	0.036	0.90	0.066	0.42	0.35	0.08	0.05	0.04	0.012	0.006
WI-38[e]	4.64	3.20	2.03	1.26	1.40	0.09	0.93	0.74	0.62	0.65

[a] Eagle's basal medium only

[b] Eagle's minimum essential medium only

[c] Rat embryo fibroblast cell culture

[d] Hep-2 cell culture

[e] WI-38 cell culture

CHAPTER IV

DISCUSSION

Nature of Attachment and Penetration of Microorganisms

Bacteria in many natural environments have a predilection for colonizing surfaces. In the mouth and elsewhere in the body (35, 110), large numbers of bacteria grow on the available surfaces. Bacterial colonization of these surfaces requires, first, that the organisms become firmely attached and, second, that they proliferate (colonize) under existing conditions.

Although some microbes can enter the host directly by bites of arthropod vectors or by trauma, most infections begin on the mucous membranes of the respiratory, alimentary, and urogenital tracts. These membranes are protected mechanically by movement of the lumen contents and by surface mucus, and often by commensal microorganisms which are part of the normal microflora (115). Protection is also provided by macrophages, killer T-cells and IgA. Early microbial attack of mucous membranes assumes several forms: attachment and multiplication without significant penetration; attachment to, and penetration of, mucosal cells in which the pathogen multiplies with little or no spread from the initial site; and attachment and penetration into the

underlying tissues (115).

1. Host and Tissue Specificity

Two of the most striking and poorly understood phemomena in microbial pathogenicity are host specificity -- the ability of microbes to attack some animal species in preference to other -- and tissue specificity -- the ability of microbes to attack some tissues in preference to other (113, 114). The specificities are determined by variations between species or tissues of the environment the host provides in relation to microbial proliferation. The most known host influences are: the nutritional environment, the nature and strength of the humoral and cellular defense mechanisms, and, in relation to the route of entry of the pathogen, the existence of host receptors for initial attachment.

There are a number of examples of selective adherence of bacterial pathogens to tissues. For example, pathogenic strains of Streptococcus pyogenes were found to attach to human pharyngeal cells better than E. coli, thus correlating with the ability of the former but not the latter to infect the oral cavity (39, 51). With regard to host specificity, S. pyogenes, which rarely infects rodents, has been found to attach more strongly to human than to rodent buccal epithelial cells (53). Also, S. salivarius and S. sanguis, which are found naturally in humans but not in rats, adhere better to human tongue cells and less well to rat tongue

cells than S. faecalis and a serum-requiring diphtheroid, both of which are found naturally in rats (55). The result of my study showed that the Jo 2 strain of L. pneumophila had greater affinity than the Chi 8 strain for all three cell lines tested, among which Hep-2 was the least associated with Legionella. This may indicate that REF and WI-38 have similar or identical surface receptors which attract Jo 2, an idea worth further pursuit.

2. Effect of Additives on Attachment

According to Ofek and Beachey (89), ligand-receptor interactions play an important role in the binding of bacteria to surfaces of tissue cells of the host. In general, the chemical nature of bacterial ligands has been found to be (a) protein in certain strains of E. coli (109), gonococci (22), and mycoplasma (67); (b) the lipid portion of glycolipid molecules such as lipoteichoic acid in group A streptococci (12); and (c) sugars and other carbohydrates in cariogenic streptococci (57). Sugar moities also have been identified as bacterial receptors on animal cells (89); those include residues of mannose for certain E. coli strains (88), fucose for Vibrio cholerae (73) and sialic acid for Mycoplasma spp. (27). Apparently the sugar residues are components of either glycolipids or glycoproteins in the cell membrane.

Results (Table VIII) of this study showed that attachment of Legionella was the best when 2DG had been added to the culture, especially when the exposure period was 24 hours rather than being continuous. The next best facilitators of attachment were L-glucose and pyruvate. This may suggest that 2DG, L-glucose, and probably pyruvate, serve as bridges between cell membrane receptors and bacterial ligands i.e., both cells and bacteria have affinity for these sugar moieties and pyruvate. Conversly, attachment of Legionella was almost entirely inhibited by D-galactose and D-mannose, probably because these two sugars compete for either receptors or ligands; both treatment modalities (24 hours and continuous) showed comparable inhibitory effects.

3. Other Factors Affecting Attachment

In my study, the Chi 8 strain of L. pneumophila was found to be less able to attach to the animal cells than was Jo 2 strain. This is interesting since that Chi 8 strain is a clinical isolate, while Jo 2 is an environmental one. A reasonable explanation for this difference is that Chi 8 may lose some of its pathogenic determinants, particularly those affecting cellular adherence, during prolonged passage in vitro. Loss of virulence of legionellae upon subculture in bacteriologic media and its restoration upon animal passage has been recognized (19, 84). Numerous investigators have shown that various species of bacterial pathogens differ

TABLE VIII

SUMMARY OF PEAK EFFECTS OF CHEMICALS ON BACTERIAL ATTACHMENT

Cell	Legionella	D-mannose				D-galactose			
		24 hrs		continuous		24 hrs		continuous	
		1 mg	5 mg	1 mg	5 mg	1 mg	5 mg	1 mg	5 mg
REF	Jo 2	-	-	-	-	-	-	-	-
	Chi 8	-	-	-	-	-	-	-	-
Hep-2	Jo 2	+++	++++	-	-	-	-	-	-
	Chi 8	-	-	-	-	0	-	-	-
WI-38	Jo 2	-	-	-	-	+	-	+	++
	Chi 8	-	-	-	-	0	-	-	-

Cell	Legionella	D-glucose				L-glucose			
		24 hrs		continuous		24 hrs		continuous	
		1 mg	5 mg	1 mg	5 mg	1 mg	5 mg	1 mg	5 mg
REF	Jo 2	-	-	-	+++	+++	+	-	-
	Chi 8	-	-	-	-	++++	+++	++++	+++
Hep-2	Jo 2	-	-	+	-	+++	+	-	-
	Chi 8	-	-	-	-	-	-	-	-
WI-38	Jo 2	0	++++	-	-	+++	++	++	+
	Chi 8	+	+	-	-	++	+	-	-

++++ 4-fold or more greater than control
+++ 3-fold greater than control
++ 2-fold greater than control
+ slight effect
0 no effect
- inhibition

TABLE VIII -- Continued

Cell	Legionella	2-deoxy-D-glucose				Pyruvate			
		24 hrs		continuous		24 hrs		continuous	
		1 mg	5 mg	1 mg	5 mg	1 mg	5 mg	1 mg	5 mg
REF	Jo 2	++++	++	++++	++	+	+++	+	-
	Chi 8	++	+++	+	-	-	-	-	++
Hep-2	Jo 2	++	++++	+	+	+	++	0	+
	Chi 8	+++	++++	+++	+++	-	-	-	+
WI-38	Jo 2	++	++++	+	+	-	-	++++	++
	Chi 8	++	++++	++	+	-	-	+	-

Cell	Legionella	β-D-(-)fructose			
		24 hrs		continuous	
		1 mg	5 mg	1 mg	5 mg
REF	Jo 2	0	0	+	-
	Chi 8	-	-	-	-
Hep-2	Jo 2	-	+	0	0
	Chi 8	-	-	-	-
WI-38	Jo 2	-	+++	-	+
	Chi 8	-	-	-	-

chemically and biologically when grown *in vivo* (112). These facts suggest that artificial subcultivation would affect adversely the attachment ability of both *Legionella* strains I studied, especially the clinical isolate, Chi 8.

Another factor that may have affected attachment in my study is trypsin since this enzyme has been demonstrated to remove some proteins from the surface of bacteria and animal cells (2). Thus, trypsinization, used to disperse the cell monolayers, may damage both cell receptors and bacterial ligands which interact in the adherence process.

Effect of Growth Condition on Viability of *Legionella*

As can be seen in Table VII and VIII, neither *Legionella* strain survived long during incubation in the tissue culture medium, whether animal cells were present or not. As a matter of fact, no bacteria were observed by immunofluorescence after two weeks of incubation.

The inability of *Legionella* to survive in tissue culture or in tissue culture media could resulted from : (a) suboptimal nutritional or other culture conditions; and (b) effects of trypsinization. The pH of the tissue culture medium employed for culturing the REF, Hep-2 and WI-38 cell lines was maintained between 7.2 and 7.4 during incubation because this range is optimum for most animal cell cultures. However, *L. pneumophila* grows optimally *in vitro* at a pH of 6.9 (108), the pH of CYE agar used to maintain *L. pneumophila*.

Also, L-cysteine and ferric ion are especially critical nutrients in media designed to support growth of L. pneumophila (43). Neither of these compounds are among the ingredients in BME or MEM, nor is L-cysteine among the free amino acids found in bovine serum (95).

Trypsin, as mentioned above, may also contribute to the failure of the organism to grow in tissue culture. Since it destroys receptors, bacteria could be detached and lost while replacing the spent medium. Also, trypsin may be toxic to the bacteria.

Cytopathic and Growth Effects of Legionella pneumophila on Animal Cells in Culture

Elongation of REF cells and microvillus formation were the only clear morphological responses observed, either by light or electron microscopy, in the tissue cultures inoculated with L. pneumophila. In general, there was no difference in cytopathic effect between the strains of Legionella except that Jo 2 had more pronounced effect on the microvillus formation than the Chi 8 strain. Rodgers et al. (103) noted the production of microvilli by animal cells infected with this organism. They believed that internalization of organisms was facilitated by the microvilli. They noted further that cell surfaces of tissue cultures without bacteria were comparatively smooth. Their findings are consistent with those of my study. In addition, some of my photomicrographs revealed adhesion of the legionellae to the

animal cell membranes, in one instance apparently by means of surface fibrils, possibly pili.

Besides causing morphological aberrations, growth of tissue culture cells were somewhat retarded by Legionella infection (Figures 52, 53 and 54). The infected cells had a shortened stationary phase, and their density dropped faster than the uninfected control cultures. In other words their numbers decreased more rapidly than the controls if not subcultured. However, if regularly subcultured, the infected cultures readily grew and the bacteria disappeared. The mechanism of retardation of growth was not investigated. Possibly Legionella produced toxins which may have affected metabolism of the host cells. Such toxins, as well as the bacteria, could have been greatly diluted following media changes and successive passages.

Postscript

Tissue culture provides a convenient model for studying the host-parasite relationship. This is a little used system for studying the cytopathogenicity of L. pneumophila. No doubt tissue cultures will be utilized increasingly in future studies of this unusual pathogen which has such obscure pathogenesis.

Intracellular multiplication of L. pneumophila in vitro in protozoa as well as vertebrate cells in cultures, has been reported (32, 103). This was not clearly established in

my study. This may due to a diminution in virulence of the "domesticated" strains of Legionella. In other words, they had lost their ability to multiplicate intracellularly through many passages on an artificial medium. To a minor extent, attachment and penetration were observed. Considering the fact that Legionnaires' disease is an intracellular infection with high mortality, one might have expected a greater degree of adherence and cell invasion than seen in this study.

Perhaps it would have been wise to have passed the two strains through a suitable experimental animal, thereby recovering cultures with enhanced virulence for use in the tissue culture model. Also, short-term growth studies avoiding trypsin should be considerder in the future. Another area requiring further exploration is the development of a tissue culture medium capable of supporting the growth of legionellae.

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