THE ASSOCIATION OF L-FORMS OF GROUP A STREPTOCOCCI WITH MAMMALIAN CELLS CULTURED IN VITRO

NEIL HUNTER B.D.S.

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GENERAL SUMMARY

There has been a considerable interest in the role of L-forms of beta-haemolytic streptococci in disease processes, for example, rheumatic fever. Central to any role in pathogenicity is, however, the ability of the L-forms to persist *in vivo*.

It was decided to investigate the possibility of cell-associated survival and/or persistence in an *in vitro* model system. The L-form strains used in this study were those that met the basic requirement of ready growth in broth culture. Attempted infection of a number of cell types was carried out under a variety of experimental conditions. The interactions of L-forms with cells were monitored by various techniques, including acridine orange fluorescence, fluorescent antibody staining, electron microscopy, quantitative adsorption studies and autoradiography.

The results pointed to a low frequency interaction of the L-forms with the cells but there was no evidence of cell-associated survival. Autoradiographic and other studies indicated that the L-forms were less readily taken up by macrophages than were the parent streptococci.

Overall, the results in this study did not fully support the concept that L-forms can survive in association with cells.

DECLARATION

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any University, and to the best of my knowledge contains no material previously published by another person, except where due reference is made in the text.

NEIL HUNTER

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GENERAL INTRODUCTION CHAPTER I

INTRODUCTION

Associated with beta-haemolytic streptococcal disease in humans are a number of phenomena that remain unexplained, including the post-streptococcal sequelae, rheumatic fever and acute glomerulonephritis. These diseases are particulary interesting because according to general opinion, the lesions are sterile in the sense that the causative microorganisms cannot be isolated from them.

This chapter contains a discussion of some of the postulated mechanisms involved in these diseases, with emphasis on findings relating to possible L-form involvement. This is followed by a consideration of certain aspects of L-form biology and a discussion on general *in vivo* and *in vitro* observations of host-parasite relationships involving L-forms of various bacterial species. The theme of the present study is based on the conclusions reached from this literature survey and the final section of this chapter describes the background to the experimental model used.

ACUTE GLOMERULONEPHRITIS

Kuttner and Lancefield (1970) have outlined the more widely accepted theories concerning the pathogenesis of this disease. Basically, these imply the lodgement of immune complexes within the glomeruli of nephritis-prone individuals or a cross reactivity between glomerulus and streptococcal component(s).

Markowitz and Lange (1964) found that soluble fractions obtained from pooled human glomeruli and the cell membrane of nephritogenic streptococci were immunologically cross-reactive. Other studies have produced evidence showing that this property could play a role in the pathogenesis of streptococcal induced nephritis. For example, Treser et al. (1971) found that fluorescein-labelled gamma-G fractions of patients with acute nephritis stained parts of the glomeruli of the same patient's renal tissue. Only proven nephritogenic streptococci blocked this staining. Streptococcal cell membranes from nephritogenic streptococci absorbed the antibody to almost the same degree as did disrupted whole streptococci, whereas membranes from other strains did not. This implied that there was an antigenic relationship between the cell membranes of nephritogenic streptococci and a glomerular component.

In another study (Markowitz et al., 1971), acute glomerulonephritis was actively induced in rhesus monkeys by immunization

with alum-precipitated soluble glycoprotein from cell membranes of nephritogenic streptococci. In addition, passive induction was achieved by intravenous injection of rabbit anti-glomerular protein antibodies. In both cases the disease was of moderate intensity and relatively short in duration; this was possibly due to the crudeness of the preparation, as it was found that only twelve percent of the immunogen employed was cross-reactive with monkey glomeruli basement membranes.

These studies emphasize the possible importance of the streptococcal cytoplasmic membrane in the pathogenesis of the disease.

RHEUMATIC FEVER

This disease occurs only as a sequel to repeated attacks of group A streptococcal pharyngitis by different M types (Kuttner and Lancefield, 1970). In acute rheumatic fever the connective tissue of the pericardium, the myocardium and the endocardium are involved in a generalized inflammation (Smith et al., 1959). The characteristic lesion of rheumatic carditis is the Aschoff body (Smith et al., 1959), which is a characteristic and specialized focus of chronic inflammation.

There have been a number of hypotheses put forward as to the basic pathologic mechanisms operating in this disease, including toxin action, antigenic relationship between the strepto-

coccus and mammalian tissue and hypersensitivity (Kuttner and Lancefield, 1970). In addition, Ginsburg et al. (1969) have advanced an hypothesis combining toxic action with the persistence of refractory microbial components within the chronic inflammatory foci. These theories are expanded in the following sections.

Toxic Action

The group A streptococcus may produce over twenty extracellular products in vivo, as judged by the immune response (Halbert, 1968). Some of these have been studied in detail, for example streptolysin O. Intravenous or paratonsillar injection of this toxin in rabbits or mice results in the production of cardiac lesions and there appears to be some tissue trophism, or at least differential tissue susceptibility to it (Halbert, 1968). The toxic action has been further studied in tissue culture by Thompson et al. (1970), who demonstrated direct cellular toxicity.

There are, however, problems in postulating a role for streptolysin O in the pathogenesis of rheumatic fever. The disease occurs only after multiple infections with different M types (Kuttner and Lancefield, 1970). Halbert (1958), has produced some evidence that streptolysin O is immunochemically similar or identical for different beta-haemolytic streptococci.

For example, partially purified group C lysin O seemed similar to a group A concentrate when run on an electrophoresis strip; in addition, the precipitin band in agar against human gamma-globulin was continuous for streptolysin O from both a group A and a group C streptococcal strain. It is probable, therefore, that a secondary antibody response would quickly neutralize any streptolysin O. Halbert et al.(1961) have suggested, however, a mechanism whereby circulating streptolysin O-antibody complexes dissociate slowly, allowing a build up to toxic levels of streptolysin O in susceptible tissues.

Streptolysin S has been similarly studied, especially so since it appears to be non-antigenic; in addition, it has been shown to possess a wide range of toxic effects (McCarty, 1972). There is, however, no direct experimental evidence to implicate this toxin in the pathogenesis of rheumatic fever.

Cross-Reacting Components

There are now several reports in the literature of antigenic relationships between some component(s) of the group A Streptococcus and mammalian tissue. For example, Kaplan (1963) noted that when streptococcal cell-wall antigen, prepared by an acid extraction procedure, was used to immunize rabbits, the resulting antisera were found by an immunofluorescence technique to react

with human cardiac myofibres, smooth muscle of blood vessel walls, and in some cases the endocardium. There was also staining of skeletal muscle. Only seven strains of fifty-two tested proved positive.

Zabriskie and Freimer (1966), using immunofluorescence, observed that antibody against isolated cell membranes of a wide variety of serological types of group A streptococci reacted with skeletal and cardiac muscle in addition to the smooth muscle of blood vessel walls. Rapaport et al. (1966) found that prior injection of 5.0 mg of purified group A cell membrane caused fifty of fifty-eight guinea pigs to exhibit accelerated homograft rejection. Group A streptococci were active as transplantation antigens in the mouse, rat and rabbit as well as in the guinea pig.

Antigenic relationship between human and bovine heart valves and group A polysaccharide was described by Goldstein et al. (1967). Antisera to group A streptococci were precipitated by valvular extracts and antivalvular antisera were precipitated by group A specific polysaccharide. Schwab (1971) showed that immunization of inbred mice with group A streptococcal protoplast membranes resulted in the production of antibody that reacted exclusively with nuclear material of isologous and homologous animals. This cross-reactive antibody was found in the serum of over two-thirds of the immunized mice. Nuclei from all cell types tested showed

homogeneous fluorescence when stained by the indirect fluorescent antibody technique. The mouse reactive antibody was completely absorbed by homologous group A membranes. Kingston and Glynn (1971) studied the properties of antisera raised in rabbits to whole group A streptococci grown in semi-synthetic medium. One serum contained no detectable antibodies to the sarcolemmal/ subsarcolemmal region of the myocardium, but gave strong immunofluorescent staining of fibroblasts, endothelial cells, smooth muscle and pia arachnoid. In addition, there was staining of astrocytes and of the stratum granulosum of the skin. Antisera to other strains also gave these reactions as well as staining of the sarcolemma. It is interesting that the tissues found to be cross-reactive are those involved in the clinical disease. The cross-reactivity of membrane components with kidney tissue was discussed under the heading of nephritis.

The cytotoxicity of these cross-reacting antibodies has recently come under scrutiny. For instance, Thompson and Halbert (1971) studied the cytotoxicity of anti-heart antibodies for pulsating rabbit and rat heart cells in tissue culture. They found that potent anti-rabbit heart antibodies in the presence of rabbit complement were without cytotoxic effects on beating rabbit heart cells. The same cells, however, were profoundly damaged by duck antibodies to rabbit heart in the presence or absence of complement. Rabbit antisera to rat, human or guinea

pig heart, all of which contained multiple auto-antibodies to rabbit heart, also failed to exert cytotoxic action on rabbit heart cells in the presence of complement. Rabbit anti-rat heart antibodies, however, were profoundly cytotoxic for rat heart cells, an effect dependent upon complement. The authors pointed out that human anti-heart antibodies may exist without detriment to the host.

Zabriskie (1970) confirmed an immunological relationship between the cytoplasmic membranes of group A streptococci and cardiac, skeletal and vascular smooth muscle of humans, rabbits and guinea pigs. Heart-reactive antibodies could be demonstrated in patients with uncomplicated streptococcal disease and in rheumatic fever patients. The rheumatic individuals always had higher titres and there was good correlation between a rise in antibody level and the onset of rheumatic fever. Case studies indicated that subsequent attacks were associated with a rise in heart-reactive antibody, but the antibody level declined over one to two years following the attack. Post-cardiotomy patients often have demonstrable heart-reactive antibody without apparent consequences. According to Zabriskie, however, this antibody appears to be unrelated to that arising following streptococcal infection.

It appears, therefore, that although the pathogenic significance of the antigenic relationship between the streptococcal

membrane and mammalian components remains to be determined, there is a growing body of evidence that indicates the widespread nature of this phenomenon.

Persistence of Refractory Microbial Components

Ginsburg et al. (1969) have advanced an hypothesis for post-streptococcal sequelae. Initial insult may occur by toxic damage to the target tissue. This could be followed by lodgement of streptococci or streptococcal components in the damaged area. Most of this material is probably carried in macrophages from the focus of infection. As already mentioned, the ability of streptococcal toxins to produce direct cardiac damage is well documented (Ginsburg, 1972). Glick et al. (1971) have demonstrated in a tissue culture model that after phagocytosis of streptococci by human monocytes, the streptococcal cell wall remains relatively intact even up to death of the phagocyte. Ginsburg (1971) showed that, following needle trauma or streptolysin O-induced heart damage in rabbits, injected streptococci or titanium dioxide particles localized in the damaged area. Most of these appeared to be localized in phagocytes. Further studies (Rickles et al., 1969) indicated persistence of fluorescein-labelled streptococci within phagocytes in inflammatory granulomata or draining lymph nodes. At this site, release of refractory material and the subsequent immunological and inflammatory reactions could well account for the histological picture seen in the post-streptococcal disease.

L-FORMS OF BETA-HAEMOLYTIC STREPTOCOCCI

In 1935, Klieneberger described variant organisms in a culture of Streptobacillus moniliformis. The term L-form was given, the "L" representing the Lister Institute where she worked. It was later shown that these forms had arisen spontaneously from the streptobacilli. L-forms have now been described for most families of bacteria and they may be considered as variant organisms that have no rigid cell wall and that possess the potential to revert to the parent organism. The basic biology of L-phase variants has been well reviewed by Hijmans et al. (1969). Transition from the bacterial phase to the L-phase is determined by the characteristics of species and strain, and by experimental conditions. There are three steps involved:

(1) The breakdown or interference in synthesis of the bacterial cell wall.

(2) Prevention of osmotic lysis.

(3) Multiplication in the L-phase.

Inducers include such diverse agents as antibiotics which have their action at the cell wall, phage associated lysin and other muralytic enzymes, high concentrations of glycine and the antibody-complement-lysozyme system.

L-forms of Gram-positive organisms require osmotic protection equivalent to twenty to thirty atmospheres. There are, however, membrane stabilizers, such as magnesium ions and spermine (Hijmans et al., 1969).

Cell division is thought to occur by a poorly controlled binary fission, there being no cell wall or mesosomes to regulate the process. The release of elementary bodies (0.1μ - 0.3μ diameter) from large forms probably occurs as well but some of these small bodies appear not to contain deoxy ribonucleic acid (DNA) and are therefore abortive elements.

Reversion to the parent form may be obtained by environmental changes, such as omission of the inducing agent, but it usually becomes more difficult as the number of passages in the L-phase increases. On initial induction, a solid medium containing 0.7 - 1.2 percent agar is required, together with adequate osmotic stabilization provided by a suitable agent, such as sodium chloride or sucrose. Horse serum at ten to twenty percent final concentration is also required for optimal growth. Transfer to broth culture is usually difficult.

Variation in properties of cell wall defective bacteria

McGee et al. (1971) have described the various cell wall defective forms. Important in their classification is the distinction between transitional phase variants, which do not propagate serially in a distinctive form on agar, and L-phase variants which give the classical "fried egg" colonies on serial propagation. Stable L-phase variants do not revert to the parent form under any conditions.

Godzeski et al. (1967) have defined L-phase variants as "reverting unstable forms" and L-forms as "stable non-reverting forms". They studied in vivo induction and infectivity of variant forms of various bacteria. Staphylococcus aureus or Escherichia coli injected intraperitoneally into mice produced a chronic infection of spleen, lung, kidney or liver. Over three to four weeks, with or without antibiotic therapy, organisms persisted, and cultures from a ten percent sucrose homogenate of tissues invariably yielded both L-phase and parent bacterial forms. However, staphylococcal L-forms did not infect mice. The authors therefore postulated differences between L-forms and L-phase bacteria (according to their definition). The properties of the organisms were thought to depend on the mode of induction as well as environmental factors.

Most authors are careful in their definition of variant forms; this seems to be essential while there is, as yet, no universal agreement on terminology. In the bulk of the literature, however, the terms "L-forms" and"L-phase variants" are used synonomously.

L-forms are thought in general to be metabolically similar to the parent form (Hijmans et al., 1969); for example, the L-forms of group A streptococci produce at least some of the toxins produced by the parent organisms.

The L-form Membrane

The possible importance of the streptococcal membrane in the aetiology of certain disease processes, has already been mentioned and it would therefore seem important to establish the immunological relationship between this membrane (that is, the protoplast membrane), and that of the stabilized L-form. Lynn and Muellenberg (1965) have carried out such a comparison using a modified latex agglutination test to compare immunological responses. Rabbits were immunized with streptococci, stable L-forms and protoplasts. Cross reactivity was demonstrable between fragmented streptococcal preparations and L-forms. Protoplasts aligned more strongly with streptococcal preparations than with L-forms. It was not possible to completely absorb any of the antisera with heterologous antigen; the authors considered that this probably reflected antigenic "masking" rather than differences in antigenicity.

The differences found between L-forms and protoplasts are not surprising in view of the findings of Cohen and Panos (1966) who compared membrane lipids from protoplasts and L-forms of group A streptococci. Significant quantitative differences were found. Hijmans and Madoff (see Hijmans et al., 1969) were unable to detect new antigens in L-forms of group A streptococci and a similar result was obtained by Havlíček (1968). It would seem likely, however, that quantitative antigenic changes could occur in transition to the stable L-form.

THE ROLE OF L-PHASE BACTERIA IN HOST-PARASITE INTERACTIONS

Human Isolations

Wedum (1970) grew biopsied and surgical specimens of hearts from patients with acute rheumatic fever for periods of one week to six months, culturing sixty specimens in all. From these a total of eight yielded L-forms; these reverted to non-haemolytic cocci or diphtheroids. The author considered that the source of these organisms could have been the serum or nutrient media.

Godzeski et al. (1968) cultured specimens of myocardium taken from patients undergoing cardiac surgery for congenital or acquired rheumatic heart disease. Tissue slices or tissue homogenates were prepared in ten percent sucrose solution. Specimens were considered positive for L-phase bacteria when classical bacteria were not grown out in the usual manner and when osmotically stabilized media gave positive L-phase growth. L-phase organisms were isolated from six of nine specimens from rheumatic heart disease and none from congenital heart disease. Multiple transfers were usually necessary to obtain reversion back to classical parent forms which comprised staphylococci, enterococci, Gram-variable rods and Gram-negative rods. Kagan (1968) found that biopsies taken from twenty rheumatic fever patients yielded thirteen positive for L-forms, four positive for transitional forms and two positive for alpha- and beta-haemolytic streptocci. Usually, typical L colonies were obtained in the second passage on media containing an inducing agent and reversion was often possible.

In Vivo Studies of L-Phase-Host Interaction

Host Resistance Factors

Kalmanson et al. (1968a) have demonstrated a cidal action of normal sera for a variety of "protoplasts". Sera tested included rat, pooled human and horse serum. The effect was concentration-dependent and inhibited by heating the serum to 56^{o}C for thirty minutes. It also tended to be inhibited at high osmolarities. Streptococcus faecalis, Strep. pyogenes, Staph. aureus and E.coli "protoplasts" were susceptible, whereas the parent bacteria were not. "Protoplasts" of Proteus mirabilis were not sensitive. McGee et al. (1972) made similar observations but found the L-phase of Pr. mirabilis to be susceptible. Killing was found to be remarkably temperature dependent. Absorbed sera containing complement failed to kill homologous species but would still kill others; antibody mediation was therefore postulated. The authors suggested that antibodies arise as a result of common infection by the parent strains of the L-phase organisms tested. L-phase killing was shown by electron microscope studies to be accompanied by lysis and fragmentation of the cytoplasmic membrane. It may be concluded that normal human sera possess a very effective cidal system for L-phase bacteria which in some cases is antibody dependent but in others is not obviously so.

Clasener et al. (1970) found that L-forms of group A streptococci and Strep. faecalis, adapted to a low requirement for osmotic protection, fared no better than those with high requirement for osmotic protection following intravenous or intraperitoneal injection in mice. However, the "low salt" strains, that is, those adapted to a low requirement for osmotic protection, were protected in intraperitoneally inserted filter boxes, and could be isolated for up to eight days following implantation. They concluded that phagocytosis was a significant factor in the clearance of L-forms in vivo. Another finding was that fresh rat serum was toxic for the L-forms, whereas old serum was not. Complement was apparently not involved and the action was not lethal because growth did occur after prolonged incubation.

Pathogenicity and Persistence In Vivo

Wittler (1968) has reviewed in vitro and in vivo studies on L-phase bacteria. The most significant overall finding was that L-forms are classically pathogenic only if they revert in vivo to the parent organism. By "classically pathogenic" is meant that organisms when introduced into a host will multiply and liberate toxins and other cell products to the detriment of the host.

In support of Wittler's summation was the observation by Freundt (1956) that L-forms of Streptobacillus moniliformis produce, upon intraperitoneal injection into mice, a latent infection which persisted until reversion occurred. The onset of symptoms coincided with the appearance of the Streptobacillus in the blood. Wittler found that in mice inoculated intranasally with the unstable L-phase of Haemophilus pertussis, death of the animals correlated well with the appearance and rapid multiplication of the bacillary form.

An exception to the findings of Wittler (1968) would appear to occur in the case of the Clostridia. Scheibel and Assandri (1959) found that L-phase variants, isolated by means of penicillin, from four toxigenic strains of Clostridium tetani, retained their capacity to produce tetanus toxin. The amount of toxin produced was of the same order of magnitude as that produced by the parent strain. This example is an unusual one, however, in that the pathogenicity does not relate to persistence of the microbe in the usual sense.

Mortimer et al. (1972) studied mice dying of group A streptococcal sepsis. L-forms could be recovered, but always in association with the parent organism. As an example of the quantitative relationship between parent and L-form, twenty-two hours after intraperitoneal inoculation of group A streptococci, 2×10^6 parent colonies were recovered as against 1.3×10^3 L-form colonies. Clasener (1970) raised a criticism of such

isolation studies because she found under certain conditions that the penicillin in the L-form agar could act as an inducing agent. Mortimer et al.(1972) reported, however, that after millipore filtration, peritoneal washes still yielded L-form colonies, that is even though any streptococci had been removed. The L-forms did not appear until late in the infection and the number of L-form units found seemed to correlate inversely with the mouse virulence of the parent strain. No potential in vivo inducers could be found, disrupted or undisrupted lysozomes from rabbit and human leukocytes had no effect in vitro nor did agents such as thorium dioxide, which depresses phagocytic function in vivo. Hydrocortisone, which stabilizes lysosomal membranes in vivo, also did not have any bearing on the number of L-form colonies produced. The administration of penicillin to mice did not apparently result in induction of L-forms for it was found that the number of L-form colonies decreased proportionately with the number of streptococcal colonies following administration of the antibiotic. Peritoneal exudate fluid apparently contained an unidentified stabilizing factor which enabled the L-forms to survive in what is, in effect, a hypotonic milieu. In mice with retained latent foci of infection, both streptococci and L-forms were isolated and in the same proportions as in the case of acute infections, that is, there was no evidence that L-forms functioned as persisters. The authors failed to demonstrate that L-forms, on intraperitoneal injection into mice, were infective.

Cook et al.(1969) injected group A streptococci and their L-forms into the knee joints of rabbits. Streptococci, L-forms and heat killed organisms produced, on single or multiple injections, a graded response from no detectable change to generalized arthritis with synovial hypertrophy, dense cellular infiltration with lymphocytes, plasma cells and giant cells in the case of L-forms only. In addition, there was pannus formation with destruction of the joint cartilage. Healing occurred by fibrosis with patches of cellular infiltration remaining. Arthritis induced by parent organisms tended to be more severe and to involve the whole joint. Overall, greater than fifty percent of injected joints showed some degree of pathological change. Protoplast membranes gave a low incidence of the L-form type lesions. Only two of thirty two animals given multiple injections of L-forms produced demonstrable antibody. L-forms were never recovered from the joint and the parent organisms were rarely recovered after they were injected. The authors suggested that the mechanism of this arthritic change might have some relationship to the ability of L-forms of group A streptococci to transform lymphocytes (Cook and Fincham, 1969).

Kagan (1968) has studied L-forms in relation to the reaction of animals directly after infection, cytopathic effect in tissue culture, sensitizing properties and experimental models of pathologic processes. The Shwartzman reaction was chosen as an

example of sensitizing properties. The immunological basis for this reaction is unknown but the phenomenon is characterized by the presence of leukocyte-platelet thrombi, particularly in blood vessels. Classically, the reaction is induced by injection of endotoxins (Davis et al., 1968a). In Kagan's work, the Shwartzman reaction could be demonstrated to occur following injection of either the L-form or the parent Streptococcus, but the reaction was stronger in the case of the parent organism. Kalmanson et al. (1968b), using the stable L-form of Pr. mirabilis, found that it also could induce the Shwartzman reaction in rabbits. In addition, Kagan (1968) found that group A streptococci or their L-forms injected intravenously or paratonsillarly, caused angina with evidence of cardiac inflammation in monkeys. Neither L-forms or bacteria were readily cultured.

Schmitt-Slomska et al. (1967) studied the infectivity for mice of two strains of group A streptococci and their stable L-forms. The strains were the Richards GIII type 3 and the GL8 strain. Reversion could be induced in the L-forms by subculture on media containing egg-yolk extract. The parent streptococci were identified by microscopic examination, colony type, serological testing and fluorescent antibody labelling. Thirty mice inoculated intraperitoneally with one thousand bacteria were dead or dying after twenty four hours, with organisms isolated from the blood in every case. Not one of one hundred and twenty six mice,

conventional or germ free, died after intraperitoneal or intravenous inoculation of L-forms. L-forms administered intravenously were isolated only in the first hour; intraperitoneally inoculated L-forms could, however, be isolated from almost all inoculated animals, usually from both blood and peritoneal cavity, for up to twenty five days at which time the experiment was terminated. Newly-isolated forms were atypical and slowly changed to yield classical "fried egg" colonies on agar. Twenty four hours after injection of L-forms, some bacterial cocci, occasionally in chains, appeared. These proved difficult to culture, but could be grown in yolk sac. After many subcultures they reverted fully to group A streptococci. Levels of L-forms apparently remained approximately the same over twenty five days, indicating that some division probably occurred.

Rickles et al. (1969) observed that fluorescein-labelled streptococci, but not L-forms, persisted within phagocytes in inflammatory granulomata or draining lymph nodes.

Bentwich et al. (1968) found that the myocardial reaction of rabbits to intramyocardial injection of living streptococci and isolated mucopeptide was essentially similar, being a granulomatous lesion. Myocardial lesions induced by L-forms were, however, considered non-granulomatous, the characteristics being early fibrosis, mononuclear cell infiltration and some accumulations of multinucleated giant cells.

In Vitro Studies of Host Cell-L-Phase Interaction

Phagocytosis of L-forms of *Strep. faecalis* and *E. coli* by human polymorphonuclear cells has been studied by Harwick *et al.* (1972). Employing acridine orange staining, the authors were able to differentially stain cell cytoplasm and organisms. Phagocytosis was carried out on cellulose-ester filters and it was noted that bacterial forms were always more readily engulfed than were their derived L-forms. This correlated with polymorphonuclear migration in Boyden Chambers where L-forms were found to be less chemotactic.

Spector *et al.* (1970), studying phagocytosis of heat-killed radioactive iodine-labelled organisms, observed ready phagocytosis of a group A streptococcal L-form. The system used was an *in vitro* model with mouse peritoneal macrophages as the phagocytes. Degradation of organisms was assessed by the release of low molecular weight labelled material from the phagocyte. Somewhat surprisingly, intracellular L-forms were degraded at a slower rate than were the parent streptococci. In fact the pattern of degradation of the L-form followed more closely that of such refractory bacteria as *Bordetella pertussis* and *Mycobacterium tuberculosis*. A possible reason for

the difference from the findings of Rickles et al. (1969) was the nature of the components labelled in each case.

Kagan (1967) compared the response of tissue culture cells to mycoplasma and L-phase bacteria. Most of the tested L-forms and mycoplasma exerted a cytopathic effect on chicken embryo fibroblasts. In the case of L-forms of haemolytic streptococci, heavily destroyed areas in the monolayer with cell vacuolization and degeneration appeared after twenty four hours.

In another study by Schmitt-Slomska et al. (1968) the stable L-form of Richards GIII type 3 was used to infect human diploid cells in culture. The tissue culture medium used did not support growth of the L-form. Diploid cells and L-forms were incubated in suspension for one hour at 37^{o}C, and at this time fluorescent antibody staining showed about ten percent of cells to have associated organisms that could be isolated as typical L-form colonies. Later, organisms were found associated with about half the cells and were isolated as atypical colonies. However, after ninety days the colonies were again typical and could be subcultured. An increase in the percentage of infected cells indicated that there was some multiplication of the L-forms which were always found to be cell-associated, extracellular forms being associated with cellular debris. No lesions were detected in the tissue culture cells.

More recently, using a similar tissue culture model, Schmitt-Slomska et al. (1972) demonstrated the induction of L-variants in human diploid cells infected by the GL8 strain of group A streptococci. If the bacterial inoculum was too large, the cells were overgrown and destroyed but at optimal ratios of bacteria to cells, the organisms were phagocytosed and progressively converted to atypical intracellular forms. At twenty four to forty eight hours, typical streptococcal colonies, typical L-form colonies and intermediate forms could all be isolated. The L-forms isolated at this stage could be propagated on agar but an eclipse phase followed until twenty five to thirty days after inoculation when variant forms could again be isolated. These, however, failed to propagate on agar.

CONCLUSIONS

Several important points emerge from this survey of the literature:

1. The cytoplasmic membrane of the group A streptococcus has obvious potential pathogenic importance. L-forms, of course, are not unique in this but it nevertheless strengthens their candidature as disease-producing agents.

2. The possible difference in properties between newly-induced and stable L-forms is of great practical importance, as much experimental work demands an organism which propagates readily in broth culture; this usually involves an extended period of transfers, which tends to produce stable forms.

3. The question of survival in the in vivo environment, which is probably osmotically unfavourable, is important. As already mentioned, Mortimer et al. (1972) described a stabilizing factor in peritoneal exudate fluid and Schmitt-Slomska et al. (1968) noted persistence intracellularly.

The bulk of opinion seems to be that L-forms of group A streptococci are incapable of infecting experimental animals or at least of producing demonstrable disease after infection. If they do play any role in disease it would seem to be that of a persisting organism, for example in the face of antibiotic therapy, or by participation in immunologically-mediated host damage. Another possibility is that of persisting in granulomatous lesions as described by Spector et al.(1970), who demonstrated refractory material in mouse peritoneal macrophages.

Against the developing literature background outlined in this chapter it appeared that one potentially fruitful line of investigation would be to study the ability of these L-forms to persist within the host, either as viable organisms or as refractory antigenic material.

THE EXPERIMENTAL MODEL

Initially it was decided to study the possibility of intracellular persistence of L-forms, the rationale being that organisms in this location would possibly be protected from the host defence mechanisms already described (see section on host defence mechanisms).

Another consideration is that of the osmotic environment. For example, Opie (1956) found that slices of liver, kidney and pancreas taken from experimental animals were in water equilibrium with salt solutions of more than twice isotonic strength, this being a property of the cells, not of the extravascular fluid. It could be concluded from this observation that some cell types may possess an internal environment which is osmotically favourable for survival of L-phase organisms (see section on L-forms - Biology).

Group A streptococci are not considered to be natural pathogens of laboratory animals although exceptions do occur, an example being the type 50 mouse epidemic strain described by Lancefield (1972). In addition, there are no really good animal models of post-streptococcal disease (Ginsburg, 1972; Thomas, 1972). Coupled with these observations is the fact that the intact animal suffers a number of disadvantages when used as an experimental system. These include:

1. Individual variation necessitating adequate sampling.

2. The mesenchymal defence system.

3. The tracing and subsequent identification of the introduced organism or product.

4. The difficulty of studying closely the interaction of organism or product and the target.

5. The ability of the animal to repair or replace damaged tissue.

It was decided therefore to study L-form - host interaction in a tissue culture model system, the rationale being similar to that of Moulder (1971), who described an L cell (a cell line derived from mouse embryonic fibroblasts) - Chlamydia psittacci interaction. Moulder defined a model as a host parasite system which was simpler than the natural infection but which retained some of its characteristics. The model should be simpler than the natural infection while retaining some of its important features, and from it, one should be able to make interpretations on a cellular and molecular level, and to predict some aspects of the behaviour of the complex host-parasite system in a new situation. Tissue culture as a model system in the study of infectious phenomena can be used to study intracellular killing and digestion of microorganisms by phagocytic cells and the effect of microbial metabolism on cells (Moulder, 1971).

The choice of cell types and their culture, together with the induction and growth of various strains of group A streptococcal L-forms, will be discussed in the following chapters.

CELL CULTURE CHAPTER II

INTRODUCTION

To avoid repetition, certain basic information, which is common to all the cell types described, has been collated at the beginning of this chapter. This is followed by descriptions of the various cell types used, together with cultural procedures.

Water Quality and Washing Procedures

To ensure a reliable high quality water supply for tissue culture, deionized - distilled water was purchased from Commonwealth Serum Laboratories, Melbourne. All glassware and coverslips were prepared for tissue culture by the usual procedures.

Media and Media Preparation

All media, unless stated otherwise in the text, were obtained from Commonwealth Serum Laboratories, Melbourne. The basic media, Eagle's basal medium and medium 199, were obtained as 10 times concentrates in 100 ml quantities and were stored

at 4°C. The supplements to these media, glutamine solution for Eagle's basal medium and solution D.G.P. (containing L-cysteine, glutathione, ascorbic acid, vitamin A, adenosine triphosphate, Tween 80 and ethyl alcohol) for medium 199, were stored at -20°C in suitable aliquots. Calf serum was stored at 4°C. Foetal calf serum was stored in suitable aliquots at -20°C. Buffers and sodium bicarbonate solution were stored at 4°C, while trypsin and trypsin-versene solutions were stored in aliquots at -20°C. Storage of serum and trypsin solutions in aliquot form eliminated the necessity for repeated freezing and thawing. Different batches of serum were available from Commonwealth Serum Laboratories and, for best results, it was necessary to test several of these and choose the one that was apparently best suited to the particular culture. The chosen batch could then be obtained in large quantity.

Media preparation was done on the open bench using the technique of flame sterilization commonly employed in bacteriology. Liquids were sucked into pipettes by mouth, but were never forcibly blown out for reasons discussed under the section on mycoplasma contamination in Chapter 3. In the case of media with an Eagle's basal medium base, fresh glutamine solution was added after about 2 weeks storage at 4°C. As a matter of policy, antibiotics were never included in media for tissue culture; the reasons for this will be discussed under the heading of contamination in Chapter 3.

Culture Manipulations

All manipulations were carried out at bench temperature, materials being either cooled or warmed to this level. General handling of cultures was carried out in a fully enclosed cabinet with glove ports for access. An ultraviolet lamp maintained sterility while the cabinet was not in use.

Storage of Cell Cultures

It was noted at an early stage, that, L cells at early confluence of the monolayer stored well at room temperature for up to 2 weeks. If necessary, the pH was adjusted with sodium bicarbonate solution. Diploid cells, on the other hand, did not respond favourably to such an interruption of the normal culture cycle. Both cell types stored well when deep frozen in liquid nitrogen. The procedure was to harvest cell monolayers in the appropriate manner into medium containing 10% sterile dimethyl sulphoxide (obtained from Merck Ltd. and sterilized by millipore filtration). About 2 ml of cell suspension containing approximately 5×10^6 cells, were then transferred to strong round-bottom ampoules, which were sealed and stored at $4^{o}C$. This was followed by freezing at $-20^{o}C$, $-70^{o}C$, and the ampoules were then lowered slowly into liquid nitrogen at $-196^{o}C$. The aim of this procedure was to obtain a slow cooling rate after the time of addition of the growth medium with dimethyl

sulphoxide. Although this procedure probably gave a cooling rate markedly different from the usually accepted optimal of 1°C per minute (Mazur et al., 1970), it was subsequently found that an acceptable proportion of the cells survived. To recover the frozen cultures, the ampoules were placed directly from liquid nigrogen into a water bath at 37°C. When they had warmed to this temperature the ampoules were opened under sterile conditions and poured into a 100 ml bottle. Twenty ml of warmed growth medium were added and the culture incubated at 37°C for 12 hours. The medium was then replaced and the cultures grown to confluence, usually within 5 days.

Sterilization Procedures for Tissue Culture

Leighton tubes and Rose chambers were sterilized with the coverslips inserted. The only plastic ware used was bought in sterile package form. For filtration sterilization, membrane filters of pore size 0.45 microns, obtained from Gelman Ltd., U.S.A. were used.

Culture Vessels

Cultures were maintained as monolayers in 100 ml flat soda-glass bottles with screw caps sealed with rubber discs. These bottles were also used to store prepared media. For experimental

purposes it was sometimes desirable to grow the cells as coverslip cultures either in Leighton tubes or Rose chambers. Most of the coverslips used were the "Gold Seal" type, produced by Clay Adams Ltd., New York. For cells with a high metabolic rate, such as primary heart cultures, polyester sheeting, 1/100" thick, could substitute for one of the coverslips in the Rose chambers. This material allows some gaseous exchange and so prevents rapid build up of acidity in the growth medium. The polyester sheeting was sterilized by immersion in 70% ethyl alcohol whereas the other components were sterilized by autoclaving. It was necessary, therefore, to assemble the chamber under sterile conditions after the separate sterilization of the components. The Rose chamber allowed high power phase microscopy of cultured cells. Both this chamber and Leighton tube cultures are commonly used in experiments involving cell cultures.

CULTURE OF HUMAN DIPLOID FIBROBLASTS

Introduction

The main reason for using this cell type was to follow closely the work of Schmitt-Slomska et al.(1968), who described an interaction between human diploid fibroblasts and an L-form of a group A Streptococcus. Because these cells are diploid with regard to their chromosome complement, they provide a more realistic model system than do the aneuploid cell lines.

The behaviour of diploid fibroblasts in culture has been well described by Hayflick and Moorhead (1961). They found that the diploid chromosomal complement was maintained throughout the life of the culture and that there was no evidence of contamination by mycoplasmas or latent viruses. They pointed out the great advantage, for experimental work, of a cell culture that is uniform in type, and they compared the diploid fibroblasts with heteroploid cell lines where, even after cloning, there may be rapid divergence of chromosomal types.

Cultural Procedure

The cells used were obtained from the Commonwealth Serum Laboratories, Melbourne, and were derived from human foetal skin. Initially, the procedure adopted was to wash a confluent monolayer culture in calcium- and magnesium-free Dulbecco phosphate buffer (Dulbecco and Vogt, 1954) and then to add a trypsin solution consisting of 0.25% trypsin (Difco 1:250) in calcium- and magnesium-free Dulbecco phosphate buffer pH 7.4. The culture was then incubated at 37^{o}C for 10 minutes and the cell layer was scraped into the trypsin solution and gently centrifuged at approximately 100g for 10 minutes. If necessary, the cell pellet was dispersed, after addition of growth medium, by gently pipetting, using a rubber teat. This gave a single cell suspension. The cells were split 1:3, and subcultured when the monolayers became confluent. The growth medium consisted of

Eagle's basal medium supplemented with 10% of heated foetal calf serum; this was replaced as necessary. Using this procedure, it was found that the cells grew well for a few passages and then the growth slowed, and finally ceased, even though the cells appeared to be in good condition, as judged by microscopic inspection.

A second procedure entailed growing cells to confluence, pouring off the medium and washing the monolayer with calcium- and magnesium-free Dulbecco phosphate buffer, warmed to room temperature. The next step was to add 5 ml of trypsin-versene solution obtained from Commonwealth Serum Laboratories as a single strength mixture comprising 0.1% trypsin and 0.02% versene. This solution was left in contact with the monolayer for 1 minute, and then poured off. After 3 minutes at room temperature, fresh medium was added to each bottle culture and the pH was maintained between 7.2 and 7.5 by addition of sodium bicarbonate where necessary (rather than changing the medium). The growth medium consisted of Eagle's basal medium with the addition of 10% of non-inactivated foetal calf serum. New glassware was conditioned for growth of diploid cells by growing L cells on it for a number of passages. Provided that these technical details were rigidly adhered to, diploid cultures flourished for several months.

PRIMARY HEART CULTURES

Introduction

Primary cultures of new born rat heart and foetal rabbit heart were established in Rose chambers and Leighton tubes. Impetus to study the interactions between L-forms and heart cultures was provided by literature reports such as those of Bentwich *et al.* (1968), who noted a direct toxic effect of streptococcal L-forms on rabbit myocardial tissue; and of Kagan (1968), who produced diffuse myocardial lesions in monkeys following intravenous or intratonsillar injection of streptococcal L-forms. It seemed that a useful *in vitro* model could be established, particularly as the pulsation of the myocardial cells in culture provided a clear indication of differentiation.

Cultural Procedure

The procedure was based on the technique described by Mark and Strasser (1966). Under sterile conditions the hearts were removed from newborn Sprague-Dawley rats killed by a sharp blow, and they were then placed in warmed Hanks solution in a petri dish. The auricles were discarded and the ventricles finely minced. The Hanks solution was carefully poured off and the minced tissue added to a 0.25% trypsin solution

(Difco 1:250) in a conical flask which also contained a sterile magnetic flea. The mixture was incubated for fifteen minutes at 37°C with slow stirring and then the supernatant was poured off, and fresh trypsin added. The procedure was repeated twice, the supernatants containing dispersed cells were retained and quickly cooled in ice water. The suspensions were gently centrifuged at approximately 100g for 10 minutes, the supernatant poured off and growth medium added. The growth medium consisted of medium 199 with a supplement of 10% non-inactivated, batch tested, foetal calf serum. Sufficient cells were added to the culture vessels to give a confluent layer on the coverslip. According to Mark and Strasser, and Kasten (1971), there are two cell types in these cultures. The muscle cells have a dense cytoplasm packed with many-sized muscle mitochondria. They have a well defined nucleus, usually containing one acentric nucleolus and surrounded by a double membrane-like structure clearly visible with phase optics at a magnification of 1000 times. Contractions, which begin a few hours after seeding the culture do not occur in the absence of myofibrils. The endothelioid cells have a clearer cytoplasm and predominantly filamentous mitochondria. The nucleus is larger and less dense than that of the myocardial cells; it contains several nucleoli and there is no double membrane visible at light microscope magnification. These cells are very

mobile and are thought to be derived from capillaries. The ratio of the two cell types present varies with the age of the culture; during the first twenty four hours it is difficult to estimate accurately as some cells are still too rounded to identify. By the second day all the cells have spread out and counts show about three muscle cells to each endothelioid cell. By three days, the ratio is about two muscle cells to each endothelioid cell. The endothelioid cells do not overgrow the muscle cells, however, even over long term cultivation. According to Mark and Strasser, the cultures can be maintained for up to eleven weeks with no loss of beating. As the culture develops after seeding, muscle cells form syncitia which beat synchronously. Kasten noted that these differentiated myocardial cells were capable of mitosis and presumably the culture is maintained by an equal rate of gain and loss of cells. Personal experience was that difficulty was encountered intermittently in obtaining multiple cultures all of which beat regularly. Variation in serum batches could well have been a contributing factor but it did appear that undefined variables were operating as well.

Rabbit heart cultures also were prepared from foetuses obtained at 22 or 23 days gestation. The pregnant rabbits were obtained from the Institute of Medical and Veterinary Science, Adelaide. The cultural procedure was the same as that described

for rat heart cultures. Rabbit heart cultures were not used to a great extent because of the expense and the fact that they were less successful overall than were the rat cultures.

EARLE's L CELL

Introduction

This cell is a prototype of continuous cell cultures. The chromosomal pattern of strain L and its sublines is markedly aneuploid and the modal karyotype varies significantly among numerous existing substrains in different laboratories (Harris, 1964). Like many other permanent cell lines, strain L has undergone morphological alterations in the course of serial culture to the point where it does not resemble normal fibroblasts in primary culture. The appearance of the cells does vary, however, with the conditions of culture. Despite the disadvantages of using a de-differentiated cell type when compared to primary or diploid cultures, this particular cell line has enjoyed wide popularity in the study of infectious phenomena. Smadel (1963) studied intracellular infections and the carrier state using an L cell - Rickettsia tsutsugamushi and an L cell - Salmonella typhosa model. Gordon et al. (1965) studied the phagocytosis and intracellular digestion of artificially synthesized deoxyribonucleic acid - protein coacervates by L cells.

Moulder (1971) and Friis (1972) used L cells in their model systems for the study of intracellular infection by Chlamydia psittaci. Stanbridge (1971), in a review of mycoplasmas and cell cultures, makes reference to L cell - virus interactions and L cell - mycoplasma interactions. It is apparent that there is advantage in using such a widely studied cell with proven ability to interact with a wide variety of infectious agents.

Cultural Procedures

Although the L cells do not resemble fibroblasts in culture (Harris, 1964), they will be referred to as L fibroblasts or mouse embryonic fibroblasts, where it is necessary to avoid confusion with L-forms. The cells were obtained from Commonwealth Serum Laboratories, Melbourne, as a line derived from clone 929. They were grown as monolayer cultures in a medium consisting of Eagle's basal medium containing 10% of non-inactivated calf serum. They were passaged at early confluence by pouring off the medium, adding a few ml of fresh medium, and scraping the cells into this by the use of a rubber policeman with a silicone rubber tip. The cell clumps were broken up by gentle pipetting. The cultures were split 1:4 twice a week with 20 mls of growth medium for bottle cultures.

MACROPHAGE CULTURE

Introduction

If L-forms of group A streptococci persist *in vivo* then it would seem reasonable to assume that they would interact with macrophages, especially at foci of chronic inflammation. An *in vitro* study of macrophage-L-form interaction therefore seemed to be worthwhile. Another reason for utilizing macrophage cultures was for comparative purposes; these cells should phagocytose L-forms and so techniques designed to follow this process in other cell systems could be assessed with more certainty. The possibility, discussed in the introduction, that macrophages may not degrade L-forms completely and may release antigenic material, was a further impetus to studies involving this cell type.

The difficulty in choosing the source of macrophages was simplified by practical demands. Perhaps the most readily tapped sources are alveolar cells, blood monocytes and peritoneal macrophages. Macrophages from different sources vary in a number of properties. These include the sources of energy used by the cells; peritoneal macrophages depend solely upon glycolysis, whereas alveolar and Kupffer cells utilize oxidative phosphorylation as well (Asiddao *et al.*, 1964). The content of some hydrolytic enzymes and the killing ability for phagocytosed bacteria vary as well (Pavillard, 1963).

The requirement of antibody for phagocytosis varies also, for example, Mouton *et al*. (1963) noted that *Salmonellae* injected intravenously into new-born, antibody-deficient pigs were taken up mainly by the spleen, not the liver. This is the reverse of the events that occur in adult, antibody-containing pigs and takes place even though the liver Kupffer cells phagocytose the majority of carbon injected into the new-born pigs. A possible conclusion from this work is that spleen cells have a lesser requirement for opsonins than do Kupffer cells. Blood monocytes are relatively few in number, for example, having a mean of about 500 per ml in human blood (Ham and Leeson, 1961). There is the additional disadvantage of heavy contamination by other cell types. The mouse peritoneal macrophage was selected because of its common usage and ready availability in large numbers. According to Cohn and Benson (1965) these cells are immature phagocytes similar to blood monocytes when first cultured, but which mature with culture, as indicated by increased size, spreading over glass surfaces and increase in the number of lysosomal granules. There appears to be controversy over the proliferation of these cells *in vitro* (Jacoby, 1965). Jacoby concluded that mammalian macrophages probably do not divide *in vitro* and this opinion is supported by Cohn and Benson (1965). The uptake and digestion of microorganisms by macrophages *in vitro* will depend on the physiological state of the cell and the presence or absence of specific antibody and complement (Vernon-Roberts, 1972).

Cultural Procedures

The technique was based on the studies by Gesner and Howard (1967) and Stuart (1967). The mice used were from 6 to 10 weeks old and were Swiss albinos. They were killed by dislocation of the neck. Between 1 and 2 ml of growth medium was injected into the peritoneal cavity and the abdomen was then gently massaged with sterile forceps. After about 2 minutes the fluid was aspirated. The cells were counted in a haemocytometer and then seeded into the appropriate culture chamber. The medium used was Eagle's basal medium with a supplement of 10% of non-inactivated calf serum. After 24 hours the medium was changed, thereby removing all unattached cells and leaving behind a population which comprised mainly macrophages. No attempt was made to increase the yield of macrophages by prior injection of irritants as suggested by Gesner and Howard (1967) as it was considered that these macrophages might be unsuitable for subsequent experiments. The reason for this was that these macrophages are "activated", that is, they have an increased lysosomal activity and phagocytic ability. Because of this it would be more difficult to detect a change when organisms were introduced to the cultures. The majority of macrophages obtained were therefore non-activated and required 1 to 2 days to spread over the glass substrate. Gesner and Howard mention typical yields from unstimulated mouse peritoneal

cavity of 2×10^5 to 1×10^6 macrophages which was in line with an average of approximately 5×10^5 cells obtained in the present study.

CONTAMINATION IN CELL CULTURES — CHAPTER III

Introduction

This was a problem of great concern and one which was very pertinent to the current studies involving attempted infection of cells in culture with streptococcal L-forms. The following section is an attempt to review the literature on this problem and to indicate how contamination could affect the response of the cells to introduced organisms.

Fogh et al. (1971) have discussed various contaminants in cell cultures as follows:

Tissue Culture Cells. Contamination of a culture with a different cell type is particularly likely when more than one cell line is being handled in the same laboratory. It can be detected by such procedures as serological or karyological diagnosis; alternatively, it is possible to distinguish two cell types by determining the levels of enzymes characteristic of each cell line. These include dehydrogenases, kinases, tranferases and esterases.

Bacteria, Yeasts and Fungi. In the presence of antibiotics, bacteria may persist as a latent or low order infection (10^2 - 10^3 bacteria per ml). Fogh et al. (1971) cited one report claiming that 27% of cell cultures tested were contaminated in this manner. Organisms isolated included Corynebacterium, Micrococcus, Bacillus and Gram-negative enteric rods. These organisms may persist without obvious effects on the cells, whereas in cultures maintained without antibiotics, bacterial contaminants will usually grow up to a level where they produce turbidity in the medium and destruction of the cells. Yeasts originating from many sources are common contaminants with some growing well only in the presence of living cells. Fungi may also be present. These groups of organisms can usually be detected by staining the cultures.

Parasites. Amoebae can produce a cytopathic effect similar to a virus. They may be detected by staining, phase contrast microscopy or by culture on agar plates seeded with a lawn of Escherichia coli.

Viruses. An important source of contaminating virus could be the tissue of origin. The presence of pathogenic viruses can be readily recognized by the destruction produced in the culture. Latent viruses are more difficult to detect but techniques

as outlined in the review by Fogh et al. are available. These include direct examination at the electron microscope level, passage to susceptible cells where the virus will produce demonstrable damage or change, and by demonstration of interference properties against known viruses.

Mycoplasma Contamination

The general field of the biology of the mycoplasmas was followed closely for a number of reasons. Firstly, there is an obvious similarity in a number of properties between L-forms of bacteria and mycoplasmas. These include structure, morphology, colony characteristics on agar, requirement for serum and ability to pass through filters with an average pore diameter of 0.45μ. Another reason was that it was hoped to glean ideas and techniques for the study of L-forms from the considerable body of literature dealing with mycoplasmas, their role in disease and their ability to infect tissue culture cells. These aspects are dealt with more fully in later sections. There has been a growing interest in the contamination of cell cultures by mycoplasmas since this was first described by Robinson et al. in 1956. In 1965, Hayflick described a medium for the laboratory culture of mycoplasmas which was supplemented with unheated horse serum and 10% of autoclaved fresh yeast extract. To test for contamination of a cell culture, the

tissue culture medium was inoculated with cells into the special medium in both broth and agar. The agar cultures were incubated both aerobically and under an atmosphere of 5% CO_2 in N_2. After a number of days incubation at 37°C the broth cultures were also inoculated onto agar. Incubation was continued for a period of six to fourteen days. Mycoplasmas were identified by their "fried egg" appearance, by virtue of the centre of the colony being firmly embedded in the agar, by requirement of serum for development and by uptake and retention of Dienes' stain. They were distinguished from bacterial L-forms by their non-reversion to bacteria. Hayflick stressed the importance of recognizing artifacts on the agar surface. These included air bubbles, water condensate, tissue culture cells and pseudo-colonies. The latter were considered to present the most serious threat to diagnosis. They are composed of calcium and magnesium soaps which form crystalline structures on the agar surface, and they can be serially propagated on the agar surface by the seeding of crystal nuclei. According to Hayflick they can be recognised by the fact that they develop in the presence of antimicrobial agents.

MacPherson (1966) considered that repeated attempts may be necessary to culture some mycoplasmas. In addition, he pointed out that freshly trypsinized cells have a reduced

mycoplasma content and suggested that a preliminary passage of mycoplasmas in mouse lymphoma cells may either produce cytopathic effect, or sufficient enrichment to ensure subsequent detection on solid media. House and Waddell (1967) compared cultural tests for mycoplasmas with a biochemical assay for thymidine cleavage, this being a property of at least some mycoplasmas. This test depends upon the demonstration of an increase in free deoxyribose in mycoplasma infected cultures, after the addition of thymidine. The media used were Chanock's medium (Difco PPLO agar base + 10% yeast extract + 20% of horse serum), and feeder cell medium. To prepare the latter, monolayers of mouse embryo cells were established in Pyrex dishes and the medium was replaced with an agar overlay of Eagle's medium + 10% tryptose phosphate + 2.5% horse serum. This was in turn overlaid with the Chanock medium described above. Cell suspensions were inoculated onto the agar and the plate incubated in an atmosphere of 5% CO_2 in N_2, with high humidity. House and Waddell found that the feeder medium gave the earliest and best results, while the thymidine-cleavage assay was of low sensitivity. They were of the opinion that this could have been due to a low level of the enzyme in some mycoplasmas or low level contamination of cell cultures, and therefore insufficient enzyme. Stanbridge (1971), in a review of mycoplasmas and cell cultures, pointed out that mycoplasmas are very heterogeneous

with regard to metabolic behaviour, in which case it would seem unlikely that biochemical tests alone would be suitable methods for screening cell cultures. He also noted that, while the bulk of mycoplasmas seem to lie in the extracellular environment in close proximity to the cell surface (although some may lie in the cytoplasm, either free or in vacuoles), the desirability of disrupting cells to release mycoplasma must be questioned on the basis of demonstrated mycoplasmicidal activity of some cell homogenates. In the present study it seemed desirable, however, in testing for contamination, to include cells as well as medium in the inoculum because of the close association between mycoplasmas and the cells. Stanbridge concluded that some mycoplasmas have become so adapted to a cellular environment that growth is negligible on solid media. He noted that other tests, such as fluorescent-antibody staining, lacked wide application and sensitivity. In the case of fluorescent-antibody staining, multivalent antisera are required to cover the many serological types of mycoplasma found as tissue culture contaminants. Demonstration of the enzyme arginine deiminase restricts the test to non-fermentors that utilize arginine as an energy source. Staining by Giemsa or May-Grünwald Giemsa also lacks the necessary sensitivity. Fogh et al. (1971) concluded, along with other authors, that it is unlikely that all species of mycoplasma would grow on media currently available.

The Source of Mycoplasma Contamination

Stanbridge (1971) concluded that the human oropharynx was an important source of contamination. This probably occurs as a result of faulty aseptic technique, particularly the practice of blowing liquid from pipettes. In recent years, there have been isolations with presumed origins from avian, swine and murine sources. Such contaminations could arise from the tissue of origin, for example, infected or contaminated mucous surfaces. Barile et al. (1973) believed that the major sources of mycoplasma contamination were of human, bovine and swine origin. They recommended the elimination of mouth pipetting and the adoption of rigid aseptic technique. They noted that commercial bovine sera or trypsin from swine sources could carry mycoplasmas at a level undetectable by the usual cultural practices but nevertheless quite adequate for infection of cell cultures. Accordingly, they proposed that bovine serum should be heated at 56^{o}C for 45 mins or filtered twice through a 0.22μ filter. Similarly, trypsin solutions should be filtered through a 0.1μ membrane or twice through a 0.22μ filter. Over a period of 14 years, Barile et al. examined 6,600 continuous cultures and 3,200 primary cultures for mycoplasma contamination. They found, by cultural techniques, that 1,374 of the cultures were contaminated, 51 of these being primary cultures where the tissue of origin was considered to be the likely source of mycoplasmas. The observation by Hayflick (1965) that mycoplasma contamination is rare in laboratories that do not incorporate antibiotics in the tissue culture medium, and

his recommendation that this should be standard procedure, found favour with other authors as well (Fogh et al., 1971; Stanbridge, 1971). They presumed that lack of antibiotic cover enforces rigid aseptic technique and thereby reduces the likelihood of contamination of any form.

The Effects of Mycoplasma Contamination on Cell Cultures

Gross macroscopic changes in tissue culture cells infected with mycoplasmas range from inapparent changes to cytopatholgy and cell destruction (Fogh et al., 1971). Cytopathic changes may be related to depletion of arginine in the medium, this being an essential metabolite for growth of mammalian cells and of some mycoplasmas (Fogh et al., 1971). It has also been postulated that some fermenting strains of mycoplasma exert their effect by a competitive utilization of nucleic acid precursors (Stanbridge, 1971). This author commented that the growth rate of infected cultures is often lower than that of uninfected control cultures. Another interesting observation was that of chromosome damage in infected cells, this taking the form mainly of chromatid breaks, but including more gross abnormalities as well. Stanbridge concluded, however, that mycoplasmal contamination did not result in the transformation of the cell population, that is to say no population with altered karyotypic characteristics arose. In contrast to this, a limited study by Fogh and Fogh (1973) revealed that alteration in study chromosome numbers and types did not occur after elimination of mycoplasmas.

Interactions of Microorganisms in Cell Cultures

Stanbridge (1971) cited numerous examples of inhibition and enhancement of viral infection in cell cultures containing mycoplasmas. Some of the inhibitory effects could be explained on the basis of arginine depletion but the overall phenomenon remains to be clarified. Taylor-Robinson and Cherry (1972) found that Mycoplasma gallinarum inhibited the ciliary-stopping effect of Mycoplasma gallisepticum in chicken tracheal organ cultures. They postulated that the mediator of this protection might be a peroxidase known to be produced by some mycoplasmas and which would prevent the damaging effect of peroxide, a possible pathogenic factor produced by M. gallisepticum.

Elimination of Mycoplasmas from Cell Cultures

Hayflick (1960) showed that maintenance of contaminated HeLa and L cells at 41^{o}C for 18 hr kills some strains of mycoplasma differentially, without injuring the cells beyond recovery. Stanbridge (1971) noted that although mycoplasmas could be eliminated from cell cultures by treatment with specific antiserum, this implied that the mycoplasma must be isolated and identified before the appropriate antiserum could be used. He went on to comment that antibiotic treatment was the most popular method of elimination. Antibiotics used included kanamycin,

tetracycline, tylosin, erythromycin and lincomycin. Another important factor stressed by Stanbridge is that much higher concentrations of antibiotic may be required to kill cell-associated mycoplasmas than are needed in direct in vitro tests. The possibility that antibiotic treatment may suppress, but not eradicate, mycoplasmas, is mentioned in the review by MacPherson (1966).

RELEVANCE OF CONTAMINATION TO THE PRESENT STUDY

In the present study, the contaminants considered to be the most significant were viruses and mycoplasmas because of the difficulty of detection and because of their possible interference with attempted infection of the cells by L-forms. In the case of mycoplasmas there was the additional hazard of confused identification in that mycoplasmas could be mistaken for L-forms. In addition, contamination of a culture by another cell type could produce great discrepancies in the response of such a culture to infection. It is obvious that even though heteroploid cell lines are far from being uniform in type (as discussed under the heading of Cell Cultures), the diversity of response would be very much less than that of a mixed culture. This problem was avoided by careful handling of separate cultures in the laboratory. As recommended by Hayflick (1965) and Stanbridge (1971), antibiotics were not added to the culture medium, it

was hoped that any bacterial contaminant would rapidly grow up and become obvious. In the present study, bacterial contamination was, in fact, rarely encountered.

Procedures for Detecting Mycoplasmas

The brief literature survey in this section indicated that although no single technique was completely satisfactory, culture on agar remained the most accepted method, other procedures lacking sensitivity or having application to only a limited range of mycoplasmal strains. The media developed for culture of mycoplasmas have, overall, the common basis of a meat infusion broth with yeast extract and horse serum as supplements (MacPherson, 1966). The medium first used in the present study was a base of 3.7% Brain Heart Infusion (B.B.L. division of Bio Quest, Maryland, U.S.A.) with Difco yeast extract, 2.5% (Bacto Yeast Extract; the water-soluble portion of autolysed fresh yeast), and a supplement of horse serum at 20% of the final volume. The horse serum was obtained from The Commonwealth Serum Laboratories, Melbourne (C.S.L.) and was described as suitable for mycoplasma culture. For agar plates, 1% of Difco Bacto agar was added. Both the horse serum and the autoclaved components were equilibrated at 45^{o}C before adding the serum. Plates were dried at 37^{o}C for 30 minutes to remove excess moisture, packaged in air-tight plastic bags and stored at 4^{o}C.

Media were always used within a few days of preparation, because experience with other microorganisms had indicated that the growth-supporting ability of agar plates could decrease significantly with storage.

Method

Most of the medium was poured off a monolayer culture and the cells were then scraped off using a rubber policeman. The resulting cell suspension was inoculated into mycoplasma broth, 1 ml of suspension to 4 ml of broth. This mixture, together with a control broth, was incubated at 37°C for 3 days; at this time the broths were inoculated onto mycoplasma agar plates and incubated aerobically in sealed plastic bags or under 5% CO_2 in N_2 in anaerobic jars. In both cases, moistened paper towels were included to maintain high humidity. The plates were incubated for a total of 18 days and were examined at intervals during this time. If microscopic examination indicated structures resembling mycoplasma colonies, the plates were stained with Dienes' Stain. The procedure used was that described by Madoff (1960), except that eosin was used as a substitute for azurII which was not available. Staining takes only a few minutes with mycoplasma colonies which are reported to stain bright blue against a faintly blue background (Madoff, 1960). Suspicious structures can be examined under oil immersion to

confirm their identity. The problem of pseudocolonies (see plate 3.1) could be partly overcome by relating the number of colonies in controls to the number on test plates. Using this procedure, one batch of L-cells yielded, on aerobic agar plates after 10 days incubation, structures about 10μ in diameter (see Plate 3.2). They were still present at 18 days and no pseudocolonies were found on control plates. They could not be subcultured by the standard technique of rubbing an inverted block of agar containing the suspected colonies over a fresh agar plate and incubating at 37°C. The procedure was repeated and the results confirmed. In relation to this, MacPherson (1966), commented that mycoplasma strains poorly adapted to growth on solid medium could form atypical colonies.

Electron Microscopy

The techniques used for transmission electron microscopy of cell cultures are discussed in detail in Chapter 6. Examination of cell sections at electron microscopic level provided a useful method for detection of contaminants. This applied in particular to the cell surface where mycoplasmas tend to aggregate (Stanbridge, 1971). It was interesting to observe that electron microscopy of the L cell batch suspected of mycoplasma contamination by cultural procedures, showed structures resembling mycoplasmas at the cell surface (Plates 3.3. and 3.4). These

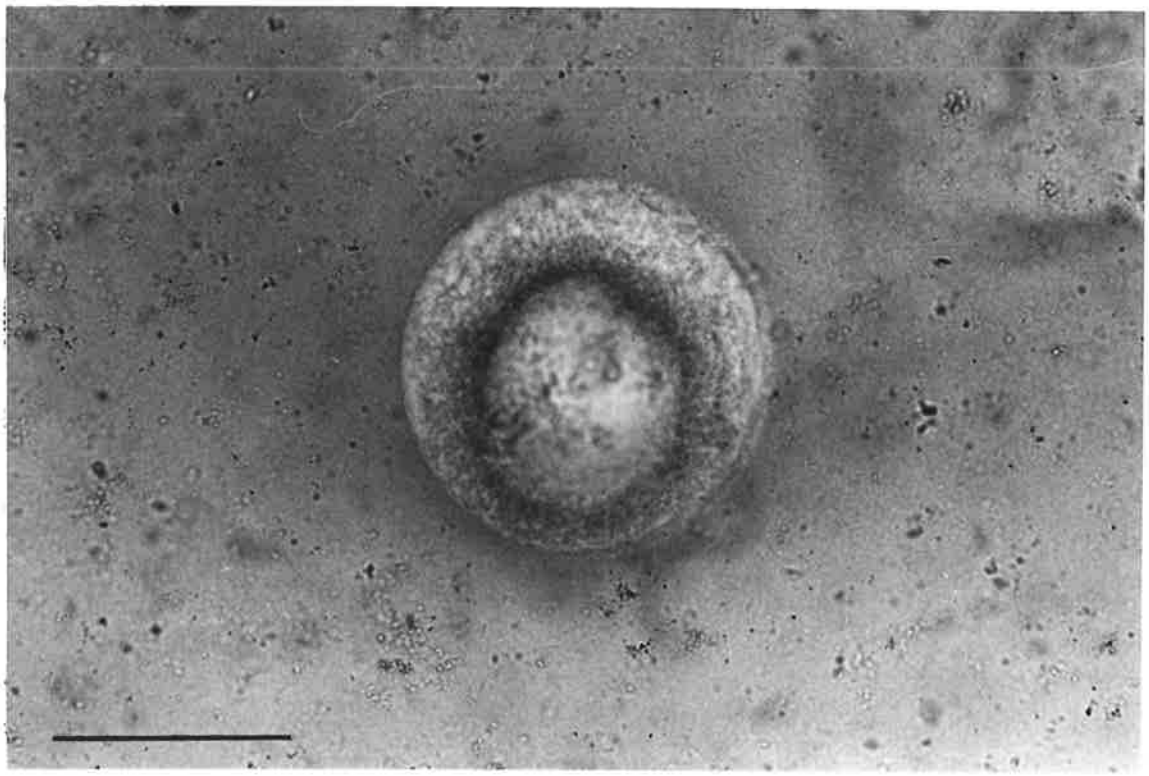


Plate 3.1

Pseudo-colony on agar, stained with Dienes' solution

Scale, 100μ

Magnification, x 310

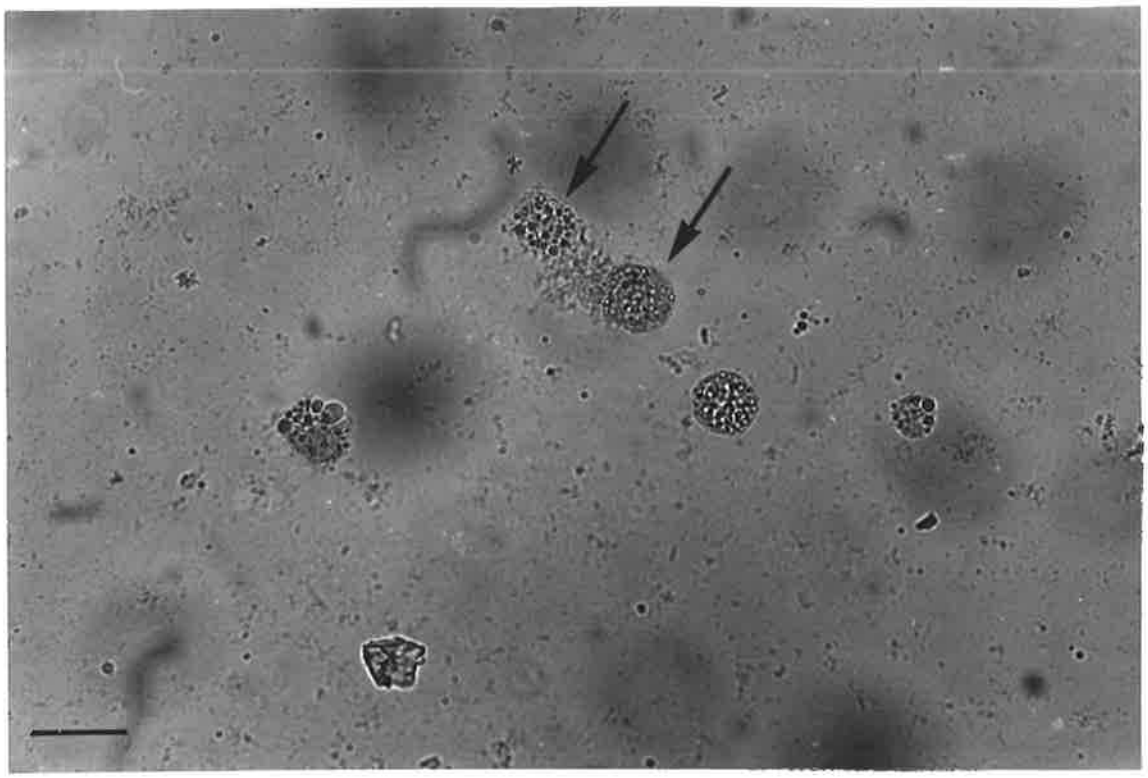


Plate 3.2

Suspected mycoplasma colonies after 18 days culture, stained with Dienes' solution

Arrows indicate colonies.

Scale, 10μ

Magnification, x 1250

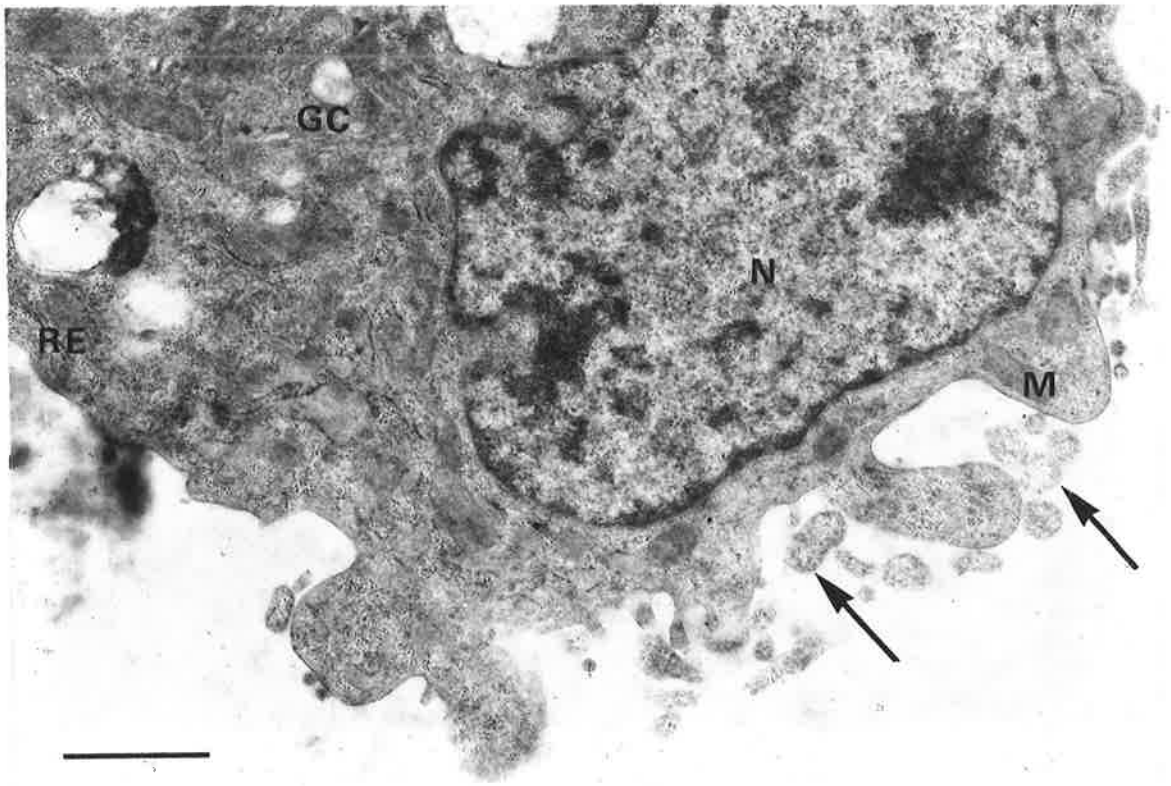


Plate 3.3

Electron micrograph of an L cell with a possible mycoplasma contamination

GC Golgi complex.

N nucleus.

M mitochondrion.

RE rough endoplasmic reticulum.

Arrows indicate mycoplasma-like structures.

Fixed in glutaraldehyde and post-fixed in osmium tetroxide.

Stained in uranyl-acetate - lead citrate.

Scale, 1μ

Magnification, x 19000

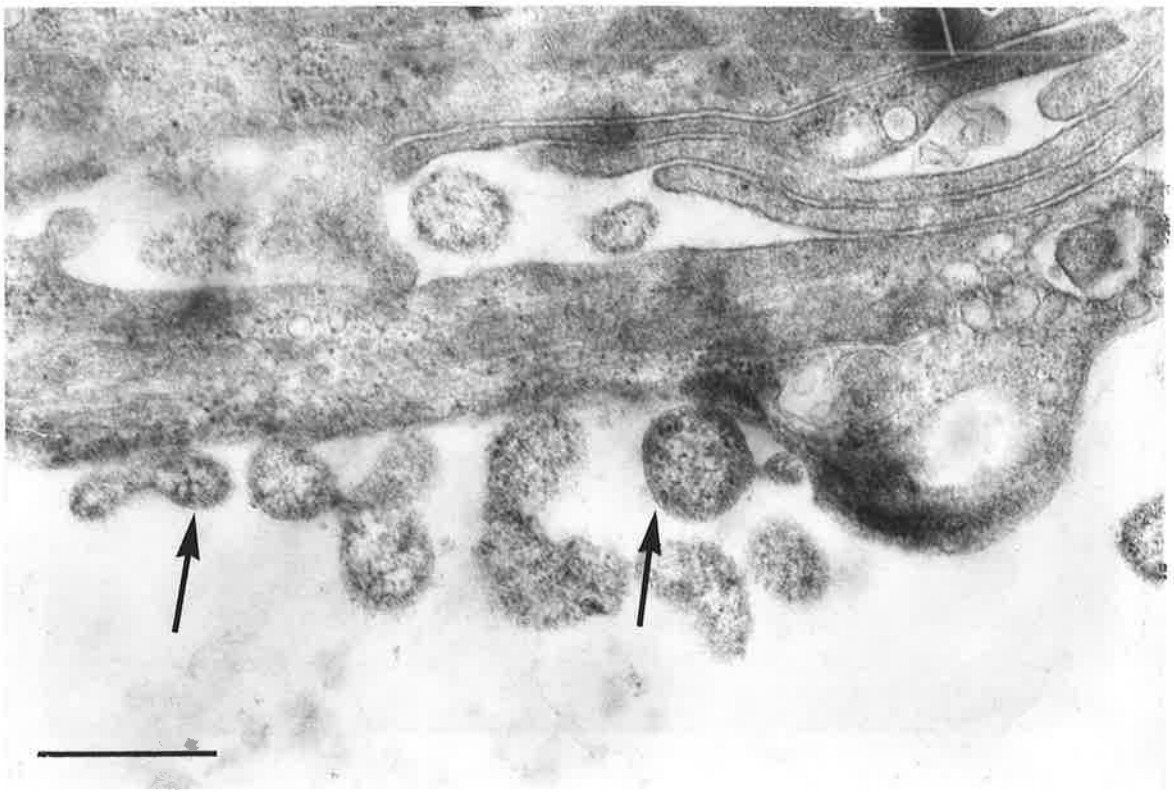


Plate 3.4

Higher power electron micrograph of a portion of an L cell with possible mycoplasma contamination

Arrows indicate mycoplasma-like structures.

Fixed in glutaraldehyde and post-fixed in osmium tetroxide.

Stained in uranyl-acetate - lead citrate.

Scale, 0.5μ

Magnification, x 47,250

structures have a similar morphology to some of the mycoplasma strains shown in the studies of Maniloff and Morowitz (1972). It appeared that these cells were infected by virus as well (Plates 3.5 and 3.6). The structures indicated are very similar to the virus-like particles shown in L cells by Cromack (1968).

It was decided to discard a culture regarded as possibly contaminated by electron microscopic examination or cultural tests, rather than to attempt to cure it of infection by antibiotic therapy. A fresh batch of cells was obtained from C.S.L.,examined by both procedures and, if considered free from obvious viral or mycoplasma contamination, was stored in liquid nitrogen. By discarding cultures at intervals and utilizing frozen stock, it was possible to maintain cultures that were apparently uncontaminated. This combined with regular cultural testing of cell cultures enabled control of this problem.

CONCLUSIONS

In the present study mycoplasmas were considered to be the most serious contaminants of cell cultures. The publications cited in this chapter indicate the growing awareness of the need for vigilance against these organisms. At present it appears that no single technique can enable positive identification of all mycoplasmas. They were considered particularly

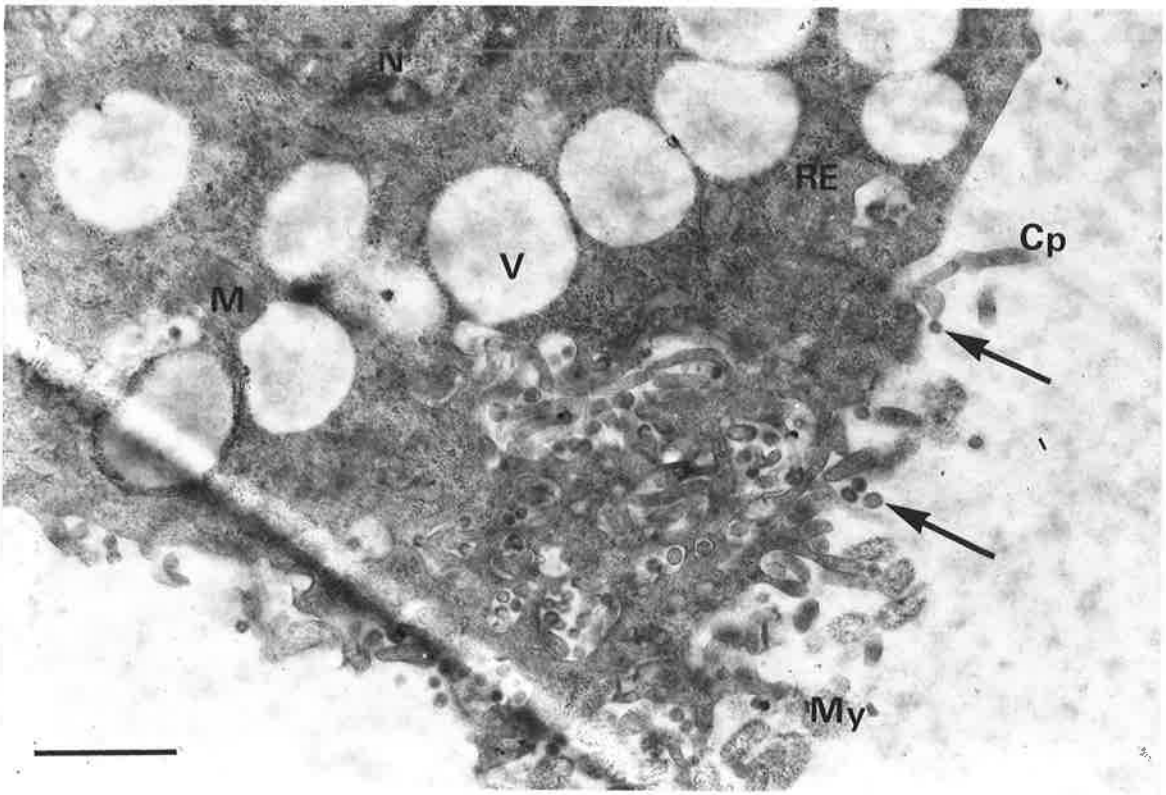


Plate 3.5

Electron micrograph of an L cell with suspected viral contamination

Cp cell process.

N nucleus.

RE rough endoplasmic reticulum.

M mitochondrion.

V probably fat aggregation.

My mycoplasma-like structure.

Arrows indicate virus-like particles.

Fixed in glutaraldehyde and post-fixed in osmium tetroxide.

Stained in uranyl-acetate - lead citrate.

Scale, 1μ

Magnification, x 19,000

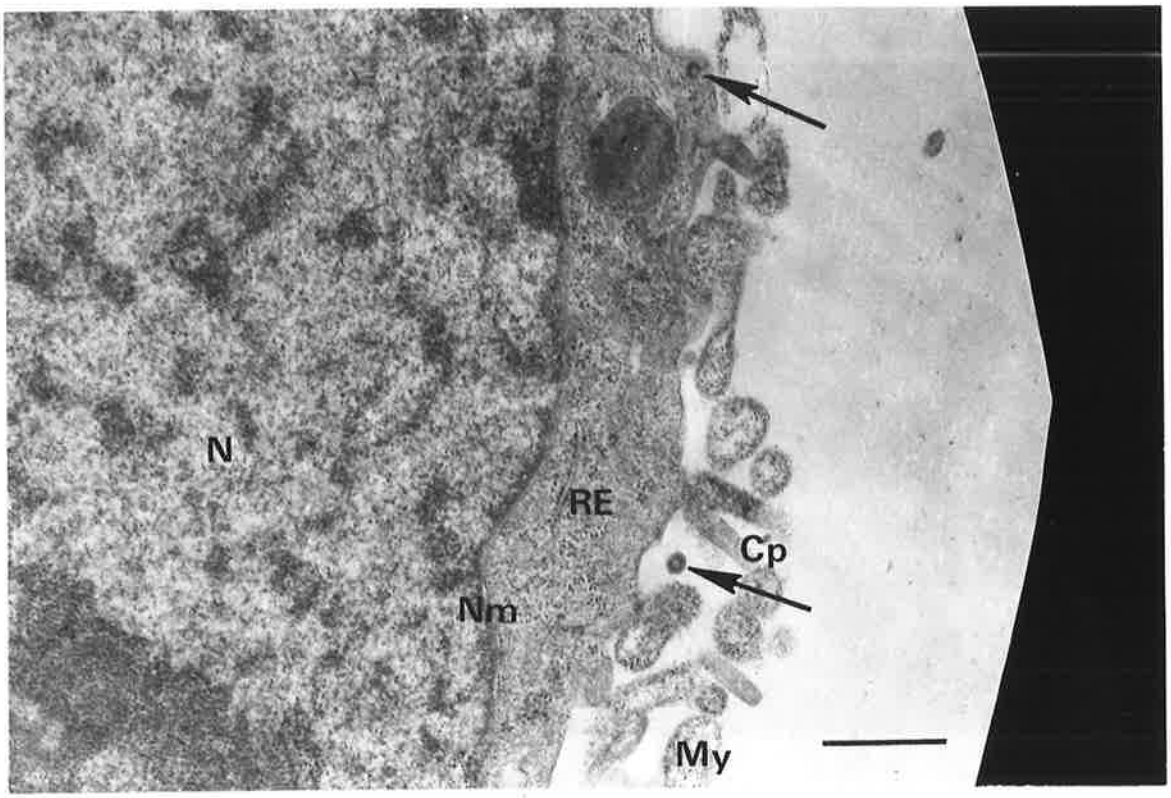


Plate 3.6

Higher power electron micrograph of a portion of an L cell showing possible viral and mycoplasmal contamination

N nucleus.

Nm nuclear membrane.

RE rough endoplasmic reticulum.

My mycoplasma-like structure.

Cp cell process.

Arrows indicate virus-like particles.

Fixed in glutaraldehyde and post-fixed in osmium tetroxide.

Stained in uranyl-acetate - lead citrate.

Scale, 0.5μ

Magnification, x 31,500

important in the present study because of possible false interpretation of infectivity experiments. This could have arisen from damage to the cells, inhibition or augmentation of L-form action on the cells or from confused morphological identification of infecting organisms.

INTRODUCTION

This chapter describes the induction and cultural characteristics of L-forms of group A streptococci. A consideration of the requirements of an L-form strain which was suitable for infectivity studies, indicates that ideally it should be induced *in vivo*, following infection with a group A streptococcus. It should also possess,immediately after *in vivo* isolation, the *in vitro* properties of rapid and uniform growth in broth culture with minimal osmotic support. Such a strain would closely mimic any probable natural parasite and would have desirable cultural properties. The majority of literature information indicates that this is a remote possibility indeed. Usually, a strain grows well in broth culture only after prolonged cultivation in a suitable medium (Hijmans, 1968). The two aims of good growth in artificial media and possession of the characteristic properties of an organism *in vivo* appear to be mutually exclusive.

The following pages describe, in a chronological manner, the attempts to obtain L-form strains which grew well in broth culture. This was considered to be an essential, albeit rather obvious, requirement for infectivity studies. The various approaches are discussed under the following headings:

1. Culture and maintenance of group A streptococcal strains.
2. Lysozyme action.
3. Phage-associated lysin.
4. Attempts to culture from in vivo.
5. Propagation of L-form strains.

CULTURE AND MAINTENANCE OF GROUP A STREPTOCOCCAL STRAINS

Group A streptococci were grown in Todd-Hewitt broth (BBL), and on Brain Heart Infusion agar. Early studies employed Oxoid Brain Heart Infusion (BHI), but later BBL or Difco products were used. The reasons for this are discussed in a later section of this chapter. Difco Bacto Agar at 1.2 to 2% final concentration was used. Distilled-deionized water was used in media preparation. For agar culture, pre-sterilized, plastic disposable petri dishes were used. It was found that growth of group A streptococci was superior under an atmosphere of 5% CO_2 in N_2, this being obtained by blowing the mixture into previously evacuated anaerobic jars. All strains were grown at 37°C and for short term incubations, a water bath was used so as to obtain greater accuracy and reproducibility.

A variety of storage forms was used for the organisms. Cultures in Cooked Meat Medium (BBL) at 4°C provided reliable growth over 2 to 3 months while early log phase cultures in Todd-Hewitt broth, held at 4°C for 1 to 2 weeks, or frozen at -20°C for several months, were also reliable. For long-term storage, cultures were

stored freeze-dried in sealed ampoules. Usually one ampoule was opened and cultured after processing each batch, to check on viability of the organisms. For L-form induction experiments, concentrated sucrose solutions were filtered through 0.45μ filters as this seemed to be the most common practice (for example, Young and Armstrong, 1969). Reagent-grade chemicals were used to prepare buffers and other solutions. When L-forms had been induced, a comparison was made between human serum (obtained from the Adelaide Blood Bank), bovine and horse serum. The latter two were obtained from The Commonwealth Serum Laboratories, Melbourne (CSL), as heat-inactivated sera. The superiority of horse serum was at once apparent, and this was the only source of serum supplement used in subsequent experiments. The media used for L-form induction and propagation are described later in this chapter.

THE ACTION OF LYSOZYME

Introduction

Abrams (1958) reported that the cell walls of *Streptococcus faecalis* were susceptible to digestion by lysozyme. In 1959, he prepared protoplasts of this organism using lysozyme digestion in the presence of sucrose as an osmotic protectant. Gooder and King (1964) were able to obtain L-form growth from lysozyme-

induced protoplasts of S. faecalis. According to Freimer et al. (1959), "lysozyme does not appreciably affect the cell walls of group A streptococci." However, the early results in the present study encouraged closer examination of this property, particularly as Maxted (1968) considered that minor cell wall damage could lead to L-form induction.

Experimental

At the commencement of the present study, the only group A streptococcal strain available was a group A type 33, obtained from the National Collection of Type Cultures, London, U.K. This was strain number 8224. It was noted that a suspension of this organism in 0.2M Tris (Sigma) buffer pH 7.2, when incubated at 37^{o}C in the presence of 200 µg per ml of lysozyme (Sigma, grade 1 from egg white) for 2 hours (Gooder, 1968), showed evidence of cell damage. This was assessed by microscopic examination of a smear stained with 1% methylene blue (BDH). The suspension yielded only a few bacterial colonies on BHI agar; it was not, however, susceptible to lysis by sodium lauryl sulphate (Barkulis et al., 1964). A series of experiments was designed to examine this phenomenon more closely. A log phase culture of group A type 33 was centrifuged and resuspended in Tris buffer pH 7.2. The suspension was divided and treated in various ways. For some samples, Tris buffer containing 0.8M sucrose was used for the lysozyme incubation. All agar plates were freshly prepared,

and the serum and penicillin were added separately before pouring. The experiment is summarized in Table 4.1. Methylene blue smears showed that in suspensions with added sucrose, the cells were apparently normal. However, without sucrose, the bacteria were obviously damaged and, in some cases, swollen cells were seen. It appeared, therefore, that the sucrose had acted as an osmotic stabilizer.

In another experiment, a bacterial suspension was incubated with a high concentration of lysozyme, namely 1000µg per ml for 14 hours. Both sucrose-protected and unprotected Tris suspended organisms were used. Tris suspended bacteria without lysozyme served as controls. After the incubation period, the suspensions were centrifuged and resuspended in ice-cold distilled water. No clearing, in comparison with the control suspension, was noted. Sodium lauryl sulphate was then added to a final concentration of 2.5% (Barkulis et al., 1964). Clearing of Tris-suspended and sucrose-protected suspensions, in comparison with the control, was observed. This provided some evidence that, in the previous experiment, the sucrose solution had protected the organisms, rather than interfered with lysozyme action. Similar experiments were performed with group A type 1 (N.C.T.C. 8198), and type 12 (N.C.T.C. R53/1077), both obtained subsequently from the National Collection of Type Cultures, London, U.K. as suitable for L-form induction. These strains did not appear to be susceptible to lysozyme attack. As L-form colonies could not be induced, these experiments were discontinued.

TABLE 4.1

Growth of *Streptococcus pyogenes* Group A type 33 (N.C.T.C. 8224) after incubation with lysozyme

Lysozyme (µg per ml)	Time Incubation	Growth on Media BHI+Ser.+Suc.	BHI+Ser.	BHI+Suc.+Ser+pen.
200	1	fair	fair	-
100	1	fair	fair	-
200 + Suc.	2	heavy	heavy	-
100 + Suc.	2	heavy	heavy	-
200	2	good	good	-
100	2	fair	fair	-
Tris suspension only	2	fair	fair	-
Control broth	2	heavy	heavy	-

BHI = Brain Heart Infusion.

Suc. = 0.8M sucrose.

Ser. = 10% horse serum.

pen. = penicillin G 1000 µg per ml.

A possible explanation for the failure to obtain growth on L-form agar plates could be that the group A type 33 was a poor converter to the L-form state under the action of lysozyme.

PHAGE-ASSOCIATED LYSIN

Introduction

The ability of a phage-free lytic factor, arising from virulent phage infection of group C streptococci, to lyse group A streptococci was reported independently in 1957 by both Maxted and Krause. Gooder and Maxted (1958) used the phage-associated lysin to prepare protoplasts of group A streptococci. These could be protected from osmotic lysis by the addition of sucrose or saline but these authors were unable to induce growth of these forms at that time, although they later obtained growth by a modified pour-plate technique (Gooder and Maxted, 1961). Freimer et al.(1959) obtained surface growth of L-form colonies with lysin-induced protoplasts using a medium which was composed of a heart infusion base, 1.5% agar, 4% sodium chloride and 10% horse serum. Gooder (1968) noted that between 10% and 100% of the colony forming units of group A streptococci treated with phage-associated lysin formed L-form colonies. This contrasted sharply with the estimate made by Hijmans (1968), of 1 in 10^6 cocci so transformed, using penicillin gradients.

It therefore appeared that the lysin method could provide an efficient means of obtaining large numbers of L-form colonies.

Experimental

Group C Strep. pyogenes (NCTC 4540) and phage from a Group C strain (NCTC 8368) were kindly supplied by Dr. W.R. Maxted, Colindale, London. A freeze-dried sample of the phage was suspended in 1 ml of Todd-Hewitt broth and added to an early log-phase culture of group C streptococci in 10 ml of broth. The mixture was incubated at 37°C and examined for lysis as described by Krause (1958). As there was no reduction in optical density after several hours incubation, it was decided to filter the broth through a Gelman 0.45μ filter and to store the filtrate at -20°C. The ability of this preparation to lyse group A streptococci was minimal, as judged from microscopic examination of suspensions which had been incubated for up to 12 hours with it. The bacteria showed little change from normal. When the lysis preparation was assayed for phage by the Soft Agar Layer Method (Adams, 1959), no plaques were seen after 24 hours incubation. These results suggested that either the phage was inactive, or else it had been absorbed by the group C streptococci, but had not completed a lytic cycle. The activity of the phage was checked in another ampoule, by the Agar Layer Method of Adams. The test plates showed confluent lysis, indicating that the phage was infective.

Preparation of High-Titre Phage

Five millilitres of Todd-Hewitt broth was added to each of the plates showing confluent lysis, the soft agar layer scraped off with a glass rod, and the sludge so formed was then poured into a 2 oz bottle and shaken vigorously.

Extraction was continued for 20 minutes, after which the mixture was centrifuged to remove the agar phase and then filtered through a 0.45μ filter to remove bacteria. The filtrate was stored in aliquots at -20°C.

Phage Assay

Todd-Hewitt broth in 1.25 ml quantities was warmed to 48°C in a water bath. To each quantity, 1.25 ml of molten 1.5% agar was added. Two drops of a logarithmic phase broth culture of group C streptococcus was added to each mixture, and the components well mixed. Duplicate phage dilutions from 10^{-1} to 10^{-7} were added, mixed, and then poured onto BHI agar plates held at room temperature. The plates were incubated under CO_2-N_2, at 37°C for 24 hrs, at which time, clear plaques 2 to 4 mm in diameter were apparent. The phage assay was approximately 7×10^8 plaque forming units (p.f.u.) per ml. Confluent lysis occurred at approximately 1×10^5 pfu per ml on 9 cm diameter petri dishes. According to Adams (1959), it should be possible to obtain a yield of 1×10^{11} pfu per ml using this technique with an E.coli system.

After some experimentation, a yield approaching this level was obtained. The significant changes from the method previously described were to increase the inoculum of the group C Streptococcus and to extract the agar sludge for 48 hours at 4°C with constant stirring.

Preparation of crude lysin

To obtain a more satisfactory lysin preparation it was considered desirable to mix the phage and the group C Streptococcus in known ratios. According to Maxted (1957), lysin production continues over a wide range of phage to bacterium ratios, but the ratios of approximately 1:4 to 1:14 gave the best production. Therefore a growth curve was constructed for the group C Streptococcus in Todd-Hewitt broth.

Procedure. From an overnight broth culture, 0.5 ml were inoculated into 25 ml of Todd-Hewitt broth warmed to 37°C. Samples were taken 2 hours after subculture and then at 30 minute intervals. The dilutions were made by mixing 0.3 ml broth culture with 2.7 ml Todd-Hewitt. In this manner serial dilutions were made, and duplicate 0.2 ml quantities were plated onto BHI agar. The plates were incubated under 5% CO_2 in N_2 and the colonies counted after 24 hours. Three ml quantities of broth were set aside at each time interval for optical density recordings at 650mμ in a spectrophotometer (Bausch and Lomb Ltd.). Todd-Hewitt

broth was used as the standard. The relationship between colony forming units (c.f.u.) and optical density readings is shown in Figure 4.1. This particular group C Streptococcus grew very rapidly and reached a high maximum census.

Even though it was impossible to quantitate the chain length of the streptococci, attempts to produce a crude lysin preparation were made with phage to bacterium ratios of the order described by Maxted (1957). The two components were incubated at 37°C for 25 minutes, with gentle shaking as described by Fox (1963). The infected culture was then chilled in ice water and centrifuged at 2,000g at 10°C for 30 mins. The packed cells were resuspended in 4 ml of phosphate buffered saline (PBS) pH 7.2, containing 5µg per ml of deoxyribonuclease (Sigma), 10^{-3} molar magnesium chloride and 10^{-3} molar 2-mercapto-ethanol. The suspension was allowed to stand at room temperature for 15 minutes and it was then centrifuged at 10,000g for 30 minutes at 10°C. The supernatant was filtered through a 0.45µ filter and stored in aliquots at 4°C or -20°C.

An attempt was made to obtain some measure of the potency of the lysin using the method described by Fox (1963). Five ml of a culture of Group A, type 12 strain was centrifuged and the pellet resuspended in the same volume of the modified buffered saline described above. To this suspension, 0.2 ml of the lysin preparation was added, and the components were incubated for 10 mins

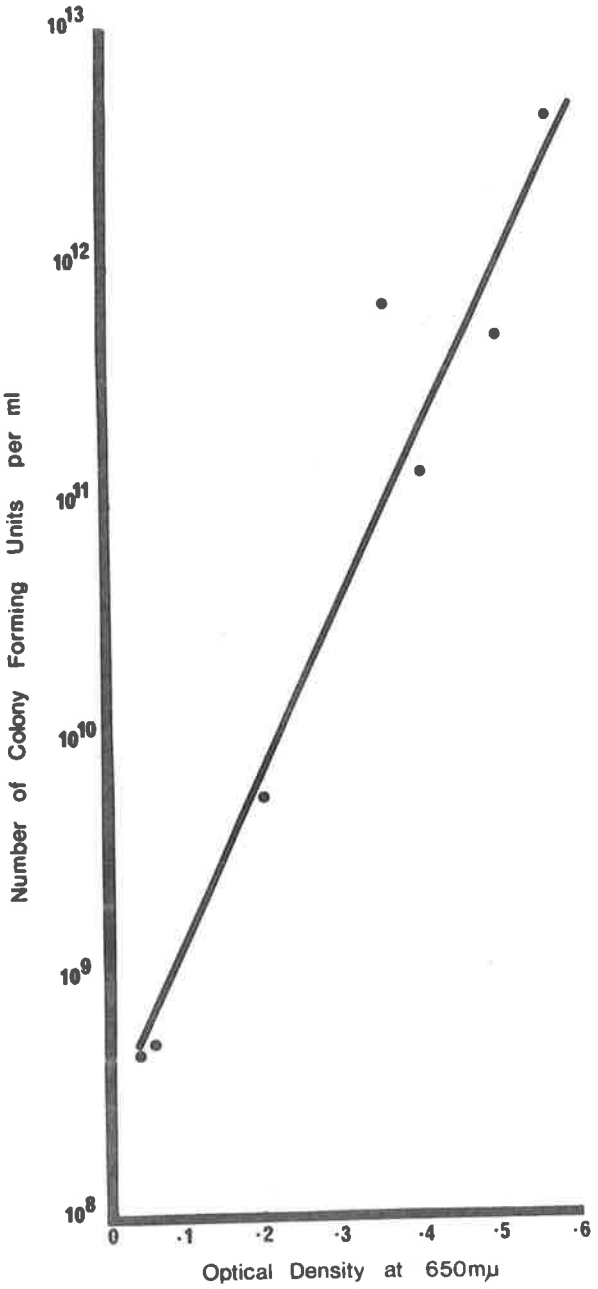


Figure 4.1

Growth - Optical Density Relationship for Group C Streptococcus

at 37°C, with occasional shaking. At this time, no reduction in optical density was noted, and the addition of 0.2 ml of a 2.5% solution of sodium lauryl sulphate did not result in lysis of the suspension. A methylene blue stained smear showed, however, that few streptococci of normal appearance remained in the suspension. A possible explanation of these findings was that although the lysin was active on the streptococcal cell walls, it had not produced significant protoplasting within the 10 minute incubation period. This method of producing phage-associated lysin was standardized as much as possible, and used to produce several batches over a period of some months. Because the requirements of the lysin preparation were strictly limited to its ability to produce protoplasting of group A streptococci, there was no attempt to purify it.

Protoplast Production and L-Form Induction on Agar

In early experiments, 2 ml of log-phase cultures of the three streptococcal strains (A1, A12, A33) were centrifuged at 2,000g for 30 mins and resuspended in 1 ml of 0.01M PBS at pH 6.5. To this, 0.5 ml of crude lysin preparation were added to give a final saline concentration of 4%. The mixtures were incubated at 37°C and smears were made at 10 minute intervals. By 30 mins, a high proportion of the suspensions comprised protoplast-like structures, with few normal cells remaining. Incubation was stopped at this time, and 0.2 ml quantities were

plated out. Experience gained from attempted L-form induction, as described below, indicated that 0.8M sucrose provided better protection for protoplasts than did the 4% saline used above.

Media for L-Form Growth

Various media were used to grow L-forms, including one described by Nimmo and Blazevic (1969). This medium consisted of Brain Heart Infusion (Difco) 5.2%, 20% sucrose, 20% horse serum, penicillin G 1,000 units per ml and agar at 1% final concentration. These authors found that this medium, designated BSYP, gave the best results in comparing growth of the L-phase of a variety of Gram-positive and Gram-negative organisms, in several media formations. The media formulations tried in the present study are shown in Table 4.2. There was no difference between heat-inactivated and non-inactivated horse serum. Oxoid Brain Heart Infusion(BHI) did not support growth of L-forms whereas both Difco and BBL BHI appeared to be equally suitable.

The L-form agar plates used were always freshly prepared with approximately 17 ml of medium per plate. Plates were dried to remove surface moisture only and then they were packaged in sealed bags. All incubations were at 37^{o}C and only two atmospheric conditions were used, namely aerobic and 5% CO_2 in N_2. Incubation was carried out in sealed containers, with wet paper present to ensure high humidity. The gas mixture was changed whenever the plates were examined for growth, usually at 48 hour intervals.

TABLE 4.2

Growth of Phage-Associated Lysin Induced L-Forms on Various Media

Medium	Brain heart infusion		Sucrose		NaCl	Horse serum		Difco bacto agar		Penicillin G 1000 units/ml	Growth
	5.2%	3.7%	20%	28%	4%	20%	10%	1%	1.2%		
1	+		+			+		+		+	some classical "fried-egg" colonies
2		+		+			+	+		+	mainly microscopic atypical colonies
3		+		+			+		+	+	" "
4		+			+		+	+		+	" "
5		+			+		+		+	+	" "
6		+			+	+		+		+	" "

Early Experience of L-Form Induction

The usual procedure was to plate 0.2 ml of a lysin treated streptococcal suspension onto a variety of media (see Table 4.2), allow this inoculum to dry, and then to incubate both aerobically and under 5% CO_2 in N_2. The plates were examined periodically under a stereomicroscope and incubation was continued for up to 3 weeks. Because of the nature of the experiments, contamination by moulds and yeasts was a significant problem. It was standard procedure to include control plates of identical composition to the L-form plates, but without penicillin. The streptococcal colonies that grew up on these plates provided evidence that viable organisms could arise from the procedures involved in attempted protoplast induction, but it is not clear whether their number reflected the adequacy of osmotic support, or the ineffectiveness of lysin action.

Results

Generally, the most common finding was that after about 5 days incubation under CO_2-N_2, small, round, embedded colonies appeared on all L-form media. These were macroscopically visible on the BSYP plates, and microscopically visible on other media, usually in smaller numbers as well. The BSYP plates contained reasonable numbers of the classical "fried-egg" colonies, whereas these were rare on other media. Other colony forms resembled

those illustrated by Young and Armstrong (1969) for L-form colonies of Streptococcus liquefaciens. Blocks were cut from the most promising areas on the L-form plates and subculture was attempted by vigorously pushing an inverted block over a fresh agar surface. After some days incubation, a sparse growth of colonies occurred on some plates. These colonies did not enlarge and they could not be subcultured. To study the suspected L-form colonies in more detail, blocks of agar were stained with Dienes' stain as described for mycoplasmas in Chapter 3. The addition of a few drops of osmotically protected broth to agar plates to facilitate subculture by the inverted block technique, did result in some increase in the number of transferred colonies, but again these failed to propagate. It was found useful to include plates that had been inoculated with sterile broth only as controls. This aided the interpretation of attempted subcultures. Pour plates were used in attempted primary culture of L-forms, but with a total lack of success. Protoplasts were incorporated with the serum and penicillin components, after ensuring adequate osmotic protection, in both full depth pour plates and as a layer over existing L-form agar (Gooder and Maxted, 1961). It is quite possible that failure with this technique was a direct result of inexperience with L-form culture and identification. Greater difficulty was experienced in attempting to identify a colony within the agar mass.

Some other points of significance that arose from these investigations were, that the Group A, type 12 strain gave consistently better results than did type 33 or the type 1, and that a good yield of induced L-form colonies could be obtained from a suspension which looked to be relatively intact. This indicated that the assessment of lysin action by microscopic examination was open to question. Another finding of great interest was the variation in yield and colony type with the storage time of the lysin preparation. Overall, lysin preparations that had been stored at $4^{o}C$ or $-20^{o}C$ for more than a week, produced low yields of L-form colonies with relatively few of these having the classical "fried-egg" structure. Some batches of lysin, however, produced good yields of L-form colonies with a high proportion of classical forms. However, even from these it was not possible to obtain subcultures which propagated.

Because the type 12 strain showed the most promise it was routinely used, and a growth curve of this strain was constructed to facilitate a more precise and reproducible estimate of lysin action. The procedure was the same as that described earlier for the group C Streptococcus, and a graph showing the relationship of colony forming units to optical density is shown in Figure 4.2. By using fresh lysin it was found that high yields of classical L-form colonies could be obtained with the type 12 strain, and these propagated well on subculture. Commonly, the

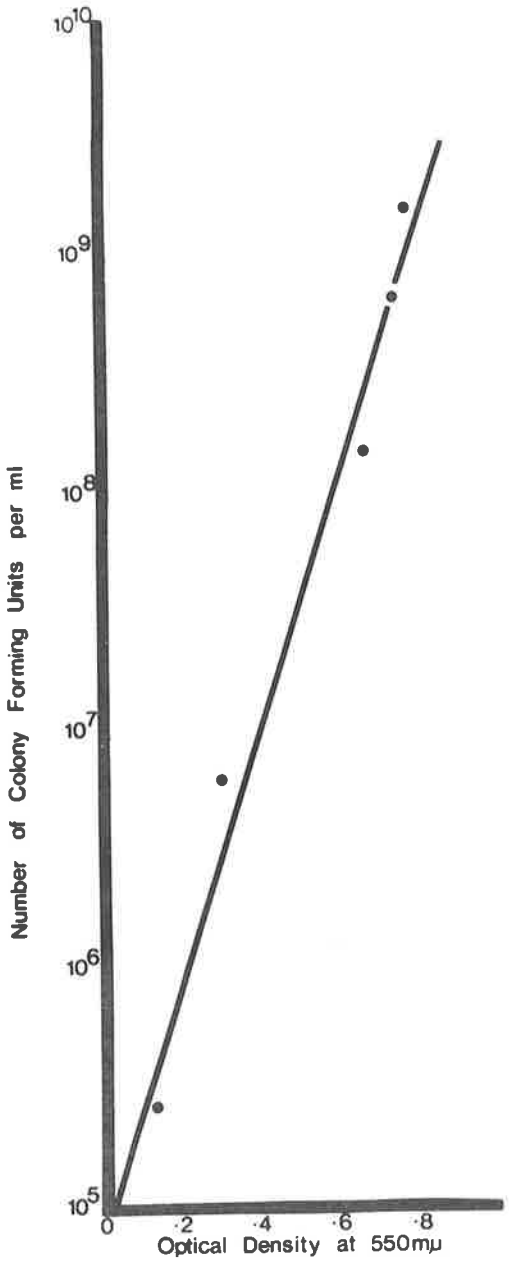


Figure 4.2

Growth - Optical Density Relationship for Group A Streptococcus

first subculture fared little better than did those arising from stored lysin, but subsequent propagation improved rapidly, in size of the colonies and particularly in their number. A large proportion of these colonies had the classical "fried-egg" morphology. This finding only became obvious after many attempts at induction and subculture. The reason why L-form colonies, induced by stored lysin, failed to propagate satisfactorily, remained unexplained, that is even though the amount of protoplasting did not decrease.

Perhaps these colonies were of a similar nature to those described by Young and Armstrong (1969), who thought such structures were a "forme fruste" of mature L-form colonies of S. liquefaciens. It was noted, however, that the classical colonies induced by fresh lysin were larger than those arising from the stored product. It could be that such colonies because of their increased size and possibly their greater bulk above the agar surface, are physically better for subculture. The impression was formed that adaptation of the streptococcal strains to growth in high sucrose broths, before attempted L-form induction, resulted in an increased yield of colonies. This finding was similar to that of Young and Armstrong with S. liquefaciens, although these authors used a different method of induction.

ATTEMPTS TO CULTURE FROM IN VIVO

Background

It was pointed out, in the introduction to this chapter, that an L-form strain induced *in vivo* would provide a realistic setting for a model system of infectivity. Because of this it was felt that an attempt to recover L-forms from mice infected with group A streptococci would be worthwhile. Mortimer (1965) obtained L-form colonies from mice that had been infected with a lethal dose of group A streptococci. Using a type 3 and a type 14 *Streptococcus*, he recovered an average of between 5 and 20 classical L-form colonies from 18% of infected mice. These colonies propagated readily using the inverted agar block technique for subculture. In another study described in this paper, Mortimer obtained L-form colonies from 10 out of 18 streptococcal strains tested. Proof of streptococcal origin was obtained by testing for haemolysin production, M protein typing and reversion to the parent organism. If the mouse peritoneal washings were filtered to remove streptococci prior to plating on a penicillin free medium L-form colonies still appeared. This indicated that conversion occurred *in vivo*, and not as a result of the penicillin induction on the L-form agar plates. Similar results were obtained by Rotta *et al.*(1965). Maxted (1968) obtained L-form colonies from two type 1 strains in a total of 21 strains tested. He found that vancomycin incorporated in the L-form agar, allowed the growth of established

L-forms of the type 1 strains,but did not induce L-forms. When applied to the in vivo situation, L-form colonies failed to grow on the vancomycin agar plates. A similar finding was noted when the amount of the osmotic stabilizer was reduced to allow ready growth of established forms, but did not induce protoplasts to develop. Maxted concluded that the organisms existed as protoplasts within the mice, rather than L-forms. Mortimer (1968) was unable to demonstrate that penicillin could act as an in vivo inducer in experimentally infected mice.

Attempted culture of L-forms from infected mice in the present study was made against the above literature background.

Experimental

Previous experience in this laboratory had shown that the three streptococcal strains used were of low-order virulence for mice. After intraperitoneal injection of between 1×10^6 and 1×10^9 colony forming units, the infected mice died over several days. In view of Mortimer's (1965) findings that low virulence strains gave higher yields of L-forms, the organisms used in the present study seemed to be potentially suitable.

Overnight cultures of group A type 12 and type 1, were centrifuged and resuspended in Ringers solution. Approximately 1×10^7 colony forming units in 0.2 ml volumes were injected intravenously into Swiss albino mice. The 50 mice used were divided into groups:

1. Received penicillin G intramuscularly in 0.5 ml volumes at the same time as the streptococcal injection,
   A. 10,000 units
   B. 1,000 units
   C. 100 units

2. These received penicillin on the following day,
   A. 10,000 units
   B. 1,000 units
   C. 100 units

3. Mice with no penicillin treatment.

4. Mice injected with Ringers solution only.

All of the mice were sacrificed 48 hours after the streptococcal inoculation. They were killed by a blow to the head and blood was collected under sterile conditions. The samples were plated onto BHI and BSYP agar and incubated under CO_2 in N_2.

Results

After 4 days incubation no bacterial colonies appeared on any of the BHI plates. It is quite possible, however, that organs such as spleen and liver contained streptococci even though blood cultures were sterile. Some of the L-form agar plates showed structures which, when stained with Dienes' stain resembled L-form colonies. Interpretation was difficult because of the large number of partly broken down mouse red blood cells and leukocytes on the agar surface.

The experiment was repeated using a larger infecting dose of streptococci, this being about 1 x 10^9 colony forming units per mouse. On this occasion some of the infected mice not treated with penicillin had died by 30 hours, but there were also 2 deaths in the group treated at 100 units of penicillin G per mouse. Again no streptococci were isolated from the blood of these mice. Blocks of agar were removed from promising areas in the L-form agar plates, stained with Dienes' stain and suspected colonies were counted. Control plates which had been incubated with normal mouse blood, were treated similarly. The counting was done in a blind-trial manner and the results showed the counts to be approximately the same. Similar negative results were obtained after 14 days subculture of these plates.

The failure to obtain classical L-form colonies which propagated well on subculture could perhaps have been predicted in view of the results of Maxted (1968), who succeeded with only 2 out of 21 strains. The experiments did, however, indicate the importance of an adequate means of identifying suspected L-form colonies with certainty. Only 2 suitable streptococcal strains were available at the time; this coupled with the evidence produced by Maxted (1968) that the organisms may exist as protoplasts, not growing L-forms in vivo, prompted the discontinuation of these experiments.

PROPAGATION OF L-FORM STRAINS

The group A types 1 and 12 propagated well on a variety of L-form media, producing "fried-egg" colonies in abundance. The type 33 propagated only poorly and was abandoned. Attempts were made to induce direct growth in broth by dropping a block of agar, containing colonies, into 10 ml of L-form broth. This broth consisted essentially of L-form agar medium without agar.

The result of this, was that the colonies merely enlarged in situ, growing as large cone shaped bodies in the agar block. Even when the agar block was mashed, by forcing it through a sterile syringe and needle, as described by Hayflick and Stinebring (1960) for mycoplasmas, the isolated colonies continued to enlarge as an integral mass. The colonies were apparently very coherent, as they resisted moderate means of disruption, such as physical agitation and even mild sonication. Diphasic media were prepared by adding L-form broth to a slant of L-form agar in a screw cap bottle. The previous experience with very coherent colonies was confirmed.

New Strains

Two L-form strains, type 12 number 416 and a number 35, derived from a type 6 streptococcus, were supplied as freeze dried cultures by Dr. W.R. Maxted, Colindale, London. The

number 416 propagated well in the L-form medium recommended by Dr. Maxted. This consisted of BHI (Difco) 3.7%, horse serum 10% and agar 1% with either 25% sucrose or 3.5% sodium chloride as osmotic stabilizers. The technique of subculture was to use the inverted block method or to spread a mashed colony preparation over the agar surface. The latter method gave superior growth but it was attended by a greater risk of contamination.

When mashed colonies of this strain were introduced into diphasic media, L-forms grew as a sticky, tenacious mass but vigorous physical agitation helped to disperse the lumpy material. Growth was at first very slow in diphasic media, with the number of colony forming units doubling only after several days of incubation. Because of the very small size of many of the L-form colonies, random areas of Dienes'-stained plates were counted under the microscope and the total area counted was then extrapolated to the whole agar plate. With continued propagation of this strain in either diphasic media or broth culture, it was possible to obtain obvious growth within 24 hours using a 1 in 20 inoculum. Another finding was that this strain grew well in the presence of sucrose concentrations as low as 10%. As the broth cultures became more vigorous with continued subculture, it was noted that larger colonies were formed on agar. Most of the colonies were macroscopic, some being as large as 2 mm in diameter. At this stage, a 40-hour broth culture contained approxi-

mately 1 x 10^5 colony forming units per ml. There was little difference in the growth rate or maximum census between cultures that were agitated and those that were not.

After some time, granularity appeared in some of the cultures. There was no apparent reason for this but it was a problem because the culture was not easily dispersed, even by agitating it in the presence of glass beads. In an attempt to disperse the culture, Tween 80 (Chemical Materials, Ltd., N.S.W.) was prepared as a 1% solution in 50% sucrose and was sterilized by filtration. To find concentrations that would disperse the organisms but not inhibit growth, broth cultures were established with Tween 80 at final concentrations of 0.1%, 0.01% and 0.001% in addition to controls. After two days incubation the broth with 0.01% Tween 80 showed turbid uniform growth, the 0.1% concentration was apparently inhibitory to growth and the 0.001% concentration was just as granular as the controls. This strain continued to propagate well in broth and was apparently stable because no sign of reversion was ever detected in penicillin-free media.

The type 12-416 strain provided the basis for all of the early infectivity studies, its use being discontinued only after a growth failure. A batch of horse serum with either toxic or lack of growth-supporting properties was probably responsible for this. Non-toxic sera were used in subsequent studies.

Experience with the L-Form Strain GL8 and 49

The restoration of the 12-416 strain coincided with the arrival of two new L-form strains, the GL8 derived from a type 19 parent and an L-form derived from a type 49 Streptococcus. These strains were kindly supplied by Dr. Janine Schmitt-Slomska of Paris, France. The 49 L-form had been induced in vivo in infected mice while the GL8 had been induced in vitro. The L-form derived from the type 3 Streptococcus (Richards strain), used in the tissue culture infectivity experiments of Schmitt-Slomska et al. (1968), was requested but was unavailable. These L-form strains were grown in Trypticase Soy Broth (BBL), yeast extract 1.5% (Difco), 1% agar (Difco bacto agar), 10% horse serum (C.S.L. labelled "suitable for mycoplasma testing"), 3% sodium chloride and penicillin G at 1,000 units per ml. The strains were found to be stable and the penicillin, because it was unnecessary, was deleted from the medium. It was soon found that the type 49 strain was a poor, slow grower, whereas the GL8 strain was quite vigorous. Characteristically, this strain produced large colonies (up to 4 mm in diameter) with an opaque centre and an irregular raised periphery. It grew well in broth culture giving an even turbidity in the absence of dispersing agents.

A growth curve was constructed in the manner described previously for the group C Streptococcus (see Figure 4.3). The excellent growth was an obvious advantage but the relationship of growth to optical density recordings was a limiting factor because it was possible to estimate growth only over a limited range (see Figure 4.4).

Attempts were made to obtain a linear relationship with absorbance readings at 260mμ for nucleic acids, and at 280mμ for proteins. The quantities of both were apparently so great that high dilutions were necessary to obtain a meter reading. Presumably, the horse serum and the yeast extract in the medium contributed to these problems. It was thought improbable that an accurate and reproducible measuring system could be devised in this case.

If an L-form broth culture was centrifuged under normal laboratory conditions of 2,000g for 20 mins, about 50% of the colony forming units were recovered in the pellet. When this pellet was resuspended in broth and centrifuged under the same conditions, most of the colony forming units were recovered. This information was useful in the preparation of L-forms for infectivity experiments as discussed in Chapter 5.

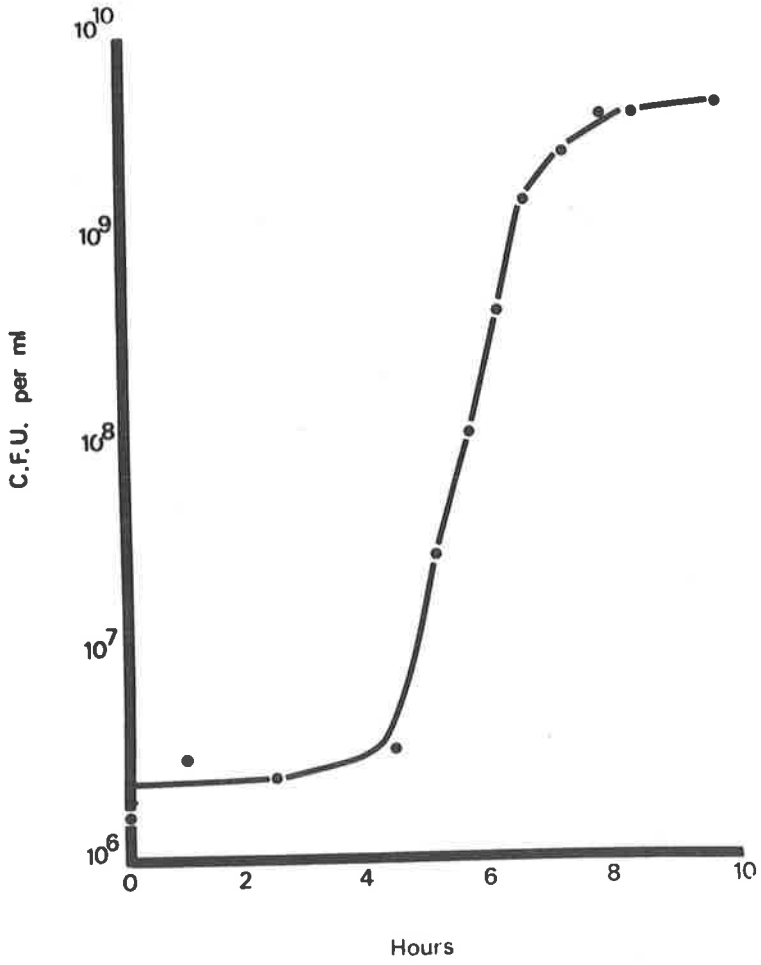


Figure 4.3

L-Form Strain GL8 Growth Curve

C.F.U. ≡ Colony Forming Units.

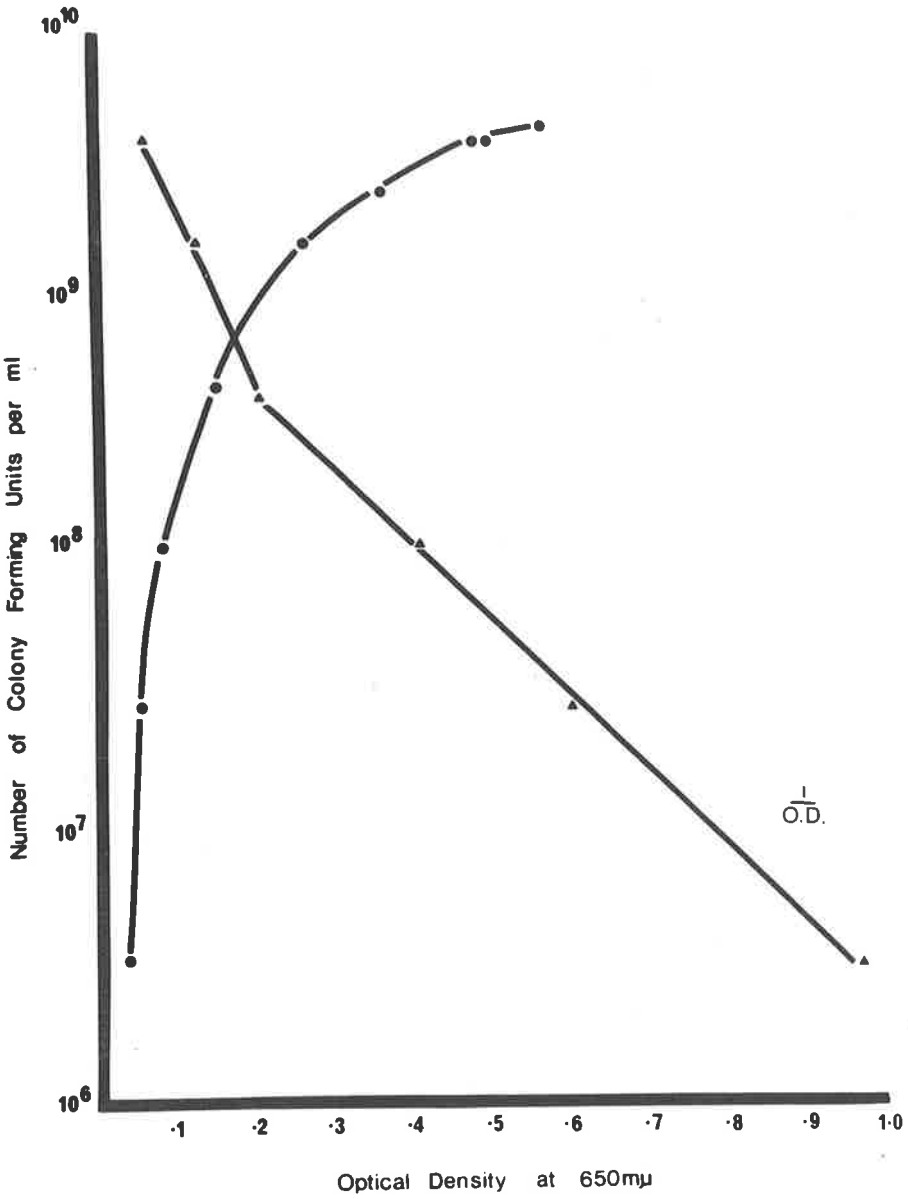


Figure 4.4

Relationship of Colony Forming Units of L-Form GL8 to Optical Density

$\frac{1}{O.D}$ ≡ Reciprocal of Optical Density Recording.

Another noteworthy observation was that the growth rate and the maximum census of the GL8 strain were very dependent upon the batch of horse serum used.

Preservation of L-Form Cultures

Agar plate cultures stored at -56°C gave good recovery rates over several months. Stewart and Wright (1970) obtained better recovery of meningococcal and streptococcal L-forms in broth culture stored at -196°C or -70°C, than from lyophilized organisms. For short term storage in the present study, L-forms were stored as broth cultures at 4°C, while deep frozen (-56°C) and lyophilized stocks provided long term preservation.

COMMENT ON THE EXPERIENCE WITH L-FORM INDUCTION AND PROPAGATION

At the commencement of this study, it was felt desirable to induce and grow L-forms from the parent streptococci. It was thought that valuable experience would be gained in the culture of L-forms and that induction from the parent would allow transfer of markers such as antibiotic resistance, these being more readily induced in parent streptococci than in the relatively slowly propagating L-forms. Although it was considered that the early work provided valuable lessons in L-form culture and behaviour, the problem of limited time forced an altered approach. The strains used tended to fulfil practical requirements such as ready and reliable growth in broth culture.

INFECTIVITY STUDIES CHAPTER V

This chapter describes the early attempts to follow possible interactions between L-forms and cells.

INTRODUCTION

The experiments were designed to provide information in the following areas:

L-form interaction with various cell types.

Adsorption of L-forms to cell surfaces.

Intracellular persistence and multiplication.

Cytopathology associated with L-forms or their products.

There was obvious need to utilize techniques capable of adequately demonstrating L-forms, both extracellularly and intracellularly, as well as identifying cell changes in response to introduced organisms. In addition, there was the need to quantitate, as far as possible, the interactions of L-forms with cells and the recovery of these organisms from cell cultures.

These aspects of the infectivity study are considered under the following headings:

Staining procedures for the L-forms.

Staining procedures for the estimation of cell changes.

The estimation of pathologic cell changes.

The quantitation of infectivity ratios and L-form recovery.

Experimental procedures.

Fluorescent antibody studies.

STAINING PROCEDURES FOR L-FORMS

Hayflick and Stinebring (1960) used the May-Grünwald and Giemsa staining procedure of Jacobson and Webb (1952) to identify intracytoplasmic inclusions of mycoplasmas in tissue culture cells. These inclusions stained red to purple against a cell background described by Jacobson and Webb as a blue cytoplasm of varying intensity and granularity, and a nucleus stained overall purple-red, but of varying colours during the different phases of the mitotic cycle. These authors produced evidence that the blue staining was specific for ribonucleoprotein, whereas purple-red staining was specific for deoxyribonucleoprotein in the resting nucleus.

Acridine orange staining was considered to be a potentially useful technique because it differentially stains deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), giving brightly fluorescing colours (Kasten, 1971). Kasten (1967) has extensively reviewed the use of acridine orange as a cytochemical stain. Despite the pitfalls pinpointed by Kasten this technique seemed to offer a

chance of obtaining differential staining of cells and organisms. The technique used for staining was that described by Lynch et al. (1969a). Acridine orange (BDH) was prepared as a 0.1% aqueous solution and stored in a dark bottle at 4°C. Bacterial smears were heat fixed while tissue cultures on coverslips were fixed in 95% ethanol. They were stained at pH 6.0 and rinsed in phosphate buffer pH 6.0 to remove unbound dye. This was followed by differentiation in 0.1M calcium chloride for varying times. The calcium chloride differentiation was found to be the crucial step in determining the colour balance produced. In the case of tissue culture cells, 90 seconds differentiation produced cells with only green nuclear fluorescence whereas 15 seconds produced a good balance of yellow-green nuclei and orange cytoplasm. Streptococcal and L-form preparations gave a bright orange fluorescence after 15 seconds differentiation, with the streptococci showing resistance to further differentiation. L-form smears tended to show this resistance as well, but less convincingly than the parent organisms (see Plate 5.1).

STAINING PROCEDURES FOR THE ESTIMATION OF CELL CHANGES

After experimenting on cell cultures with different fixation and staining procedures, glutaraldehyde fixed, Heidenhain's haematoxylin-stained cell cultures were found to produce good

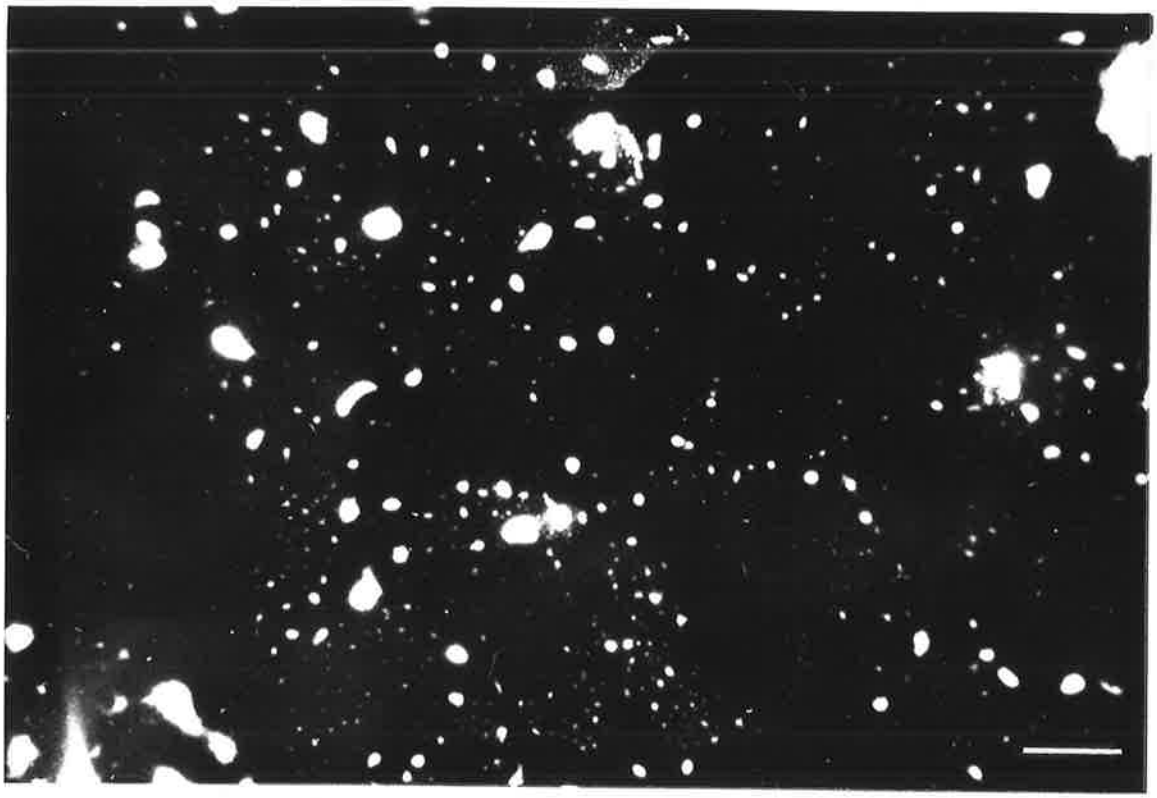


Plate 5.1

Acridine orange stained suspension of L-form strain 416.

Showing fluorescent L-forms after 2 minutes calcium chloride differentiation.
There is a wide range in the size and the intensity of fluorescence of the bodies.
There is no obvious characteristic morphology.
Scale: 10μ
Magnification, x 1,250

cytological detail (see Plate 5.2). Tissue cultures were rinsed with buffer prior to fixation in 4% glutaraldehyde (AJAX) in 0.2M cacodylate buffer (sodium cacodylate, BDH), pH 7.4. The cacodylate buffer was prepared according to Giegy Scientific Tables, while the fixative was prepared according to Hopwood (1967). The cultures were stained by Heidenhain's haematoxylin method as described by Lynch et al. (1969b).

In the case of rat heart cell cultures, it was thought desirable to have a means of rapidly identifying the two cell types present, namely, the myocardial cells and the endothelioid cells. This was achieved by the Van Gieson procedure described by Conn et al. (1960). The morphology of the myoblasts and endothelioid cells was discussed in Chapter 2. The Van Gieson stain aided differentiation of the two cell types by staining the cytoplasm of the myoblasts yellow and that of the endothelioid cells red-brown.

ESTIMATION OF PATHOLOGIC CELL CHANGES

General

The response of cells to noxious agents varies from mild changes, such as slowing of the rate of division, to sudden death. Retrogressive cell changes have been described by many authors but

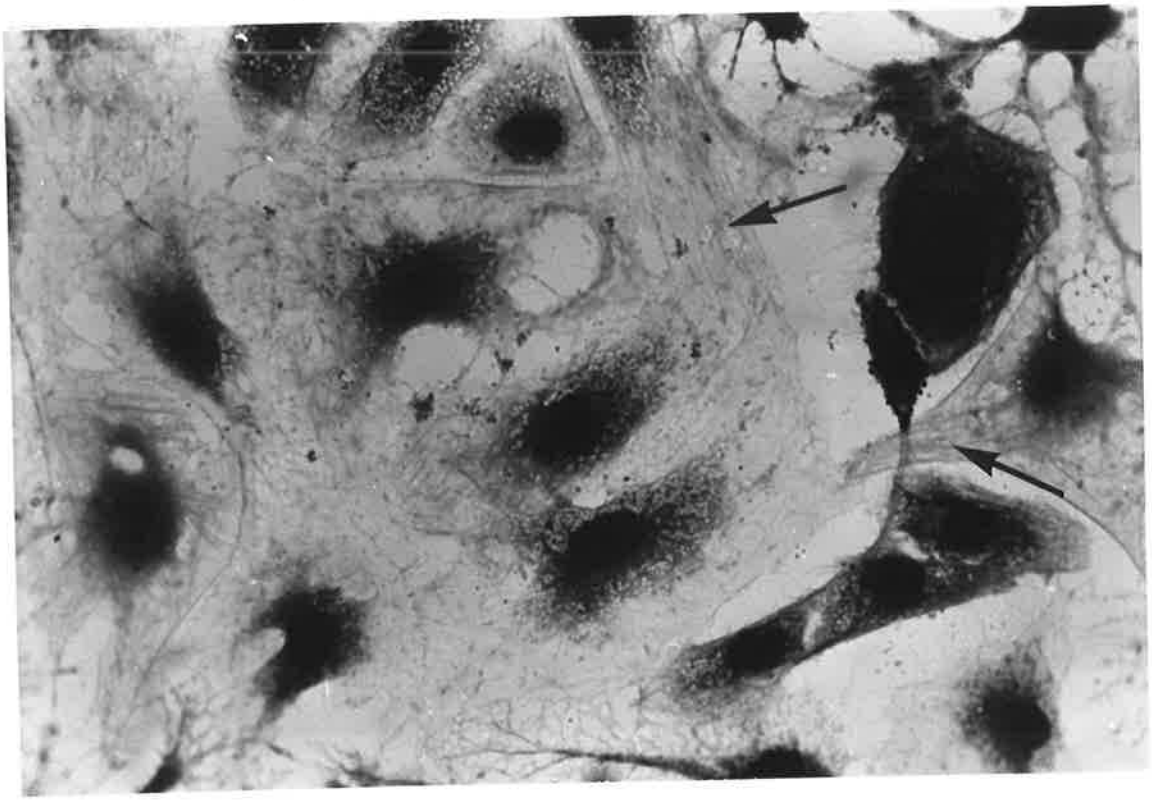


Plate 5.2

A Heidenhain's Haematoxylin stained rat heart cell culture

Arrows indicate myofibrils.
At this degree of differentiation nuclear morphology is obscured.
Magnification, x 1,250

Scarpelli and Trump (1971) listed the most common response of cells to diverse forms of injury as:

Loss of cell volume control.

Decrease in the optical density of the cytoplasm.

Appearance of lipid droplets in the cytoplasm.

Violent movements of the plasma membrane followed by the appearance of blebs.

Nuclear oedema.

Pyknosis or condensation of the nuclear material.

Karyolysis.

These changes are progressive and reversible up to a certain stage. According to Scarpelli and Trump, lipid accumulation could result from an imbalance in normal lipid metabolism, or from a breakdown of cell lipid, particularly in membranes.

Malinin and Perry (1967) listed a variety of procedures used for estimation of cell viability. Some of these that are appropriate for a tissue culture system are:

Phagocytosis - pinocytosis.

Dye exclusion (for example, trypan blue).

Mitosis.

Enzyme activity.

pH changes in the media.

Electron microscopic changes.

Volume changes.

Uptake of tritiated thymidine.

Histochemistry (for example, of enzymes or lipids).

Cell attachment to glass.

Phagocytosis would perhaps be easier to measure than pinocytosis in appropriate cells,for example, cultured macrophages; a damaged cell would presumably have a lower uptake of given organisms than a normal control. Quinn and Lowry (1967) used trypan blue exclusion as an index of cell viability in a tissue culture system. A decreased mitotic index and a change in the levels of activity of certain enzymes could also provide evidence of cell damage. A slower fall in medium pH could be coupled with a changed mitotic index, while electron microscopic examination could confirm cell changes such as those listed by Scarpelli and Trump. Histochemistry, using for example, Sudan black staining for lipid, could provide a semi-quantitative measure of cell changes. On the other hand, Malinin and Perry (1967) noted one report indicating that incorporation of a nucleotide, such as tritiated thymidine, did not necessarily require a living cell.

Examples of Assessment of Cell Change

Kagan (1967) studied the cytopathic effect in cell cultures induced by L-forms of Salmonella typhi and beta-haemolytic streptococci. Cells with clear vacuolation, pyknotic and karyorrhectic

nuclei, areas of cell lysis and denuded areas in the cell monolayer were the cell reactions described, with differing time sequences and severity noted for different organisms. Quinn and Lowry (1967) observed the effect of Strep. pyogenes on cultured cells. They noted cytoplasmic blebs, thickening of the nuclear membrane, coarse cytoplasmic granulation, distortion of nucleoli, cell shrinkage and a failure of the cells to stick to the glass substrate. Jones and Schwab (1970),in a study of the effect of cell wall fragments of group A beta-haemolytic streptococci on a kidney cell line, measured the cell population and average cell size as criteria for a cytopathic effect. Thompson et al. (1970) noted the following changes in rat heart cell cultures after addition of streptolysin O. By phase contrast microscopy, the myocardial cells were found to stop pulsation less than 20 seconds after administration of 8,000 haemolytic units per ml of streptolysin O. Cytoplasmic blebs extruded from the cell membranes and cytoplasmic organelles became intensely granular. After 5 minutes, the double nuclear membrane appeared to be swollen with the nuclear contents showing disorganization. These findings were confirmed and extended by electron microscopic studies.

Methods used in the Present Study

Gross damage could be detected by marked shrinkage of both cytoplasm and nuclei with some areas of the monolayer being

denuded. The sloughing of the cells from the glass gave rise to a great increase in the number of dead cells floating in the medium. Sudan black staining of lipid accumulations was attempted by the method of Lynch et al. (1969c), but it was difficult to estimate a minor change because many apparently healthy cells showed evidence of lipid accumulation. The degree of this was possibly related to the batch of serum used as a medium supplement (see Plate 5.3). More subtle changes in the cell population were detected by slowing of the growth rate and lower metabolic rates as indicated by a slow fall in the pH of the medium. These changes were followed more closely with electron microscopic examination.

The electron microscope was used to follow other than retrogressive changes as well, namely the change in cell organelles following ingestion of a foreign particle. These events have been well described by Gordon et al. (1965) for L cells and DNA-protein coacervates. This aspect of cell behaviour is considered more fully in Chapter 6.

The distribution of cell changes over an entire monolayer had an important bearing on the interpretation of experiments. It was commonly noticed that in both experimental and control cultures variation in cell morphology, staining intensity and cell density were apparent. In the case of L cell cultures, the lack

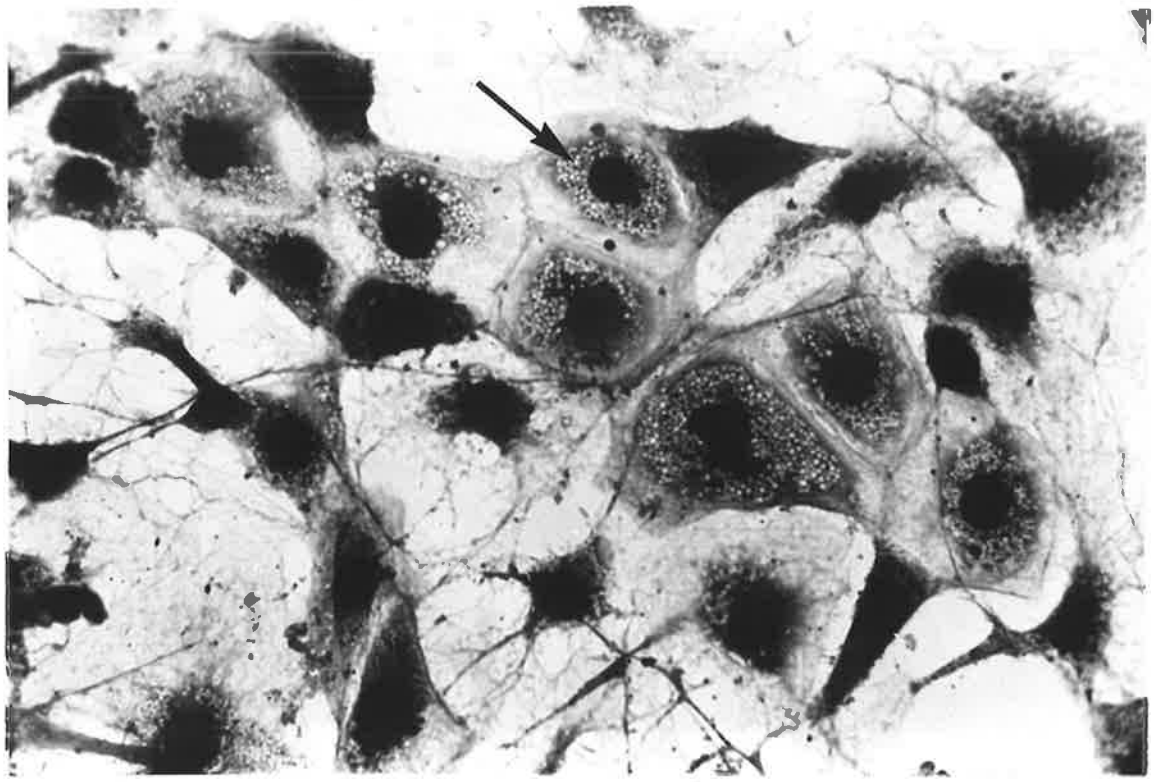


Plate 5.3

Heidenhain's Haematoxylin stained rat heart cell culture

Arrow indicates cytoplasmic vacuolation, possibly due to lipid accumulation.
This was commonly seen and was thought to be related to batch of serum as well as the age of the culture.
Magnification, x 1,250.

of true homogeneity in the cell population could explain why small areas, possibly derived from a single cell, showed atypical reaction (see Plate 5.4). Other cases could be due to a lack of uniformity in the nature of the glass substrate or to physical factors. An example of the latter could be uneven distribution of introduced organisms as shown below in Figure 5.1.

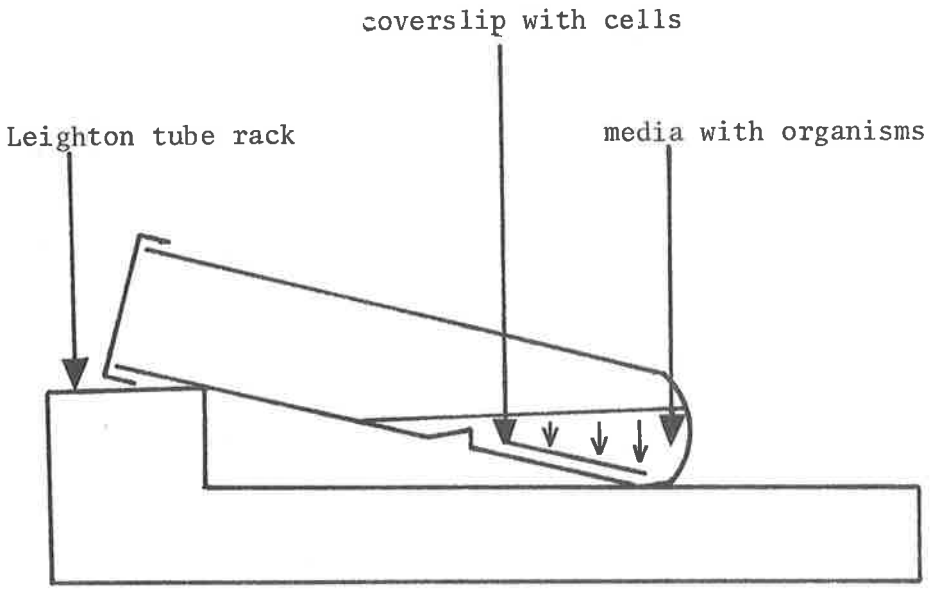


Figure 5.1. Possible uneven settling of organism on the coverslip.

A slope like this could result in most of the organisms settling on a small proportion of the cell population. Findings like these highlighted the need for careful design and evaluation of experiments.

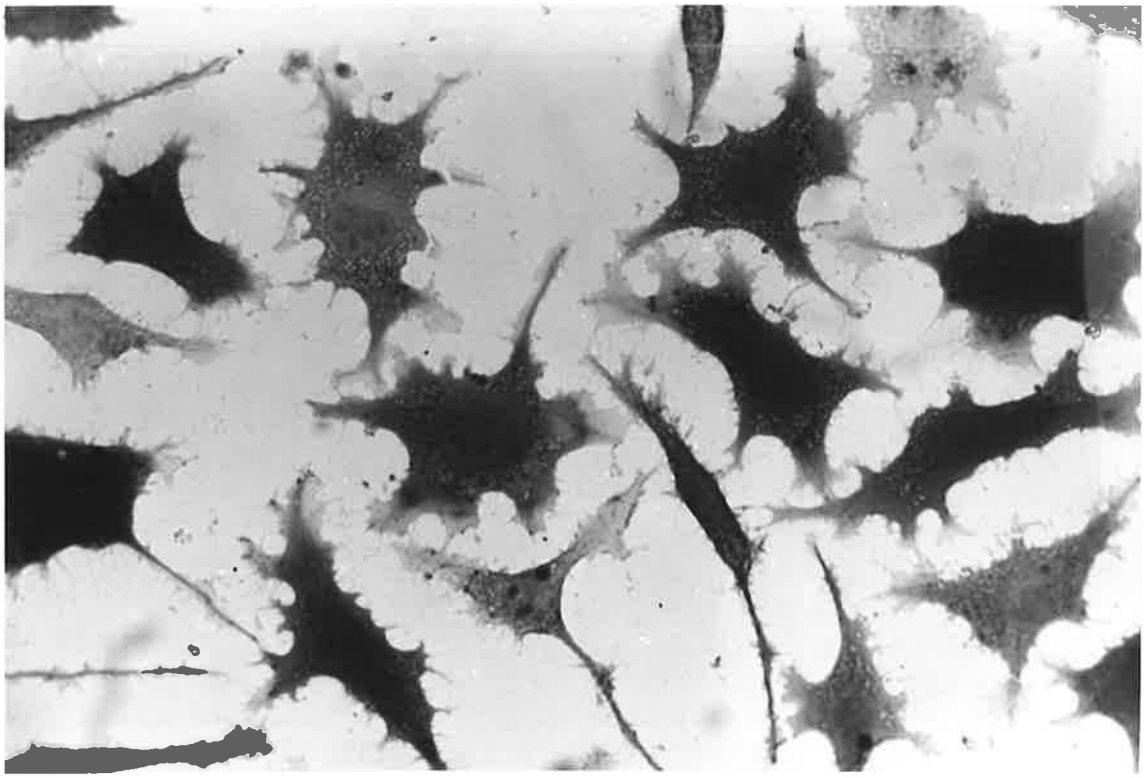


Plate 5.4

Heidenhain's Haematoxylin stained L fibroblast culture

Showing the variation in staining intensity between cells.
Magnification, x 1,250

THE QUANTITATION OF INFECTIVITY RATIOS AND L-FORM RECOVERY

The Quantitation of Infectivity Ratios

As noted in Chapter 4, the 12-416 L-form had a variable growth pattern. The two main considerations relating to this were that the growth in broth slowly improved and that the culture was often not completely uniform, that is to say, a minor degree of clumping was not uncommon. In the early experimental work, growth peaked at about 40 hours with a maximum census of about 1×10^5 cfu per ml. Figure 4.4, relating growth of L-form GL8 to optical density, shows that for this organism at least, there was no appreciable increase in readings until a level of about 1×10^8 cfu per ml was reached. Attempts to correlate organisms counted under phase contrast or acridine orange-stained smears with colony forming units failed. For these reasons, 40-hour cultures with approximately 1×10^5 cfu per ml were used with plate counts giving accurate numbers after the experiment had been performed.

With the GL8 L-form, counts based on optical density recordings were possible over a limited range. Accurate recordings made by plate counts could be obtained after the experiment as in the case of the 12-416 strain.

Cell populations could be estimated by counting in a haemocytometer. If cells and L-forms were to be mixed in suspension, the

cells were counted prior to addition of L-forms, whereas if L-forms were added to monolayers the cell population could be estimated by harvesting from a number of coverslips and averaging the count.

L-Form Recovery

Viable L-forms might occur free in the medium, attached to cell debris or dead cells floating in the medium; alternatively they could be associated with cells in the monolayer. Because of this, samples were plated onto L-form agar from culture medium, from the supernatant of a centrifuged suspension and from the resuspended cell pellet. Samples from control cultures and L-form broth cultures were plated for control purposes.

EXPERIMENTAL PROCEDURES

Early experiments involved attempted infection of rat heart cell and human diploid cultures with the 12-416 L-form strain. The main aim was to evaluate the techniques described in the introduction to this chapter. Another important consideration was the basic rationale applied to osmotic pressure requirements. Because of the requirements of L-forms of Strep.pyogenes for osmotic protection, they do not survive in normal tissue culture

media. The population decline of L-forms suspended in tissue culture media is considered in detail in Chapter 7. In contrast to this, tissue culture cells do withstand considerable changes in the osmolarity of the medium, provided that the changes are not too rapid (White, 1963). It was considered that L-forms induced from the parent organism *in vivo* could be located in the relatively hypotonic environment of the host's body fluids, or in the variable environment of a cell. Mammalian fluids appear to have certain stabilizing properties for L-phase organisms. These include the peritoneal exudate factor described by Mortimer *et al.* (1972) and the membrane stabilizing action of spermine (Hijmans *et al.*, 1969).

It seemed, therefore, that at least some experiments should involve presentation of protected L-forms to the cells.

Osmotically Compatible Media

Gooder (1968) used 8% polyethylene glycol to stabilize lysozyme-induced protoplasts of *Strep. faecalis*. Trials with 8% polyethylene glycol, as carbowax 4,000 (Selby Ltd.), indicated that the cell cultures tolerated this level quite well. It was found, however, that L-forms did not appear to survive in this medium because no growth resulted from a suspension with 3×10^4 cfu per ml added, when 2 hour and 24 hour samples were plated and incubated for 3 days. If cells and L-forms were suspended in tissue culture medium (TCM) with sucrose at a final concentration

of 8 to 12%, good survival of L-forms resulted. Incubation of tissue culture cells for 1 to 2 hours in a medium containing 8% to 12% sucrose also resulted in an acceptable level of cell survival. Incubation of cells with sucrose for longer periods resulted in a marked loss of viability, as judged by trypan blue exclusion techniques. This is discussed further in Chapter 7.

Combinations used in Infectivity Studies

These are summarized in Table 5.1. In addition, the following points are worthy of note. With some infections of monolayers, the organisms were not removed after 1 or 2 hours incubation. L-forms added without osmotic protection were introduced as L-form broth cultures or suspensions washed in phosphate buffer with 3% sodium chloride. Sucrose was preferred to a salt as the osmotic protectant because it was thought that any ionic material might interfere with adsorption of the organisms to the cells.

Cell Inoculum

This was determined by the duration of the experiment and the cell type used. Rat heart cell cultures did not increase in number significantly, therefore the inoculum of cells was sufficient to give a good cover of cells over the coverslip. Human diploid cells, on the other hand, continued to divide and so the

TABLE 5.1

The Conditions for Infectivity Experiments with L-Form Strain 12-416

Cell Presentation	Infectivity Ratios		Osmotic Support		Incubation Times	Agitation		Monitor System			Reculture
TCM suspension	1 cfu/ 10 cells	100-500 cfu/cell	8-12% sucrose	none	1 or 2 hours	gentle mixing	none	May-Grunwald-Giemsa	Acridine Orange	Heidenhain's Haematoxylin	Attempted
Monolayer	1 cfu/ 10 cells	100-500 cfu/cell	8-12% sucrose	none	1 or 2 hours	none	none	May-Grünwald-Giemsa	Acridine Orange	Heidenhain's Haematoxylin	Attempted

inoculum size was adjusted to give confluent control cultures at the end of the experiment. This was necessary because cultures soon showed signs of degeneration after confluence had been reached. The adjustment of pH, where necessary, was carried out by the addition of sodium bicarbonate rather than by changing the medium.

Results

These are summarized in Table 5.2. The experiments were continued for up to 10 days when most of the cultures showed signs of deterioration. At earlier times, both test and control cultures remained healthy. L-forms of the 12-416 strain were recovered quantitatively only up to 24 hour sampling when 2.5% to 10% were recovered from the cell pellet and 10% to 15% from the supernatant. It is possible that those recovered with the cell pellet were not actually cell-associated, but were merely carried down with the cells. Although a detailed examination of the survival of this strain in hypotonic media was not carried out, it was thought from limited observations that the numbers recultured reflected approximately the survival rate in tissue culture medium. May-Grünwald-Giemsa staining did not reveal any cytoplasmic inclusions and acridine orange gave variable results. Some test cultures showed discrete orange-fluorescing objects against a pale cytoplasmic background as shown in Plates 5.5, 5.6 and 5.7. In only one rat heart cell culture, after 7 days incubation, were a few streptococcal chains seen

TABLE 5.2

Summary of the Results of Infectivity Experiments with L-form strain 12-416

Cell type	Effect of incubation in suspension or as monolayer with or without agitation	Effect of osmotic support for the L-forms	Effect of infectivity ratios		Acridine orange	Giemsa	Heidenhain's Haematoxylin	Reculture
			Low	High				
Heart cell cultures	none	none	no interaction	observed interaction	orange fluorescing granules against pale background	no inclusions	test cultures in good condition	only at levels comparable with death rate in this medium
Human Diploid Fibroblasts	none	none	no interaction	observed interaction	orange fluorescing granules against pale background, but at lower frequency	no inclusions	test cultures in good condition	only at levels comparable with death rate in this medium

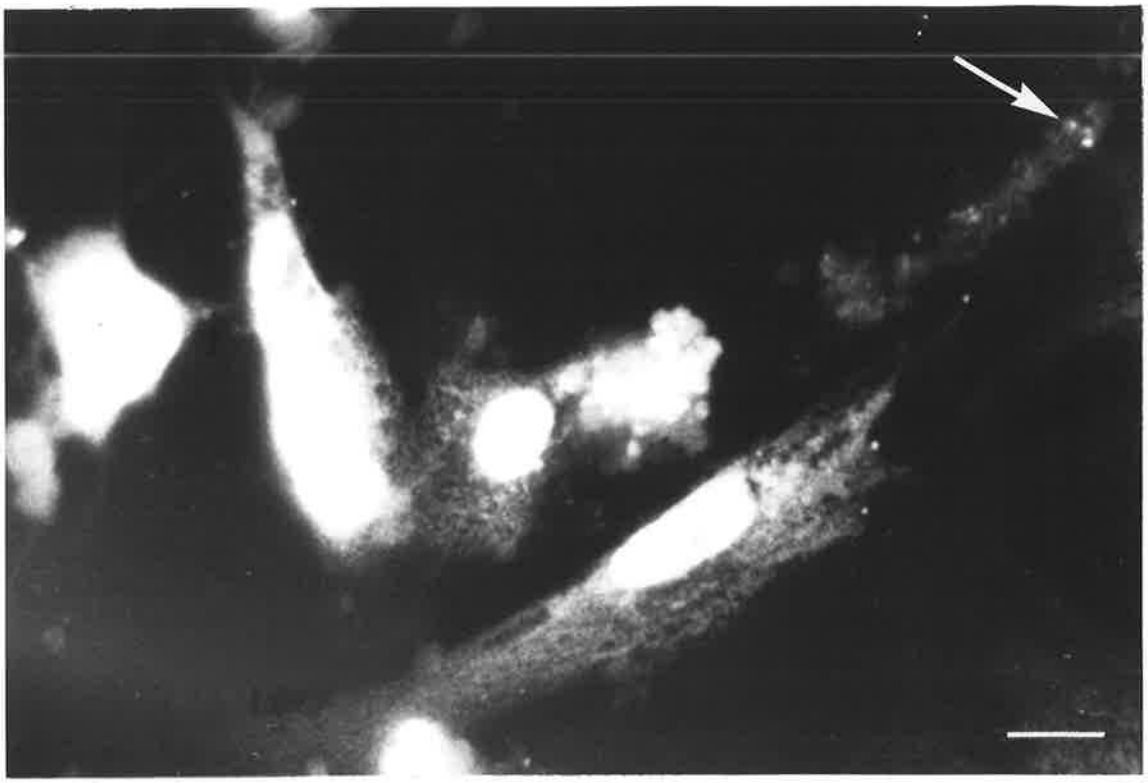
Plate 5.5

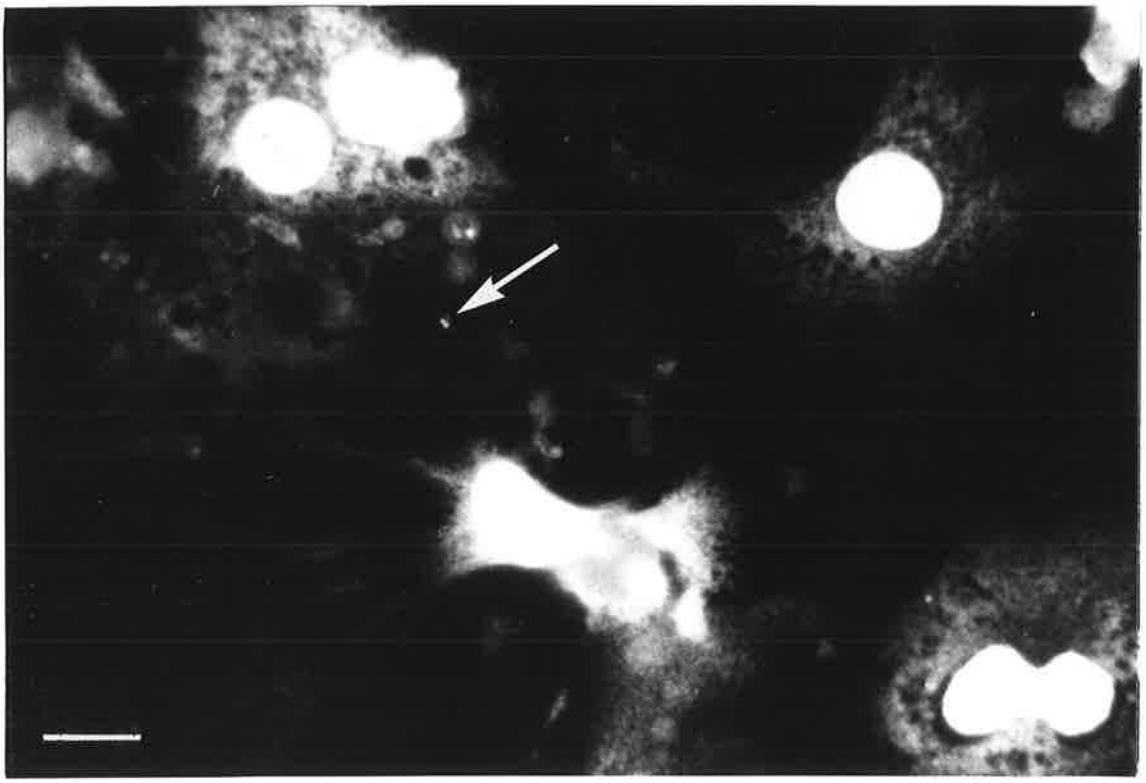
Acridine orange stained rat heart cell culture infected with L-form 12-416 at 100 cfu per cell for 2 hours

Arrow indicates fluorescent bodies, possibly L-forms.
Differentiated in calcium chloride for 1 minute.
Scale: 10μ
Magnification, x 1,250

Plate 5.6

As above.
Scale: 10μ
Magnification, x 1,250





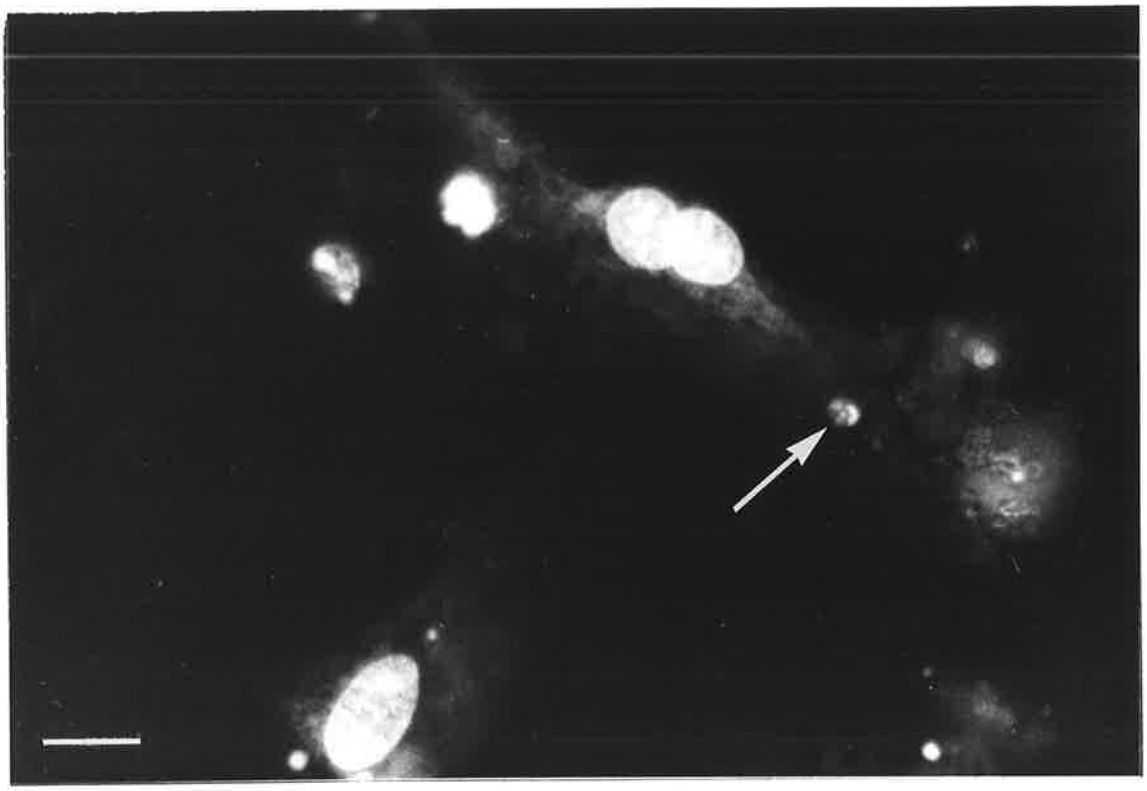


Plate 5.7

As for Plate 5.5

Scale 10μ

Magnification, x 1,250

in relation to the cells. These were most probably reverted forms.

Staining reactions were never completely uniform over a whole coverslip culture; for example, a few cells would retain brilliant orange cytoplasmic fluorescence while the majority were almost colourless. There was also a variation in staining intensity in different areas of the coverslip cultures despite careful staining and washing procedures. To avoid preparations with large amounts of debris, it was found necessary to rinse the cultures adequately in buffer prior to fixation and to agitate during fixation. Another finding related to acridine orange fluorescence was that discrete green-fluorescing structures were quite common in the cytoplasm of cells. Kasten (1971) considered that these were probably mitochondria or lysosomes. In addition, there were areas in the cytoplasm of many cells which seemed to retain orange fluorescence. Only when attention was confined to discrete orange fluorescing granules could any real difference between control and test cultures be detected.

Further experiments with the GL8 L-form strain and L fibroblasts were carried out in a manner identical with that described for the 12-416 strain with rat heart cell cultures and human diploid fibroblasts. The results were essentially the same with the only real indication of interaction being the presence of brightly orange fluorescing granules following acridine orange staining.

Reculture of the GL8 strain from infectivity experiments was comparable only to the survival in tissue culture medium shown in Figures 7.1 and 7.2. The lower recovery rates of this strain compared with the 12-416 strain were attributed to the adaptation of the latter to growth in media with low osmotic support.

At this time there was an obvious need for a technique that would allow identification of single organisms in relation to the cells. This is considered further in the next section.

FLUORESCENT ANTIBODY STUDIES

Introduction

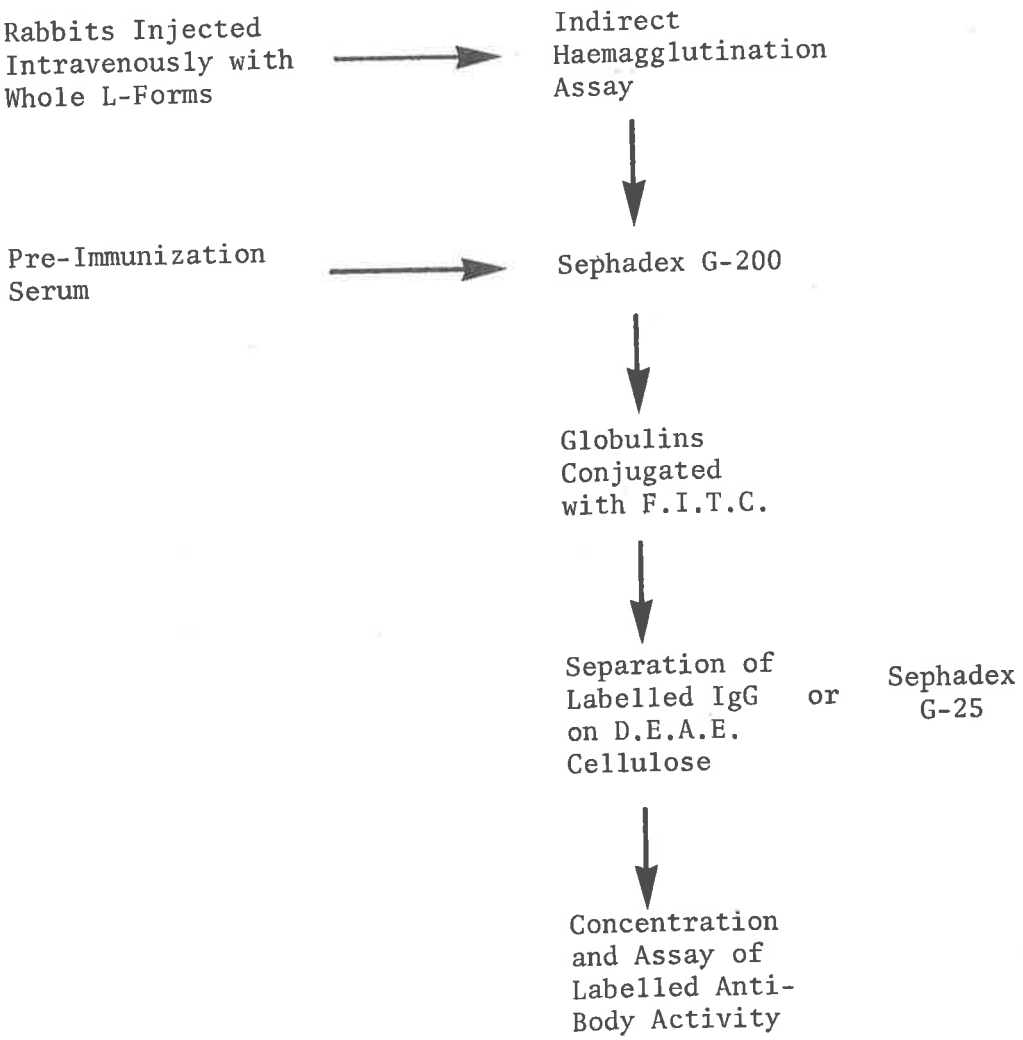
This is a widely used and sensitive technique dependent upon immunological specificity for its use as a biological tracer. Because of this, it was thought that fluorescent antibody might help to overcome some of the ambiguities that arose with techniques described earlier in this chapter.

Experimental

The preparation of fluorescent antibody is shown in Figure 5.2.

FIGURE 5.2

Preparation of F.I.T.C. Labelled Antibody



Antibody Production

Fluorescent antisera were prepared against the L-form strains 12-416 and GL8. Lop-eared rabbits were selected on the basis of ready availability of blood from the marginal ear veins. The serum was removed and stored at $-20^{o}C$.

Antigen was prepared by washing log-phase cultures of the L-forms twice in phosphate buffer with 3% sodium chloride as osmotic stabilizer and finally resuspending in this solution. Because the growth media contained a large amount of antigenic material, particular emphasis was placed on thorough washing, this being limited, however, by the difficulty of getting firm pellets on recentrifuging. Rabbits were injected intravenously at 3-weekly intervals, the quantity of antigen being steadily increased from 1×10^{6} cfu to 1×10^{11} cfu over six injections. For antibody estimation rabbits were bled 7 days following antigen administration.

Estimation of Antibody Titre

Krosgaard-Jensen (1971) described the measurement of anti-mycoplasmal antibody using the indirect haemagglutination technique. He used 3 types of antigen preparation, namely, a whole suspension of mycoplasmas in PBS, a sonicated suspension, and the supernatant from a centrifuged sonicate (34,000g for 1 hour). There was no significant difference in the indirect haemagglutination titre when these antigen preparations were compared.

The antigen preparation used in the present study was obtained by resuspending a washed suspension of L-forms in phosphate buffer without an osmotic protectant, so as to encourage some lysis thereby exposing antigenic groups below the cytoplasmic membrane.

The indirect haemagglutination assay described by Herbert (1967) was closely followed. The basic reaction is the agglutination of antigen-coated, tanned sheep red blood cells by specific antibody. The controls used were antigen-coated cells plus saline, antigen-coated cells with normal rabbit serum (N.R.S.) from the same rabbit used for antibody production, uncoated cells plus saline, uncoated cells with normal rabbit serum, and uncoated cells plus antibody.

It seemed that the antisera possessed non-specific agglutinins because the uncoated cells with antibody gave agglutination up to 1 in 4,000 dilution. This was overcome by heavily absorbing the antisera with the particular batch of sheep red blood cells, prior to preparing the test. This absorption did not appreciably lower the titre in the test system where titres of 2,000 to 30,000 were regarded as acceptable. This titre corresponded with a gamma globulin level of 7 to 8 times that in normal rabbit sera.

Conjugation with Fluorescein Isothiocyanate

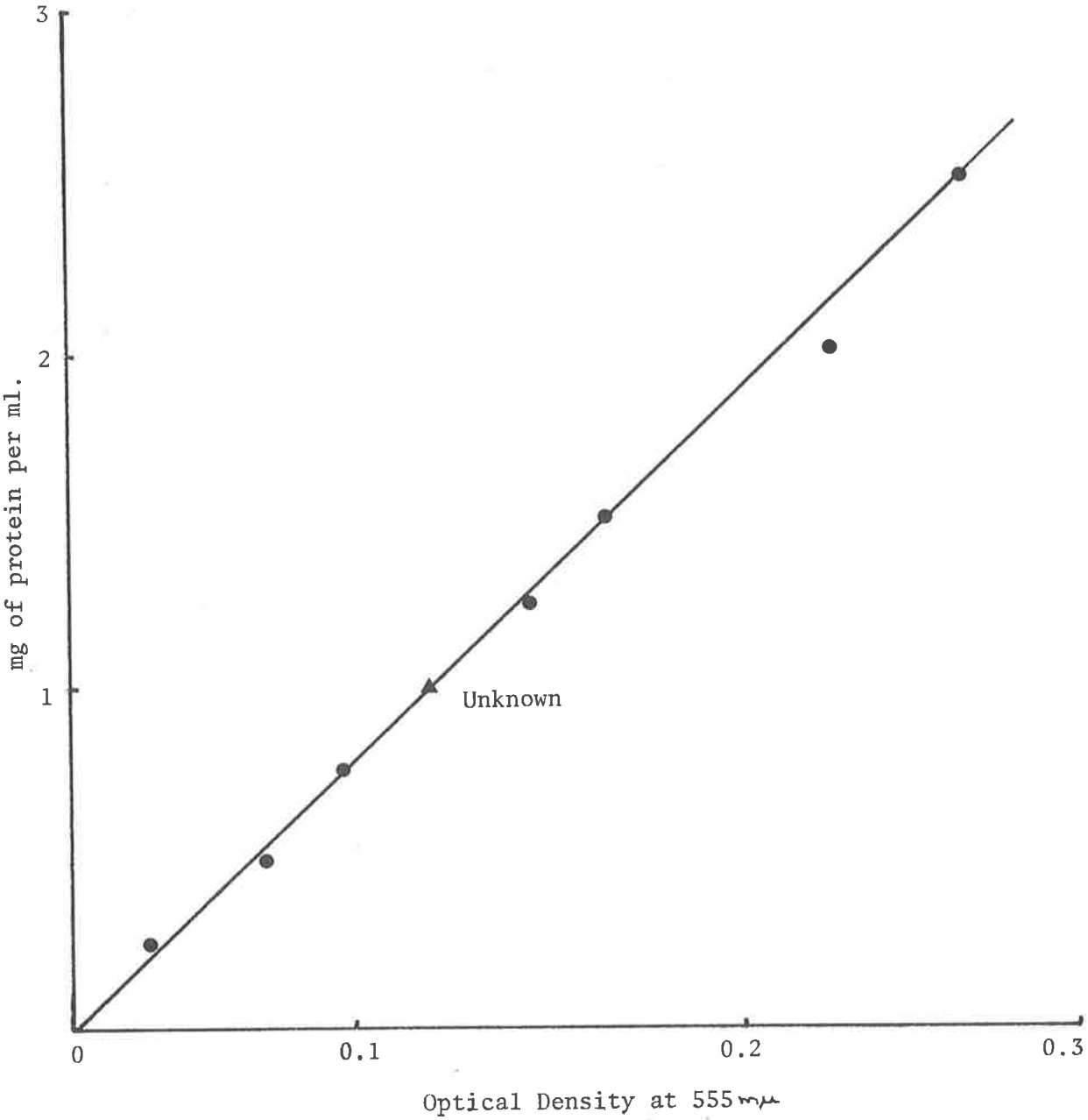
The general procedure was as outline in Figure 5.2. Two rabbits were used for each L-form strain and the serum from each was processed separately. It was considered that if one of the rabbits produced antibody with poor specificity, or the tendency to give non-specific reactions, this would be better kept separate because it could spoil more useful sera.

Fahey and Terry (1967) described the fractionation procedures available for protein separation. The procedure adopted was to isolate the gamma globulin pool by Sephadex G-200 (Pharmacia Uppsala, Sweden) column chromatography, and then to purify the conjugate by running on a DEAE cellulose (Whatman) column. Five to 8 ml of antiserum was loaded onto a 3cm x 100cm column with Sephadex G-200, and then eluted in 1.0M sodium chloride in 0.1M Tris buffer at pH 8.0, using a reverse flow eluent up the column so as to obtain better separation.

The gamma globulin fractions were pooled in dialysis tubing (Visking Co.) and concentrated with carbowax (Selby Ltd.). The protein content of the eluent was estimated by the Biuret reaction, as described by Kabat and Mayer (1961), with bovine serum albumin fraction $\overline{V}$ as the standard (see Figure 5.3).

The pooled gamma globulin was conjugated with fluorescein isothiocyanate isomer I,according to the method described by

FIGURE 5.3



Estimation of the Protein Content of Conjugated Anti-GL8 L-Form Serum, Purified by Sephadex G-25 Chromatography.

Bovine Serum Albumin Fraction 5 used as a Standard.

Nairn (1969a). The conjugated globulin was purified by running it through a DEAE cellulose - ion exchange column dimensions 35 x 2.5cm, set up according to the manufacturer's instructions (Whatman information leaflet 1L2). The eluting buffer was 0.15M sodium chloride in 0.01M phosphate pH 7.1 and, according to Fahey and Terry (1967), the conjugated globulin G (IgG) should pass directly through the column under these conditions.

Using the method of Nairn, conjugated antisera were prepared against the L-form strains GL8 and 12-416, with conjugated-pooled normal rabbit globulin prepared for control purposes.

Estimation of Protein Recovery and Fluorochrome-Protein Ratio.

The amount of protein recovered as conjugated globulin was estimated by the Biuret reaction. This was commonly about 30% of the protein used for conjugation, usually about 100mg. The fluorochrome-protein ratio was estimated by the method described by Fothergill (1969a).

$$\frac{\text{Moles of fluorochrome}}{\text{Moles of protein}} = X \frac{\text{O.D } (\lambda\text{max. for FITC})}{\text{conc.protein in mg per ml}}$$

X for fluorescein isothiocyanate (FITC) = 2.8

λ max. for FITC = 495mμ

The ratio was usually about 1 to 1 in contrast to the 4:1 FITC:protein ratio described by Fothergill as adequate for tracing work. It appeared therefore that not only was the yield of conjugated protein low, but it was probably inadequately labelled as well. To rectify this matter the conjugate was purified by a different technique.

Purification of the Conjugate on Sephadex G-25

This material allows the separation of low molecular weight substances, such as unreacted fluorochrome, from high molecular weight molecules. Fothergill (1969b) recommended this procedure for removal of unreacted fluorochrome. The conjugates were run on a column approximately 1cm x 25cm which resulted in a protein recovery of about 50% and a fluorochrome to protein ratio of 3.7 to 1.

Evaluation of Techniques

Under the conditions used in this study, Sephadex G-25 gave a higher yield of adequately labelled globulin. The DEAE cellulose separation, on the other hand, probably yielded a more uniform class of globulin molecules with light labelling. This could have been advantageous in view of the findings of Fothergill (1969a) that heavily labelled globulins are more likely to give non-specific staining reactions. A disturbing finding related

to the conjugation process was that indirect haemagglutination titres fell sharply in comparison with identical sera stored at $4^{o}C$. After correcting for protein loss on columns, titres fell, for example, from 12,800 to 800 and from 25,600 to 1,600. The reason(s) for this loss of antibody activity were not apparent, but the explanation could have been denaturation either during conjugation or by the fluorochrome. However, Fothergill (1962) reported that conjugation with fluorochrome often reduces the antibody titre by 50% and in some cases much more than this.

General Considerations of Antibody Staining

Nairn (1969b) considered the intact cell membrane to be impermeable to molecules the size of gamma globulin. It was noted, however, that FITC-labelled antibody did appear to penetrate liver parenchymal cells in oxygen-deprived rats. The usual methods of ensuring penetration are somewhat more drastic than this, involving procedures such as freezing of cells or fixation. According to Nairn, the usual fixatives are acetone or 95% ethyl alcohol, with the choice depending on the nature of the antigenic material to be stained. If the antigens were removed or significantly altered by the fixative, the staining procedure would fail. As an alternative to direct staining of antigen by labelled antibody, it is possible to increase the sensitivity of the system by an indirect or "sandwich" technique. Nairn stressed that the

increased sensitivity is obtained at the expense of decreased accuracy and more non-specific staining reactions. The general problems of non-specific staining can be overcome by prior absorption of the conjugated antisera with tissue homogenates (Nairn, 1969a) and by background staining with 0.1% Evans blue (Nairn, 1969b). Because of the requirement for good specificity in the present study, only direct staining was used.

Experiments and Results

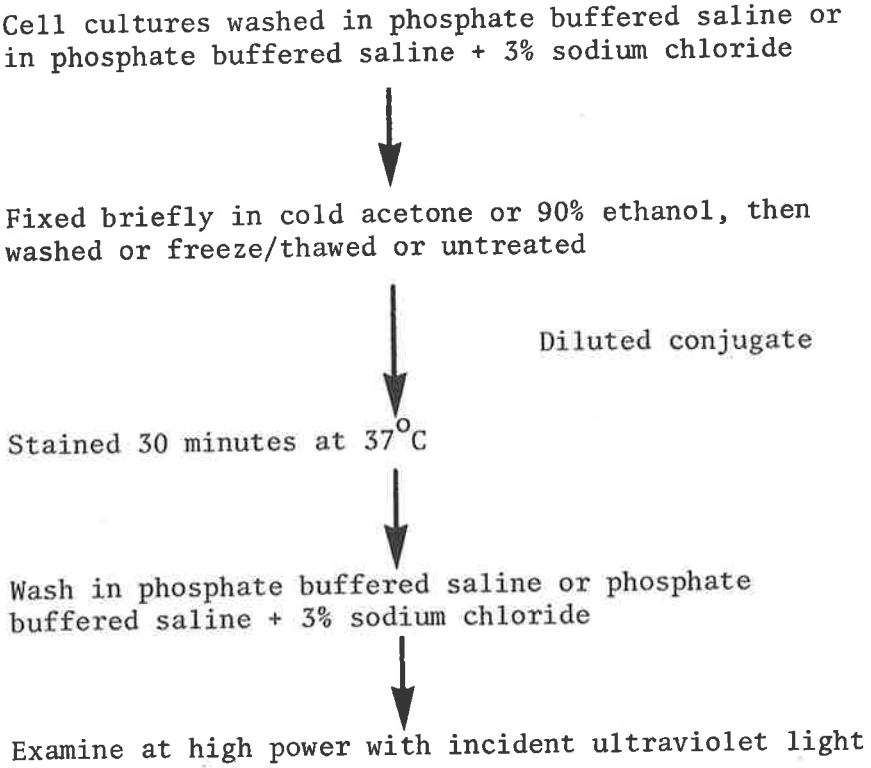
Most of the fluorescent antibody work involved infectivity studies of L-forms GL8 and 12-416 with L fibroblasts and mouse peritoneal macrophages. Rat heart cell cultures were not used in this study because they were time consuming to establish and did not give promising results in the infectivity studies described earlier in this chapter. Similarly, human diploid cultures did not show promising results and were therefore not used in many experiments. The experimental procedures used were the same as those for the earlier infectivity studies, outlined in Table 5.1. The method used to stain the cultures is shown in Figure 5.4.

Results

Antisera were tested for specificity by attempting to stain parent group A streptococci and unrelated oral streptococci. These

FIGURE 5.4

The Procedure for Fluorescent Antibody Staining of Cell Cultures



were stained unfixed, as suspensions in buffer. L-forms were stained as suspensions in PBS or PBS + 3% sodium chloride. The L-forms appeared as faintly fluorescing halos while the other organisms were unstained. Similar results were obtained for organisms heat fixed, stained and washed, although the preparations were inferior by this method.

In the experiments, the washing was done with either PBS or PBS + 3% sodium chloride. It was thought that the PBS could cause distension of L-forms at the cell surface with subsequent reduction in staining intensity, because of its relative hypotonicity.

With both the L fibroblasts and the mouse peritoneal macrophages it was necessary to heavily absorb the antiserum with cell suspensions prior to staining. This removed troublesome non-specific staining. The nuclei of the L fibroblasts in fixed preparations, however, strongly bound antiserum (Plate 5.8). This was interesting in view of the findings of Schwab (1971), discussed in Chapter 1, who noted that streptococcal cytoplasmic membranes absorbed mouse anti-nuclear antibody.

Overall, in the test cultures, both L fibroblast and macrophage, some cells had fluorescent granules associated with them (Plates 5.9 and 10). The findings are summarized in Table 5.3.

Reculture was not attempted from these cultures because of the previous findings that there was no evidence of cell-associated survival.

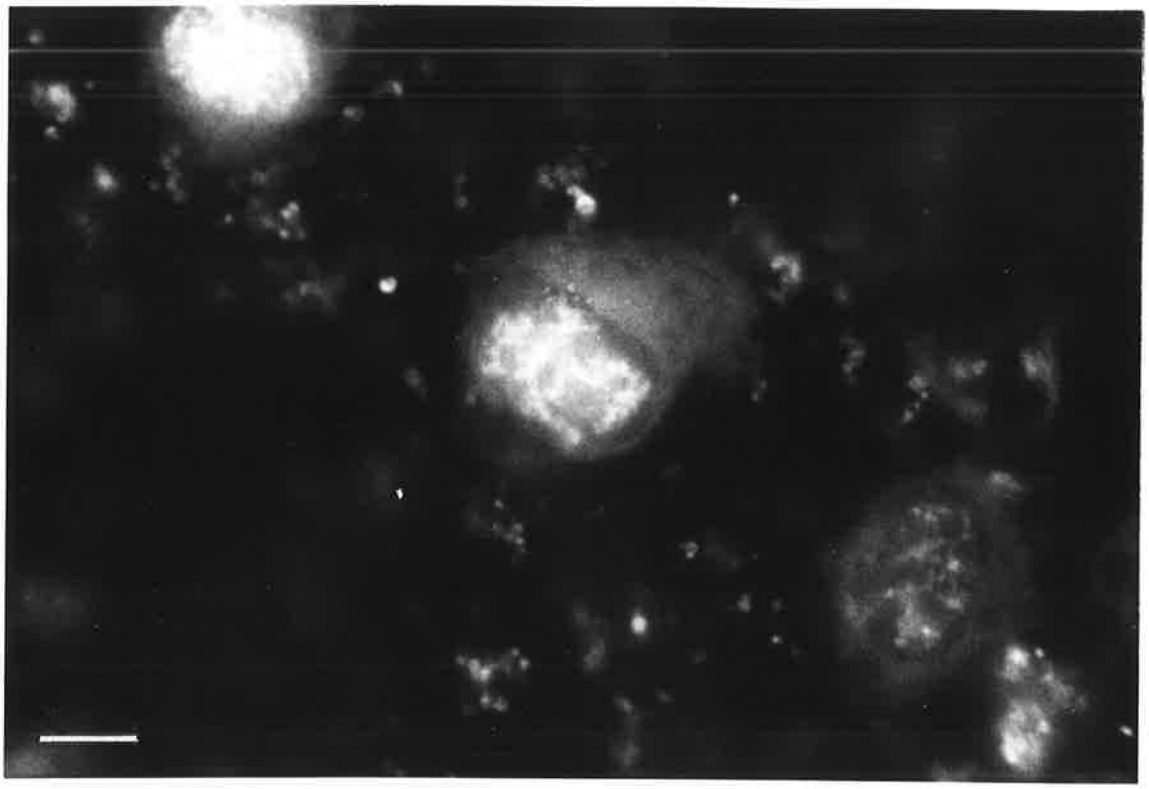


Plate 5.8

L-cell culture stained with anti L-form 12-416 conjugate

Showing fluorescent nuclei.

Scale: 10μ

Magnification, x 1,250

Plate 5.9

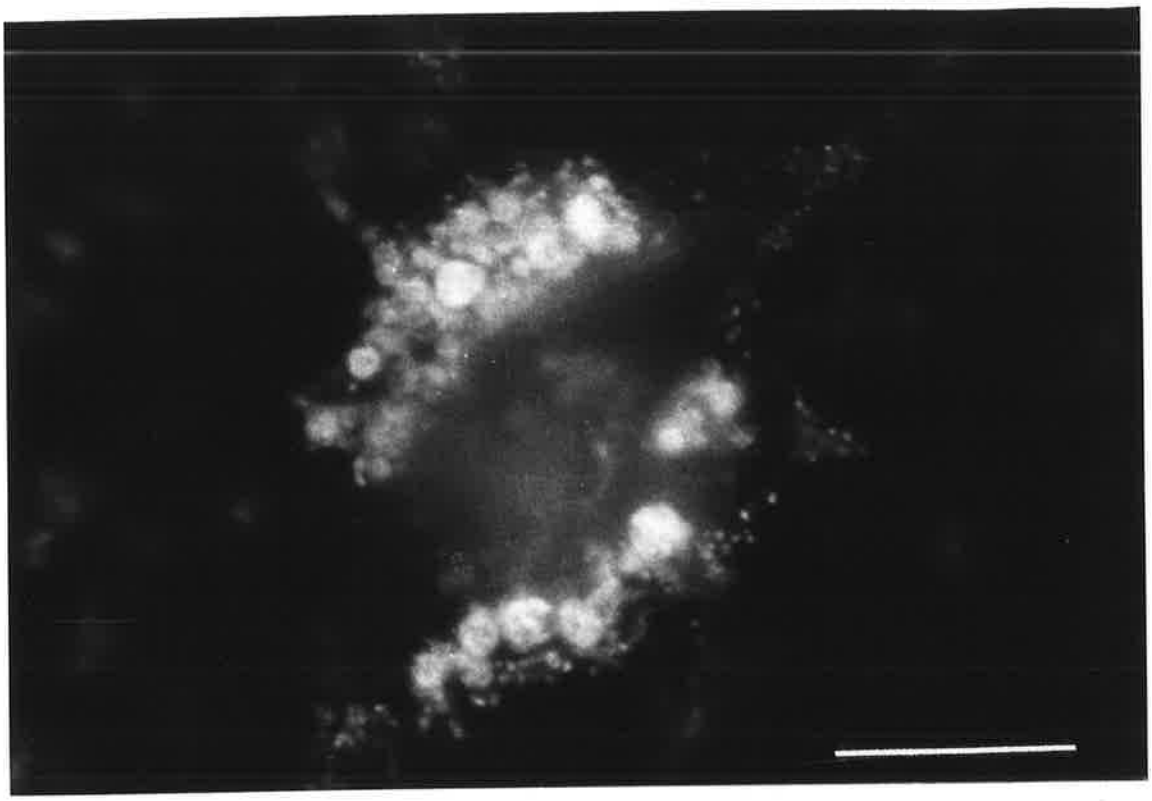
L-cell culture infected with L-form 12-416 at 500 cfu per cell for 5 days and stained with fluorescent antiserum

Fluorescent granules associated with cell.
Scale: 10μ
Magnification, x 3,100

Plate 5.10

Mouse peritoneal macrophage culture infected with L-form 12-416 500 cfu per cell for 18 hours

Cell associated fluorescent granules apparent after staining with conjugated antibody to L-form 12-416.
Scale: 10μ
Magnification, x 3,100



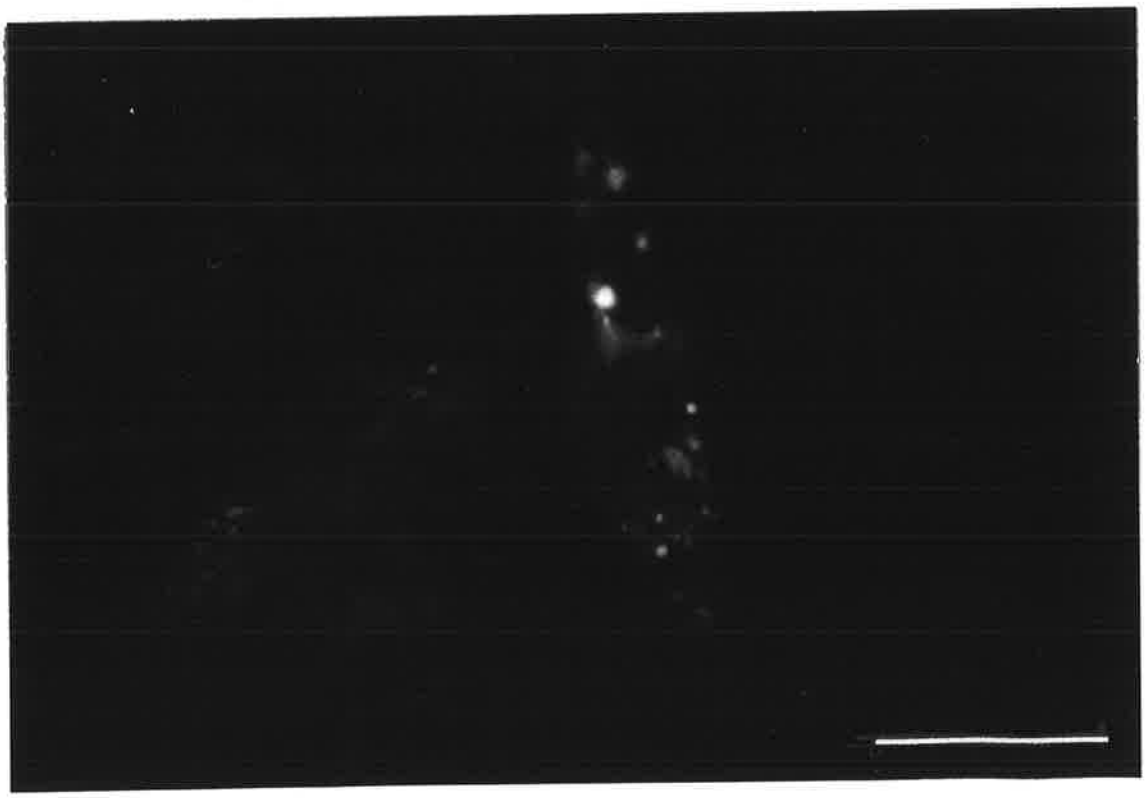


TABLE 5.3

The Results of Fluorescent Antibody Staining in Infectivity Experiments

L-form strain	Background cell fluorescence		The appearance of stained L-forms	Differences in staining of anti-sera prepared by DEAE cellulose or Sephadex G-25 fractionation	Controls	Test cultures	Differences in test results with acetone fixed, alcohol fixed, freeze-thawed or untreated cell cultures
	Mouse peritoneal macrophage	L fibroblast					
12-416	-	fluorescent cell nuclei in fixed preparations	fluorescent circles more distended in hypotonic media	none	normal cell cultures	fluorescent cell-associated granules	none
GL8	-	fluorescent cell nuclei in fixed preparations	fluorescent circles more distended in hypotonic media	none	normal cell cultures	fluorescent cell-associated granules	none

DISCUSSION

Evaluation of Staining Procedures

From the results obtained, it appeared unlikely that the May-Grünwald-Giemsa stain would enable detection of single L-form units at low frequency in relation to the cell cultures. This procedure is probably of more use in the detection of aggregates of organisms, such as inclusion bodies. Heidenhain's haematoxylin was a useful stain, enabling a general cytological assessment of the cell cultures. The cells were not obviously affected by the introduction of L-forms.

Although acridine orange staining is not absolutely specific for nucleic acids (Kasten, 1967), this technique was adapted to provide useful information regarding L-form-cell association. Allowing for the non-uniformity of fluorescent intensity in the cell cultures,it was possible to distinguish L-forms as brightly fluorescing objects against a pale background. This observation was made possible by the discovery that L-forms were less susceptible to the leaching action of calcium chloride. Suspected L-forms were never seen as large clusters in association with the cells but as single fluorescing units or two or three in a group.

Other workers have utilized acridine orange fluorescence in

attempts to identify L-forms in relation to cells. Chattman et al. (1969) commented on the difficulty of staining L-forms but claimed success in identifying L-forms in relation to cells in blood smears. Pohlod et al. (1972) found that cell wall defective organisms stained orange against a pale background of red blood cells. Harwick et al. (1972), in a study of phagocytosis of L-forms by leukocytes, found that cell nuclei and L-forms fluoresced orange. They were able to detect L-forms in the cytoplasm of leukocytes as discrete fluorescing granules. The reversal of colour patterns from those found by other authors and in the present study was attributed to different techniques in the staining procedures.

Reculture of L-Forms from Infected Cultures

The results presented for the short term studies indicated that the re-isolation of L-forms really reflected only their survival in normal tissue culture media. Because there was no indication of quantitative survival of L-forms in the cell cultures, there was no detailed attempt made to specifically identify organisms re-isolated from cells. Had there been any indication of long term survival, positive identification would have been mandatory. Probably the best way to do this is to induce antibiotic resistance into the L-forms and to couple this with fluorescent antibody staining of the colonies. Combined with a require-

ment for hypertonicity for growth and characteristic colonial morphology, this should give reasonably certain identification. There is, of course, the possibility that re-isolated organisms may grow as atypical colonies which do not propagate readily on agar. This would increase the difficulty of positive identification.

Fluorescent Antibody Staining of L-Forms

Arising from the present study are a number of points of interest regarding preparation of fluorescent antisera and the specificity of staining. The technique used for antibody production involved a series of injections over 4-5 months. While the indirect haemagglutination titres and the increase in the amount of gamma globulin were satisfactory, it is possible that the procedure resulted in antibody of relatively poor specificity because of the long time period of immunization (Davis et al., 1968b). Sera from different rabbits were treated separately in case any one of these had undesirable non-specific staining properties; for example, if the conjugated pre-immunization serum stained the tissue culture cells. There did not, however, appear to be any real difference in the sera from individual rabbits or as a result of differing purification procedures. The reduction of antibody activity following conjugation with the fluorochrome was disturbing but unexplained; interference with critical determinant groups is, however, one

possibility. The need to heavily absorb conjugated sera with various cell types highlighted the troublesome non-specific staining experienced.

There are two published studies that have a strong bearing on the present findings. Kagan (1967) found that L-forms of haemolytic streptococci produced a cytopathic effect in chicken embryo fibroblast cultures. Of a number of cell types tested, this cell line was found to be the most sensitive to the presence of organisms. The infectivity ratio is not stated but inoculation of L-forms produced destruction of areas of the monolayer with clear vacuolization and degeneration of cells. There was a strain difference amongst the L-forms in the rate of appearance of these changes. Although different cell types and L-form strains were used, these observations are in contrast to the findings in the present study where inoculation of cultures with organism-cell ratios as high as 500 cfu per cell did not result in cytopathic effect.

Schmitt-Slomska et al. (1968) found that the streptococcal L-form of the Richards III type 3 strain could infect human diploid fibroblast cultures. Again the infectivity ratio was not specified, but commonly the organisms were rapidly taken up by the cells within 1 hour, resulting in a cell culture with approximately 10% of the cells containing L-forms. A photo-micrograph of a fluorescent antibody stained fibroblast culture shows

numerous fluorescent granules up to about 15μ in diameter. Presumably this implied the presence of clusters of L-forms in relation to some of the cells. Antisera were prepared by injecting rabbits intravenously 3 times weekly for 6 weeks. Conjugated sera were purified by DEAE cellulose chromatography. This should have given a good staining preparation, but the authors did not state the specificity of the conjugate. Typical L-form colonies, identifiable by fluorescent antibody staining, were isolated for the first 10 days after infection and then not until 90 days. In the interim only atypical non-propagating forms were isolated. The yield of L-forms in relation to the inoculum size is not stated.

These observations are very different from the results in the present study, particularly with respect to the number of infected cells and the number of organisms associated with each cell. In the present study, inoculation of cells with a wide range of infectivity ratios and experimental conditions resulted in a few cells in the culture usually having a low number of suspected organisms associated with them. This was found for both the 12-416 L-form strain adapted to low osmotic pressure requirements and the GL8 strain. Because of this, it is unlikely that the use of a strain adapted to a low osmolarity medium in the study by Schmitt-Slomska et al. could explain the difference in results. One obvious difference between the two studies is

the L-form strain used, but it is unlikely that this alone could account for the contrasting results.

One important question arising from the present studies is the exact location and form of cell-associated organisms. This is considered in the next chapter.

ELECTRON MICROSCOPIC STUDIES — CHAPTER VI

The results obtained from the infectivity studies described in the previous chapter, indicated the possibility of L-form-cell interaction. In this study there were, however, at least two obvious areas of deficiency:

1. Under the conditions used, the techniques did not allow L-forms to be identified with certainty in relation to cells, that is, there was some ambiguity in interpretation.

2. Structures thought to be L-forms could not be accurately localized in relation to the cells; for example, it was often not possible to tell if they were extra- or intra-cellular.

For these reasons an electron microscopic study was attempted.

INTRODUCTION

The most important contraindication for electron microscopy was the apparent low frequency of L-form-cell interaction. Transmission electron microscopy is not a technique suited to mass screening; in fact only a very small fraction of the experimental sample can be examined at one time. In addition to screening

only a relatively few cells, only a small part of each cell is in turn examined. This is perhaps easier to appreciate by example:

If a cell is regarded as a disc of 10μ diameter and 3μ thick, its volume would be $\pi r^2 h$ or 25 x 3

= approximately 250 cubic μ

A cross section of 0.1μ thickness would have a volume

π 25 x 0.1

= approximately 7.5 cubic μ

that is only 3% of the total cell volume.

Because of this, experiments for electron microscopy employed infectivity ratios of the order of 500 cfu per cell. The examination at electron microscopic level consisted of looking for L-form attachment to cell surfaces and for intracellular location, either free or in vacuoles. The activation of the Golgi complex and lysosomal system was monitored as a sign of contact with a parasite. Basic concepts of this system and electron microscopic appearance in heterophil granulocytes and macrophages have been described by Cohn and Fedorko (1969). It has been similarly described by Gordon et al. (1965) for L cells.

Experimental

The study involved the following investigations:

1. Morphology of L-form strains 12-416 and GL8 from broth cultures.

2. Population studies concerning the distribution of various-sized bodies relating to the different phases of the growth cycle.

3. The effect of exposure to hypotonic conditions over varying time intervals, thereby enabling a better evaluation of L-form morphology in infectivity experiments.

4. Electron microscopy of L cell coverslip cultures and cell pellets.

5. Electron microscopy of human diploid cell pellets.

6. Infectivity studies with human diploid and L cells over varying time intervals, using high ratios of L-forms to cells.

Materials and Methods

Buffers

Sørenson's phosphate buffer pH 7.3, prepared according to Geigy Scientific tables, was used as a standard buffer.

Fixatives

All preparations were fixed initially in 2.5% glutaraldehyde (Fluka) in phosphate buffer and post-fixed in 2% osmium tetroxide in the same buffer.

Dehydration and Embedding

Fixed specimens were dehydrated in graded alcohols: 30%, 50%, 75%, 95%, absolute ethanol. They were then passed through propylene oxide - alcohol mixtures to propylene oxide. The epoxy resin used as an embedding agent was epon. The specimens were taken from propylene oxide through mixtures of this with epon and finally they were embedded in epon in open ended gelatin capsules.

Sectioning

Sections were cut from gold to silver with glass knives on a Sorvall Porter-Blum ultra microtome.

Staining

Sections were stained in a 5% aqueous solution of uranyl acetate and lead citrate solution. They were then examined with an Hitachi model HU-IIE-1 electron microscope.

The detailed techniques for various specimens together with the difficulties encountered, are considered individually in the following sections.

PREPARATION OF L CELL COVERSLIP AND PELLETED CULTURES

Coverslip Preparation

The idea was to embed the cells *in situ* and then to strip away the coverslip, leaving the cells embedded but unaltered in the epon matrix. The cells were identified by the osmium tetroxide stain so that it was possible to prepare a suitable face for cutting.

Because of the difficulty of separating glass from epon, the cells were grown on melinex O (polyester) 400 gauge (O'Brien Ltd., South Australia) coverslips prepared according to Firket (1966). The cells grew poorly on this substrate demonstrating both clumping and failure to spread out. Various modifications in washing and conditioning procedures did not result in significant improvement.

For electron microscopic examination the cultures were fixed in glutaraldehyde for 30 minutes, washed in phosphate buffer for 10 changes and post-fixed in osmium tetroxide. They were then dehydrated and embedded. The embedded cells were so orientated as to cut parallel to the flat cell surface. Great difficulty was experienced in getting good sections. In addition, there was only a one cell thickness to cut through. The aim of this procedure was to obtain cell preparations with correct spatial arrangements and freedom from distortion.

Cell pellet preparations were obtained by centrifuging washed suspended cells at 100g for 5 minutes. The loose pellet was fixed in glutaraldehyde, washed in phosphate buffer and post-fixed in osmium tetroxide. Repeated centrifugation was necessary to maintain the pellet. Sectioning of the embedded pellet was comparatively easy, resulting in preparations with an appearance very similar to those obtained on coverslip embedding. Because it did not appear that centrifugation produced significant distortion of the cells the cell pellet method was used in subsequent experiments.

The procedure was simplified by resuspension of centrifuged cells in buffer containing 3% molten ion agar (Difco). The mixed suspension was poured over a cleaned glass slide and allowed to solidify when it was cut into small blocks. It was essential to maintain a size of the order of 1 cubic millimetre, as fixation and embedding in larger pieces were incomplete because of poor penetration. These agar blocks were more conveniently manipulated than were flocculent cell suspensions.

HUMAN DIPLOID CELL PREPARATIONS

These were prepared in a manner identical to that described above, with the cells being harvested by scraping rather than trypsinization. It was considered that trypsinization would

produce cells with a false morphology. This was based on the findings of Kasten (1971) who, in an electron microscopic study, described the damage caused by trypsin to primary rat heart cultures.

PREPARATION OF L-FORM PELLETS

Because of similar problems with loose pellets as described for cell cultures, L-form broth cultures were centrifuged at 8,000g for 15 minutes. The fixed pellet retained its integrity throughout the subsequent manipulations, with the morphology of the organisms being similar to that prepared by light centrifugation (2,000g for 30 minutes).

A similar procedure was used by Anderson and Barile (1965) for mycoplasma broth cultures. They pelleted the organisms at 27,000g for 30 minutes and obtained good results. Fixation prior to centrifugation did not offer any advantages over fixation of the pellet.

L-FORM MORPHOLOGY

A typical morphological appearance is shown in Plate 6.1. From the plate it can be seen that there is an enormous range in size of the elements; the possible significance of this is discussed later in this chapter. It is necessary to appreciate, however, that interpretation of dimensions from a section could

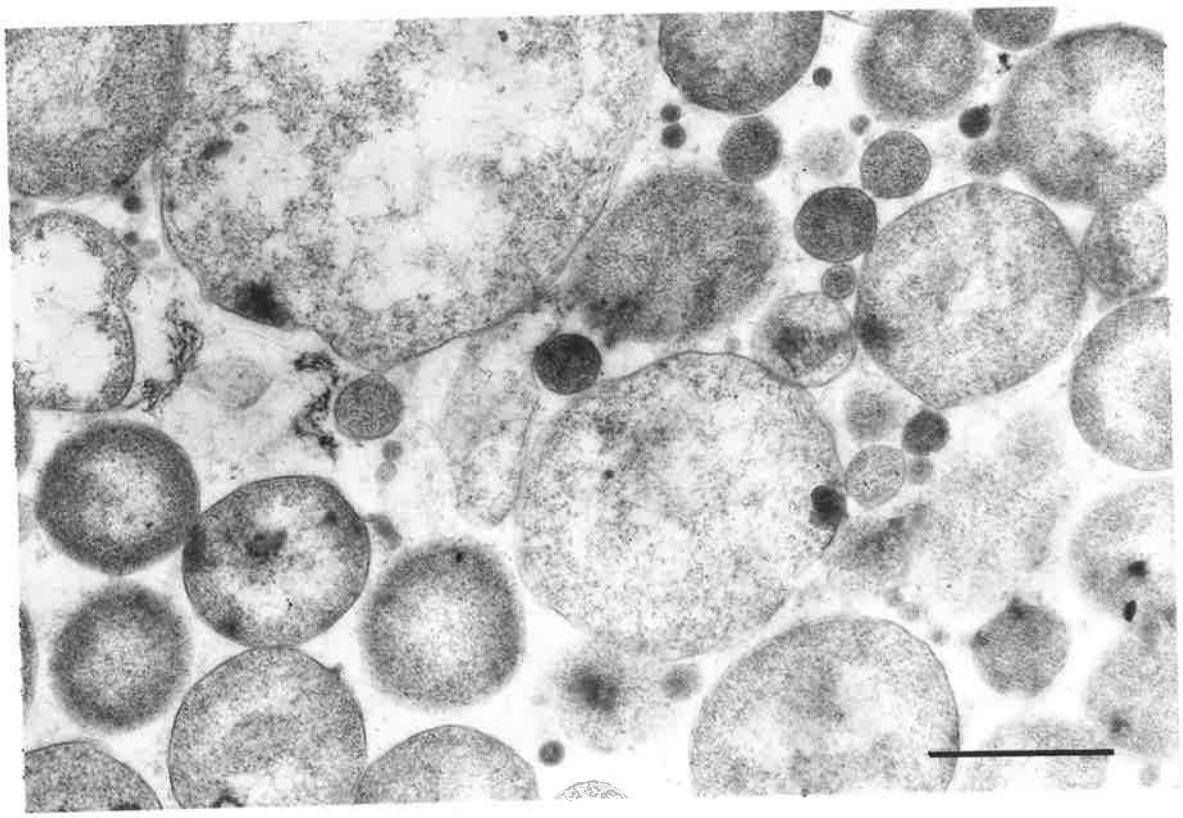


Plate 6.1

Electron micrograph showing the typical morphology of a pelleted broth culture of L-form GL8

Fixed in glutaraldehyde and post-fixed in osmium tetroxide.
Stained: uranyl acetate and lead citrate.
Scale: 1μ
Magnification, x 24,700

be misleading for reasons outlined below.

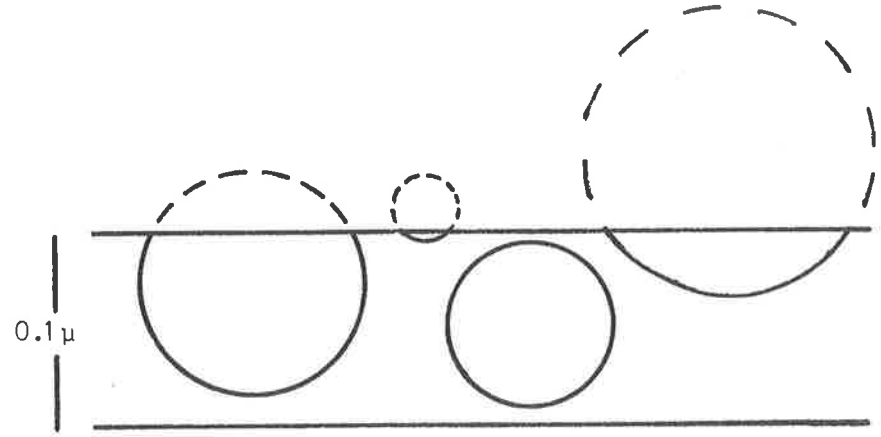


Figure 6.1. The Appearance of Organisms in a Section.

If a section of 0.1µ is considered then the appearance of the organisms would be as shown in Figure 6.1, assuming that all of the units were spheres. In other words, because the plane of section through the individual structures was unknown it was not possible to state the diameter from examination of one preparation. All that could be stated was that the size range appeared to lie between 0.1µ and 5µ.

The method of replication in L-forms has been a topic of much debate, with binary fission, budding and internal formations of daughter elements all being claimed as possible modes (Hijmans et al., 1969). Plate 6.2 shows possible binary fission while plate 6.3 shows the formation of small forms within a large element. It would seem, however, from the nature of the

Plate 6.2

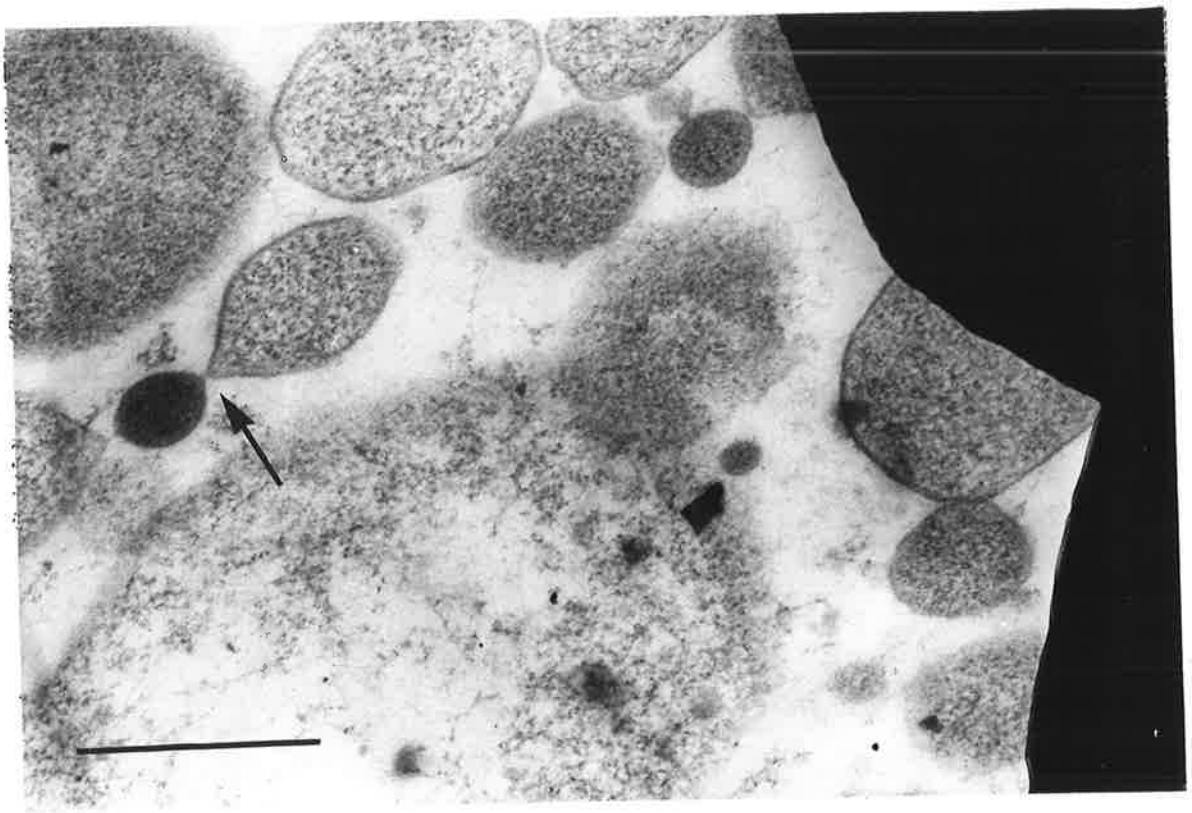
A high power electron micrograph of L-form GL8 showing possible binary fission

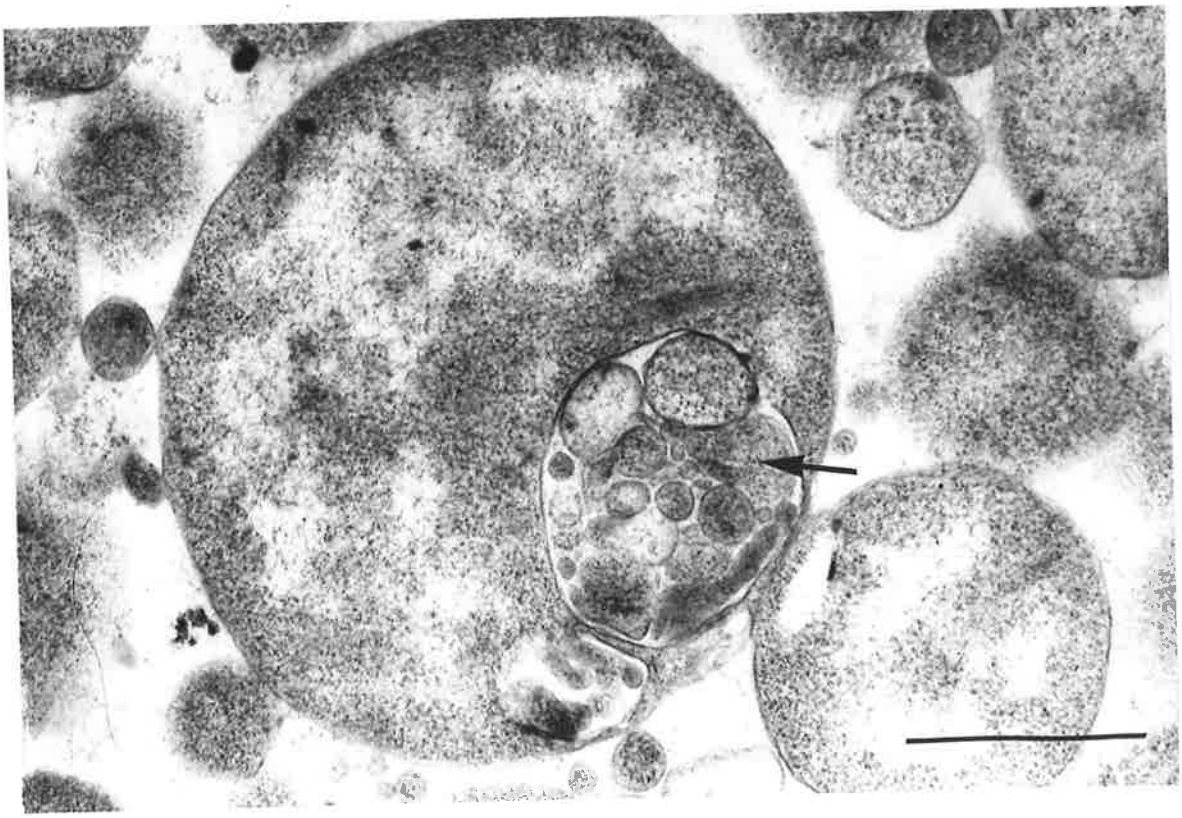
Arrow indicates possible binary fission.
Fixed in glutaraldehyde and post-fixed in osmium tetroxide.
Stained: uranyl acetate and lead citrate.
Scale: 0.5μ
Magnification, x 63,000

Plate 6.3

A high power electron micrograph of L-form GL8 showing internal formation of daughter elements

Arrow indicates daughter cells.
Fixed in glutaraldehyde and post-fixed in osmium tetroxide.
Stained: uranyl acetate and lead citrate.
Scale: 0.5μ
Magnification, x 63,000





L-form growth curve (for example, see Figure 4.3) that binary fission would be the major mechanism of multiplication.

Population Studies

It was thought that the distribution of different sized forms might vary with the different phases of the growth cycle. Additionally, it seemed quite probable that different-sized bodies could vary in a characteristic and reproducible manner in their response to hypotonic conditions. For example, the very large forms could be more labile than the elementary bodies. Another consideration was that only certain species might be infective for cells. Because of these considerations it was decided to measure the populations at different stages of the growth cycle.

Procedures. This work, using strain 416, was based on the assumption that the different sized forms would be randomly distributed throughout the centrifuged pellet. An attempt was made to verify this by taking samples from different portions of the pellet as shown in Figure 6.2.

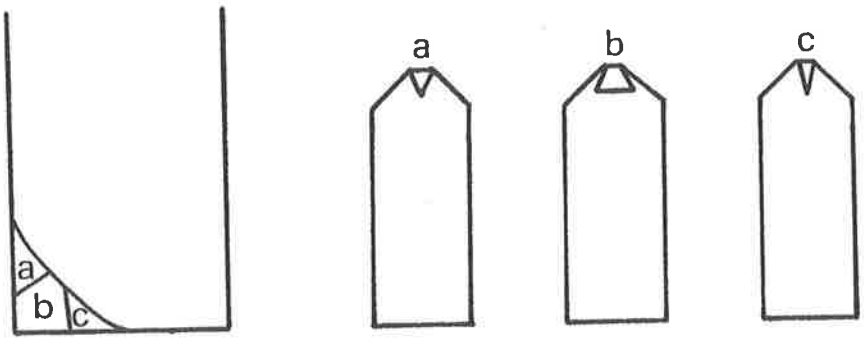


Figure 6.2. Showing a Pelleted L-Form Suspension.

Preparations were made at early log-phase, mid-log-phase and stationary-phase as estimated by optical density relationships to growth. To avoid biased results, areas in the sections were photographed randomly. Several hundred measurements were made for each sample by estimating the greatest diameter of all structures on each print.

Results. A histogram relating the percentage of the population to diameter of the organism for the 3 phases is shown in Figure 6.3. When the whole populations of the three phases, early log-phase (group I), mid-log-phase (group II) and stationary-phase (group III) were subjected to the Student 't' test, there

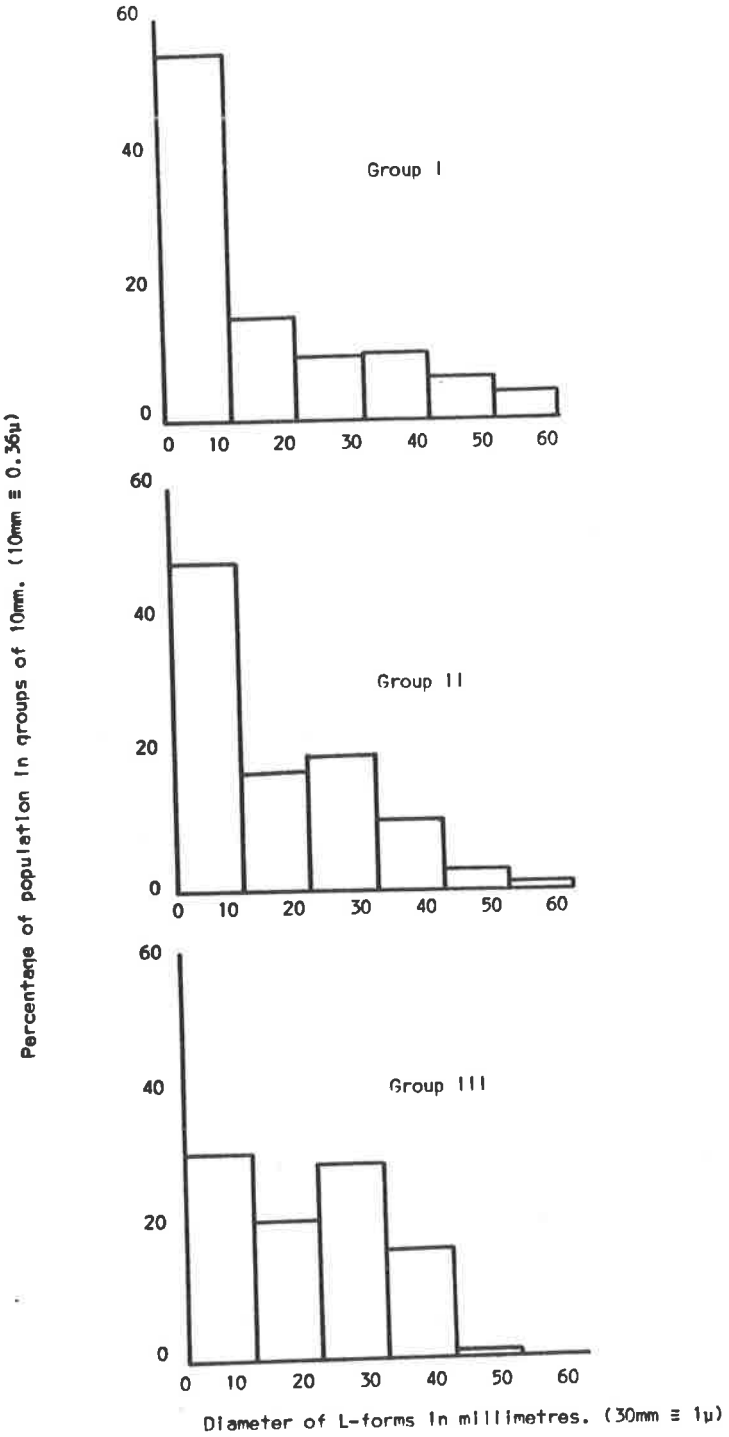


Figure 6.3. Size distribution of 3 groups of L-form 12-416 (taken from electron micrographs).
Group I ≡ early log-phase culture.
Group II ≡ mid log-phase culture.
Group III ≡ stationary log-phase culture.
The significance of the differences in the populations are described in the text.

was no apparent significant difference between them with respect to comparisons of mean diameters (see Table 6.1). However, when organisms of less than 10mm diameter (10mm ≡ approximately 0.3μ) were compared, for the three phases, significant differences were found between the log-phase and the stationary-phase cultures (see Table 6.2). The elements of 0.3μ or less were chosen for comparison because of the interest in the relationship of these elementary bodies to growth of L-form populations. It was of interest to note their higher frequency in growing cultures. The random nature of the distribution of the various elements was verified by the absence of a significant difference in the different portions of the L-form pellet.

These studies indicated that provided log-phase cultures were used for all experiments, there should not be significant size differences in the populations of L-forms offered to the cells. This is not to say, however, that the log-phase populations were necessarily the most infective.

Effect of Hypotonic Conditions on L-Form GL8

Forty ml of a log-phase culture were centrifuged and resuspended in tissue culture medium. Samples were taken from suspensions held at 37°C at 0 time, 90 minutes, 7 hours and 20 hours. They were centrifuged at 4,000g for 15 minutes.

TABLE 6.1

Showing the Comparison of the Three Growth Phases of L-Form 416 by the Student 't' Test

Groups	T value	P value	Significance
I & II	0.1709	0.4 to 0.45	no significant difference
I & III	0.2898	0.35 to 0.4	no significant difference
II & III	0.4593	0.3 to 0.35	no significant difference

Group I ≡ early log-phase

Group II ≡ mid log-phase

Group III ≡ stationary phase

TABLE 6.2

Comparing the elements of 10 mm diameter or less by Chi^2 2 x 2 contingency test

Groups	Total	Counts of 10mm or less	Value	P	Significance
I	410	218			
II	499	253			
III	280	107			
I & III			14.9372	<0.01	high
I & II			0.5496	0.5	none
II & III			11.2513	<0.01	high

Group I ≡ early log-phase

Group II ≡ mid log-phase

Group III ≡ stationary phase

Plates 6.4 to 6.7 show the typical morphology of the L-forms at these times.

Visual observation indicated that there was little reduction in pellet size up to 20 hours, that is, there was apparently no mass lysis of the organisms. The micrographs do indicate, however, that there has been lysis of the large forms. Under the conditions used in this experiment, the organisms show obvious distortion and a progressive decrease in internal density.

This experiment demonstrated the progressive change in morphology with time of exposure to hypotonic conditions. It provided useful information on the electron microscopic appearance of L-forms in tissue culture media.

THE ELECTRON MICROSCOPIC APPEARANCE OF STRAIN L FIBROBLASTS AND HUMAN DIPLOID CELLS

L Fibroblasts

These have been described in detail by Gordon et al. (1963) and Bensch et al. (1964). The appearance of the Golgi apparatus was found to change after phagocytosis (Gordon et al.,1965). These authors also noted dense bodies in the immediate vicinity of phagocytic vacuoles. The cells used in the present study had a similar morphology when they were infected with L-forms (see Plates 6.15 and 6.16).

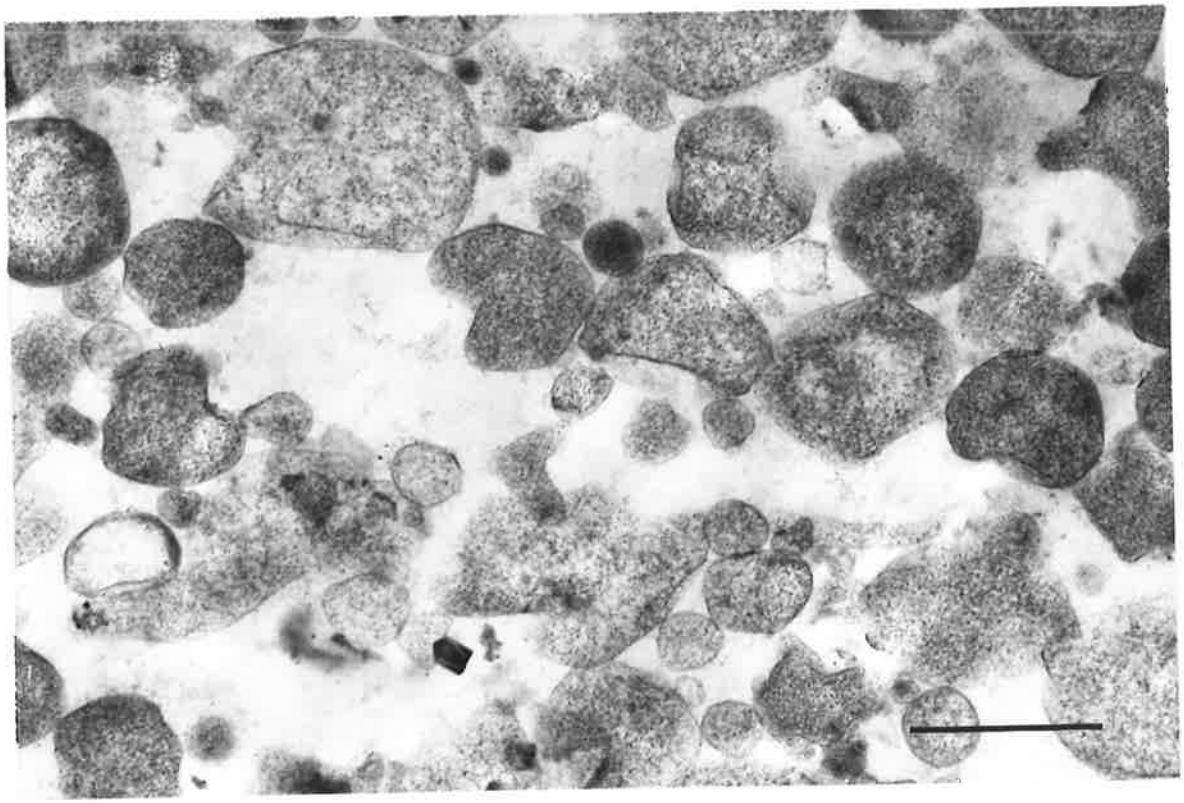
Plate 6.4

Electron micrograph of GL8 L-forms exposed to hypotonic conditions at zero time

Fixed in glutaraldehyde and post-fixed in osmium tetroxide.
Stained: uranyl acetate and lead citrate.
Scale: 1μ
Magnification, x 25,200

Plate 6.5

As for Plate 6.4 but 90 minutes after exposure to tissue culture medium



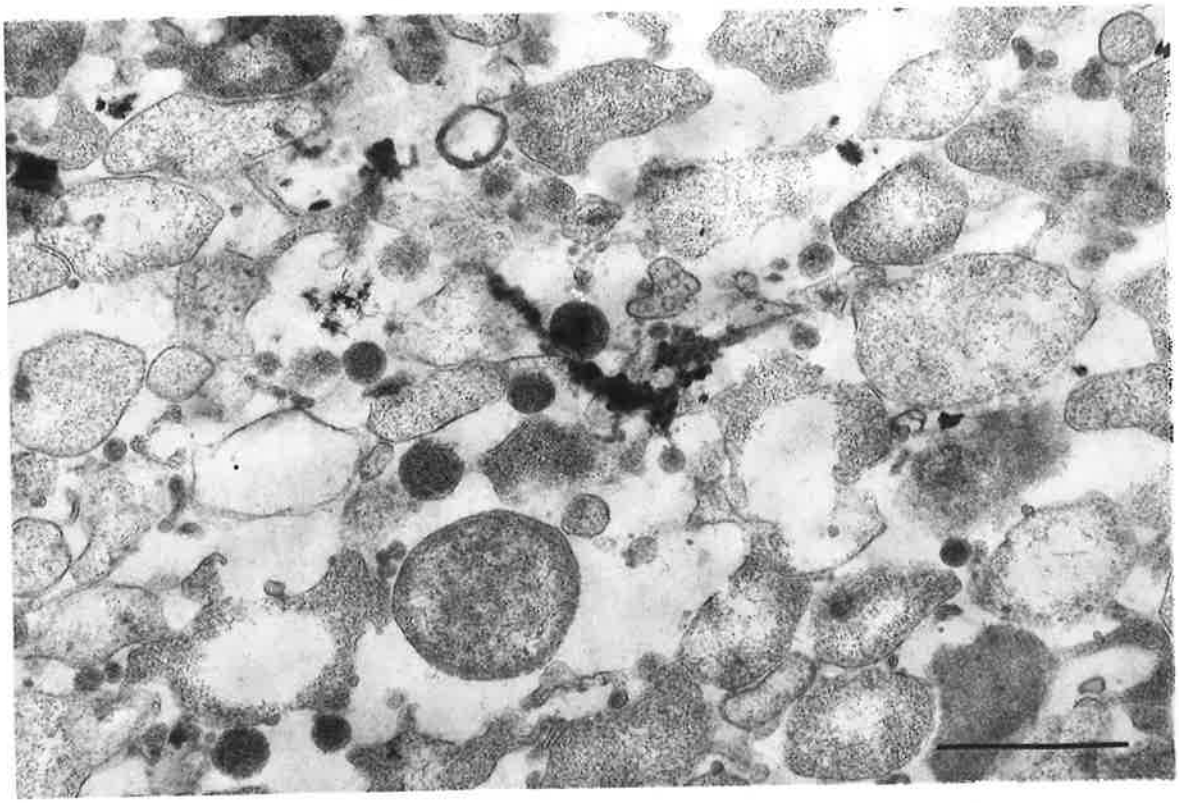


Plate 6.6

Electron micrograph of GL8 L-forms exposed to hypotonic conditions after exposure for 7 hours

Procedure as for Plate 6.4

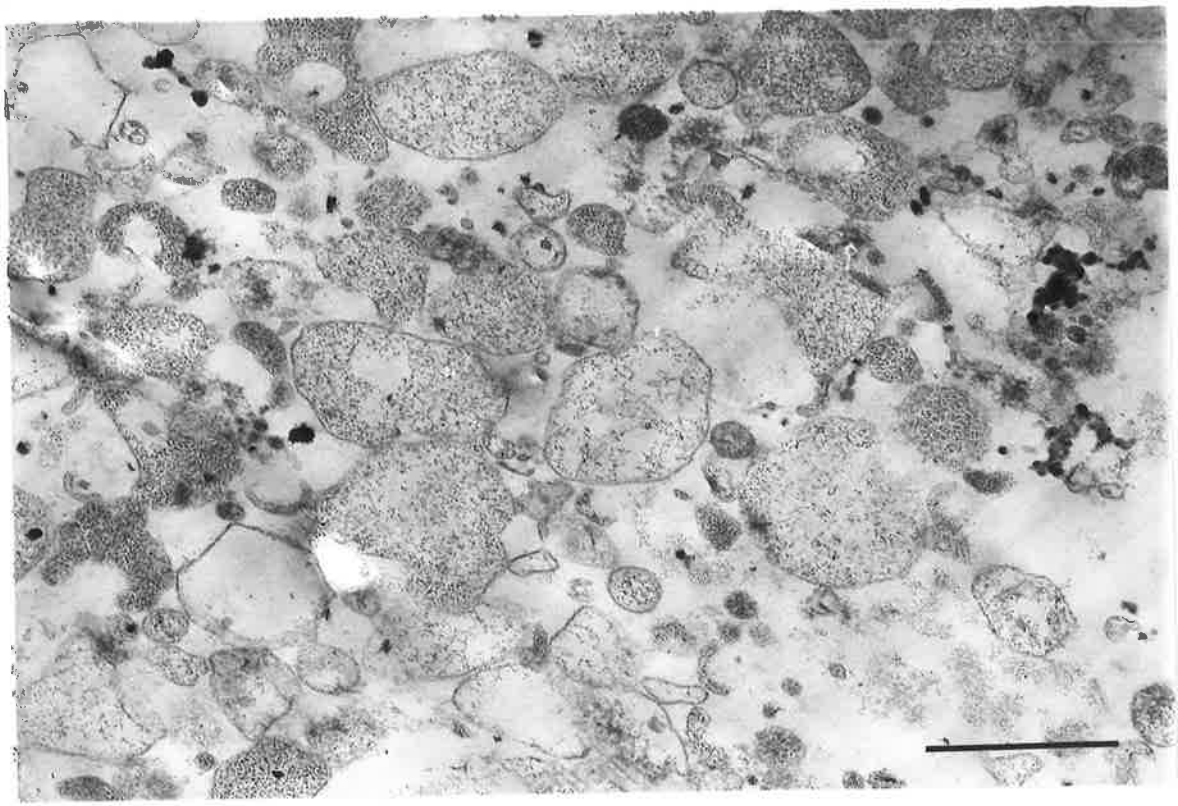
Scale: 1μ

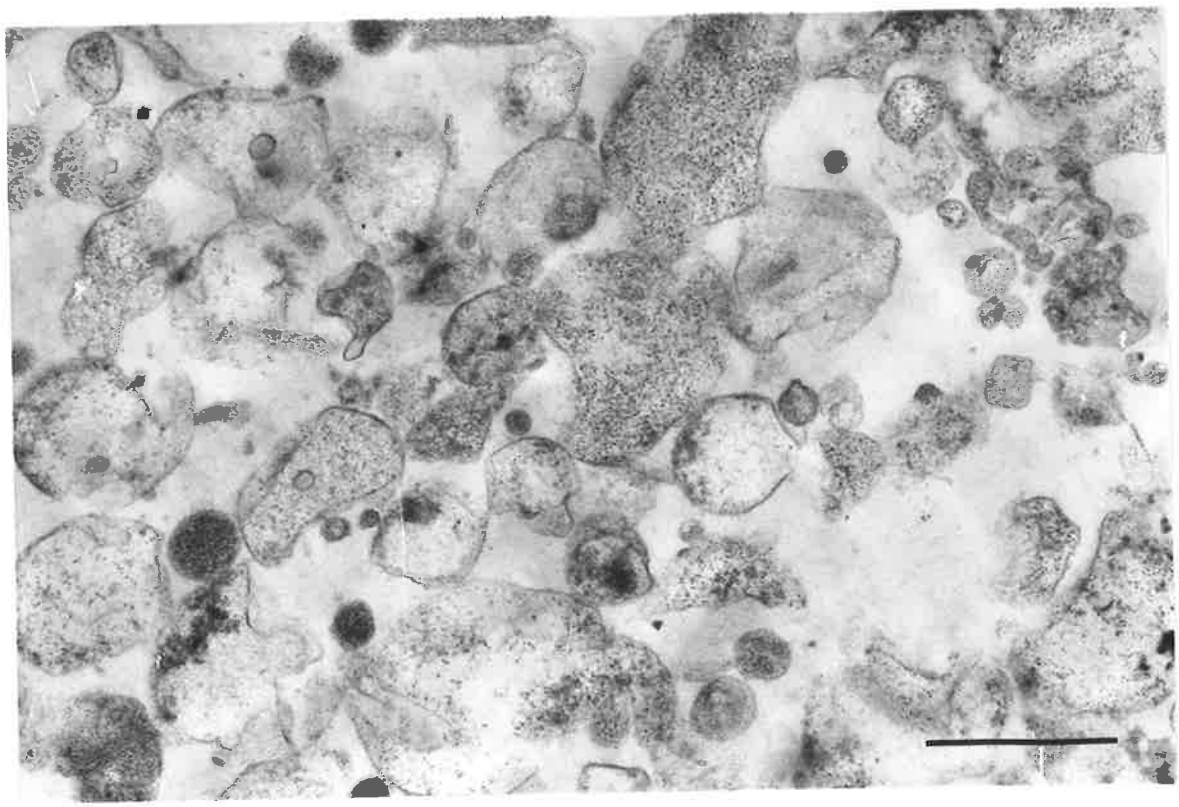
Magnification, x 25,200

Plate 6.7

As for Plate 6.6 after exposure for 20 hours

Over the time of exposure there has been progressive degeneration within the culture.





Human Diploid Cells

The surface morphology is shown in Plates 6.8 and 6.9. These cells obviously have an enormous surface area and the difficulty of identifying an organism in relation to the cell surface can be well appreciated.

INFECTIVITY EXPERIMENTS

Human Diploid Cells

The experimental procedure was similar to that outlined in Table 5.1, except that only high infectivity ratios were used for reasons already discussed earlier in this chapter. Cell cultures to which organisms were possibly adhering were rinsed with buffer prior to fixation. Experiments were continued for up to 48 hours with samples being taken at intervals. Giemsa-stained preparations provided light microscopic parallels. Controls were usually uninoculated cultures. No attempts were made to re-culture organisms from the cell cultures because previous efforts were unsuccessful.

Results

No changes in the cells were apparent and no organisms were detected in relation to the cells in any of the experiments. This confirmed the findings of Chapter 5 in relation to this cell type.

Plate 6.8

Electron micrograph of a human diploid cell preparation showing enormous surface area

N nucleus.

cp cell processes.

Fixed in glutaraldehyde and post-fixed in osmium tetroxide.

Stained: uranyl acetate and lead citrate.

Scale: 1μ

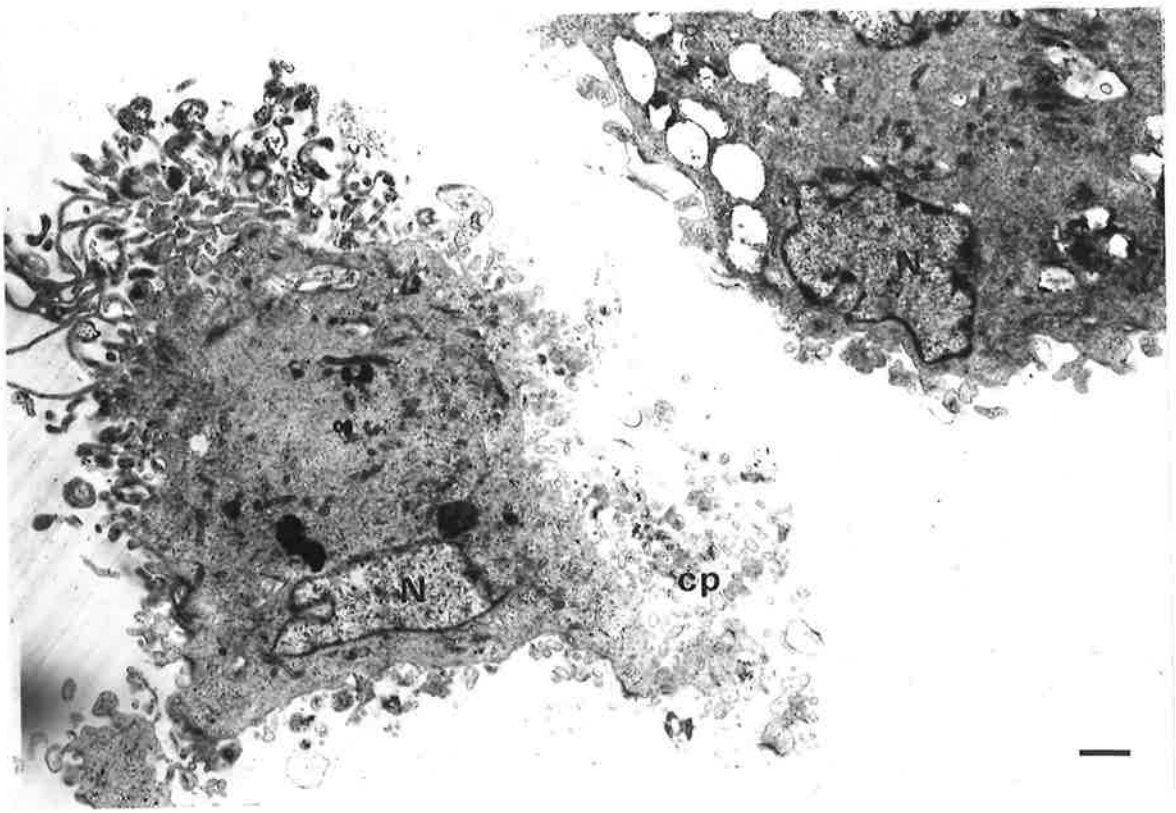
Magnification, x 6,700

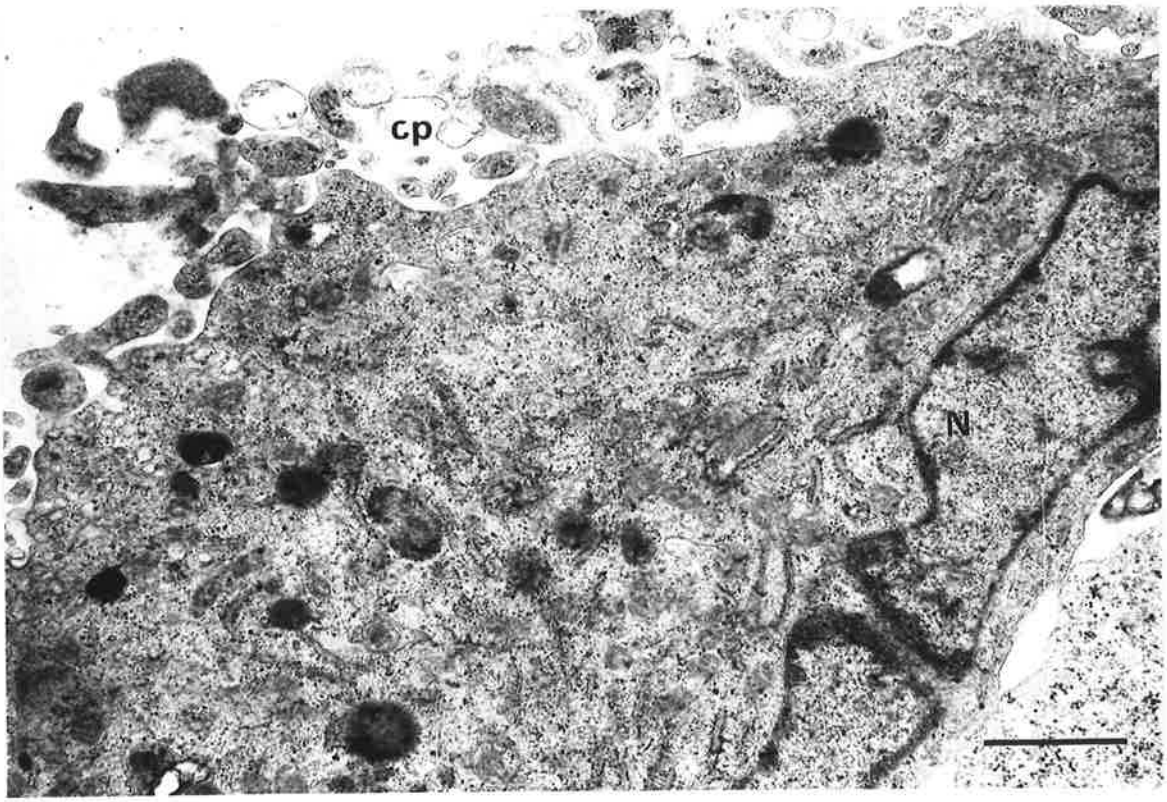
Plate 6.9

Higher power micrograph

Scale: 1μ

Magnification, x 19,000


N
N
cp


cp
N

L fibroblasts. The experimental procedures were the same as described for human diploid cells.

Results

A number of contributing factors complicated the course of these experiments. For example, early experiments were apparently complicated by an undetected mycoplasma infection. The appearance of a culture infected with 500 cfu per cell and incubated for 20 hours is shown in Plates 6.10 and 6.11. There was gross destruction of the culture with a very large population of mycoplasma-like structures present. Control cultures seemed to be normal and healthy with no obvious contamination. Giemsa-stained parallel cultures are shown in Plates 6.12 and 6.13. Heat killed L-form controls were not included. It remained unknown if there was active "collaboration" between the L-forms and the "mycoplasma" or if the phenomenon was merely that of a slight toxic effect of the L-forms on the cells upsetting the balance with the other organisms. After some effort, atypical colonies were cultured as described in Chapter 3, from these cells. In some other cultures, with an apparent light infection of mycoplasmas, the introduction of L-forms did not result in destruction of the cells. L-forms were not seen in relation to the cell surface, but some cells showed vacuoles filled with L-form like structures (see Plate 6.14).

Plate 6.10

Showing an L fibroblast preparation infected with 500 cfu L-form GL8 for 20 hours. Cell degeneration and numerous mycoplasma-like bodies are apparent.

N nucleus.

V probably fatty aggregations.

My mycoplasma-like structures.

Fixed glutaraldehyde, post-fixed in osmium tetroxide.

Stained: uranyl acetate and lead citrate.

Scale: 1μ

Magnification, x 6,700

Plate 6.11

Higher power micrograph showing a dead cell with numerous mycoplasma-like structures.

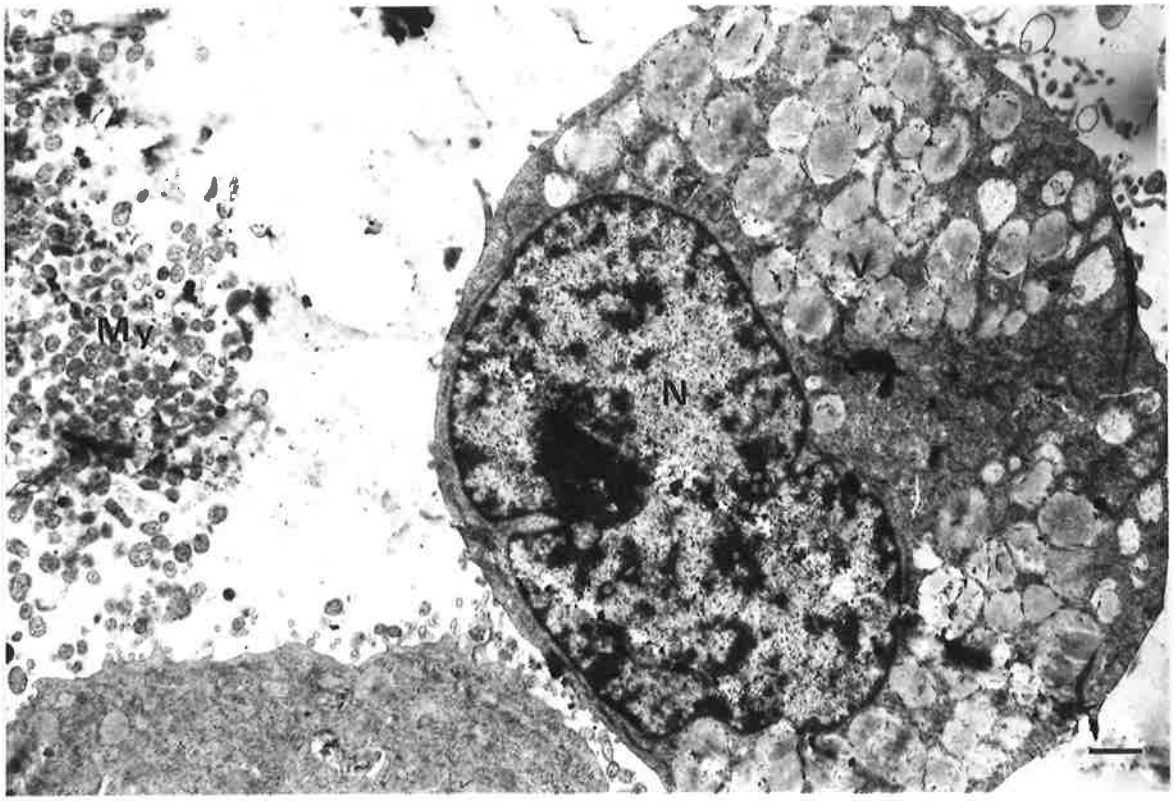
N nucleus.

M mitochondria.

My mycoplasma-like structure.

Scale: 1μ

Magnification, x 24,700


My
N
V

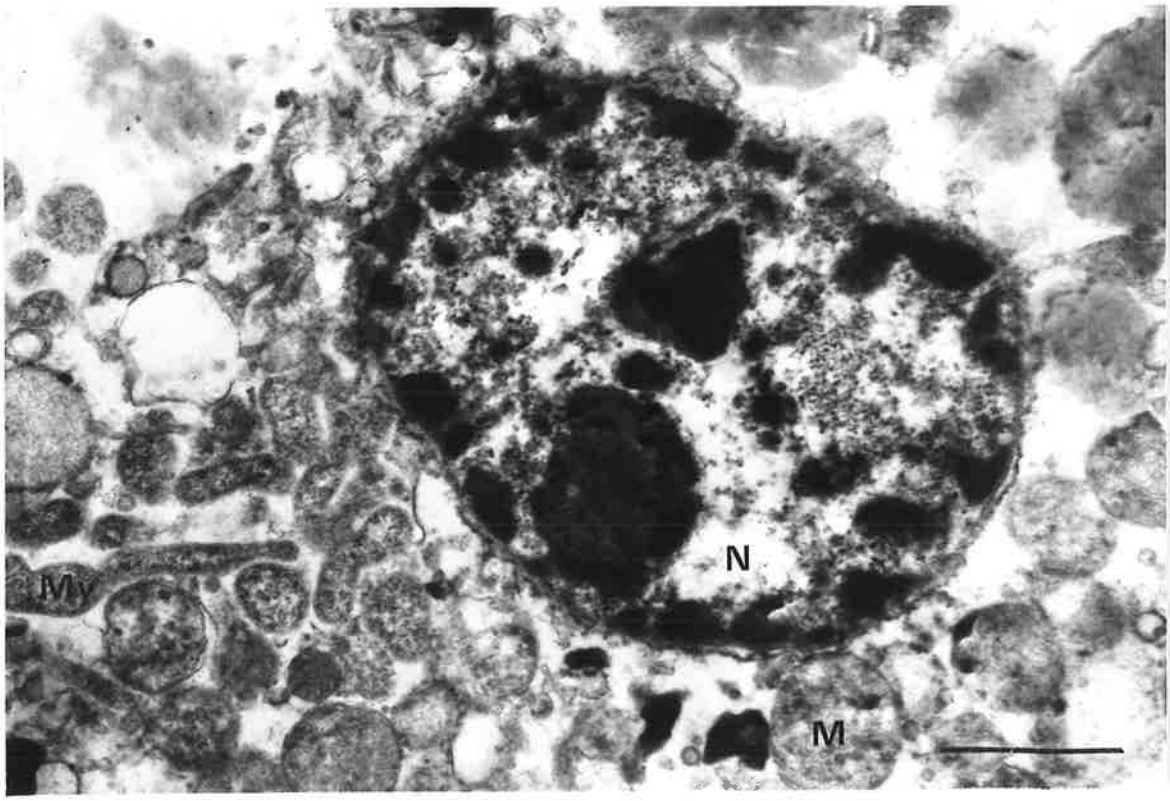

My
N
M

Plate 6.12

A light microscopic parallel to Plates 6.10 and 6.11, showing a Giemsa stained control culture

Scale: 10μ

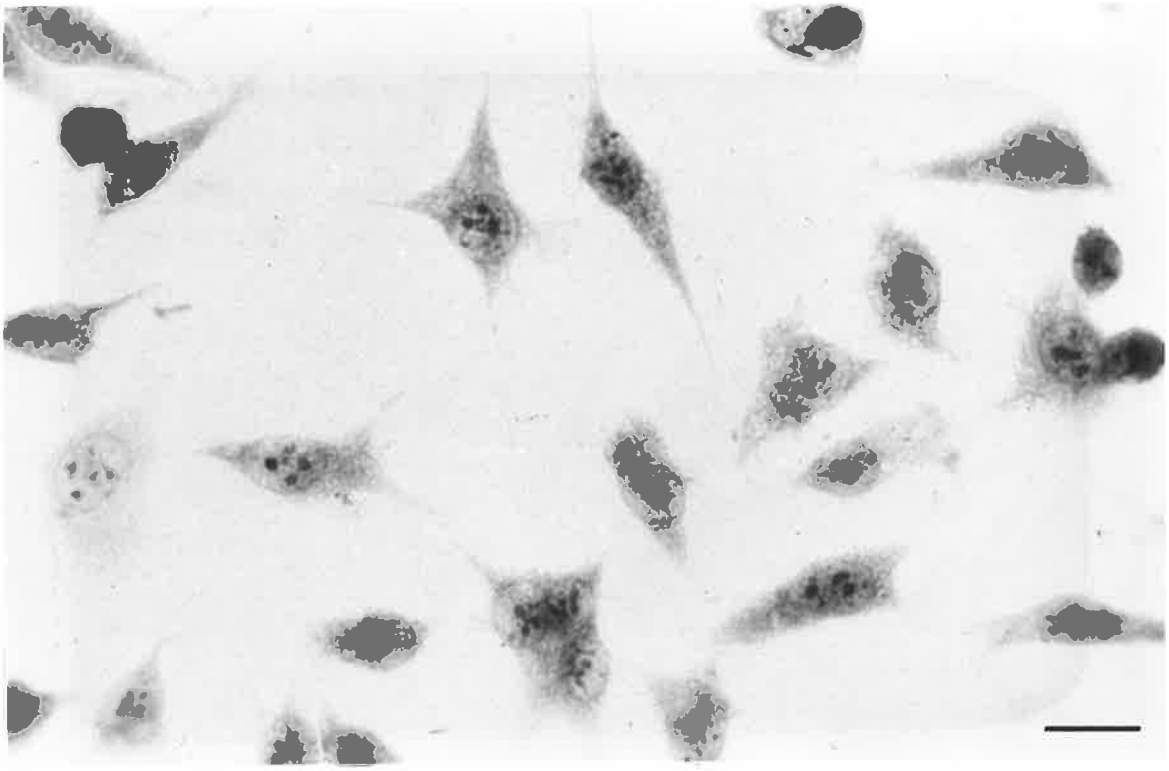
Magnification, x 1,250

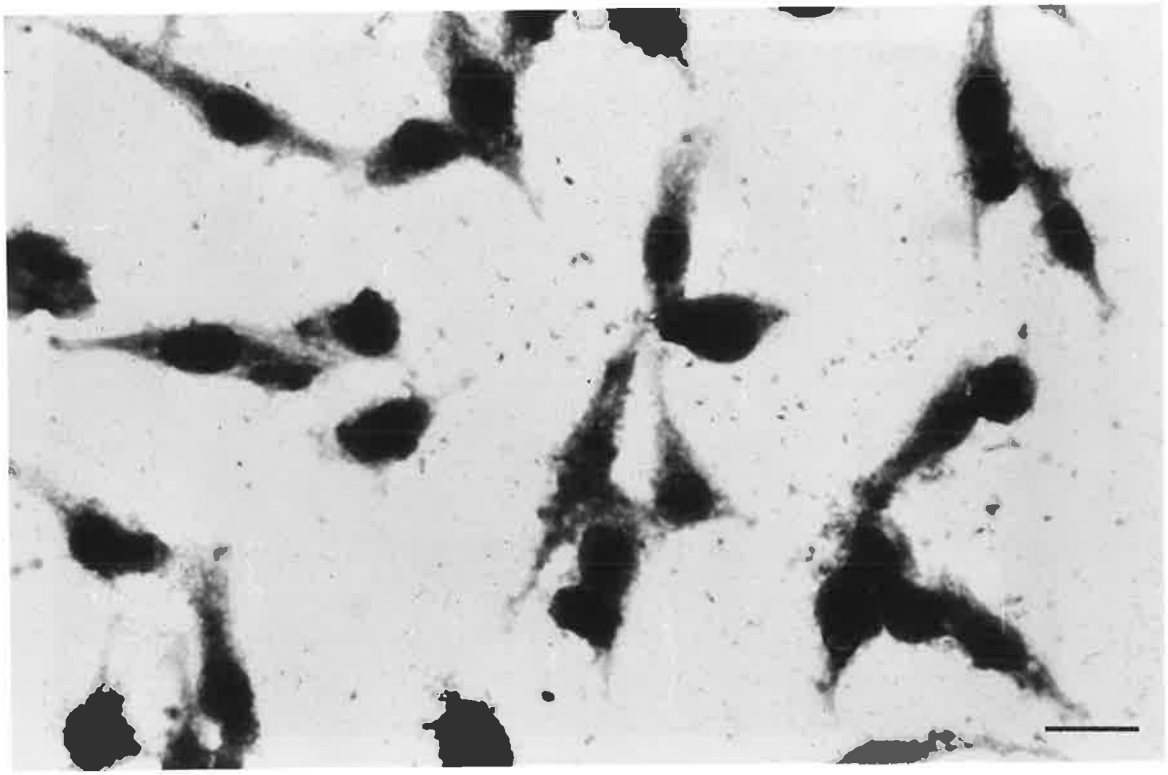
Plate 6.13

A Giemsa stained test culture showing marked cell degeneration with much debris

Scale: 10μ

Magnification, x 1,250





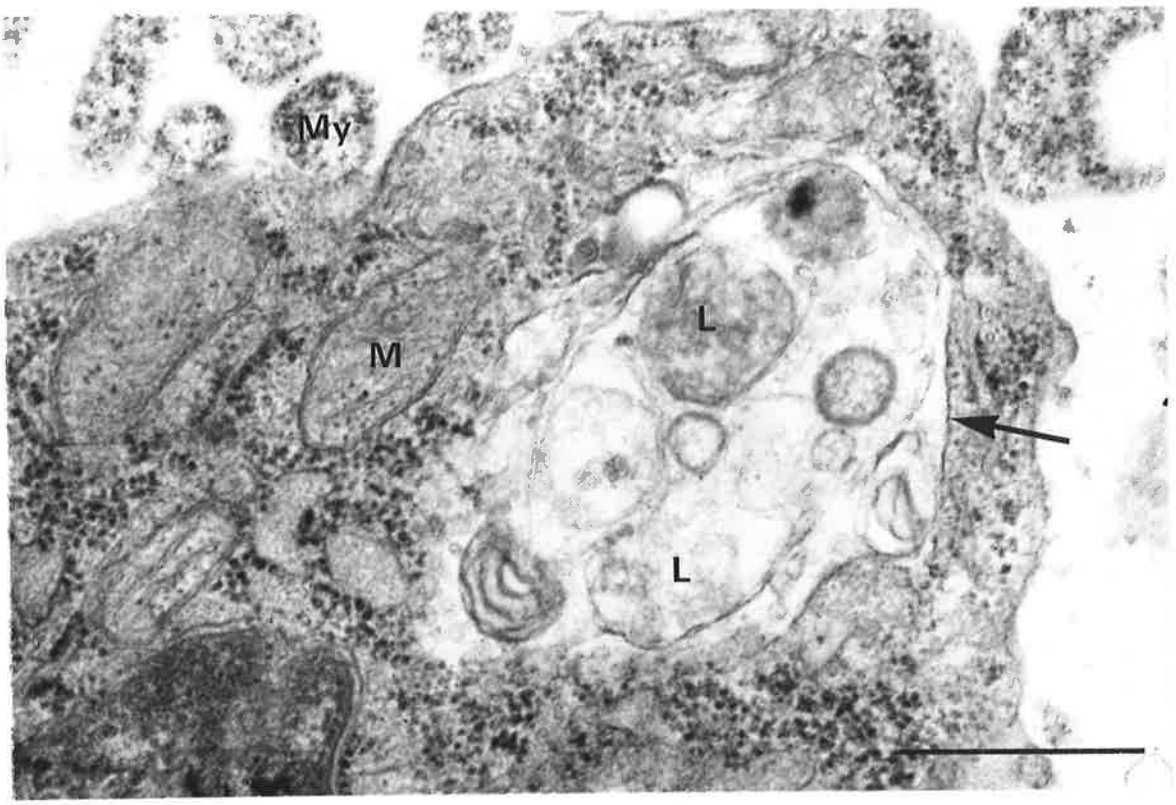


Plate 6.14

Electron micrograph of an L cell from a culture infected with L-form GL8 at 500 cfu per cell

The area shown contains a vacuole with L-form-like structures. There also appears to be mycoplasma contamination.

M mitochondria.

My mycoplasma-like body.

L L-form-like structures.

Arrow points to vacuole membrane.

Fixed glutaraldehyde, post-fixed osmium tetroxide.

Stained: uranyl acetate and lead citrate.

Scale: 0.5μ

Magnification, x 67,500

When a batch of L cells, apparently free of contamination as judged by cultural tests and electron microscopic examination, was infected with L-forms at high ratios, some of the cells showed obvious signs of degeneration. This included the loss of normal cytoplasmic structure and the appearance of large numbers of dense bodies. Other cells had areas that were apparently vacuoles filled with L-form-like structures and surrounding these, dense bodies. These changes are shown in Plates 6.15 and 6.16.

A control batch of L cells, also apparently free from contamination, showed a cytoplasm full of dense bodies. These cells multiplied rapidly and were apparently quite healthy. The typical electron microscopic appearance is shown in Plates 6.17 and 6.18. These bodies were thought to be lysosomes but to provide more information the cells were stained for acid phosphatase, an enzyme found in abundance in these organelles (Daems et al., 1969).

Acid Phosphatase in L Fibroblasts

Procedure. The technique used was based on that described by Burstone (1962) with naphthol AS-BI phosphate (Sigma) as substrate for the enzyme. Cultures were fixed in cold acetone for 45 seconds, washed in physiological saline and incubated with substrate at 37^{o}C for 1 hour. Controls were either fixed for

Plate 6.15

An electron micrograph showing a portion of an L fibroblast infected with L-form GL8 at 500 cfu for 5 hours and showing increased numbers of dense bodies

N nucleus.

M mitochondria.

Db dense bodies

Fixed glutaraldehyde, post-fixed osmium tetroxide.

Stained: uranyl acetate and lead citrate.

Scale: 1μ

Magnification, x 19,000

Plate 6.16

Showing a portion of another cell from the same preparation as Plate 6.15. Dense bodies surround an apparently vacuolated area with possible L-forms

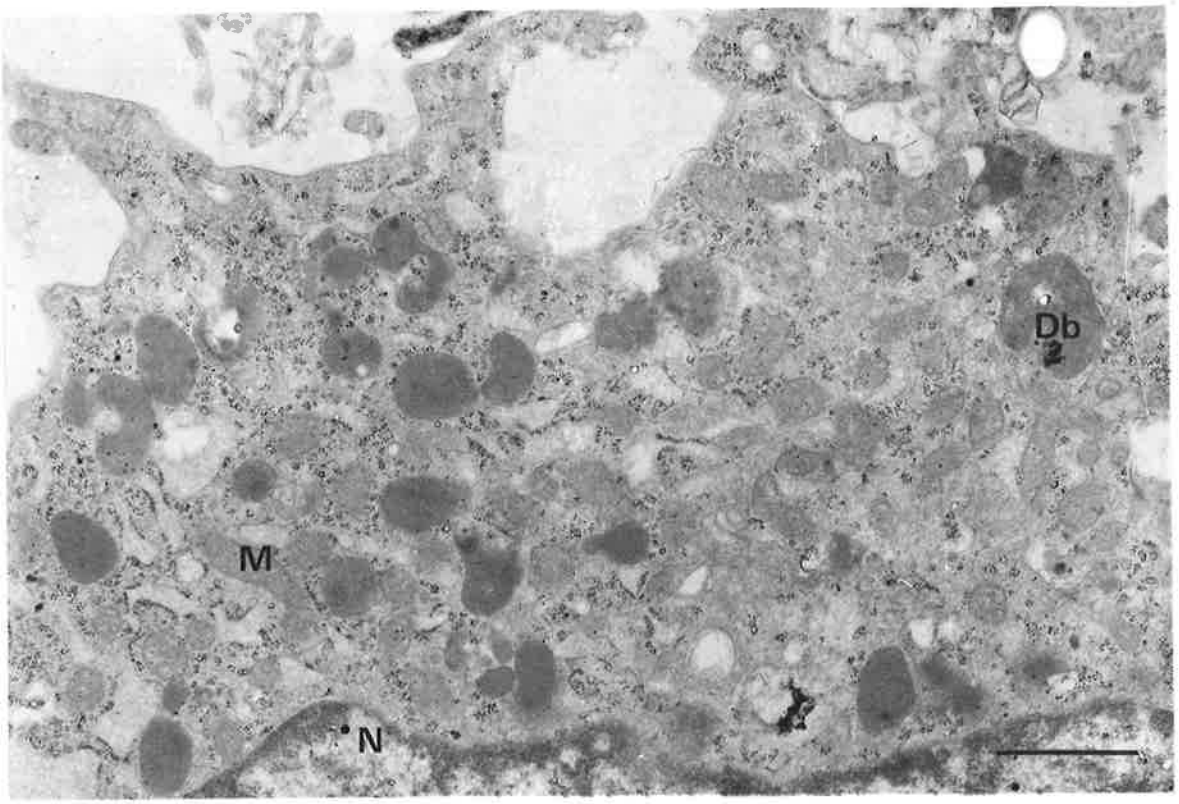
M mitochondria.

Db dense bodies.

L possible L-form.

Scale: 1μ

Magnification, x 19,000


Db
M
N

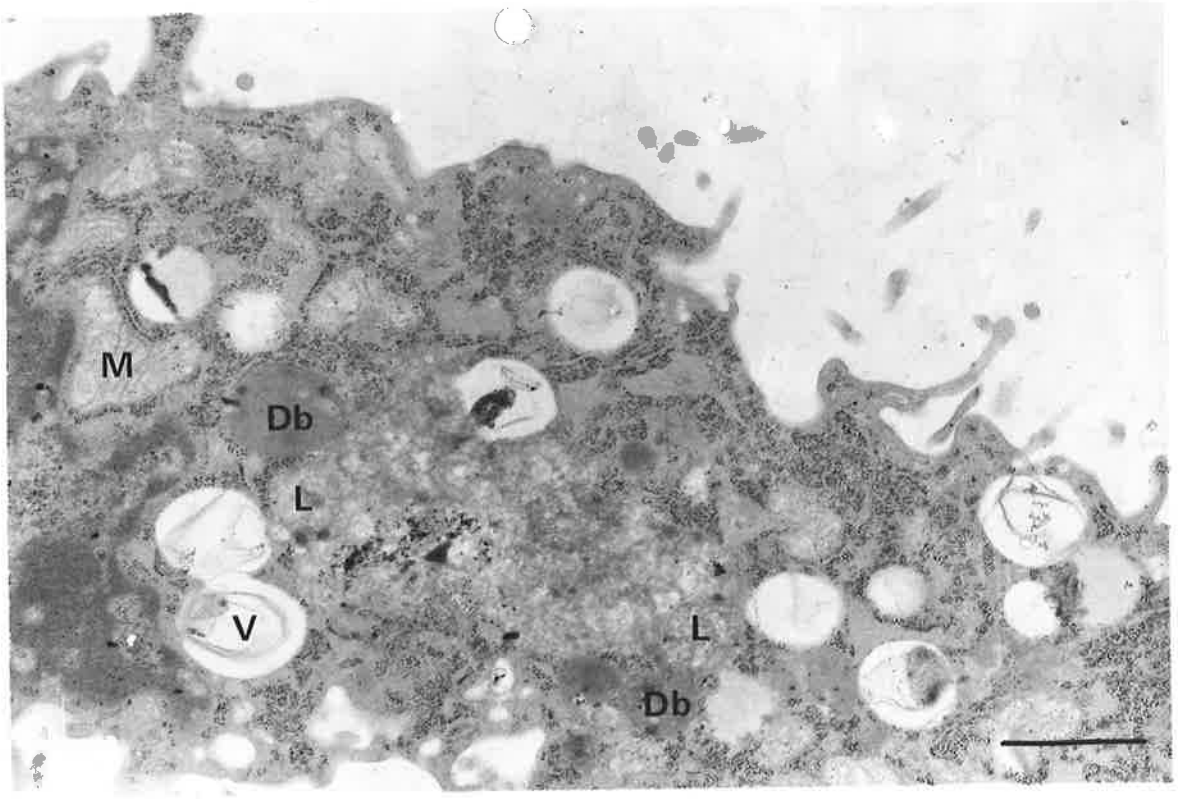

M
Db
L
V
L
Db

Plate 6.17

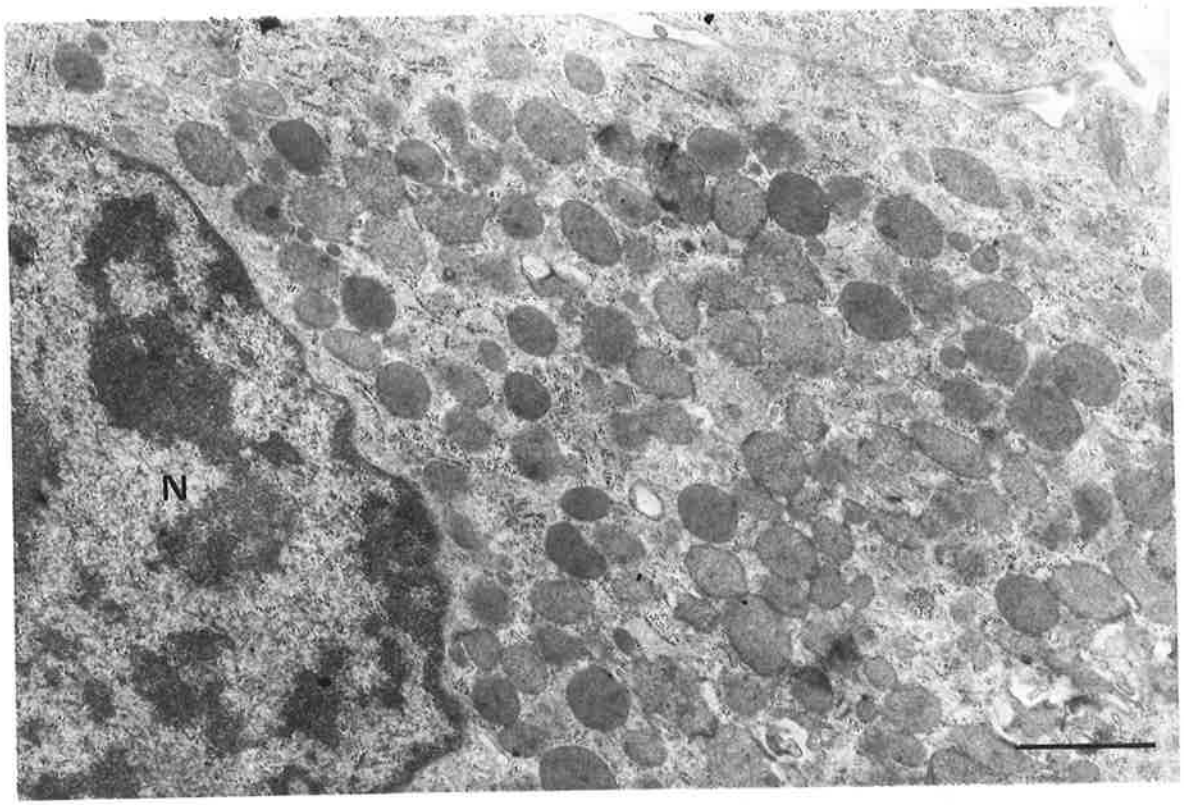
An electron micrograph of L fibroblasts apparently free from contamination but containing large numbers of cytoplasmic dense bodies

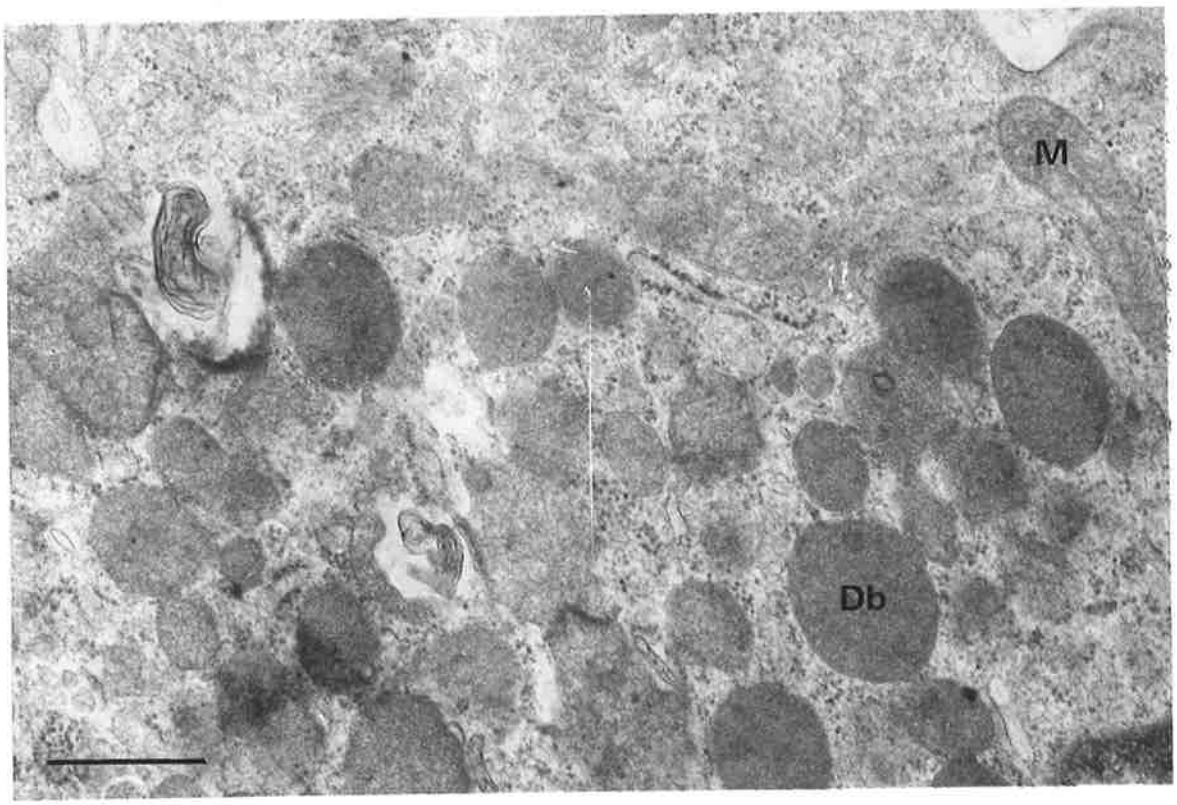
N nucleus.
Fixed glutaraldehyde, post-fixed osmium tetroxide.
Stained: uranyl acetate, lead citrate.
Scale: 1μ
Magnification, x 18,000

Plate 6.18

A higher power electron micrograph showing the contrast between mitochondria and dense bodies

M mitochondrion.
Db dense body
Scale: 0.5μ
Magnification, x 42,000


N


M
Db

1 hour in acetone at 37°C to inactivate the enzyme before proceeding as for test cultures or fixed for 45 seconds in cold acetone and incubated in Red-Violet without substrate. After incubation in Coplin jars the slides were rinsed in distilled water for 10 seconds and mounted in phosphate buffered glycerol jelly pH 7.2.

Results. Most of the cells in test cultures contained a well-defined red-staining zone in close proximity to the nucleus. No controls showed such areas. Examination at high power failed to relate the pattern to dye-uptake to granules like those seen in electron microscopy. The fact remained that these cells were apparently rich in acid phosphatase, in which case it was quite probable that the granules were actually lysosomes. The results of staining for acid phosphatase are shown in Plates 6.19 and 6.20.

Electron microscopy of these cells with introduced L-forms revealed a situation similar to that described previously with some cells containing L-form like structures apparently held in phagocytic like vacuoles. In these particular cells, however, it was very difficult to estimate changes in cell lysosomes.

Plate 6.19

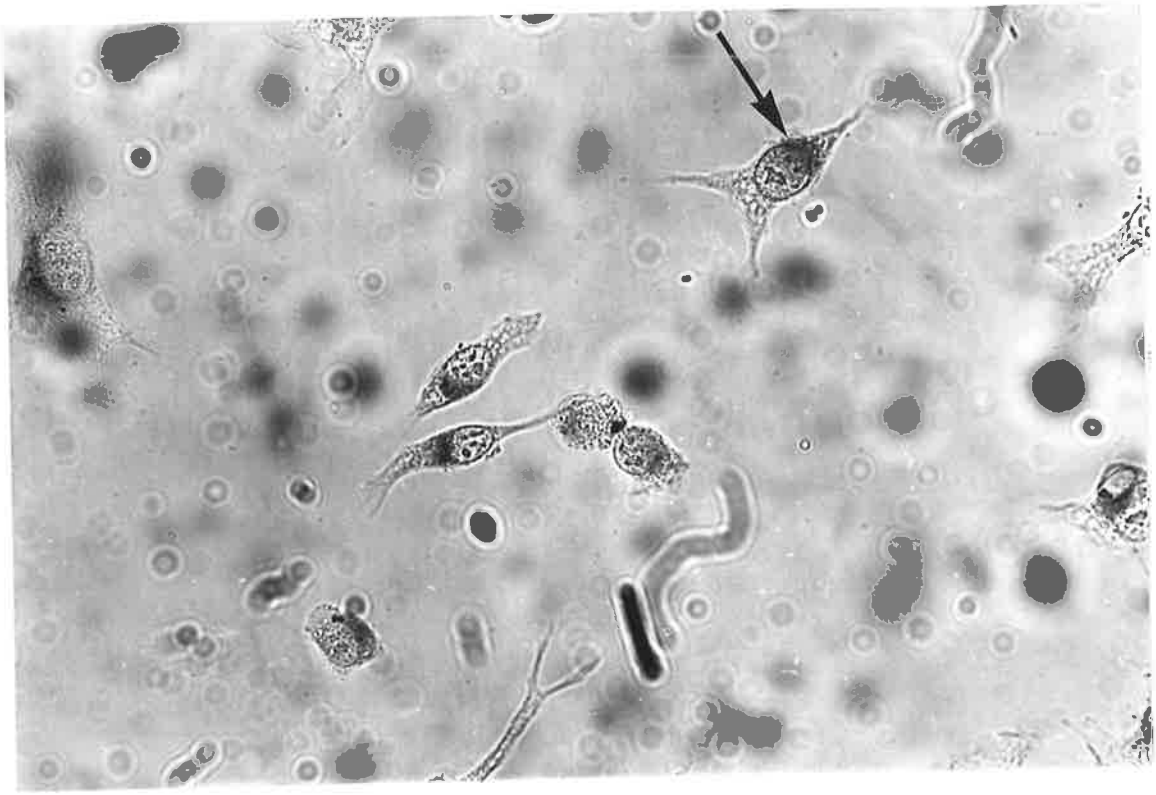
A light microscopic examination of the cells shown in Plates 6.17 and 6.18 with the cells stained for acid phosphatase

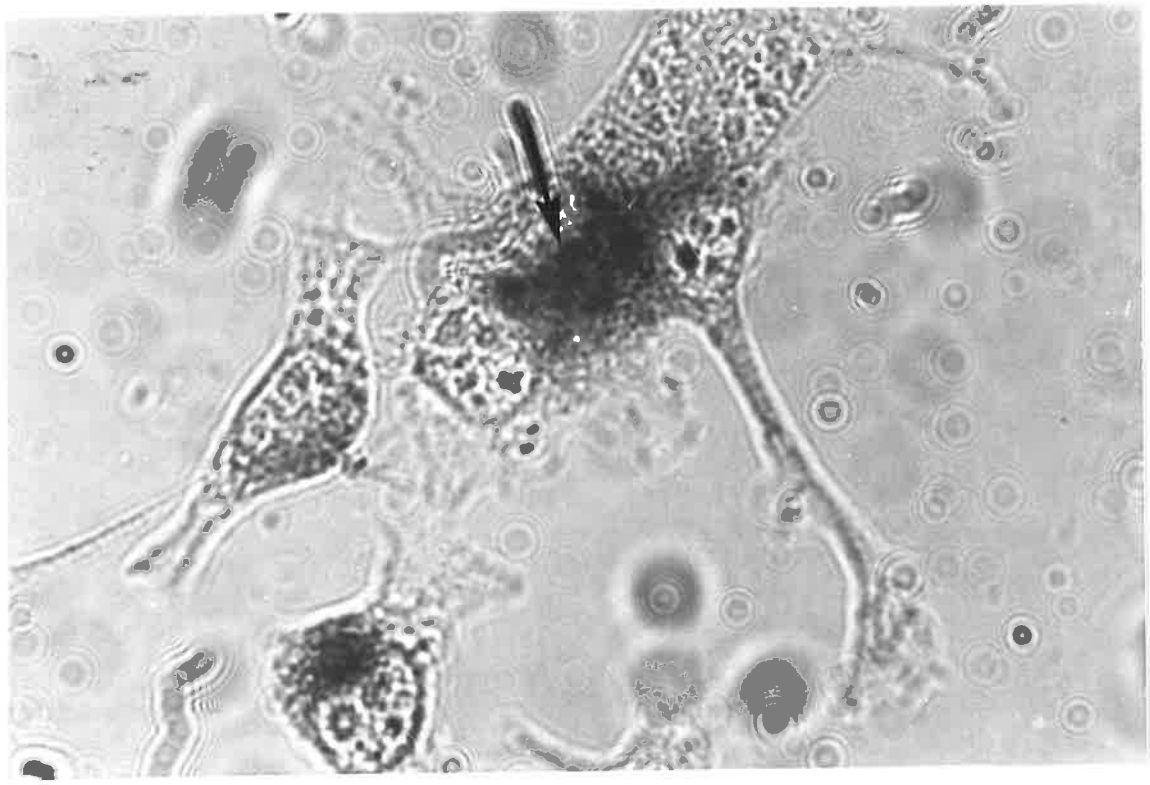
The arrow points to a stained area in proximity to the nucleus.
Magnification, x 750

Plate 6.20

A high power photograph showing that the staining zone (arrow) does not appear to be related to granules

Magnification, x 3,100





DISCUSSION OF EXPERIMENTAL PROCEDURE AND RESULTS

The findings described in Chapter 5 for infectivity studies with human diploid cells were confirmed. That is, there was little detectable interaction between L-forms and cells. The findings described in L fibroblast experiments stressed the need for vigilance against contamination. Because the events described for cells apparently infected with mycoplasma were considered to be peripheral to the theme of the present study, they were not pursued. It would, however, have been extremely interesting to have explored the phenomenon further as a possible example of microbial "co-operation". In this context, it was unfortunate that heat killed L-form controls were not included to enable a better evaluation of the nature of the role played by L-forms in this process; for example, the L-forms may play an active role or they may have a passive, toxic effect.

Evidence was presented for intracellular degradation of L-forms in L fibroblasts and for the apparent activation of the lysosomal system in some cell sections where no L-form like structures were seen although the cells were apparently infected. A large number of sections were screened over many experiments but L-forms were never identified in relation to the cell surface, despite the fact that samples were taken from the time of introduction of L-forms through to several days. This observation

must be balanced against the very small fraction of each cell examined in one section. The possibility of serial sectioning was considered, but it was very difficult to obtain good sections of the cells at all of the chosen intervals. In addition, this procedure does not overcome the problem of limited sampling of the specimen.

The study by Pham Huu Trung et al. (1968), in which human diploid fibroblasts infected with L-forms of haemolytic streptococci were examined by electron microscopy, contrasts with the findings in the present study. Detailed technical descriptions are not given, but the organisms used were the Richards G III (type 3) and the GL8 strain. When the cells were examined 2 to 3 months after infection it was found that both extra- and intracellular L-forms were present. The cell cultures were checked for the presence of mycoplasmas, but the L-forms are not clearly indicated.

In the present study, the desirability of labelling the L-forms, for example, with ferritin-tagged antibody, was emphasized by findings such as those shown in Plate 6.14, where it is claimed that L-forms are present within a vacuole, while mycoplasma can be seen at the cell surface. The presence of electron dense particles in relation to the supposed L-forms would have helped to eliminate ambiguity.

Ferritin labelling was not carried out for a number of reasons, including the anticipated time taken to adequately establish this technique. Another consideration was that the labelling might be weak, with subsequent difficulty in interpretation. There was also the problem of the apparent low frequency of intracytoplasmic organisms. At this time it was considered more important to collect a different type of information, namely, the quantitative uptake of L-forms by cells.

ADSORPTION STUDIES — CHAPTER VII

INTRODUCTION

The work in Chapters 5 and 6 described, in a largely morphological manner, the findings relating to L-form cell association. Although it was not possible to make any definite conclusions from these experiments, it seemed, in the case of L fibroblasts, that there could be some form of interaction.

This chapter describes an attempt to verify these findings by study of adsorption of L-form GL8 to L fibroblasts. It was considered that any interaction taking place would imply a phase of adsorption to the cell surface. Accordingly, experiments were designed to quantitate the removal of organisms from suspension by the cells. As in previous chapters, the procedures are described and discussed in a largely chronological manner because approaches were varied depending upon experience gained. The work is discussed under the following headings:

Adsorption of L fibroblasts to colonies of L-form GL8.

The characteristics of the suspending medium for adsorption studies.

The experimental technique for adsorption of L-forms to L fibroblasts.

Adsorption experiments and results.

Growth behaviour of L-forms in tissue culture media containing cells.

Adsorption of L-forms by mouse peritoneal macrophages in suspension.

Adsorption experience with the L-form parent, Streptococcus 63X.

ADSORPTION OF L FIBROBLASTS TO L-FORM GL8 COLONIES

This method of studying cell-organism interaction was based on the work of Sobeslavsky et al. (1968) and Manchee and Taylor-Robinson (1969) who studied adsorption to mycoplasma colonies by a range of cell types. The basic technique used by both groups was to flood the mycoplasma colonies in agar with a cell suspension, to incubate under a variety of conditions and following this, to wash the agar surface with buffer to remove unattached cells. Adsorption was indicated by clustering of cells about the colonies against a clear agar background. Various cell receptors were considered to play a role in this process, including N-acetyl neuraminic acid, this being implicated because of reduced adsorption after neuraminidase treatment of the cells. Sobeslavsky's group found that adsorption to colonies of Mycoplasma pneumoniae was specifically inhibited by homologous antimycoplasmal serum, but not by heterologous antimycoplasmal antibody. It was also

found that it was of the same order of magnitude after incubation at 4°C, room temperature or 37°C, for 15 to 30 minutes.

Experimental

In the present study, blocks of agar containing L-form colonies were flooded with a cell suspension of about 1×10^6 per ml in PBS. After incubation for 1 hour at 37°C the preparations were washed liberally in PBS and examined, unstained, by low-power light microscopy.

To test the effect of anti-L-form serum, colonies were incubated with antiserum prepared and assayed as described in Chapter 5, or with pre-immunization serum from the same rabbit. After incubation for 1 hour at 37°C, the preparations were washed in PBS and flooded with a cell suspension as described above. Experiments involving anti-L fibroblast serum were not carried out.

Results

A reproducible finding was that many L fibroblasts were observed clumped about the periphery of most colonies with fewer cells being attached to the central part of the colony. The microscopic appearance is shown in Plates 7.1 and 7.2. It was noticed that with large L-form colonies, rinsing in PBS removed the friable outer aspects of the colony with its adherent cells.

Plate 7.1

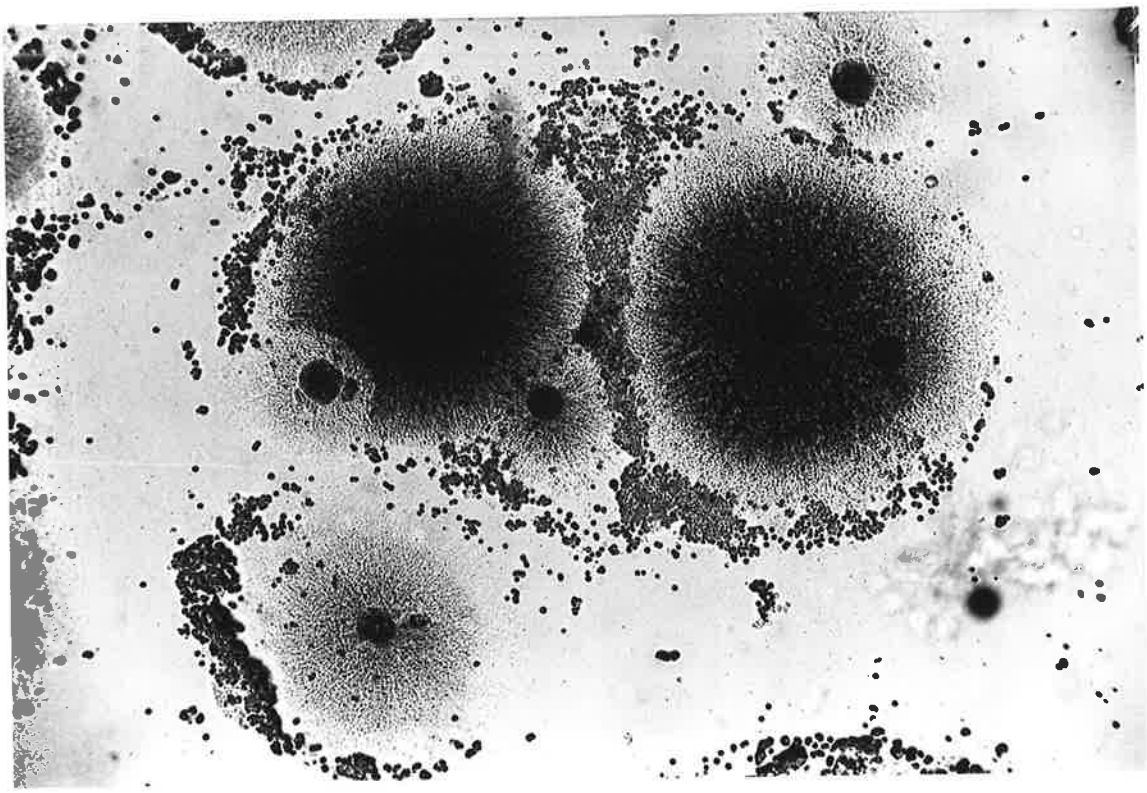
Photograph of L cell adsorption to L-form GL8 colonies

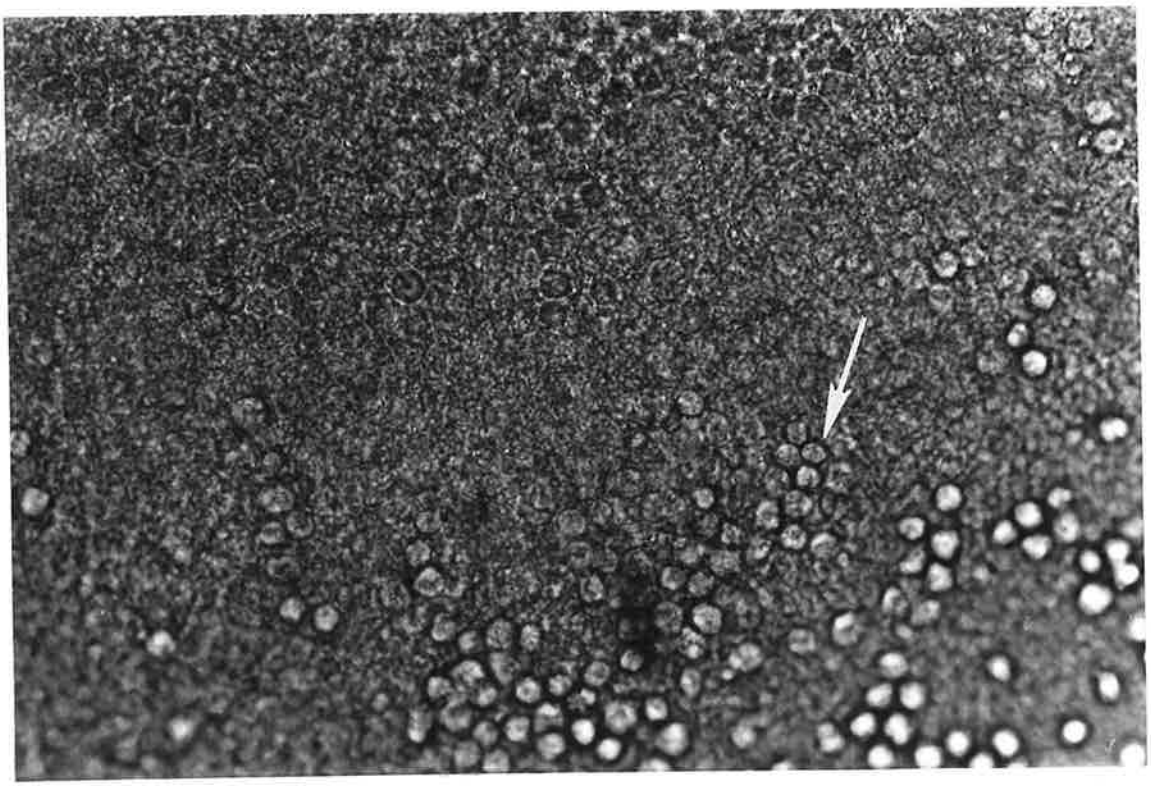
Cells appear to be aggregated mainly at the colony periphery. Magnification, x 75

Plate 7.2

Higher power photograph showing L cell adsorption to the centre of a colony

Arrow indicates L cells in relation to the colony mass. Magnification, x 750





Prior incubation with anti-L-form serum or pre-immunization serum had no effect on the adsorption rate. Presumably, the antigenic determinants bound to antibody were not essential binding sites in the adsorption of L fibroblasts to the colonies.

It was at first considered that the cells might merely be trapped by irregularities in the colony surface or at the colony-agar border. However, it was found that even vigorous washing failed to dislodge the bulk of the cells; from this it was concluded that some definite form of adhesion had occurred. The nature of the adsorption was not studied further, but these experiments did give encouragement for further work on adsorption using L-forms in suspension.

THE MEDIUM FOR ADSORPTION IN SUSPENSIONS

The requirements of the suspending medium were that:

1. L fibroblasts should remain viable.

2. Adsorption should not be inhibited.

3. L-form counts should not decrease too rapidly over the period of the experiments because it would make interpretation of differences between test and control populations difficult.

If the L fibroblasts died rapidly in the medium, the experiment could be affected in two ways by lysis of the cells. Firstly, diminished adsorptive ability by cell fragments might occur, and secondly, cell components, toxic for the L-forms, could be released.

Pethica (1961), in a consideration of the nature of the forces involved in cell adhesion, mentioned the importance of the ionic strength of the medium in relation to this process. It could be, for example, that the presence of high concentrations of salt to provide osmotic protection for the L-forms, could affect adhesion. This seemed quite likely in view of the apparent importance of ionic events in cell adhesion (Pethica, 1961).

If the rate of L-form death was so great as to give a marked fall in control and test populations over the experimental period, then it would have been difficult to detect a difference. This implies that under the conditions used, the populations should remain static or at least decline only slowly.

Experimental

1. L-Form Survival in Tissue Culture Medium

A late log-phase culture estimated to contain, by optical density measurements, 4×10^9 cfu per ml was centrifuged at 450g for 20 minutes. The pellet was resuspended in Eagle's basal

medium with 10% calf serum (EBM + 10% CS) using the same volume as the original broth. The suspension was then held at 37°C. In addition to counting this suspension, samples were taken from the original broth at the time of centrifugation and from an identical pellet resuspended into L-form broth. Plates were freshly prepared as described in Chapter 4. The techniques of diluting samples and plating were described earlier (Chapter 4).

Results

The results of these experiments are shown in Figures 7.1 and 7.2. The most striking feature of these graphs is the high death rate in the first hour with only about 10% of the colony forming units remaining at that time. There appears to be a plateau between 1 and 5 hours and following this, a slow decline with approximately 0.0001% of the viable cfu remaining after 25 hours. The difference in the rate of the early falls was attributed to the fact that the L-form pellet was easier to resuspend in the experiment represented by Figure 7.2. It was considered that vigorous agitation had an additive effect when coupled with hypotonic conditions. The levelling off of the curves over 1 to 4 hours, if truly representative, could represent greater resistance to hypotonic conditions by a proportion of the population. This could well be related to the size of the organisms.

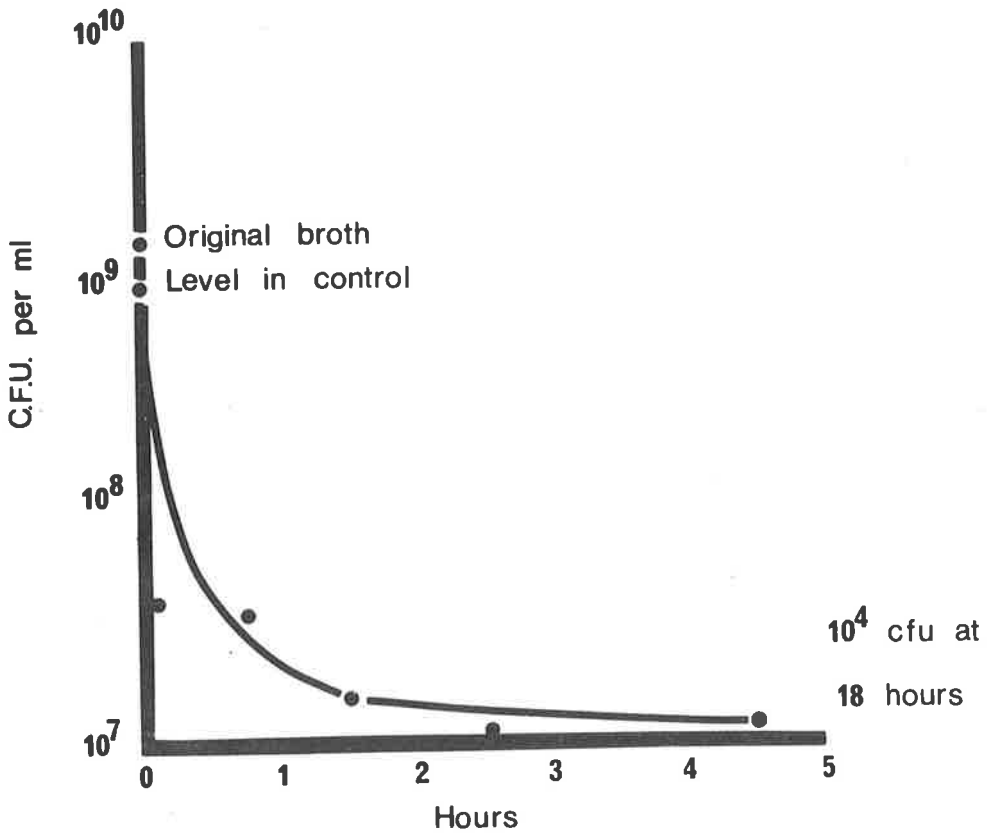


Figure 7.1

Survival of L-Form GL8 in Tissue Culture Medium (Eagle's Basal Medium + 10% Calf Serum).

C.F.U. ≡ Colony Forming Units.

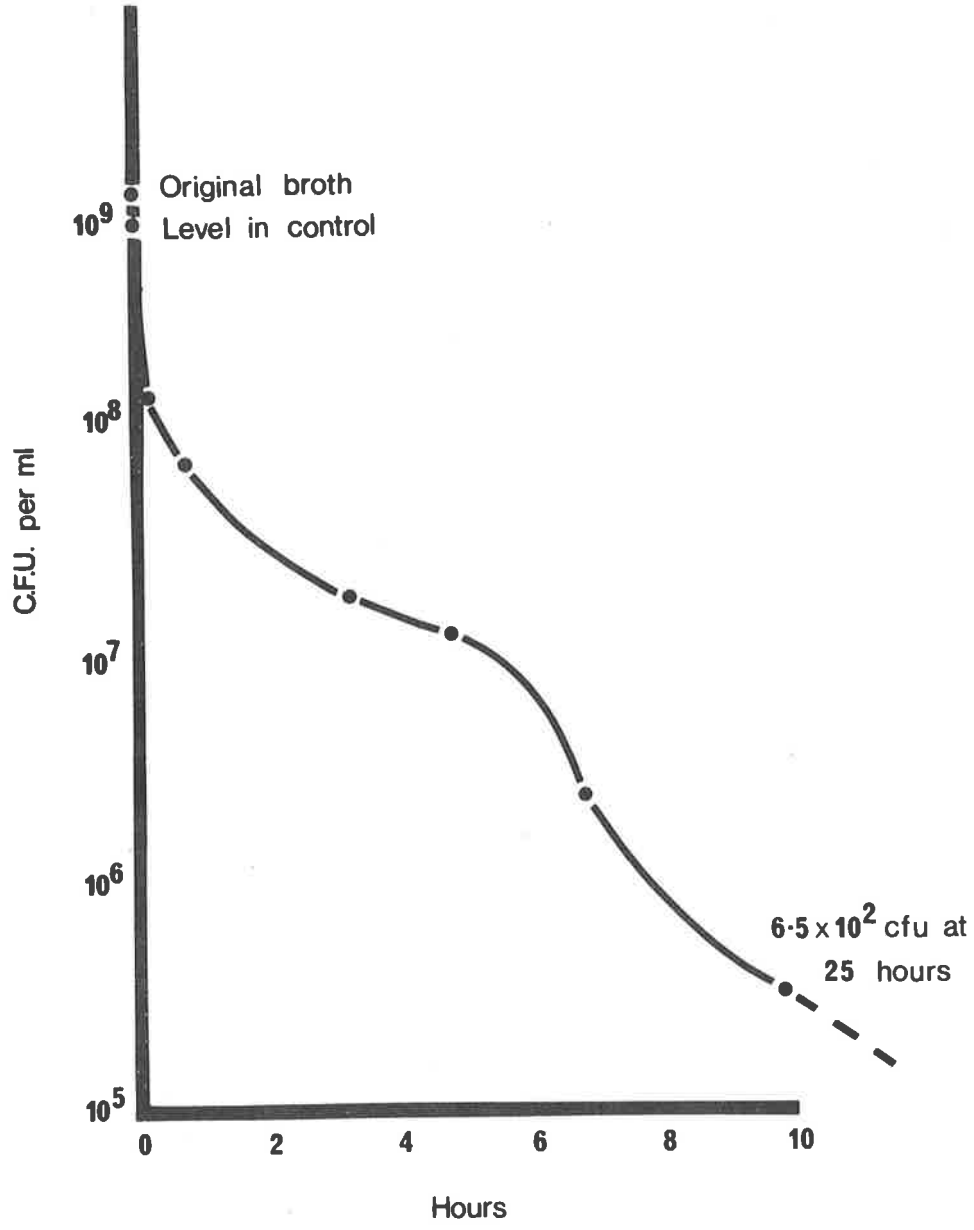


Figure 7.2

Survival of L-Form GL8 in Tissue Culture Medium (Eagle's Basal Medium + 10% Calf Serum)

C.F.U. ≡ Colony Forming Units.

2. A Hypertonic Medium

With the requirements of the suspending medium in mind, it was decided to increase the osmolarity of the tissue culture medium. The agents considered were polyethylene glycol, sodium chloride and sucrose. The failure of polyethylene glycol to provide osmotic support for the L-forms was described in Chapter 5, and sodium chloride was unsuitable because of its ionic effects. Sucrose was therefore selected and after some trials 15% was chosen as a level which gave adequate support for the L-forms while still being tolerated by the L fibroblasts.

Experimental

Effect on viability of L fibroblasts of EBM + 10% CS with sucrose at 15% concentration. Centrifuged cells were resuspended in this medium and controls in EBM + CS. They were gently agitated at 37°C for 6 hours with samples being taken at 3 hours and 6 hours. Trypan blue (0.1 ml of a 0.4% solution added to 0.5 ml of a cell suspension) exclusion was used as a measure of cell viability. The cells were examined in a haemocytometer after 10 minutes staining with stained cells and total cell counts being recorded. The results of this test are shown in Table 7.1. Although the level of viability was disappointingly low, microscopic examination of the suspension after 6 hours incubation indicated that the cells were still intact.

TABLE 7.1

The Effect of Sucrose on Viability of L Cells as judged by Trypan Blue Uptake

Time	Ratio of stained to total cells		% of cells dead or damaged	
	EBM+CS+Suc*	EBM+CS	EBM+CS+Suc	EBM+CS
3 hours	58/128	15/59	45	25
6 hours	142/145	48/102	approx. 100	47

* EBM = Eagle's basal medium

CS = 10% calf serum

Suc = 15% sucrose

3. Toxicity of Damaged L Cells for L-Form GL8

Although the tissue culture medium with 15% sucrose seemed to be the best compromise for a suspending medium, it was considered important to test the effect of damaged cells on L-form viability. This high level of sucrose was necessary because the organisms were not adapted to growth in media with low osmotic support.

Experimental

The effect of filtrates from an L fibroblast suspension that had been frozen to kill the cells and from a suspension exposed to sucrose for 8 hours, were examined. Organism concentrations were of the order of 1×10^6 cfu per ml. Trypan blue staining of the frozen cells showed that all cells took up the dye. The cells from both test samples were centrifuged at 100g for 10 minutes and the supernatants filtered through 0.45μ (Millipore) filters. In the case of the frozen cell filtrate, sucrose solution was added to 15% concentration. The filtrates were inoculated with L-forms and incubated at $37^{o}C$ with gentle shaking. Samples were removed at given time intervals and plated in duplicate.

Results. These are shown in Figure 7.3. Over the time period shown there did not appear to be any toxic effect of the cell lysate on the L-forms.

THE EXPERIMENTAL TECHNIQUE FOR ADSORPTION

The procedure was to incubate L-forms with cells in EBM + CS + 15% sucrose at 37^{o}C with gentle agitation. L-form counts were estimated by optical density recordings and final values were obtained by plate counts after the experiment. Ratios of 1 cfu per cell to 30 cfu per cell were used. Because of the deleterious effect of sucrose on the cells over several hours, most experiments were conducted over 4 to 8 hours. Volumes of 10 ml were used because small volumes tended to produce frothing on agitation and, in addition, 10 ml allowed a moderately concentrated suspension of about 2 x 10^{6} cells per ml. The rationale was that these conditions should provide a favourable environment for adsorption. Controls were L-form inoculated media without cells. At selected time intervals, usually 3 to 5 for each experiment, 2 ml samples were removed and centrifuged at 100g for 10 minutes. Tests showed that at least 85% of cells were sedimented under these conditions. Samples of 0.3 ml were carefully taken from the top of the supernatant and dilutions were made at 0.3 ml into 2.7 ml in TSB + YE + 3%NaCl and 0.1 ml samples plated onto TSB + YE + 3% NaCl + HS.

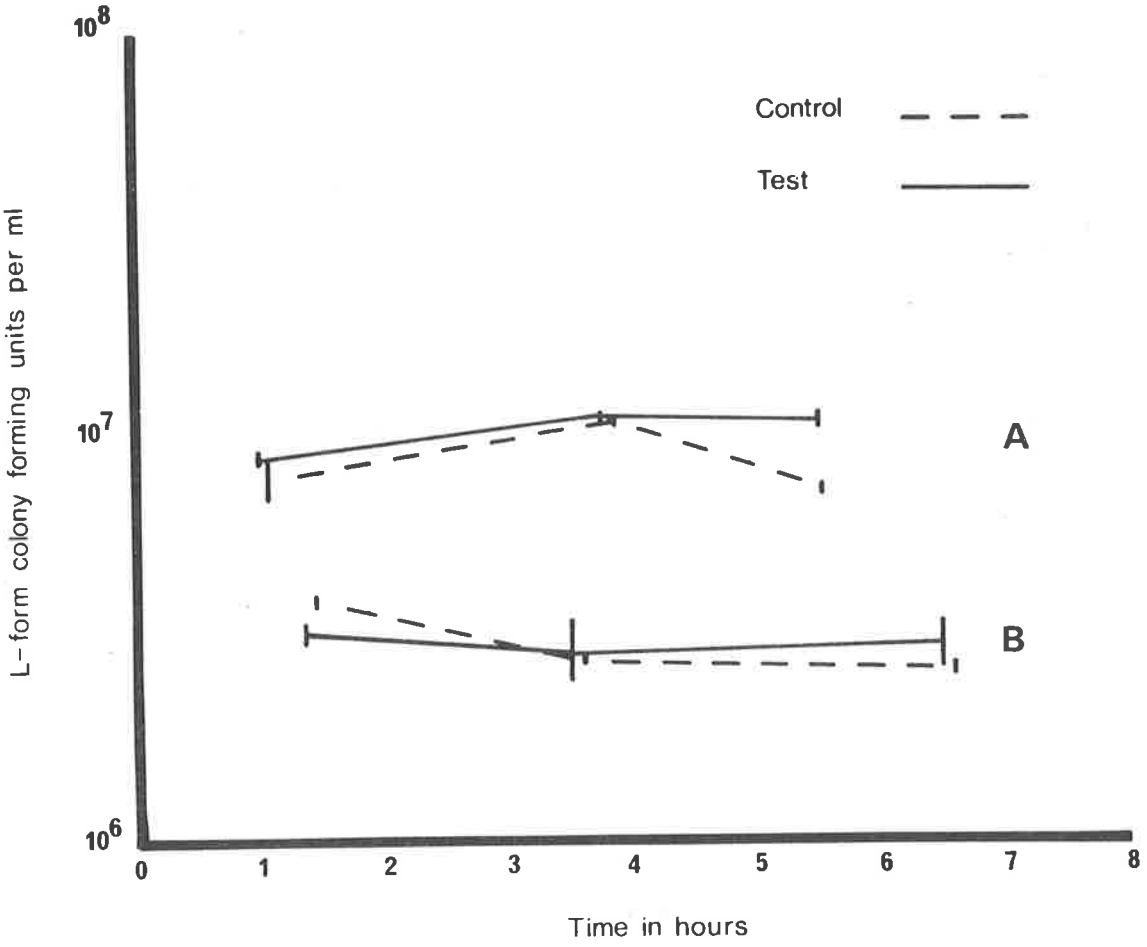


Figure 7.3. The Effect of L-Fibroblast Preparations on the Viability of L-Form GL8 Suspended in Eagle's Basal Medium + 10% Calf Serum + 15% Sucrose.

A. The effect of an L fibroblast suspension previously exposed for 8 hours to tissue culture media with 15% sucrose.

B. The effect of a filtrate from a freeze-thawed cell suspension.

A note on plating procedures. Earlier experience with L-form GL8 exposed to hypotonic conditions had indicated that some of the colonies took a minimum of 48 hours to become visible, whereas the majority were seen by 24 hours. Therefore, to obtain accurate counts it seemed to be necessary to incubate at 37°C for at least 48 hours. This had to be balanced against the very large size of some of the colonies at this time, causing partial confluence with subsequent difficulty in counting. There was no completely satisfactory solution to this problem as the use of low colony number plates for counting increased the risk of error.

The 0.1 ml sample size was probably the most important source of error in the experiments, but it was preferred to larger volumes because of moisture considerations. As was described in Chapter 4, L-form agar plates were only dried for a short time to enable optimum growth. Such plates did not readily accept larger volumes of liquid; that is to say, it was necessary to dry the plates for some time after inoculation or risk the running of the inoculum across poorly-dried plates.

ADSORPTION EXPERIMENTS AND RESULTS

Figure 7.4 shows the result of the first adsorption experiment using approximately 30 cfu per cell. At 4 hours 70% of the population appears to have been adsorbed, while at 8 hours the test sample had only 2% of the control population; that is, there was almost complete adsorption of the organisms. Figure 7.5 shows adsorption at 12 cfu per cell and 3 cfu per cell. At 12 cfu per cell the test population was 50% at 1.5 hours and 30% at 3.5 hours. With 3 cfu per cell there was no difference at 1.5 hours, but the test population was only 25% of control at 3.5 hours.

The overall results of the early experiments are shown in Table 7.2 including adsorptions carried out with cells killed by freezing.

Presentation of Results

The trends of the L-form populations, as illustrated in Figures 7.4 and 7.5, are shown by lines connecting points that are the average of the duplicate plate counts. Vertical bars show the spread of the two values.

It was difficult to decide the level at which differences in the populations of test and control samples were meaningful, this interpretation depending very largely on the magnitude of the error inherent in the technique.

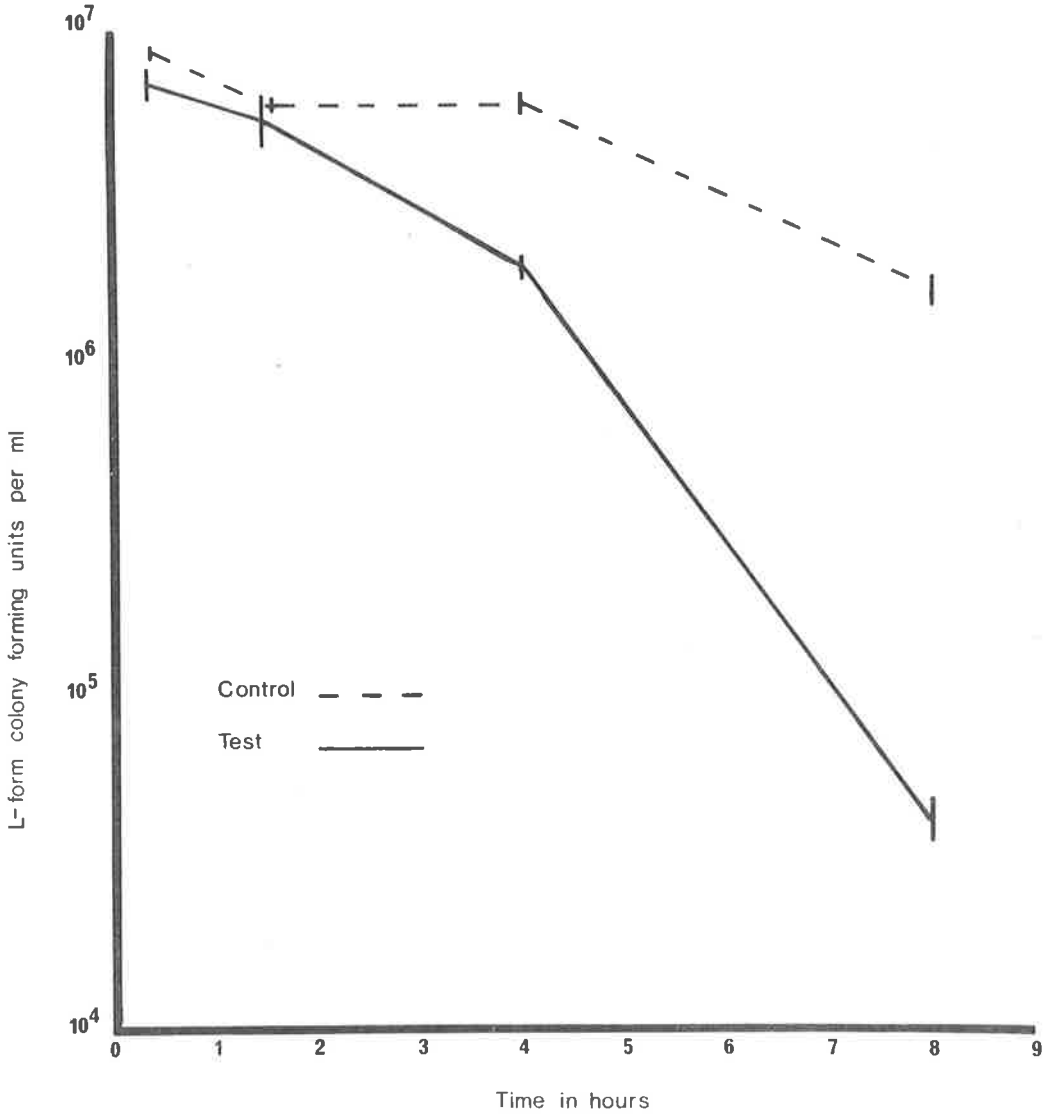


Figure 7.4. Adsorption of L-Form GL8 by L Fibroblasts in a Suspending Medium of Eagle's Basal Medium + 10% Calf Serum + 15% Sucrose (30 Colony Forming Units per cell).

The test populations are compared with controls (Control ≡ L-forms suspended in Eagle's Basal Medium + 10% Calf Serum + 15% Sucrose).

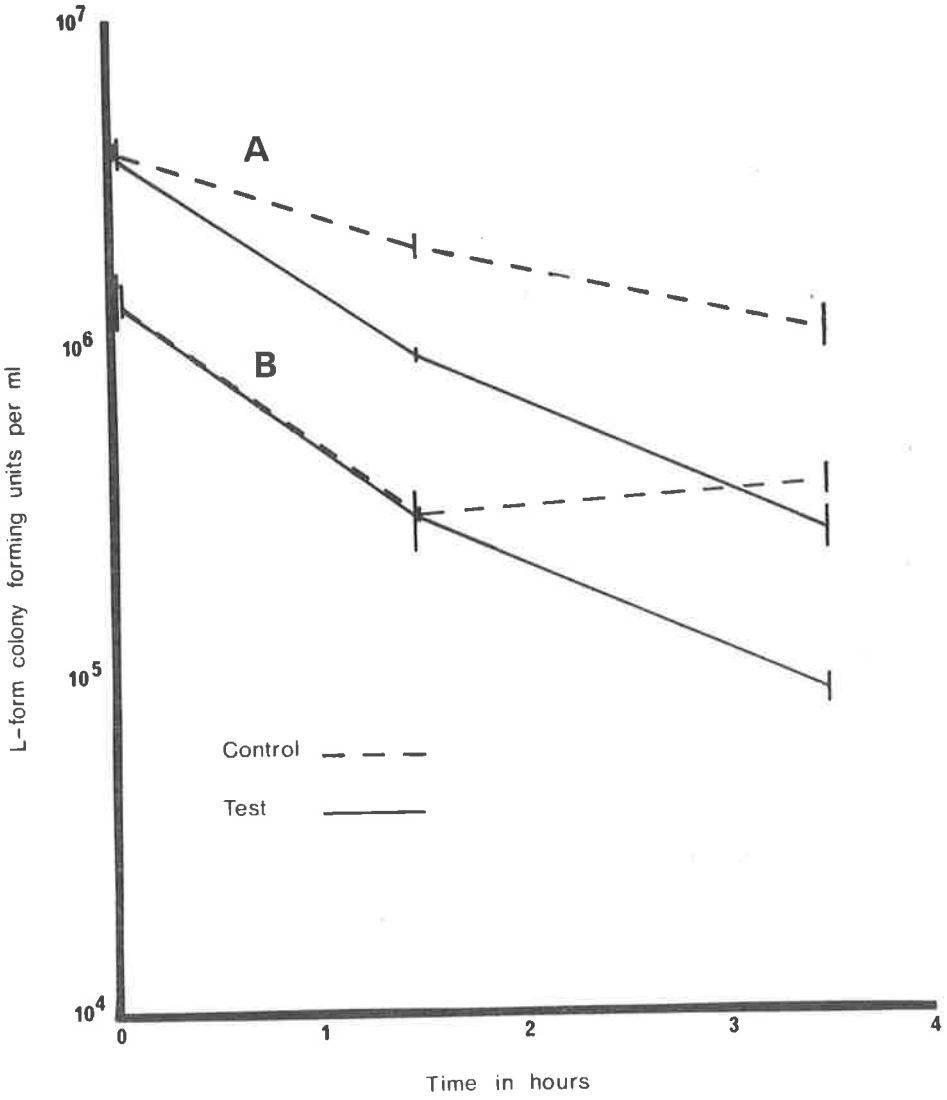


Figure 7.5. Adsorption of L-Form GL8 by L Fibroblasts in Eagle's Basal Medium + 10% Calf Serum + 15% Sucrose

Controls suspended in Eagle's Basal Medium + 10% Calf Serum + 15% Sucrose.

A. Adsorption at 12 colony forming units per cell.
B. Adsorption at 3 colony forming units per cell.

TABLE 7.2

The Population of Non-Adsorbed L-Forms in the Test Mixtures Expressed as a Percentage of the Control Population

Experiment number	cfu to cell ratio	Time in hours									
		0.75	1	1.5	2	2.5	3	3.5	4	5	8
1	30			-					30		2
2	15				-				50		
3	12			50				30			
4	10	-					80				
5	5	-					-				
6	3			-				25			
7	2		-			-			25		
8	1			-			-			170	

Experiment number	cfu per freeze-thawed cell										
1	10	-					80				
2	5	-					60				
3	2	60				30				25	
4	1		-			-			30		

The horizontal bars (-) represent overlapping counts; that is, where the minimum or maximum values for test and control population overlapped.

The figures used to provide the percentages are the averages of duplicate counts.

To enable an evaluation of the results shown in Table 7.2, it was necessary to have an idea of the magnitude of this error. When minimum and maximum plate counts were compared by the Student 't' test for a number of observations in the 1×10^6 and 9×10^6 cfu per ml range, a t-value of -0.47688 was obtained with 16 degrees of freedom. Therefore, over the range considered, there did not appear to be a significant spread in the plate counts ($p > 0.30$). It was possible to obtain a quantitative estimate of the error in the method, by using the test described by Dahlberg (1940). This test makes use of the fact that double determinations made for each observation enable the standard deviation, occasioned by the random errors of the method, to be calculated. The standard deviation of a single observation is computed according to:

$$\text{Standard deviation} = \pm\sqrt{\frac{\Sigma d^2}{2N}}$$

where d = the difference between the first and second plate counts.

N = number of pairs of observations.

To apply this method to the adsorption experiments, 52 paired recordings lying in the range of 1×10^6 cfu to 9×10^6 cfu per ml were randomly selected over several experiments. This gave the following result:

$$d^2 = 34.6 \times 10^{12}$$

$$N = 52$$

Therefore, Standard deviation $= \sqrt{\frac{34.6 \times 10^{12}}{2 \times 52}}$

$= 5.77 \times 10^5$ or approx. 6×10^5 cfuper ml.

This indicated that the probability was about 68% that a single determination would not differ by more than $\pm 6 \times 10^5$ from the true population value.

The arithmetic mean of the 104 observations was 3.66×10^6 cfu per ml. Therefore the standard deviation of a single determination, used as an indication of the experimental error would be:

$$\frac{6 \times 10^5}{3.66 \times 10^6} \times 100$$

or approx. 16% of the mean value.

If this value is taken as the overall magnitude of error in the technique, then from Table 7.2, observations of test populations that were of the order of 70% or less of the control populations, were unlikely to have arisen by experimental error and probably indicated that adsorption had taken place.

One source of concern with regard to the experimental technique was that test and control samples were centrifuged under different conditions, in that test samples had cells present

as well. The question arose as to the possibility of the cells functioning as "umbrellas" in dragging unattached L-forms down with them. This would have resulted in a lower count in the test supernatants. To test this possibility, an additional control was included in the form of an equivalent quantity of cells added to control broths just prior to centrifugation. Counts from these samples were compared with ordinary controls and were found to be very close in all cases. The fact that none of the adsorption experiments had shown marked removal of L-forms at very early times, suggested that this control was valid and that the presence of cells did not constitute a source of error at centrifugation.

The variation between experiments in the rate of decline of both test and control populations was attributed to differences in centrifugation conditions.

Growth of L-Forms in Media Containing Cells

In the section on selection of a medium for adsorption studies, one of the main considerations was that this medium should preserve the viability of the L-forms over the experimental period. It was found, however, that following an inoculum of 3×10^6 cfu per ml at 1 cfu per cell, there was a two log increase in counts after 12 hours, while counts in inoculated tissue culture media remained approximately constant over this time.

In view of the previous difficulty in adapting L-forms to grow in new media, this result was completely unexpected. The pattern of growth in tissue culture media with cells is shown in Figure 7.6.

It seemed quite feasible that despite a possibly lengthy lag-phase, growth could have been occurring in the time intervals used in the adsorption experiments. This would indicate that the results obtained were the result of a balance between adsorption and growth.

After some experimentation it was found that calcium- and magnesium-free Dulbecco phosphate buffer plus 15% sucrose did not give rise to growth in the presence of cells. Figure 7.7 shows the result of an experiment at approximately 1 cfu per cell using this suspending medium. There was no difference between test and controls.

The explanation of this result was not clear in view of the earlier results under less favourable conditions; the removal of L-forms by adsorption was counteracted by the occurrence of growth of the organisms in the suspending medium. It could be that the medium used in this experiment did not provide a favourable environment for adhesion. Another factor in this experiment was the low infectivity ratio used.

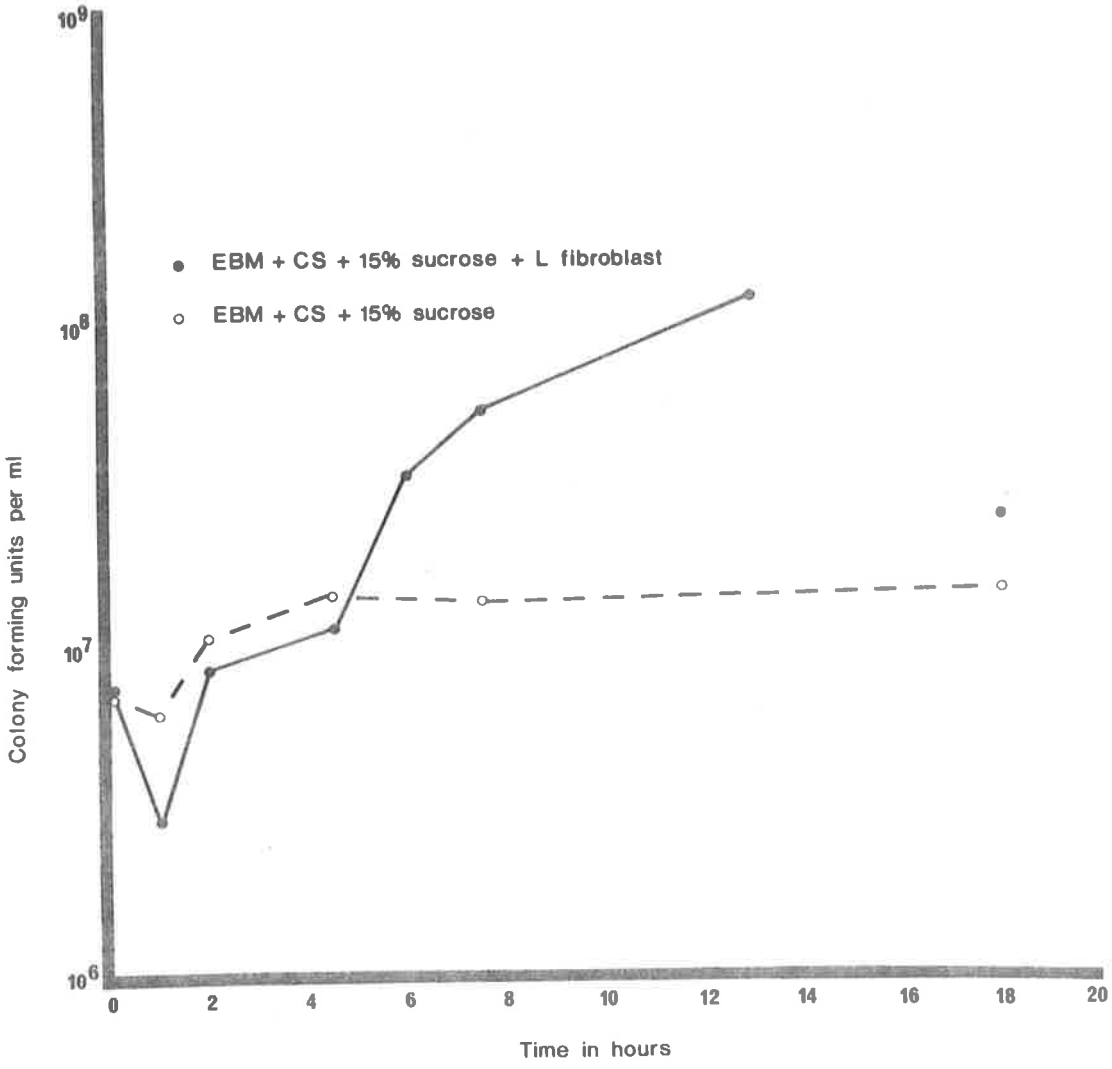


Figure 7.6. Growth of L-Form GL8 in Eagle's Basal Medium + 10% Calf Serum + 15% Sucrose, also containing L Fibroblasts at 1 x 10^6 per ml

Control ≡ Growth behaviour of L-form GL8 in Eagle's Basal Medium + 10% Calf Serum + 15% Sucrose.

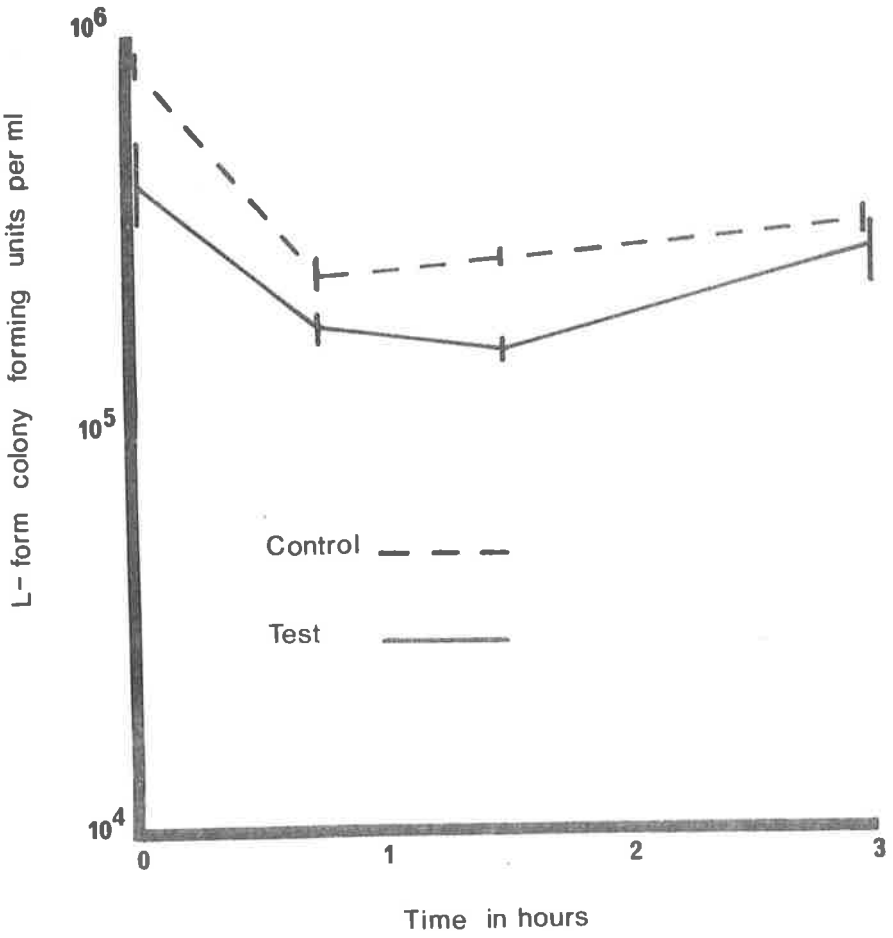


Figure 7.7. Adsorption of L-Form GL8 by L Fibroblasts in Dulbecco Phosphate Buffer + 15% Sucrose (1 colony forming unit per cell).

Control ≡ L-Form GL8 in Dulbecco Phosphate Buffer + 15% Sucrose.

Adsorption of L-Form GL8 by Mouse Peritoneal Macrophages

This was carried out for two reasons; firstly, there was interest in macrophage - L-form association and, secondly it was thought that the use of these cells could provide a measure of the efficiency of the adsorption technique for reasons discussed in Chapter 2. The experimental conditions were the same as those used for early adsorption studies with L cells, using EBM + CS + 15% sucrose as the suspending medium. Macrophages, obtained from 5 week-old Swiss albino mice, were harvested by scraping the cells from the glass substrate after 24 hours incubation in EBM + CS. The results of an experiment with approximately 4 cfu per cell are shown in Figure 7.8. The test and control points were nearly identical.

The failure to obtain a difference between test and control points could have been due to a steep decline in both test and control L-form populations over a short time. Overall, the results with macrophage cultures indicated a level of adsorption that was less than the rate for L fibroblasts.

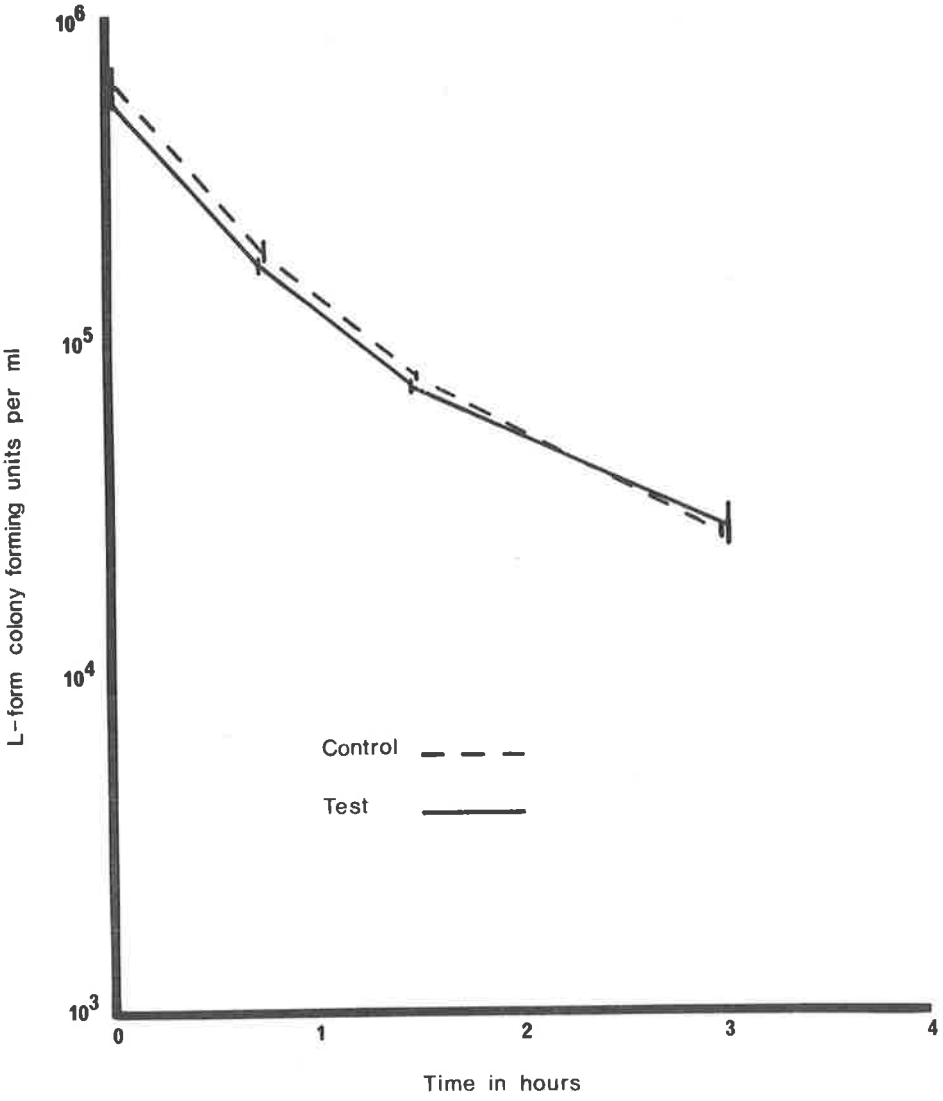


Figure 7.8. Adsorption of L-Form GL8 by Mouse Peritoneal Macrophages in Eagle's Basal Medium + 10% Calf Serum + 15% Sucrose (4 colony forming units per cell).

Control ≡ L-form GL8 suspended in Eagle's Basal Medium + 10% Calf Serum + 15% Sucrose.

ADSORPTION EXPERIMENTS WITH STREPTOCOCCUS 63X

These experiments were carried out with the aim of providing a parallel between the L-form and the parent organism and to allow a better evaluation of the technique used for adsorption. It was possible to eliminate any deleterious effects produced by hypertonic conditions and to correlate adsorption rates with microscopical examination of stained preparations.

Morphological Observations of Interactions Between the Streptococcus and Cells

Streptococci were added to L cells and mouse peritoneal macrophages (MPM) in Leighton tubes at 50 to 100 cfu per cell and then incubated at 37°C. At 1, 24 and 36 hours cultures were washed in phosphate buffer, fixed briefly in 90% ethanol and stained with 0.1% methylene blue (Gurr). Macrophage cultures showed streptoccal chains in relation to most cells at all times (see Plate 8.6), whereas organisms were observed in relation to L cells only at 24 and 36 hours. At these times, both macrophage and L cell cultures were very degenerate. The relationship of the organisms to the cells involved at least firm adsorption, as it was unaffected by thorough rinsing prior to fixation. There was no obvious reason why whole micro-organisms did not associate with L cells at 1 hour.

Adsorption of Streptococcus 63X at 37°C (100 cfu per cell), by an L fibroblast monolayer in EBM + CS and EBM + CS + 15% sucrose, showed, that over 1 to 2 hours adsorption was somewhat reduced in the hypertonic medium. This suggested that the hypertonic conditions necessary in the L-form adsorption studies could have reduced the efficiency of the techniques.

The Medium for Adsorption in Suspensions

Figure 7.9 shows the rapid early growth of the Streptococcus in EBM + CS with and without cells. Figure 7.10 shows the pattern obtained with calcium- and magnesium-free Dulbecco phosphate buffer (DPB) with 10% CS as the suspending medium. The control population remained approximately constant while the presence of either L cells or MPM allowed some growth. When a suspending medium consisting of DPB without CS was used, the streptococcal population in the presence of MPM and L cells at 2 cfu per cell declined slowly over 20 hours, whereas there was a marked early drop in the census of washed streptococci suspended in DPB alone. There was no attempt made to construct a detailed survival curve in DPB, but this rapid loss of viability was confirmed over three experiments. Such behaviour was unexpected, with the only logical contributing factor being the lack of divalent cations in this buffer.

The best suspending medium, to this time, appeared to be DPB + CS. It was decided to work with this medium rather than to test other buffers for their ability to maintain the viability of the streptococci. One reason for this was that the serum could have played a role in adsorption and, in addition, such a system more closely resembled an in vivo situation.

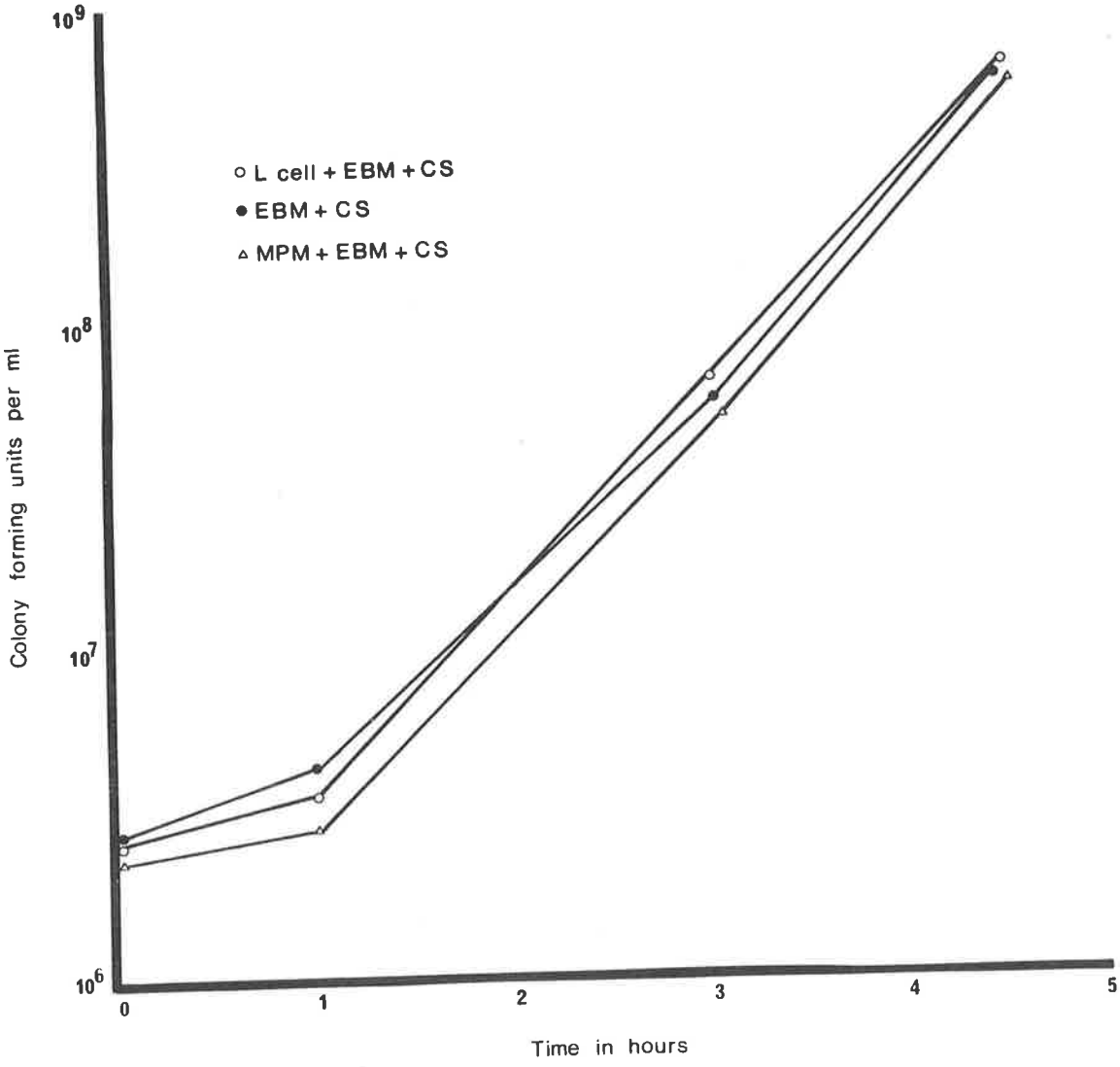


Figure 7.9. Growth of Streptococcus 63X at 37°C in 3 Media

E.B.M. ≡ Eagle's Basal Medium.
C.S. ≡ 10% Calf Serum.
L cell ≡ L cell at 1 x 10^6 per ml.
M.P.M. ≡ Mouse Peritoneal Macrophage at 1 x 10^6 per ml.

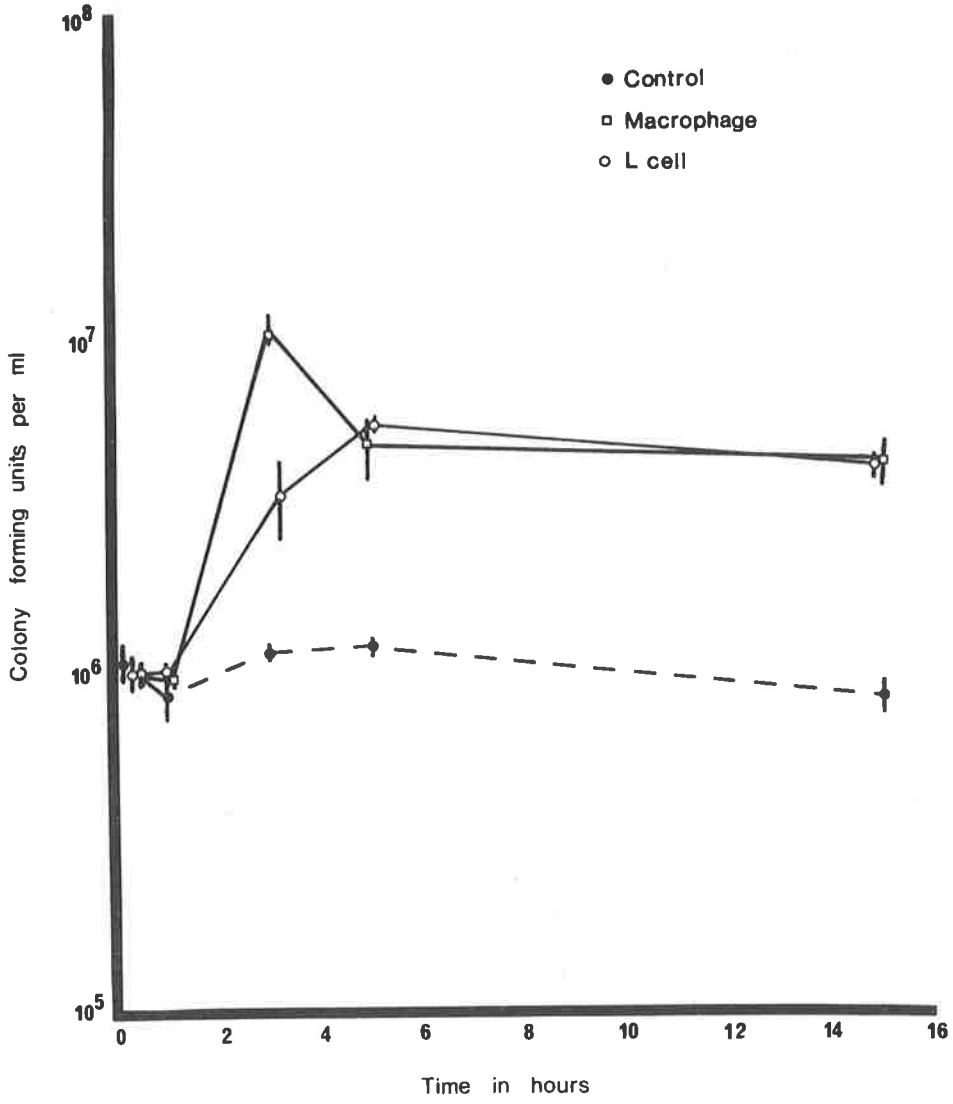


Figure 7.10. The Effect of Cells (1 cell per 3 colony forming units) on the Viable Counts of Streptococcus 63X Suspended in Dulbecco Phosphate Buffer + 10% Calf Serum.

Control ≡ Organisms suspended in Dulbecco Phosphate Buffer + 10% Calf Serum.

The problem of the growth-promoting effects of cells on streptococcal populations. There appeared to be two ways of overcoming this: firstly, to carry out the adsorption at lower temperatures or, secondly, to utilize levels of antibiotics that were bacteriostatic. This latter method would have involved testing a number of concentrations of a variety of antibiotics to find a combination that was truly bacteriostatic over the period of the experiment. The organisms would then be centrifuged and resuspended in fresh medium without antibiotic. From this they would grow up normally on agar plates because the antibiotic would have been greatly diluted by this time. It seemed quite likely that different bacteriostatic concentrations would have to be found to accommodate differences such as the cell type present in the suspending media. This method was likely to be a protracted procedure and, therefore, an alternative technique was chosen.

Adsorption at reduced temperatures. It has been pointed out in the case of macrophages (Vernon-Roberts, 1972) that adsorption does not require significant energy expenditure by the cell. One important consideration is, however, that Brownian movement is reduced at lower temperatures, with the result that the chances of two particles colliding or at least coming into close proximity would be lessened. In other words, although there are probably no energy barriers to adsorption at low temperatures, it would be expected to occur at a slower rate.

Growth Behaviour of Streptococcus 63X at 37°C and Room Temperature

Log-phase cultures were washed in phosphate buffer and inoculated into Todd-Hewitt broth, DPB + CS and DPB + CS + L cells (2 cfu per cell). The results of incubation at 37°C and at room temperature are shown in Figure 7.11. There appears to have been some initial growth in all of the combinations. This result was confirmed by another experiment depicted in Figure 7.12.

Adsorption of Streptococci at Different Temperatures

Log-phase streptococci were added to MPM cultures in Leighton tubes at 50 to 100 cfu per cell. Tubes were incubated at 37°C, room temperature and 4°C. At 2 hours and 11 hours, the cultures were washed in phosphate buffer, fixed briefly in 90% ethanol and stained in 0.1% methylene blue. The results are shown in Table 7.3. At 11 hours, the cells in the 37°C and room temperature incubations were very degenerate.

It seemed from the results, that a reasonable level of adsorption could be expected at 4°C.

Adsorption of Streptococci by an L Cell Monolayer at 4°C

On this occasion, streptococci were diluted to 3 cfu per cell in EBM + CS, and added to macrophage monolayers in Leighton tubes. No agitation was used for this experiment. At the time chosen for sampling, the medium was gently rocked to obtain an even suspension.

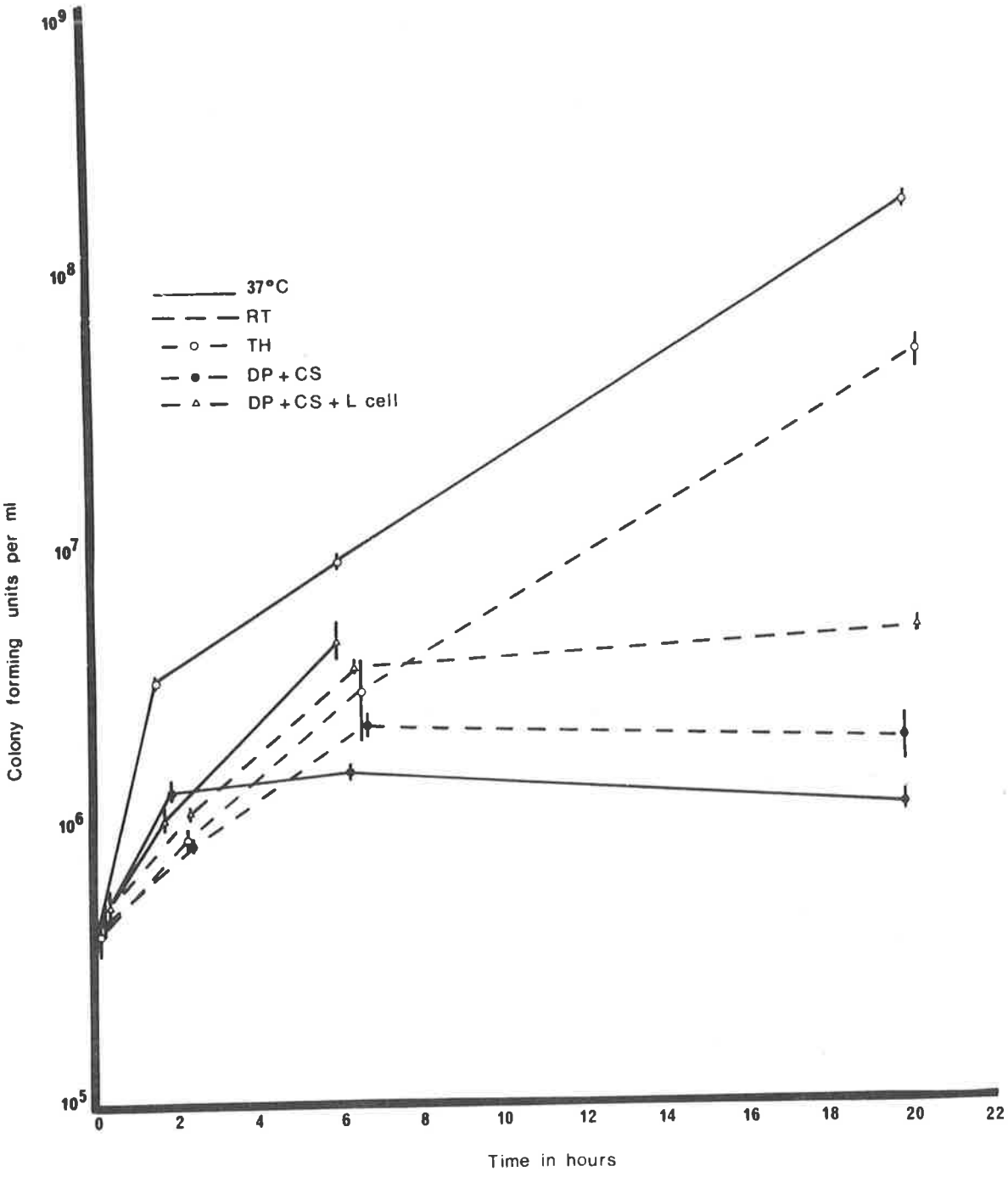


Figure 7.11. Growth of Streptococcus 63X at 37°C and at Room Temperature in Todd-Hewitt Broth (TH), Dulbecco Phosphate Buffer + 10% Calf Serum (DP + CS) and Dulbecco Phosphate Buffer + 10% Calf Serum + L Cells at 2 colony forming units per Cell (DP + CS + L Cell).

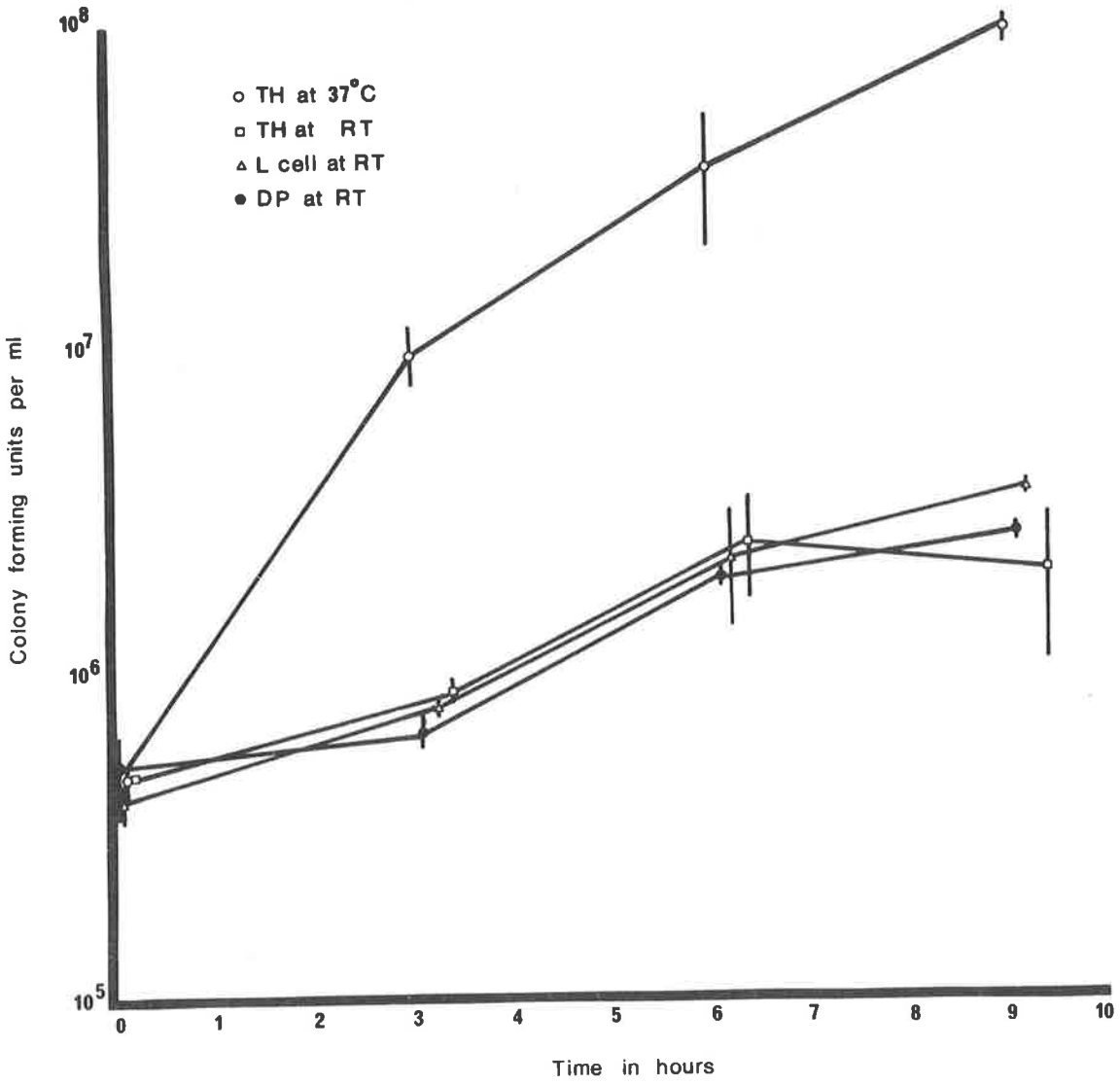


Figure 7.12. Growth of Streptococcus 63X at 37°C and at Room Temperature in Todd-Hewitt Broth (TH), Dulbecco Phosphate Buffer + 10% Calf Serum (DP at RT), and Dulbecco Phosphate Buffer + 10% Calf Serum + L Fibroblasts at 2 colony forming units per Cell (L Cell at RT).

TABLE 7.3

The Percentage of Mouse Peritoneal Macrophages with Adherent Streptococci at Different Temperatures (50 to 100 cfu per cell)

Time	Temperature		
	37°C	Room temperature	4°C
2 hours	94	88	38
11 hours	100	100	44

The results are shown in Figure 7.13. There was no difference between test and control populations over 20 hours. At this time, the cells were washed in phosphate buffer, fixed in 90% ethanol and stained with methylene blue. There were no streptococcal chains in relation to the cells.

Effect of Organism-Cell Ratio on Adsorption

Streptococci were added to Leighton tube cultures of MPM at 100 cfu, 10 cfu and 1 cfu per cell. The tubes were incubated at room temperature and 4°C. At 2.5 hours they were washed in phosphate buffer, fixed in 90% ethanol and stained with methylene blue. In both room temperature and 4°C cultures with 100 cfu per cell, there was good adsorption at this time, whereas there was little to none at lower ratios.

This result pointed to the inefficiency of the adsorptive process, at least under the conditions employed.

DISCUSSION

The adsorption study, although conceptually simple, was unexpectedly complicated. The early experiments with L-forms in EBM + CS + 15% sucrose demonstrated removal of organisms by the cells. It was later shown that growth occurred in the presence of cells. Although the relationship of growth to the density

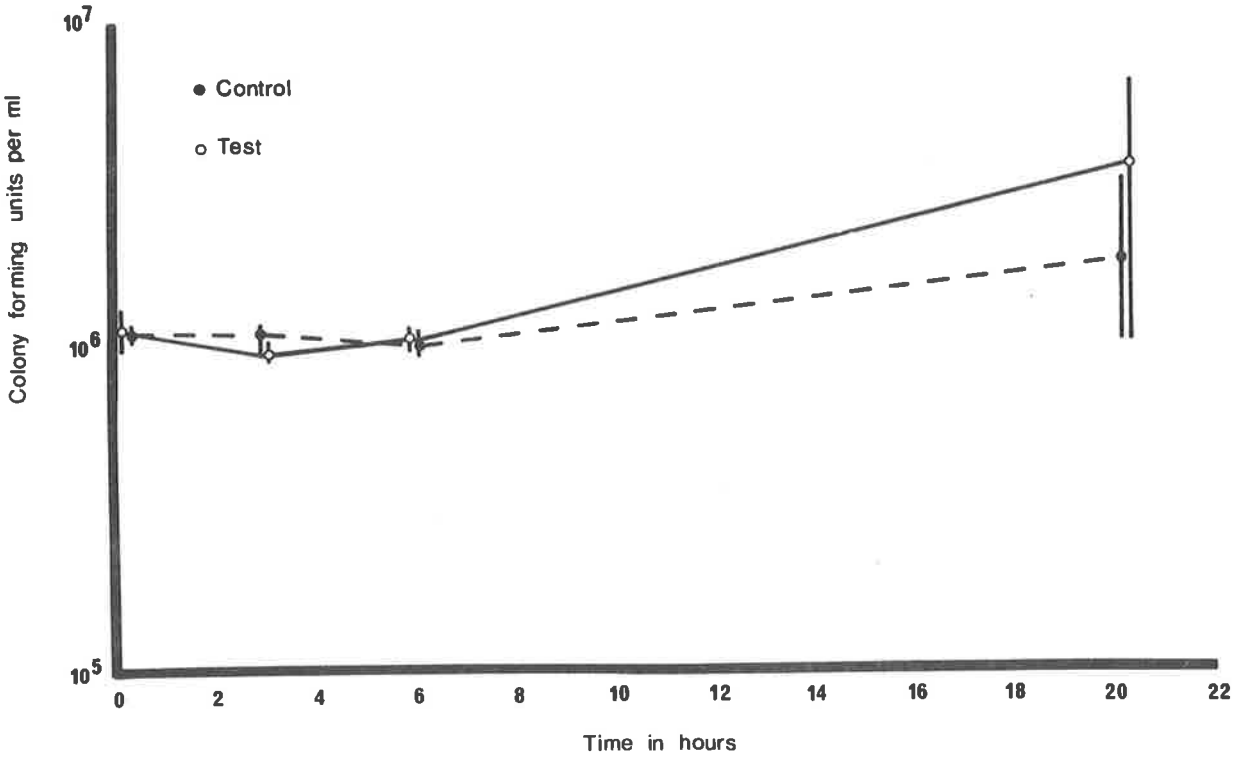


Figure 7.13. Adsorption of Streptococcus 63X by an L Cell Monolayer at 4°C (3 colony forming units per cell), in Eagle's Basal Medium + 10% Calf Serum

Control ≡ Organisms suspended in Eagle's Basal Medium + Calf Serum.

of cells was not examined, the cell population used in the growth promotion study was similar to that used throughout the adsorption experiments, and therefore it seemed reasonable to conclude that growth was a factor to be considered in all experiments. From the limited amount of work done in relation to this phenomenon, it was not possible to make an accurate assessment of the lag-phase in this medium, but it was quite probable that multiplication was occurring over the time period of the experiments. This factor would lend support to the significance of a drop in the test populations shown in Table 7.2.

It was at first considered that experiments with low L-form - cell ratios would give the greatest relative drop in L-form populations if the cells had limited binding sites for the organisms. There was, however, no trend for this in the tests summarized in Table 7.2. A possible explanation could be that there is more chance of cell-organism contact at high ratios to balance any restrictions made by limited binding sites. The failure to get adsorption in more simple suspending media could have been due to the unsuitability of this medium for adhesion between cells and organisms.

Perhaps the most significant finding in the work with the parent Streptococcus was that adsorption seemed to be an inefficient process under the conditions used. That is, it was

not possible to demonstrate, morphologically, a high ratio of adhesion with infectivity ratios used in the experiments, that is, 1 to 30 cfu per cell. High ratios were not used in these studies because it was considered unlikely that a small percentage change in the organism population could be detected even though the absolute fall might be large.

Relation to Literature Findings

Recently, Drach and Schmitt-Slomska (1973) found that phagocytes removed a significant proportion of the viable L-form counts in a suspension of cells and organisms in normal tissue culture media. Although they used a strain of L-form grown in media of high osmolarity (20% sucrose) they found an initial drop of 50% in viable counts and following this, a slow decline over several days. This was in contrast to the findings with the GL8 strain used in the present study, where the initial fall in viability following exposure to hypotonic media was very large (Figures 7.1 and 7.2).

There are also differences in growth patterns of the organisms in various media. In the present study, the parent Streptococcus 63X grew readily in Eagle's basal medium with 10% calf serum (see Figure 7.9) whereas Schmitt-Slomska et al. (1972) found that this strain did not multiply in EBM with 2% CS, but they did not describe the behaviour in EBM + 10% CS. Quinn and Lowry (1967)

thought that group A streptococci did not grow in EMB + 10% CS although they did not present evidence for this.

The demonstration of L-form - cell adhesion in the present study indicated that there was some form of interaction; it did not of course provide information on any subsequent events. The characteristics and the rate of L-form association with mammalian cells are further considered in the next chapter.

AUTORADIOGRAPHIC STUDIES CHAPTER VIII

Introduction

The use of autoradiography as a tracing technique depends upon the ability of radiation emitted from a labelled particle to generate silver atoms in a silver halide emulsion that covers the preparation. These silver atoms catalyse the formation of visible silver grains on exposure to photographic developer (Rogers, 1973a). Although the technique is sensitive, the accuracy of localization can vary quite widely. Because the silver grains are formed within the emulsion layer and not in or on the specimen, the relationship of the silver grains to the radioactive material is not always obvious. Large silver grains may also obscure the site and the morphology of the labelled material. Despite disadvantages of this type, however, the technique is very useful, enabling the tracing of precisely-labelled material.

Background to the Present Study

Autoradiography was not used earlier in this study for reasons concerning the specificity of labelling. The ideal label for the L-forms would be one that was incorporated into an organism-specific component; that is, a compound not found in mammalian

cells. The rationale relating to this was that if the organisms were labelled, for example, with tritiated thymidine, this might be released by lysing organisms in an infectivity experiment and following this it could be taken up by the cells to give false results on autoradiographic development. This was particularly important in regard to L-forms because of the difficulty in staining these organisms and therefore the lack of an alternative means of identifying them in relation to the cells. Slabyj and Panos (1973) found that a stabilized L-form of Strep. pyogenes contained glycerol teichoic acids at 0.1% of the dry cell weight. This was located mainly on the cell membrane and some also possibly located in the ribosomes. It seemed likely that labelled teichoic acid would remain particulate in a degradation or lysis situation and therefore would not be taken up, as soluble material, by the cell. The problem was to find a label that would go specifically or at a high percentage into the teichoic acid polymers. Labelled glycerol was perhaps the most obvious choice, but a percentage of this compound would presumably have entered the general carbon pool with resulting indiscriminate labelling of many cell components. The failure to find a precursor that would specifically label the cell membrane was disappointing in view of the potential importance of this structure as discussed in the introduction to this thesis.

The Use of Labelled Thymidine

In the preceding section it was emphasized that labelled material released from the L-forms, could be taken up by the host cells to give a false result. Bensch and King (1961) noted that L fibroblasts grown in suspension culture were able to phagocytose particles containing high molecular weight deoxyribonucleic acid (DNA) and protein. On the other hand, soluble DNA did not enter the cells in appreciable amounts. The uptake of DNA into the cytoplasm was followed by acridine orange staining and into the nucleus by tritiated thymidine labelling. The possibility that the DNA uptake observed was due to the degradation of particles at the cell membrane, with subsequent uptake of soluble DNA, was negated by experiments utilizing a "cold trap". This was achieved by the presence, in the medium, of unlabelled thymidine at 10 mg per 100 ml; this enormous quantity being considered as a metabolic trap, preventing the uptake of tritiated thymidine released from degraded particles. In relation to the present study, this work indicated that while it was not possible, even if desirable, to eliminate uptake of particulate DNA by the cells, it should be possible to eliminate the uptake of soluble DNA by the presence of a metabolic trap. It appeared, therefore, that with labelled thymidine, the presence of silver grains in relation to the cells would enable the possible interpretation that L-forms, or fragments of them, were cell-associated. Another

important advantage of thymidine is that it goes directly into DNA only, that is, it is a specific label.

Although thymidine is specific, it must be remembered that breakdown of the molecule occurs. Evans (1966) listed the modes of decomposition of tritium labelled compounds, with radiation-induced and chemical decomposition both playing an important role. For the present study, it was concluded that although tritium is a relatively stable isotope, tritiated thymidine that had deteriorated with storage was likely to be a source of non-specific label. To minimize the likelihood of this, the labelled material was obtained fresh for these experiments and stored at $4^{o}C$.

AUTORADIOGRAPHIC TECHNIQUE

Materials Used

Thymidine (Methyl - ^{3}H) was obtained from New England Nuclear of Boston, Mass., U.S.A. This was received as a sterile aqueous solution with an activity of 6.7 Curies per millimole, and was stored according to the manufacturer's instructions. Ilford K2 nuclear emulsion was chosen (Rogers, 1973b). It was stored at $4^{o}C$ and for use it was warmed at 40 to $45^{o}C$ and diluted 1:1 in distilled deionized water containing 2% glycerol (BDH). The emulsion was used until, with length of storage, it developed an unacceptably high background.

Kodak D19 developer was used and fixation was carried out in a fixing solution diluted 1 to 5 in distilled water.

Staining was carried out in 0.1% toluidine blue (Gurr) in acetate buffer pH 4, as described by Baserga and Malamud (1969a).

Experimental Procedure

The general approach was modelled on the techniques described by Baserga and Malamud (1969b) and Rogers (1973c). Log-phase cultures of L-form GL8 and Streptococcus 63X were centrifuged and resuspended in fresh medium with tritiated thymidine. After incubation for selected time intervals, the cultures were washed in buffer and resuspended in broth with added "cold" thymidine. They were then reincubated to ensure that all the labelled thymidine had been incorporated into DNA. Labelled organisms were then added to monolayer cultures of L fibroblasts and mouse peritoneal macrophages, after they had been thoroughly washed twice in buffer. In this study hypertonic conditions were not used to maintain L-form viability because it was assumed, from the observations in Chapter 6, that massive lysis of the organisms was unlikely to occur over the planned time period of the experiments and therefore labelled DNA should be largely cell-associated. The tissue culture medium contained thymidine at 100 µg per ml as a "metabolic trap", as discussed in a previous section.

After incubation, the tissue cultures in Leighton tubes were washed in phosphate buffer and fixed. The fixation procedure was varied but most experiments used the method described by Baserga and Malamud (1969c) for tissue culture monolayers. This involved fixation in 3:1 ethanol:glacial acetic acid, followed by 70% ethanol and sometimes this was followed by 0.3N perchloric acid. In other experiments the fixative used was methanol. The aim of fixation, stated by Baserga and Malamud, was to preserve the preparation and to remove non-incorporated labelled material; for example, to dissolve out thymidine but not DNA.

The fixed coverslip cultures were then soaked in distilled water to remove fixative, dried and mounted, cells uppermost onto slides with Xam (Gurr) as the adhesive. The slides were dipped into diluted emulsion, allowed to dry at the dark room temperature for one hour and then packaged into sealed boxes containing silica gel as a drying agent. After exposure at $4^{o}C$ for variable time periods, the slides were developed and stained with toluidine blue.

EXPERIMENTAL CONDITIONS

Initially, it was necessary to determine the optimal conditions for these experiments.

Uptake of Label by Organisms

It was found necessary to grow both L-forms and streptococci overnight in 10 to 30 micro Curies (μ Ci) per ml of ^{3}H-thymidine to obtain adequate uptake of label (Plate 8.1). This could have been due to relatively high levels of thymidine inherent in the media used to grow these organisms.

Uptake of Thymidine by Cells and the Effectiveness of the "Metabolic Trap"

L cell cultures given a 2 hour pulse of thymidine at 5 μ Ci per ml showed a high uptake of label (Plate 8.2). The presence of "cold" thymidine at 100μg per ml completely inhibited the uptake of label by the cells (Plate 8.3).

Exposure Period

Under the conditions used, exposure times of 4 to 6 weeks seemed adequate for good labelling of streptococci.

Developing Times

For each batch of slides processed, samples were developed over 2 to 6 minutes. The aim was to achieve good labelling with the minimum of background. Even small background grains were undesirable as much of the screening was done at high power.

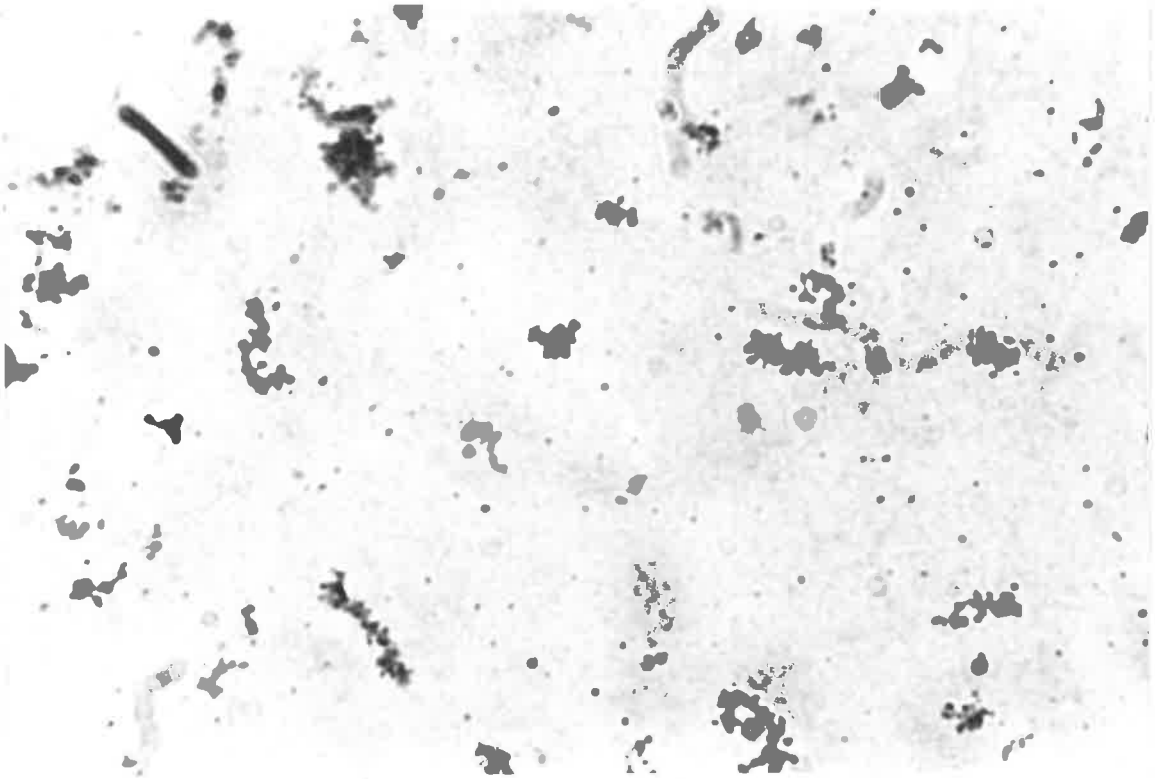


Plate 8.1

Autoradiograph of Streptococcus 63X in broth culture; incubated in the presence of ^{3}H-thymidine at 10μCi per ml for 12 hours

Exposed 4 weeks at $4^{o}C$. Stained 1% methylene blue.
Showing adequate labelling of the streptococcal chains.
Magnification, x 3,100

Plate 8.2

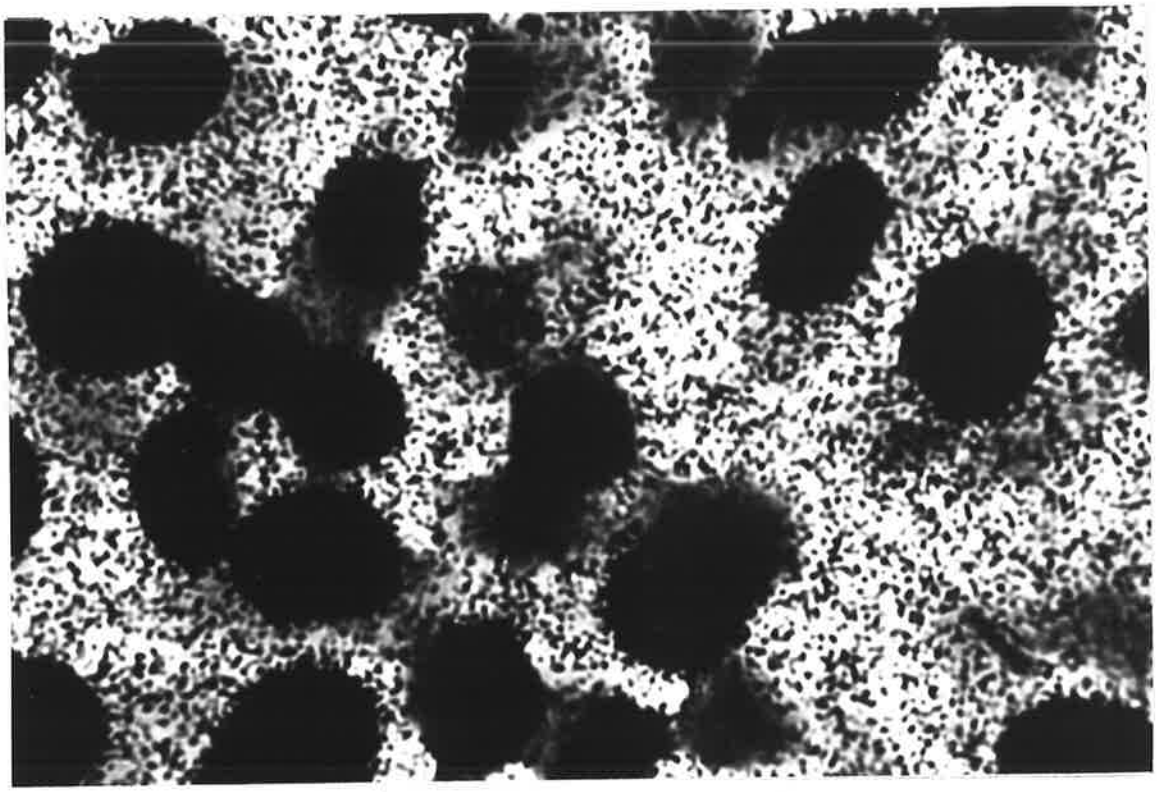
Autoradiograph of an L cell culture incubated in the presence of ^{3}H-thymidine, 10μCi for 18 hours

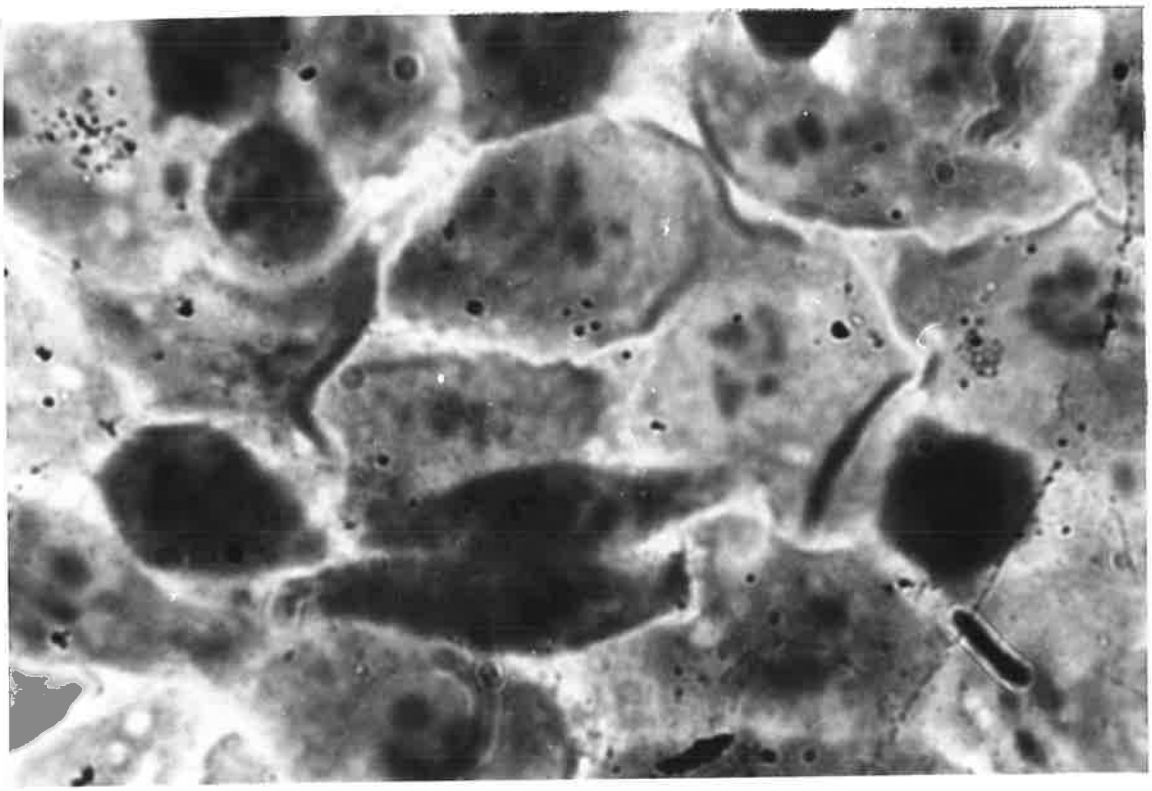
Stained 0.1% toluidine blue for one hour.
There has been massive uptake of label.
Magnification, x 3,100

Plate 8.3

Autoradiograph of an L cell culture incubated in the presence of ^{3}H-thymidine, 10μCi for 18 hours, also containing "cold" thymidine at 100 μgm per ml

Stained 0.1% toluidine blue for one hour.
The effectiveness of the "metabolic trap" is clearly indicated by the absence of labelling. There is, however, some background graining present.
Magnification, x 3,100





The Problem of Background Grains

Despite an attempt to vary the conditions of emulsion coating, drying, exposure and development, the only satisfactory method of ensuring low background graining was to use fresh emulsion and to develop for the minimum time consistent with adequate grain size in the labelled preparations.

Experimental procedures. The experimental procedures are summarized in Table 8.1. In the case of streptococcal - cell studies, incubation was not usually continued beyond a few hours because growth of the organisms resulted in destruction of the cell cultures (Plate 8.4). Antiserum was prepared and assayed as described in Chapter 5 with 0.1 ml being added per Leighton tube culture. Anti-streptococcal sera were prepared by injecting rabbits intravenously twice weekly with heat-killed organisms. The dosage was gradually increased with each injection. Antisera were estimated by the indirect haemagglutination assay with a sonicated streptococcal suspension as the antigen preparation.

Results

Meaningful interpretation could only be made from preparations with low background grainings. In some cases there were areas in both test and control cultures that showed high background. It was considered invalid to choose areas in these cultures for examination.

TABLE 8.1

The Experimental Conditions used for Autoradiographic Study of Streptococcus 63X and L-form GL8 - Cell Association

Label	Chase with "cold" thymidine	Infectivity ratio	Cell types used	Anti-streptococcal Anti-L-form serum	Controls	Incubation	Fixative		Exposure period
Overnight culture in media with ^{3}H-thymidine at 10-30 µCi per ml	1 hour	100-500 cfu per cell	Mouse peritoneal macrophages L cells in isotonic medium	Present or Not Present	Normal cell cultures without organisms	30 min to 20 hrs at 37°C in tissue culture medium with "cold" thymidine at 100µg per ml	Glacial acetic: ethanol, 1:3 70% ethanol -3 changes 0.3N cold perchloric acid	Methanol	4 to 6 weeks

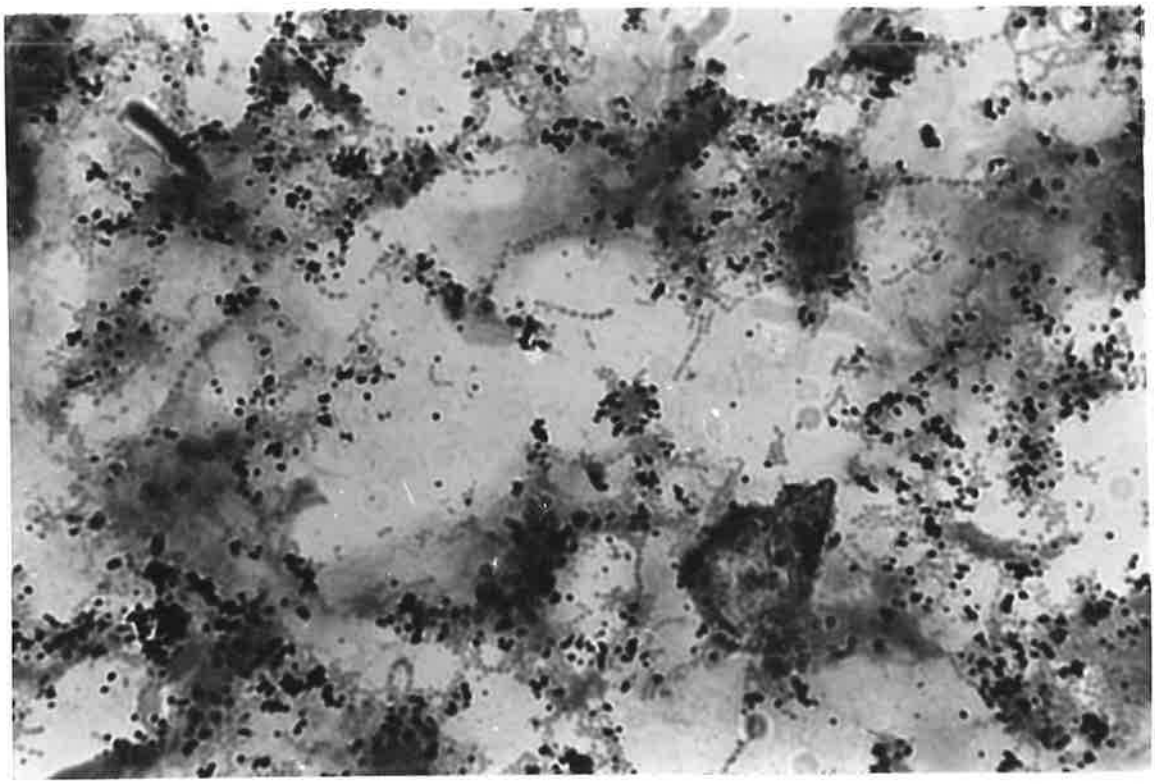


Plate 8.4

Autoradiograph of an L fibroblast culture 18 hours after infection with ^{3}H-thymidine labelled streptococci

Showing over-growth and destruction of the cell culture
Magnification, x 3,100

Control macrophage cultures usually showed cell-associated graining even in the presence of a clear background (Plate 8.5). This was not seen with L cell cultures and occurred in the macrophage cultures despite a change in fixation procedures. It seemed unusual that chemography, that is, the chemical interaction between the cells and the emulsion, occurred in the presence of macrophages alone, but there was no other obvious explanation for this. Under the experimental conditions used, the streptococcal-cell autoradiographs usually showed large amounts of graining associated with the cells (see Plate 8.6). On the other hand, parallel cultures with L-forms usually showed only a few grains per cell, as shown in Plates 8.7 and 8.8. Because most of the experiments involving streptococci produced large amounts of graining in relation to the cells, grain counts were either difficult or impossible. The impression gained was, however, that the presence of antiserum had little effect on the rate of adsorption of streptococci by the cells. This is perhaps not surprising because Glick et al. (1971) found that human monocytes, in the presence of normal human serum, readily phagocytosed a "laboratory strain" of group A Streptococcus. In the case of L-form infected cultures, the low grain counts enabled a quantitation of the results. Over several experiments there was no detectable influence of antiserum on the rate of uptake by L fibroblasts or mouse peritoneal macrophages.

Plate 8.5

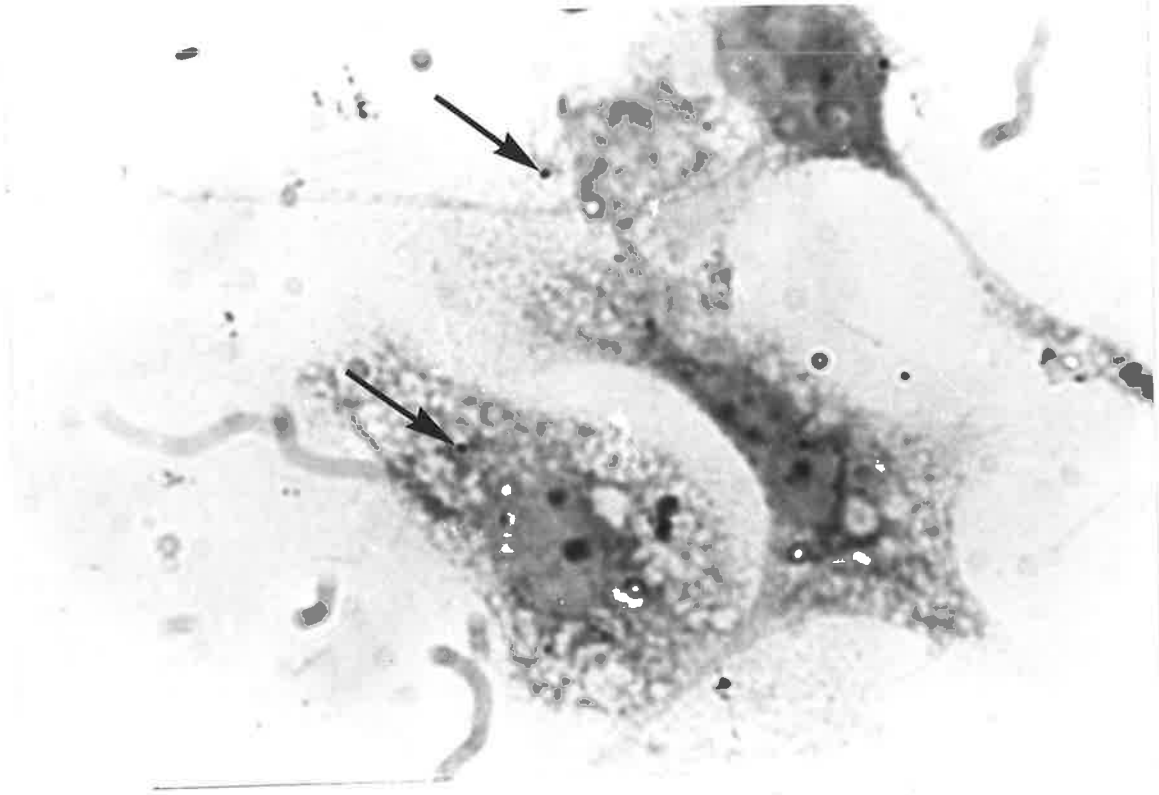
Autoradiograph of a control mouse peritoneal macrophage culture

Stained toluidine blue.
Arrow indicates grain.
These background grains are mainly cell-associated.
This was a problem peculiar to macrophage cultures.
Magnification, x 3,100

Plate 8.6

Autoradiograph of a mouse peritoneal macrophage culture infected with Streptococcus 63X at 100 cfu per cell for 2.5 hours

Stained toluidine blue.
Heavy uptake of label is obvious.
Magnification, x 3,100



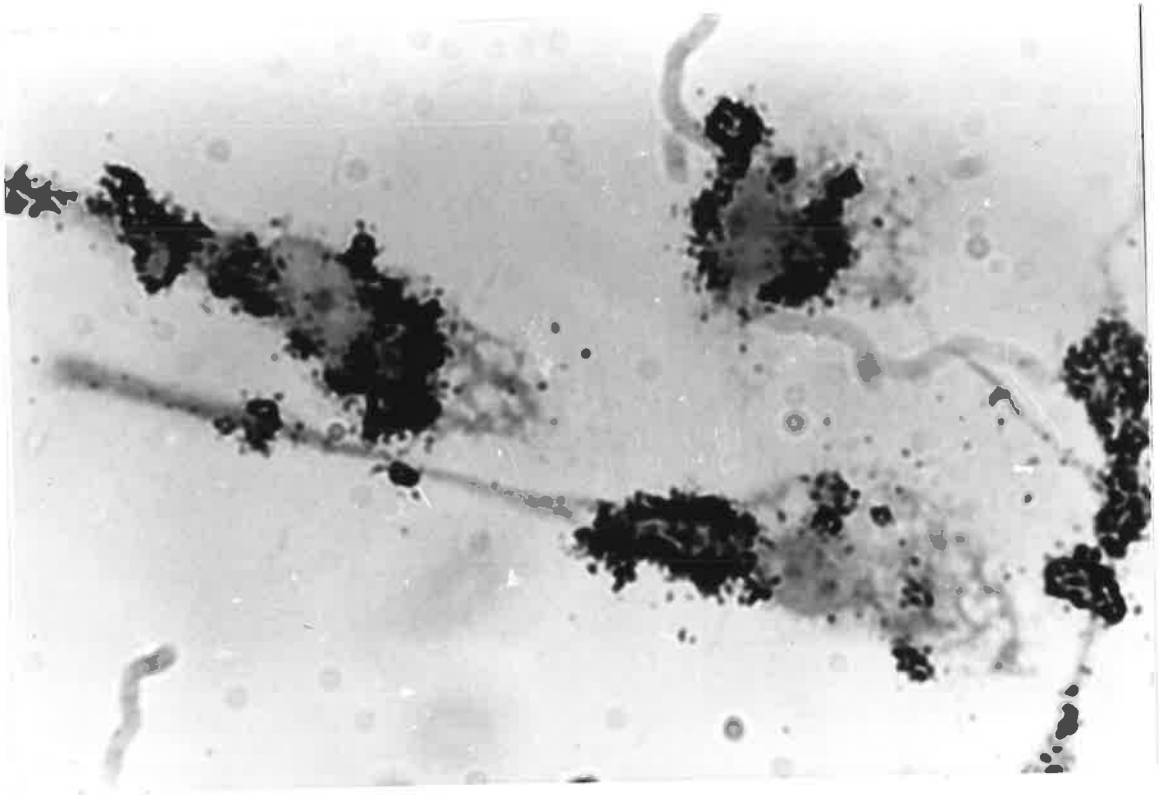


Plate 8.7

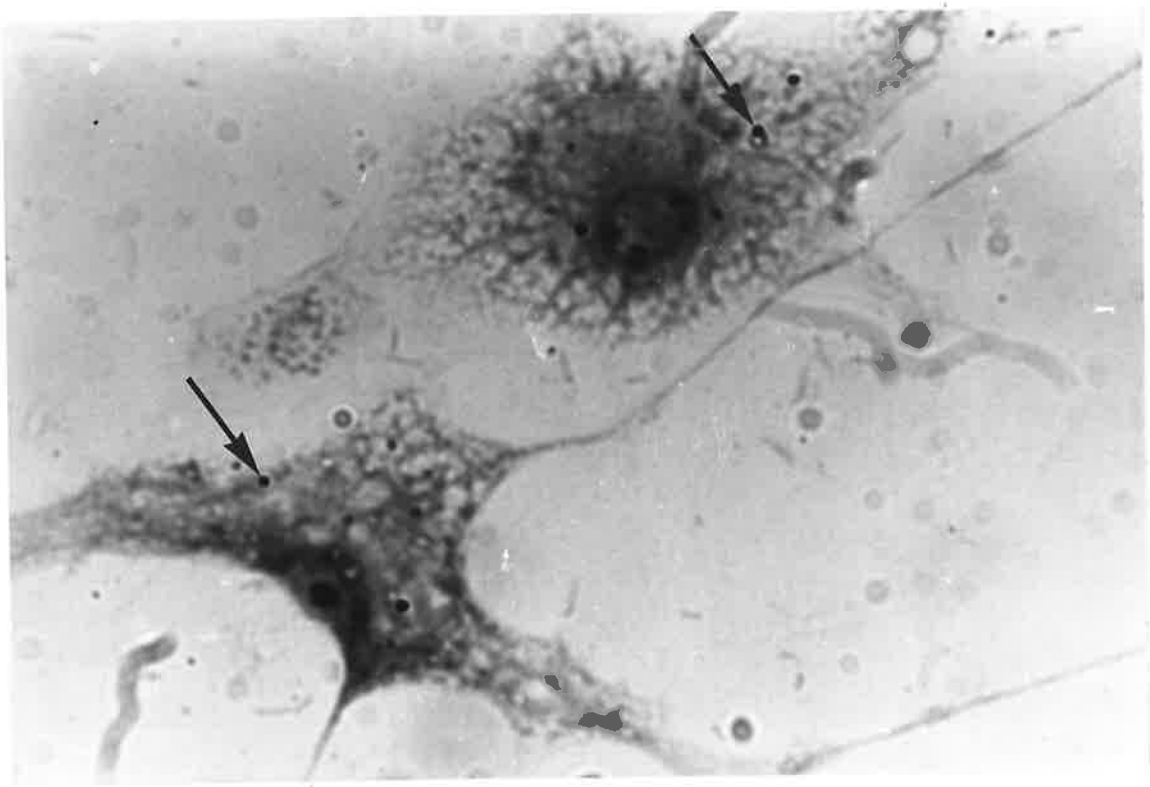
Autoradiograph of a mouse peritoneal macrophage culture infected with L-form GL8 at 100 cfu per cell for 2.5 hours

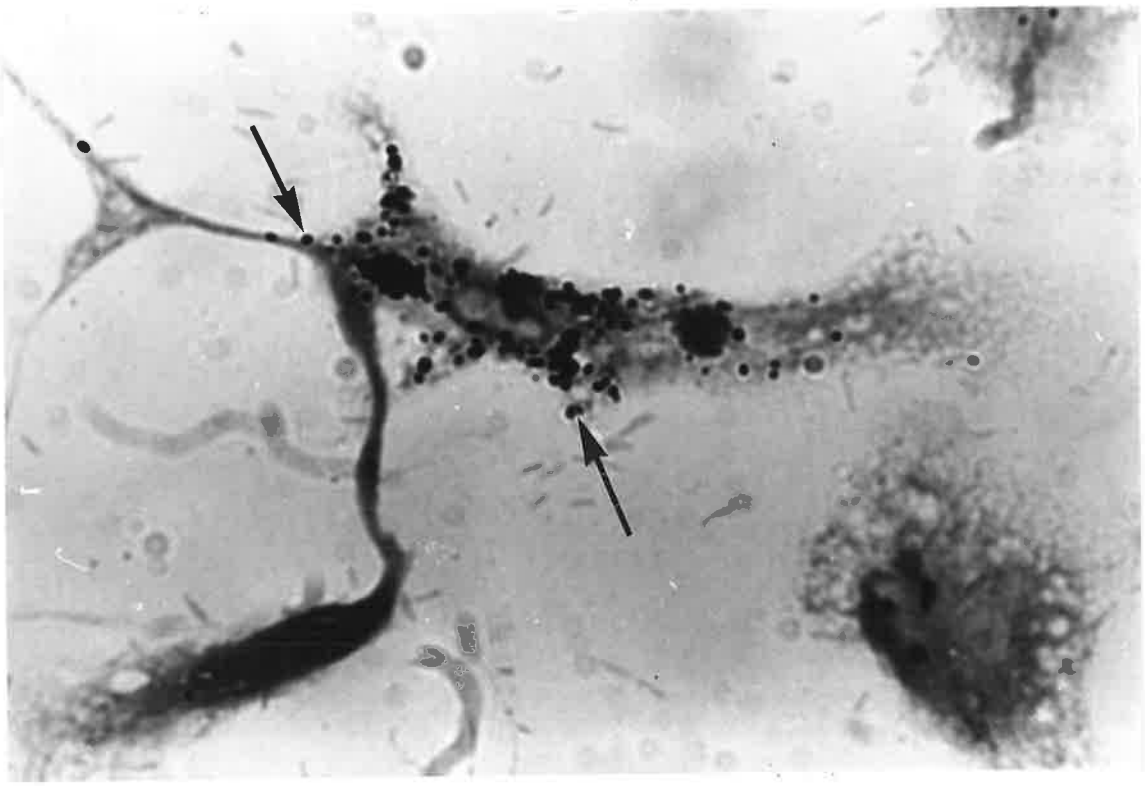
Stained toluidine blue.
Arrows indicate grains. Some of these were background (on average 2.5 per cell) but others were presumably associated with the presence of labelled L-forms.
Magnification, x 3,100

Plate 8.8

Cells from the same preparation as in Plate 8.7

The central cell shows heavy uptake of label. It was common to find a heavy labelling in a small percentage of infected cells.
Magnification, x 3,100





Because of the background in macrophage cultures, it was only possible to conclude that interaction with L-forms had occurred by counting grains at random over cells from separate cultures. An example of the findings overall, is shown for one experiment in Table 8.2. It could be concluded from this table that there was a significant difference between the test and control counts, with an average count, probably due to L-forms, of 2.5 in test cells. In other words, in cells with 5 associated silver grains an average of 2.5 would be background grains. Although L fibroblast background tended to be lower, so were the number of grains in test cultures. Therefore, overall, the fibroblasts appeared to have a lower rate of interaction than did the macrophages,as might be expected. Because the grains were usually well separated, it was possible to conclude that each one represented the presence of a labelled L-form or L-form fragment.

DISCUSSION

Perhaps the most striking feature of this study was the apparent contrast in the rate of interaction of streptococci and of L-forms in relation to the cells. It seemed necessary, however, to exercise caution in making such a comparison, for the following reason. In the adsorption studies (Chapter 7) and this chapter, parallel studies with the parent organism were made with infectivity ratios comparable, at least by inference, with those used in L-form studies. For example, comparison was made by equating

TABLE 8.2

A Comparison of Grain Counts in Autoradiographs of L-Form GL8 Infected Peritoneal Macrophage Cultures and Control Cultures

Cultures	Number of cells examined	Student 't' test		Mean	Standard deviation
		't'value	P value		
L-form infected (100 cfu per cell)	160	7.3205	<0.0005	5.05	3.76
Controls	160			2.49	2.69

numbers of colony forming units. In fact the colony forming unit of the parent organism is a chain of perhaps 20 to 30 units average length, whereas the L-form colony forming unit is unknown with respect to the relationship to the total population as seen by electron microscopy. It could be argued that accurate quantitation would be achieved by using numbers of colony forming units equated on the basis of dry weights, but again the relationship of this to adsorption to cells, or quantitative uptake of cells of tritiated thymidine remains uncertain. One possible method of achieving more comparable levels of streptococci and L-forms in the autoradiographic study could have been to equate the populations on the basis of total DNA content. However, because the growth of the GL8 L-form (Figure 4.3) was comparable with the growth of the parent organism, there did not appear to be any reason why they would not take up thymidine.

A reason for doubting the validity of comparison between grain counts due to streptococci and L-forms was the difference in the growth media. It was illustrated earlier in this chapter that high levels of "cold" thymidine in the medium could block the uptake of labelled substrate by tissue culture cells. It therefore seemed quite possible that differences in the media used to grow the organisms could affect the uptake of labelled thymidine. For example, the streptococci were grown in Todd-Hewitt broth, the main component of this being beef heart infusion

(Manufacturer's specification). Bridson and Brecker (1970) presented a table illustrating the analysis of beef muscle extracts. The level of purines was indicated by hypoxanthine at 2.5% of the weight of the extract. A consideration of the formula of hypoxanthine shows that nitrogen comprises approximately 40% of the molecular weight. Therefore, purine nitrogen, according to this table, would comprise about 1% of the weight of the extract.

L-form GL8 was grown in Trypticase-Soy broth, 1.5% of Yeast Extract and 10% horse serum. A general analysis of Yeast Extract paste by Bridson and Brecker (1970) showed that purine nitrogen comprised 0.27% of the weight. According to Giegy Scientific Tables, xanthine and hypoxanthine are found at 1 to 2 mg per litre in human blood with only traces of nucleotides being found in plasma. From this it could be reasonably expected that the 10% horse serum used as a medium supplement would not contribute significantly to the purine levels. In Trypticase-Soy broth, the soy digest makes up only 10% of the weight of the medium (Manufacturer's specifications). Although the purine levels of soy digest were not available, it seemed unlikely that they would contribute greatly to the final level in the medium.

On this basis, purine nitrogen in the streptococcal medium would be approximately 3 x 10 mg per 100 ml of medium (medium used at 3%) or 30 mg, whereas in the L-form medium it would be

4.5 mg contributed by the Yeast Extract and an unknown, but probably small quantity contributed by the soy digest. Although it is realized that these levels are gross approximations, the above does provide some evidence that purine levels in the L-form medium probably were not greater than those in the streptococcal medium and therefore, inhibition of uptake of label would not be expected.

From these findings then, it seemed valid to conclude that the L-forms should have taken up the label under the conditions used.

It was stated earlier that for these experiments osmotic support was not provided for the L-forms because massive lysis, with subsequent release of labelled DNA, was not expected over the time course of the experiments. If the results were assessed on the presumption that adequately labelled and intact organisms were present over the period of the experiment, then it would appear that there was an uptake, albeit low, of L-forms by the cells. This was in marked contrast to the obvious heavy interaction of cells with the streptococci, although the validity of direct comparisons is questionable, as mentioned earlier.

Relationship to Literature Findings

Perhaps the most interesting findings from this study were that antisera were not required for uptake of L-forms or parent

streptococci by the cells and the very much reduced uptake of L-forms in comparison to the streptococci. Harwick et al. (1972) noted phagocytosis of group D streptococci and their L-forms in serum free media and Schmitt-Slomska et al. (1973) found that while specific antibody increased the early rate of uptake of group A streptococci by phagocytes, it did not alter the final number of organisms phagocytosed. The interesting comparison between the study by Harwick et al. and the present study is that while they observed greater phagocytosis of parent organisms than for the L-forms, both were quite readily taken up, whereas in the present study few L-forms were taken up in comparison with the parent organism. The intense association of the Streptococcus 63X with L fibroblasts could be explained on the basis of the findings that cell cultures from human tonsillar tissue "held" streptococci by means of cell processes (Lowry and Quinn, 1964).

In summary, the findings described in this chapter confirm the results of Chapters 5, 6 and 7. Under the experimental conditions used, the L-forms were shown to have a low affinity for a variety of cell types. The characteristics of the demonstrated association between the L-forms and cells were not examined in depth; related to this, there was no evidence, however, that L-forms remained viable when cell-associated.

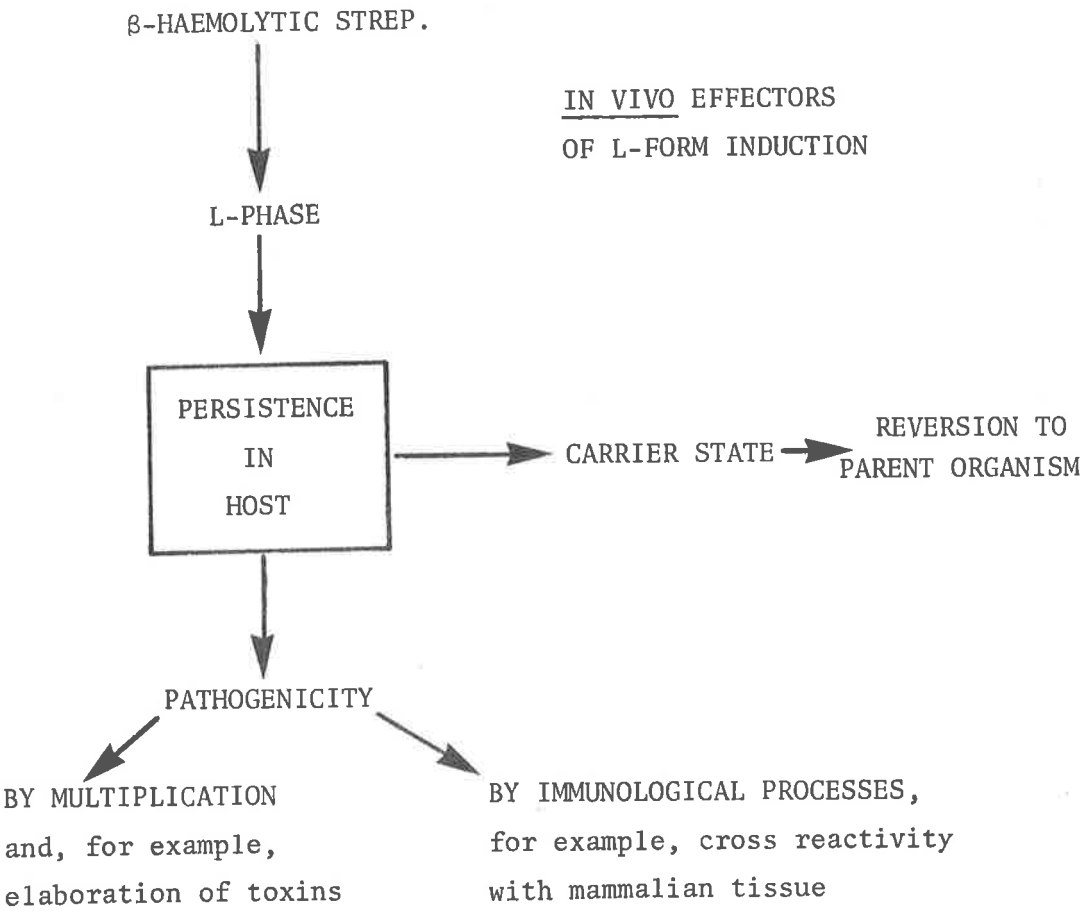
GENERAL DISCUSSION — CHAPTER IX

A postulated role of the L-phase of group A haemolytic streptococci in disease is shown in Figure 9.1. Possible *in vivo* inducers include antibiotics that interfere with cell wall synthesis and muralytic host enzymes (Hijmans *et al.*, 1969). It is obvious that the L-forms must be able to survive in the host or at least persist as undegraded material if they play any role in disease processes. Mortimer *et al.* (1972) demonstrated that the L-phase can be isolated from mice infected with group A haemolytic streptococci, although Maxted (1968) produced evidence that the organisms exist *in vivo* as protoplasts and not as growing L-forms.

If L-forms are induced *in vivo* and if they are able to survive or persist, then there are various possibilities for their participation in disease processes. McDermott (1958) put forward the concept that L-forms could function as a reservoir of latent infection under circumstances unfavourable to the parent organism; for example, penicillin therapy. This is an hypothesis that remains to be confirmed by experimentation although some reports suggest that it could be valid (Hatten and Sulkin, 1968). Another possibility is that the L-forms could participate

FIGURE 9.1

POSSIBLE HOST-MICROBE INTERACTIONS
INVOLVING THE L-PHASE OF β-HAEMOLYTIC STREPTOCOCCI



directly in host-parasite interactions by multiplication and elaboration of toxins, for example, streptolysin O (Hijmans et al., 1969) or by participation in immunologically mediated tissue damage. Related to this latter aspect is the demonstrated antigenic relationship between the cytoplasmic membrane of group A streptococci and mammalian components (see Chapter 1).

The conclusions reached from the literature survey in Chapter 1 were that the mechanisms of in vivo persistence and the possible role of persistent undegraded microbial components were important issues in a consideration of the role of L-forms of beta-haemolytic streptococci in host-parasite relationships. It was postulated that if, as found by Mortimer et al. (1972), L-forms were induced and survived in vivo, then they must have some means of avoiding the host defence mechanisms (see Chapter 1). It therefore seemed worthwhile to examine the possibility of cell-associated survival, particularly as this had been described by Schmitt-Slomska et al. (1968). The role of persistent antigenic material appeared to be important in view of the findings with mouse peritoneal macrophages cultured in vitro (Spector et al., 1970) and of Ginsburg et al. (1969) who postulated a role for non-degraded antigenic material in the inflammatory process. The reasons for the use of an in vitro model in the present study were discussed under the heading of "The Experimental Model" in the first chapter.

The present study was in fact limited to investigation of possible cell-associated survival or persistence of L-forms.

The early infectivity studies were described in Chapter 5. Attempts were made to find methods of adequately identifying L-forms in relation to the cells and to follow cell changes after the introduction of organisms. The infectivity system seemed to be a reasonable one although there was no well defined precedent to provide a basis for techniques. The experiments were quantitated as accurately as possible within the limits of the methods used and it soon became apparent that high infectivity ratios were necessary to enable detection of any interaction between organisms and cells. With acridine orange stained rat heart cell cultures, fluorescent structures, possibly L-forms, were seen in relation to some cells. The cells did not appear to be affected by the presence of organisms. Fluorescent antibody studies were mainly confined to L cell and mouse peritoneal macrophage cultures for reasons described in Chapter 5. The difficulty in removing non-specific staining of the cells by anti L-form conjugates was described, in addition to the intense nuclear staining of L fibroblasts by anti-L-form serum. Once again, fluorescent bodies occurred only at low frequency even with macrophage cultures. Attempts to re-culture L-forms from the "infected" cultures had limited success.

The plate counts really reflected only the survival of the organisms in tissue culture media with the higher survival of the 12-416 strain over the GL8 being attributed to the adaptation of the former strain to growth in media with reduced osmotic support. Long term infectivity studies were not carried out because of the limited evidence for, and the apparent low frequency of, L-form-cell interaction.

In the electron microscopic study described in Chapter 6, evidence was presented for uptake of L-forms by L fibroblasts, while there was no detectable interaction with human diploid cells. The results of this study pointed to a low frequency interaction with, in some cases, damage to the host cells subsequent to the appearance of numerous lysosomes.

An attempt to quantitate uptake of L-forms by cells was described in Chapter 7. This study was confined to the limited objective of measuring adsorption of L-form GL8 to cells in selected suspending media. The use of mouse peritoneal macrophages and a parallel series of experiments with the L-form parent Streptococcus 63X, provided information on the efficiency of the techniques used. Adsorption of the L-forms to the cells was demonstrated in an experimental system that was probably inefficient. The reduced adsorption of Streptococcus 63X in media containing 15% sucrose provided indirect evidence for this.

Chapter 8 described the attempts to follow interactions by autoradiography after the addition of labelled organisms to the cell cultures. As in Chapter 7, the parent organism Streptococcus 63X was used for comparative reasons, in order to provide information on the efficiency of labelling and the degree of uptake by cells. Antiserum did not give an increased uptake of organisms for either L-forms or streptococci. This study provided information only on uptake of organisms, it did not allow any evaluation of subsequent events.

Overall, the findings in these studies pointed to a limited interaction of the L-forms with a variety of cell types, including phagocytes. There was no evidence that cell-associated organisms were viable.

Evidence in the literature for interaction of group A streptococcal L-forms with cell types other than those of the phagocytic defence system, comes from the work of Schmitt-Slomska's group (Schmitt-Slomska et al., 1968; Pham Huu Trung et al., 1968; Schmitt-Slomska et al., 1972). These authors have demonstrated, by fluorescent antibody staining, reculture and electron microscopy, that strains of group A streptococcal L-forms could infect cultures of human diploid fibroblasts without apparent ill effect on the cells. They also demonstrated that group A streptococci could, at a defined infectivity ratio, infect human fibroblasts in culture and become transformed into the L-phase state.

These findings were supported in part by the present studies utilizing fluorescent antibody staining, acridine orange fluorescence, quantitative adsorption of organisms to cells and autoradiography (see above). L-forms were taken up by a limited number of cells in the cultures; this occurred with a variety of cell types, but was least marked with human diploid fibroblasts. Acridine orange fluorescence and fluorescent antibody staining demonstrated interaction at high infectivity ratios only.

Clasener (1972) in a review of the pathogenicity of L-phase bacteria pointed out that "Observations by different authors are seldom fully comparable, especially when little consensus exists about criteria and methods. Moreover, many of the observations are isolated ones that have not been followed up, and are sometimes badly in need of confirmation". Although this comment applies to a comparison between the findings of the present study with Schmitt-Slomska's work, it is nevertheless desirable to analyse differences where possible. Detailed comparisons were made in the appropriate chapters, but it is worthwhile to re-emphasize particular differences at this stage.

Their finding was that about 10% of the cells had organisms associated with them shortly after infection. As judged by the fluorescence micrograph shown (Schmitt-Slomska et al., 1968; Figure 3), large aggregates of organisms were associated with many of these cells, in contrast to the present findings. These

showed a variable association with relatively few cells having evidence of massive clusters of L-forms. In their study they do not state the yield of L-forms re-isolated from the cells. Classical colonies were isolated up to 10 days after infection and then again after 90 days. Because a strain adapted to growth in media with low osmotic support was used, it could be that the organisms isolated in the first 10 days reflected survival in tissue culture medium, although they were cell-associated. The classical colonies isolated after 90 days were probably not mycoplasmal colonies because they stained with anti-L-form serum. In relation to this the authors prepared antisera by injecting rabbits intravenously 3 times weekly for 6 weeks. Conjugated sera were purified by DEAE cellulose chromatography. These procedures should give a good preparation but thereis no indication of the specificity of the conjugate. This would seem important in view of the experience with non-specific staining in the present study (Chapter 5).

The electron microscopic study by her group involved examination of preparations of human diploid fibroblasts 2-3 months after infection with group A streptococcal L-forms. They presented information that both extra- and intracellular association of L-forms with the cells occurred. The resolution of some of the photomicrographs has suffered in reproduction and does not

permit definitive interpretation. Moreover, the L-forms are not indicated. It is interesting that the preparations show cells with a relatively smooth outline in sharp contrast to the appearance of human diploid fibroblasts in the present electron microscopic study (Plates 6.8 and 6.9).

In another study by Schmitt-Slomska's group (Schmitt-Slomska et al., 1972), group A streptococci were apparently converted to the L-phase within human diploid fibroblasts and this is quite significant. L-form colonies isolated from these cells gave strong fluorescence with anti-L-form sera. This is good evidence, providing that the staining was completely specific. The authors stressed the careful search made for mycoplasma contaminants both by cultural procedures and by electron microscopic screening. However, the experience described in Chapter 6 points to the great care necessary in ensuring contamination free culture. Here an L fibroblast culture after infection with L-forms was destroyed by a suspected massive growth of mycoplasma that had been previously undetected by cultural and electron microscopic screening. It appeared in this case that the cells had a very light infection with mycoplasma and it may be that the balance was upset by addition of L-forms. The ready interaction of group A streptococci with cells was supported by observations in the present study. An additional observation made by Schmitt-Slomska's group was that at certain

infectivity ratios the host cells can phagocytose and thereby remove all the attached organisms. This was not followed up in the present study.

Harwick et al. (1972) compared the phagocytosis of a group D Streptococcus and its L-form by polymorphonuclear leucocytes in serum-free media. With an infectivity ratio of 10 organisms per cell they observed ready phagocytosis of L-forms and parent organisms, although the parent forms were consistently phagocytosed at a faster rate. This is in contrast to the present results (Chapter 8) where there was a great discrepancy between uptake (by macrophages and L fibroblasts) of streptococci and L-forms. In fact, even the presence of anti-L-form serum did not appreciably affect the rate of uptake of the L-forms by the cells. It is of interest to compare these findings with those of Simberkoff and Elsbach (1971) for phagocytosis of Mycoplasma hominis and Mycoplasma arthriditis by polymorphonuclear leucocytes. They found that there was virtually no uptake of these organisms even in the presence of specific antiserum. It should be pointed out that the validity of comparison between the L-form and the parent Streptococcus in the present study, depended upon demonstrating adequate uptake of labelled thymidine by the L-forms using autoradiography. While evidence was presented that there was no apparent reason why the L-forms

should not take up the label, a more satisfactory demonstration may have been to measure the amount of radioactivity taken up by the organisms using scintillation counting. This was precluded by the facilities available.

However, the findings in relation to macrophage-L-form association in the autoradiographic study, correlate well with previous findings by fluorescent antibody staining and adsorption estimates. Interaction was demonstrated by staining at infectivity ratios in excess of 100 cfu per cell and was not related to the tonicity of the suspending medium.

Recently, phagocytosis of group A streptococcal L-forms, by human polymorphonuclear leucocytes (PMN) and mouse peritoneal macrophages (MPM),was demonstrated to occur very readily (Schmitt-Slomska et al., 1973). In that study, infectivity was performed in suspension with agitation, the concentration of cells being comparable with that used in adsorption experiments in the present study. Organisms were labelled with fluorescein isothiocyanate and added to the cell suspension at 1 cfu per 10 cells. Even after allowing for a larger number of organisms being present than were estimated by colony counts, it is difficult to envisage how nearly 100% of the cells had organisms associated with them by 90 minutes. From the photographs presented, it appeared, in addition, that many of these cells had large numbers of fluorescent bodies associated with them. The inoculation was carried out in isotonic media (Eagle's basal medium + serum) using an

L-form strain grown in media with high osmotic support, namely 20% sucrose. It is interesting to note that the presence of specific antibody increased the early rate of uptake of organisms by the cells but that it was not necessary for phagocytosis. This was confirmed in the present study to the extent that the low rate of uptake observed was independent of the presence of specific antibody. The incubation technique was similar for their electron microscopic study in which an infectivity ratio of 1 cfu per 5 PMN was used. They noted that there was no gross change in the appearance of free L-forms after exposure for 1 hour in the hypotonic tissue culture media. It was found that cells containing numerous L-forms were readily observed as early as 10 minutes after introduction of the organisms. Infected cells usually exhibited numerous vacuoles with variable content. Commonly, these were empty or contained a little granular material or else they contained vesicular bodies. The electron micrographs show dense bodies, presumably lysosomes, in relation to these vacuoles but there was apparently no fusion or discharge of lysosomal contents. From the micrographs shown, however, it is most unlikely that the vacuoles contained viable L-forms. It appeared therefore that either the L-forms were taken up as degraded forms or else this had occurred within the phagocyte.

Once again these results contrast with some of the observations in the present study. The inefficiency of transmission electron microscopy as a screening technique was stressed in the introduction to Chapter 6 and yet, at a very low infectivity ratio, Schmitt-Slomska's group observed an abundant interaction. The fact that the PMN's remained healthy after phagocytosis of the L-forms differed from the findings in the present study where L-fibroblasts often showed signs of degeneration following supposed interaction with L-forms.

In a follow-up paper, Drach and Schmitt-Slomska (1973) examined the survival of the L-form strain used in the previous experiment, both in tissue culture media and in tissue culture media containing phagocytic cells(PMN and MPM). It was found that suspension of this organism in EBM + CS resulted in an immediate 50% loss of viable counts. Following this, however, the viable counts fell slowly over several days, for example, it was about 5% of the original after 2 days. Incubation with phagocytic cells at 1 x 10^6 per ml with 1 cfu per 10 cells resulted in an appreciable removal of L-forms over the first few hours. At this time about 1% of the viable units were cell-associated, whereas about 50% of the viable population had been removed with the MPM on centrifugation. After 48 hours incubation, the viable organisms isolated from MPM comprised about 1 to 5% of the population in the control media, whereas, after 5 days, this relative

percentage had increased slightly. However, it should be pointed out that the number of organisms isolated from the macrophages at this stage comprised only about 0.1% of the original number in EBM + CS at zero time. It could be argued that differences in cfu at these very low levels are not significant in view of the original population size. The small number of viable units remaining as cell-associated forms at this time, was attributed to the inability of phagocytic cells to kill all of the organisms.

A postulate put forward by Spector et al. (1970) may have some relevance to these results; namely, that such observations could be due to the presence in a macrophage culture of a small number of cells that are incompetent with respect to their ability to degrade organisms.

Two more points of interest arise from the study by Drach and Schmitt-Slomska. Firstly, the survival of the type 49 strain in hypotonic media was much greater than that of the GL8 strain used in the present study (see Figures 7.1 and 7.2) and secondly, the requirement for long incubation periods to allow all colonies to develop. This was also found to be important in the present study when organisms had been exposed to hypotonic conditions.

Although direct comparisons between the studies by Schmitt-Slomska's group and the present study are difficult due to differences in organisms and techniques, it is nevertheless necessary to attempt to find or suggest reasons for apparent discrepancies and this has been attempted.

As was pointed out previously, Clasener (1972) stressed this difficulty, but she also commented on the need for confirmation of many isolated studies. The findings of the present study did not fully confirm those of Schmitt-Slomska's group.

From the experience gained in the present study and from published work it may be concluded that future work in the area of cell-association with both phagocytic and other cell types should be assessed very carefully. There is a need for specific labelling of L-forms and L-form components. Experimental techniques should be described in detail to enable ready comparison of results. Quantitative studies involving the tracing of radioactive-labelled components should be highly informative, particularly for investigations on the uptake and fate of L-forms within phagocytic cells. The problem of atypical colony forms obtained from re-isolation attempts may well be resolved as the nomenclature in this field becomes standardized.

Finally, in a field beset with controversy and unsubstantiated hypotheses, it is essential to define rigidly the basic parameters before any real progress can be made. For example, as shown in Figure 9.1, the whole basis for a role of streptococcal L-forms in disease is the ability to persist. Therefore, an examination of the possible mechanisms of pathogenicity, such as immunologically-mediated tissue damage, is of doubtful utility until persistence is adequately demonstrated. Even when this has been done, as with any information derived from an *in vitro* model, it can do no more than provide a reasonable basis for further investigations in the complex *in vivo* environment.

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