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ROLE OF MYCOPLASMA MEMBRANES IN THE PATHOGENESIS OF PRIMARY ATYPICAL PNEUMONIA

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13. ABSTRACT

Primary atypical pneumonia, caused by Mycoplasma pneumoniae, is a significant health problem among military recruits. The current study was designed to yield information on the mechanism of pathogenesis in this disease. Special processes for the cultivation of the causative agent have been developed. Organisms were collected and membrane fractions were prepared. When membranes were evaluated for cytotoxic potential in the hamster tracheal ring/organ culture system, they displayed active biological activity. The necrosis induced in the ciliated respiratory epithelium was grossly equivalent to that induced by viable cells, and was dose-dependent. The data indicate that the cell membrane of this pathogen may be the site of the toxic factor. Further biological and biochemical identification of this toxic factor could ultimately play a key role in the development of a suitable vaccine to reduce the high morbidity rate associated with this disease.

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Role of Mycoplasma Membranes in the Pathogenesis of Primary Atypical Pneumonia

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Summary

Primary atypical pneumonia, caused by Mycoplasma pneumoniae, is a significant health problem among military recruits. The current study was designed to yield information on the mechanism of pathogenesis in this disease. Special processes for the cultivation of the causative agent have been developed. Organisms were collected and membrane fractions were prepared. When membranes were evaluated for cytotoxic potential in the hamster tracheal ring/organ culture system, they displayed active biological activity. The necrosis induced in the ciliated respiratory epithelium was grossly equivalent to that induced by viable cells, and was dose-dependent. The data indicate that the cell membrane of this pathogen may be the site of the toxic factor. Further biological and biochemical identification of this toxic factor could ultimately play a key role in the development of a suitable vaccine to reduce the high morbidity rate associated with this disease.

Foreward

In conducting the research described in the report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

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Report

I. GENERAL STATEMENT OF THE PROBLEM

Primary atypical pneumonia, caused by Mycoplasma pneumoniae, is a recognized health problem for civilian and military populations. The pathology and epidemiology of the disease are understood, but the precise mechanism of pathogenesis has yet to be defined. Hydrogen peroxide had been considered a likely potential toxin, but recent data indicate that additional toxic factors are involved. Several studies indicate that mycoplasma membranes may be toxic or play a role in certain pathologic processes. This investigation will establish whether or not structural cell components of Mycoplasma pneumonia participate, directly or indirectly, in the pathogenesis of primary atypical pneumonia.

II. BACKGROUND

Mycoplasma pneumoniae is the etiologic agent associated with the cold agglutinin positive form of primary atypical pneumonia (11). The disease is characterized by fever, headache, sore throat, malaise, and pulminary infiltration lasting several weeks. It has a low mortality rate, but a high morbidity rate which often necessitates hospitalization. Thus, loss of work or duty time for any population is significant. Complications of primary atypical pneumonia, PAP, include mucocutaneous diseases (31,35), meningoencephalitis (34), and meningitis (1). M. pneumoniae may also display a synergistic effect with other respiratory pathogens (23).

Mycoplasma pneumoniae is responsible for 4 to 10% of the acute respiratory tract infections in children, and for 9 to 22% of the pneumonia in adults (20). The incidence values and severity are increased for highly susceptible groups like very young children (13), persons in institutional housing (25) and military recruits (6). M. pneumoniae infections are a special problem for the Armed Forces due to the highly susceptible age group involved. The risk is especially great for new recruits because of confined living conditions and the constant introduction of susceptible hosts into a population

in which the disease is endemic (4). Numerous studies on the epidemiology and control of PAP include incidence rates of 20% (18), 25% (19), 38% (3), and 45% (24) of all pheumonias in military base hospitals. An understanding of the pathogenesis, and hence control of primary atypical pneumonia is therefore crucial to the efficient utilization of military manpower.

The search for toxic factors which may mediate the pathogenicity of M. pneumoniae has concentrated thus far on peroxide formation and cytadsorption. Data from a variety of experiments lead most investigators to conclude that the pathogenesis of PAP involved adsorption of the organisms to ciliated respiratory epithelium, followed by direct damage to the cells by peroxide (4). However, Lipman, Clyde, and Denny (21,22) recently compared homologous virulent and attenuated strains of M. pneumoniae and failed to find a consistent correlation between pathogenicity and peroxide formation or cytadsorption. They concluded that these characteristics were not entirely responsible for virulence, and suggested the existence of accessory steps and/or factors possibly including a toxin.

While studying the "lethal toxicity" syndrome induced in mice by M. fermentans, we recently encountered a unique mycoplasma toxin (14,16,17). High concentrations of cell membranes from recent isolated of M. fermentans induced the same lethal pathologic response as that following IP injections of viable organisms. Membranes dissolved in detergents and then reaggregated by dialysis in the presence of divalent cations were also toxic. Preliminary studies indicated that the toxin associated with the cell membrane was heat stable and contained both protein and lipid. This was the first reported instance of a pathologic response attributed to a purified mycoplasma cell fraction. However, Piercy (26) recently reported a bovine synovitis following the intra-articular injection of heat-inactivated whole cells of M. mycoides. He deduced that the toxin constituted some integral part of the cell, analogous to the endotoxin of gram-negative bacteria. Williams et al. (36) isolated M. fermentans from patients with rheumatoid arthritis, and used purified cell membranes as the antigen for leukocyte migration-inhibition tests. They found that the mycoplasma membranes would strongly

inhibit leukocyte migration and induce production of the macrophage migration inhibition factor when used with cells from rheumatoid patients. Even more significant was their observation that mycoplasma membranes bound immunoglobulin G, which may account for the appearance of autoimmune phenomena including the production of rheumatoid factor.

There is presently one report in the literature (Collier and Clyde, Infect. Immun. 3: 694, 1971) which indicates that non-viable M. pneumonia cells are not toxic in tracheal organ cultures. The data is not entirely convincing, however, because heat inactivated preparations were used. The process used for inactivating cells thus may have simultaneously inactivated a labile toxic factor associated with the membrane. During our 3-year study of the toxic membranes of M. fermentans, we developed a cell-lysis protocol which is gentle enough to allow retention of toxicity and enzymatic activity.

Previous failures to induce toxicity with inactivated preparations may have also been due to the common error of inadequate toxin. Most reported calculations fail to consider the fact that multiplication is precluded, and one must add material (membranes) equivalent to the CFU titer one would find at the height of an acute infection. The article mentioned above notes that over 100 CFU may be attached to each infected epithelial cell, and hence larger amounts of non-viable preparations must be evaluated for toxicity.

It is significant that the proposed study will be the first to evaluate the combined effect of mycoplasma membranes and various host cellular and humoral factors. Clyde (Infect. Immun. 4: 757, 1971) recently noted the significant interaction of epithelial cells and leukocytes in the hamster model. This study will examine the influence of host cells and serum on the toxicity of M. pneumoniae membranes in the tracheal ring organ culture model.

III. Approach

This pilot study is designed to evaluate the toxigenic potential of structural cell components of Mycoplasma pneumoniae. Specific goals include the following:

A. CULTIVATION of M. pneumoniae on a massive scale, using methods to be developed in this study.

B. FRACTIONATION of cells after osmotic lysis and differential centrifugation, followed by reaggregation.

C. EVALUATION of toxicity of membranes and reaggregated membranes using organ cultures of hamster trachea, recently developed as a sensitive biological assay for M. pneumoniae virulence.

A. Cultivation

M. pneumoniae is normally cultivated in submerged broth culture (12) or attached to glass (29,33), using numerous 1 to 5 liter vessels. The large-scale growth of mycoplasmas has not been reported. To provide the large numbers of organisms needed for fractionation and chemical assays, M. pneumoniae will be propogated on glass surfaces. This provides a convenient method for preparing cells which are relatively free of serum contaminants from the medium, and one which can furnish the relatively "clean" cell preparation needed for the proposed pathology and biochemical studies.

Animal cell cultures have been propogated on glass for several years, and large scale growth is commonly achieved by using various types of "roller apparatus." Basically, such a system employs large round bottles (1-3 liters in volume; 800-1, 400 cm^2 in surface area) which are slowly rotated on rollers. The bottles contain a minimal amount of medium to bathe the cells which cover the entire inner surface of the bottle. After incubation and growth, the medium is decanted, the cell sheet is gently washed, and the cells are then scraped into the required diluent. This greatly facilitates the cleaning and concentration of large masses of cells.

This methodology should have direct application to the growth of mycoplasma. Accordingly, a cell-roller production apparatus (Flow Co., Model FCR-15, with a 15 bottle capacity

and a total internal glass surface of 12,600 cm^2) has been purchased with U. of I. funds for this purpose. Thus, many of the problems normally associated with fermentation "scale-up," protein contamination, and centrifugation of cells from several liters of media will not be encountered. The method of Purcell (29) will be used to produce glass-adherent cells, and after scraping and washing, the cells will be treated using methods previously developed in this laboratory to yield a membrane fraction with essentially no cytoplasmic contamination.

Parameters to be evaluated with regard to cell yield on the toller apparatus include speed of chamber rotation, type of medium and supplements, pH, temperature, aerobiosis, and optimum size of inoculum and time of incubation. An accurate estimate of the potential cell yields under the proposed conditions is complicated by the inexplicable lack of data concerning surface area in previous publications. However, it is known that cell culture yields of 2×10^8 cells/bottle are common when using BHK kidney cells in the apparatus described (E. Reichmann, personal communication). In regard to mycoplasma, if one estimates the surface area of a Povitsky bottle (used extensively by Somerson, 33) to be approximately 500 cm^2 and uses cell yield data of ref. 29 and 33, extrapolated yields of M. pneumoniae would be 1.6×10^{12} CFU/roller bottle, or 2.4×10^{13} CFU per full complement of 15 bottles in the apparatus to be used. This represents a final yield of approximately 1-3 grams per 15-bottle batch. The development of this methodology will result in an improved method for large-scale cultivation of mycoplasmas.

B. Fractionation

Cells will be collected from culture media by centrifugation at 12,000 x g for 30 min. Lysis will be induced by a combination of osmotic shock and sonication (15,17). The osmotic pressure of cells will be increased initially by incubation in 2 M glycerol, which is then followed by rapid injection into deionized water. Ensuing steps are: incubation, centrifugation of unbroken cells, sonication of the sediment, centrifugation, and pooling of

the osmotic and sonic lysates. Fractionation of cells into soluble and particulate, i.e. membrane, components will involve differential centrifugation using the methods of Gabridge (17) and Pollack et al. (7). Basically, membranes will be collected at a force of 19,000 x g, leaving the supernatant or soluble fraction which will be cleared of polyribosomes and cellular debris by centrifugation at 40,000 x g for 2 hrs, and by filtration through 0.2 μm pore filters.

Reaggregation is a process in which detergents temporarily separate the protein and lipid components of membranes, thereby facilitating removal of nuclear and cytoplasmic contamination and yielding a completely non-viable, purified membrane preparation. The proper combination of detergents allows one to increase or decrease the relative protein content of the membranes which reform after dialyzing out the detergents. Reaggregation techniques were originally developed using the saprophyte, M. laidlawii, but have recently been adapted to parasitic species like M. fermentans (17) and M. pneumoniae (7,30). The membrane fraction obtained as described previously will be solubilized in a mixture of sodium deoxycholate (2.0 mg/mg protein) and sodium dodecyl sulfate (0.5 mg/mg protein). After incubation at 37°C for 30 min, the partially cleared (Tris, β-mercaptoethanol, NaCl, pH 7.8) containing 0.02 M $MgCl_2$ and merthiolate (1:10,000). Flocculant material, i.e. reaggregated membranes, forming in the dialysis bag will be collected by centrifugation at 34,000 x g for 30 min. The reaggregated membranes will then be washed 6 times in β-buffer, resuspended in same, and stored at 4°C.

C. Evaluation

The toxicity of viable cells, whole lysates, membranes, and reaggregated membranes of M. pneumoniae will be evaluated using organ cultures of hamster trachea. Such a model system was recently developed by Collier et al. (9,10) for use with M. pneumoniae, and is sensitive, reliable, inexpensive, and relatively simple to perform. Human embryo trachea cultures have also been used (2,8) but present obvious difficulties in regard to procurement, sterility, and standardization. The Syrian hamster has been used for several years as an in vivo model for human pneumonia, and now the hamster tracheal ring

is serving as a convenient *in vitro* model. Tracheas will be removed aseptically from adult Syrian hamsters, placed in mycoplasma broth with 20% serum, and cut transverasIly into 1 mm sections, each containing one cartilage ring. The individual organ cultures will be maintained in broth contained in plastic culture dishes. Incubation will be at 36°C in an atmosphere of 5% CO_2 in air. Ciliary activity, which is indicative of respiratory epithelium activity, will be examined using an inverted microscope at 100-200 X magnification. Control and treated tissue will be examined daily, and samples will be processed (*i.e.*, fixed, embedded, sectioned, stained) at daily intervals for 8 days to determine the extent of cellular damage. Particular attention will be given to evidence of vacuolization, cytoplasmic eosinophilia, nuclear swelling, and loss of architecture in the ciliated respiratory epithelium as described by Collier *et al.* (8,9,10). Experiments will include negative controls (broth only), positive controls (viable *M. pneumoniae*) and treated samples (whole lysates, cytoplasm, membranes, reaggregated membranes). It noteworthy that the tracheal ring model system is especially amenable to fluorescent antibody and electron microscopy studies which may be conducted at a later date.

It is anticipated that the membrane fractions of *M. pneumoniae* will display toxicity, and induce pathophysiological alterations in ciliated respiratory epithelium identical to those induced by viable cells. Such results would indicate that respiratory tract infection by this organism is a membrame phenomenon mediated by the overt toxicity of structural cell components.

It is conceivable, however, that host factors may play a significant role in the pathogenesis of PAP. Cell membranes will be added to hamster serum and cell preparations to evaluate their combined effect. A positive result in this case may indicate that the necrosis in tracheal ring organ cultures by viable cells alone is an artifactual phenomenon, and that a more valid *in vitro* model for pathogenicity studies would involve more than one type of host cell.

IV. RESULTS AND DISCUSSION

A. Cultivation

Several studies were conducted in order to determine the feasibility of growing mycoplasma on slowly rotating glass surfaces. Various parameters were evaluated, including: speed of rotation (0.05 RPM to 5 RPM); media (Hayflick's, SSR1, M199); serum supplements horse, calf, and bovine serum fraction at various concentrations); preincubation regimens (static for 24 hrs followed by rotation); and periodic alterations of the static and dynamic cycles throughout the cultivation period. Measurements were made on the pH of the culture fluids, the optical density (520 nm) of the washed cell suspension, the number of colony forming units (CFU) and the dry weight of cells.

In general, growth was very poor and cell sheets obtained from rotating surfaces did not approach the quality (in terms of thickness and tendency to adhere) of those obtained in the usual static incubation using flat surfaces. Total yields occassionally approached 20 mg dry wt per 300 ml of medium, but were normally much less (<10 mg). Results, were remarkably inconsistent, even though standardized techniques, conditions, and inocula were employed. After almost one year of effort at examining combinations and permutations of the variables listed above, we conclude that growth of mycoplasma on moving glass surfaces is not feasible at this time.

In the course of these studies, we evaluated the ability of mycoplasma to attach and grow on solid surfaces other than glass or plastic. We found that mycoplasma will form dense layers of growth on cellulose ester and cellulose polyacetate substrates. These materials are manfactured and sold in sheet form, and this could serve as a convenient method for the mass cultivation of cells to be used as antigen for vaccines.

M. pneumoniae, strain FH (obtained from Dr. J. Tulley, N.I.H., Bethesda, Md.) was used along with species purchased from the American Type Culture Collection: ATCC 14192 (Acholeplasma laidlawii); ATCC 19610 (M. gallisepticum); ATCC 19611 (M. arthritidis); and ATCC 14027 (M. hominis). These were grown in standard Hayflick's broth containing 20% horse serum. Solid medium (15 ml per 60 x 15 mm Petri dish) contained an additional 0.75% Ionager 2S (Colab Laboratories, Greenwood, Ill.) Chlorazol Black E plates contained 0.1% of the dye (Matheson, Coleman, and Bell, Cincinnati, Ohio) in addition to agar.

Millipore filters (Millipore Filter Corp., Bedford, Mass.) of mixed cellulose esters, or Sepraphore III electrophoresis strips (Gelman Instrument Co., Ann Arbor, Mich.) of cellulose polyacetate were autoclaved and placed on the agar surface. An aliquot (0.1 ml) of a log phase culture was spread evenly over the filter (47 mm diameter disk or 25 mm square) with a sterile pipette. Plates were incubated 7-10 days in 5% CO_2, 95% air at 37°C. Filters from Chlorazol Black plates were then air dried without further processing. Filters from standard agar plates were air dried, stained in 2% Dienes Stain for 10 sec, rinsed in phosphate buffered saline (PBS, pH 7) for 30 sec, and air dried.

A. laidlawii grew well, regardless of whether the inoculum yieled confluent growth or isolated colonies. Filters with less than 200 CFU had coloneis 1-2 mm in diameter, while confluent growth was composed of pin-point colonies similar to those seen when organisms are plated directly on agar. Filter disks placed on Chlorazol Black plates yielded similar results. However, colonies were colored dark black, while the dried filters were light gray. This method, in which the dye is incorporated in the agar and is later concentrated in the colonies during growth, permits easy visualization without fixation and staining, and may be valuable in other applications.

The ability to grow on Millipore filters was not unique to Acholeplasma, since M. pneumoniae, M. gallisepticum, M. hominis, and M. arthritidis grew in a similar fashion

(Fig. 1). Differences in morphology were apparently due to differences in cell density. Sepraphore III cellulose polyacetate membranes, as well as millipore filters of various mean pore sizes (0.10, 0.22, 0.30, and 0.65 μm) all supported growth.

Organisms could be recovered by mechanically scraping the colonies from the surface into PBS. Starting with an inoculum of 9×10^3 CFU, the average yield (n = 15) was 3×10^8 CFU/47 mm filter. A relatively high protein content (5.0 mg/disk) and dry wt (5.7 mg/disk) indicated that media or filter fragments contaminated the cells. A rinsing step, using a standard millipore vacum apparatus, would be advisable. Sonication for 15 or 30 sec did not significantly increase cell recovery from scraped filters.

The manner in which the mycoplasmas grew on cellulose ester substrates was investigated using scanning electron microscopy. Samples of isolated colonies of M. pneumoniae were fixed using 2.5% glutaraldehyde in PBS for 2 hr at 4°C. They were washed 15 min in PBS, rinsed 2X in distilled water, and air dried.

Examination of critical point dried specimens, using a Jeolco U3 microscope, indicated that the colonies were round, flat, and raised slightly (Fig. 3). Organisms could apparently "weave" themselves into the interstices of the filter so that the colony gave the filter a slight increase in mechanical rigidity. Note that the colony in Fig. 2 extends over the fracture plane. The filter cleaved around the colony instead of through it. (The porous area to the lower right of the pancake shaped colony of M. pneumoniae is the exposed cellulose ester matrix from within the filter).

The benefits of using this method for the cultivation of mycoplasmas on cellulose ester substrates may be similar to those of glass-surface culture methods. Masses of cells can be washed and collected without recourse to high speed centrifugation of protein-rich media. In addition, the methodology described here should prove useful in studying

. the morphology and development of mycoplasma colonies. This technique also has potential application as a replica plating process to be used in mutation studies, and as a means of transferring cells to fresh media for induction or labelling experiments.

Another potential method for increasing efficiency of cell production involves the use of glass beads as a substrate. Glass fragments of various sizes and shapes have been used for cultivating tissue culture cells, but have not been used in conjunction with mycoplasma. A roller bottle containing 500 gm of sterile glass beads under the medium (SSR1) gave an extremely large surface area which supported growth. The surface area of a single Povitsky bottle is approximately 286 cm^2, compared to a surface area of 2,190 cm^2 for 500 gm of 6 mm diam. beads. With 200 ml of medium, our pilot study yields averaged 60 mg (dry wt of washed M. pneumoniae cells with minimal media carry over) compared to 40 mg from similar bottles without beads (static) and 20 mg without beads (rotating).

We are currently developing a vessel and value/pump assembly such that media replacement, cell washing, and cell collecting can be facilitated. Once the protocol which gives optimal yields is established, we will determine the purity of these preparations by using specific antisera and radioactive labels. Data currently available indicate that this method may prove extremely useful in the mass production of mycoplasma cells, and further results will be available soon.

B. Fractionation

While developmental studies of the cell production phase have been underway, cell fractions were obtained from organisms grown in static glass surface culture. Several 5 liter batches of M. pneumonia, strain FH, were prepared in Povitsky bottles. Cells were grown in 300 ml SSR1 medium, and after a confluent cell sheet had attached (approx. 3-5 days at 37°C with a 20% inoculum of refrigerated cells),

the medium was decanted and the cell sheet was washed 2X in PBS. The sheet was then scraped into fresh PBS and cells were stored at -20°C. When the equivalent of 20 liters of cells was attained, the resuspended cells were thawed and centrifuged at 9,000 g x 30 min. The pellet was resuspended in 25 ml 2M glycerol and incubated at 37°C for 20 min. The suspension was then injected into 300 ml of sterile deconized water, and the mixture was incubated at 37°C for 30 min.

Unbroken cells were removed by centrifugation at 3,500 x g for 3 min. The cell lysate contained in the supernatant was saved. The pellet was resuspended in 15 ml of deconized water and sonicated at 20 H_2. (125 watts) for 5 one minute periods in an ice bath. The cell debris was again removed by slow centrifugation, and the osmotic and sonic lysates were pooled. Membranes were collected at 20,000 x g, and were washed 6X in alternate rinses of 0.05 M NaCl and β-buffer.

C. Evaluation of Toxicity

Primary atypical pneumonia, caused by Mycoplasma pneumoniae. This disease lends itself well to pathogenesis studies because organ cultures serve as a convenient assay of biological activity. The trachea or wind pipe can be removed asptically from adult hamsters, and can be sliced into rings of tissue. When placed in a rich medium such as PPLO broth with sodium bicarbonate, and glucose, and then incubated in 5% CO_2, the ciliated respiratory epithelium on the inner surface of these tissue rings continues to beat for several weeks. The ciliary activity can be continually monitored microscopically. Previous work by several laboratories indicates that the mycoplasmas localize on the cell surface and in the intercellular spaces, represented in Fig. 3 by the letters TB for terminal bar.

To date, no toxin has been isolated. We have recently begun experiments to evaluate the effect of M. pneumoniae membranes in this organ culture system. Ciliary activity,

which is directly related to degree of necrosis in the epithelial layer, can be quantitated either by measuring the percent of the surface with beating cilia, or by subjective evaluation of the vigor of activity. We have combined both to give us a parameter called "relative activity." It is defined as the product of the percent of intact epithelium (0-100%) and the vigor of activity 0-4+) to give a maximum value of 400. Normally, 4-8 rings were used in each plate, and mean values were derived.

The cell lysis procedure described above was applied to M. pneumoniae, strain FH, to yield membranes, which were then tested in the tracheal organ culture system along with viable cells. See Fig. 4. The control (top line) shows high activity (i.e. 200) which is an indication of a reasonably healthy, intact epithelum. The lysate and cytoplasm treated rings behaved similarly and displayed little change. Membranes, however, had very potent activity. This membrane preparation, obtained from glass grown cells and containing approx. 10 µg/ml of protein, destroyed the epithelial surface in 3 days. If the lysate was lyophilized and reconstituted to an equivalent protein concentration, toxic activity (apparently due to the membrane content) was obvious. The slightly lower activity and slower necrosis can be explained by the fact that this new protein concentration represents both the cytoplasm and membrane present in the lysate. Hence, it is consistent that activity would be slightly lower than that of purified membranes.

The data in Fig 5 indicate that membrane preparations can give the same time-response relationship as viable cells. These curves represent 3 sets of plates and organ cultures. The top (or control) curve represents uninoculated media. The lower curves, essentially identical in effect, represent tracheal rings exposed to 1 µg/ml of purified membranes, or 3×10^6 CFU/ml. Grossly, the sequence of changes, and the specific alterations, were the same. We are currently processing similar specimens to determine whether or not the histopathology of the necrosis induced by membranes and viable cells is also identical.

The fact that the biological effect seen in the tracheal rings is due to membranes themselves can be seen in the gradient observed with various concentrations. As shown in Fig. 6, when the concentration of membranes is adjusted to 10, 1, and 1/10 μg protein per ml, a family of curves is generated. The degree and speed of necrosis is directly related to the concentration of membranes in the preparation. This dose-effect relationship indicated that the epithelial necrose is either directly or indirectly membrane-related.

D. Prognosis

Current studies are designed to show that the histopathologic alterations induced in tracheal organ culture by membranes are the same as those induced by viable cells. Simultaneously, we are preparing reaggregated (i.e., purified) membranes to determine their cytoxicity.

In the course of the past year, we found it desirable to test our hypothesis (cellular, and not soluble, components are cytotoxic) with the aid of a diffusion chamber. A chamber in which one can separate the mycoplasma and organ cultures with a semipermeable membrane was developed (See Appendix A). It has recently been modified into an extremely simple yet effective device which will play a key role in our studies, and which may be valuable in the study of other biological systems.

On the basis of the progress recorded in this report, it is our recommendation that the study of the role of Mycoplasma pneumoniae membranes in the pathogenesis of primary atypical pneumonia the vigorously pursued, in hopes that a definition of this involvement will play an integral role in the prophylaxis and treatment of this disease.

E. SIGNIFICANCE

1. An understanding of the toxic capabilities of Mycoplasma pneumoniae will facilitate defining the mechanism of

pathogenesis in primary atypical pneumonia, a prerequisite for effective prophylactic and therapeutic treatment. Knowledge of how the organism exerts its pathologic effect will dictate the optimal approach to negate the cytotoxicity and to promote natural host defense mechanisms. This will reduce the high morbidity rate which is so costly in terms of loss of duty time for military personnel.

2. Identification of the toxic factor will aid in selecting avirulent mutants to be used for vaccine development. Cultures could be mutagenized, and mutants displaying a metabolic block in the formation of the toxic component could be used for actively inducing growth-inhibiting antibody without the possibility of disease production.

3. The methodology to be developed for the mass culture of organisms will be of direct benefit in ultimate vaccine production. Large-scale culture of mycoplasma will be required for vaccine production regardless of the mechanism of pathogenesis, identity of the toxin, or type of vaccine used.

4. General benefits will accrue to the field of mycoplasma research because of data which will add to our knowledge of cultivation, metabolism, and membranes of M. pneumonia, as well as to the pathogenesis of primary atypical pneumonia.

V. FIGURES

Fig. 1. Cellulose ester filters (0.45 μm pres) with colonies of M. hominis (upper left), M. pneumoniae (upper right), M. gallisepticum (lower left), and M. arthritidis (lower right).

Fig. 2. Scanning electron micrograph of an M. pneumoniae colony on the cleavage plane of a cellulose ester filter (0.45 μm pres). Approx 200X.

Fig. 3. Diagram of hamster tracheal ring, gross specimen and enlargement of epithelial surface.

Fig. 4. Relative cytotoxic activity of viable M. pneumoniae cells, whole lysate, cytoplasm, and membranes.

Fig. 5. Relative cytotoxic activity of M. pneumoniae cells (3×10^6 CFU/ml) and membranes (1 mg/ml).

Fig. 6. Gradient of cytotoxic effects observed when various concentrations of M. pneumoniae membranes are tested in the hamster tracheal ring/organ culture system.

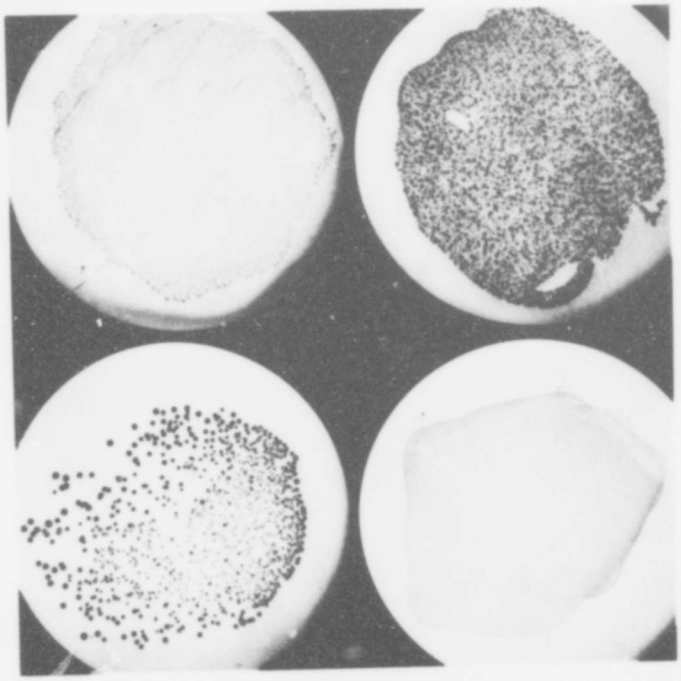
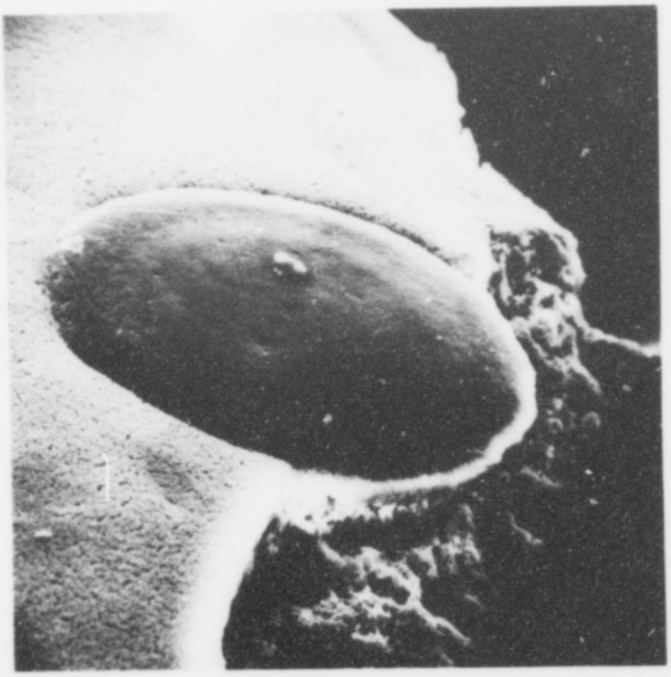


Fig. 1.



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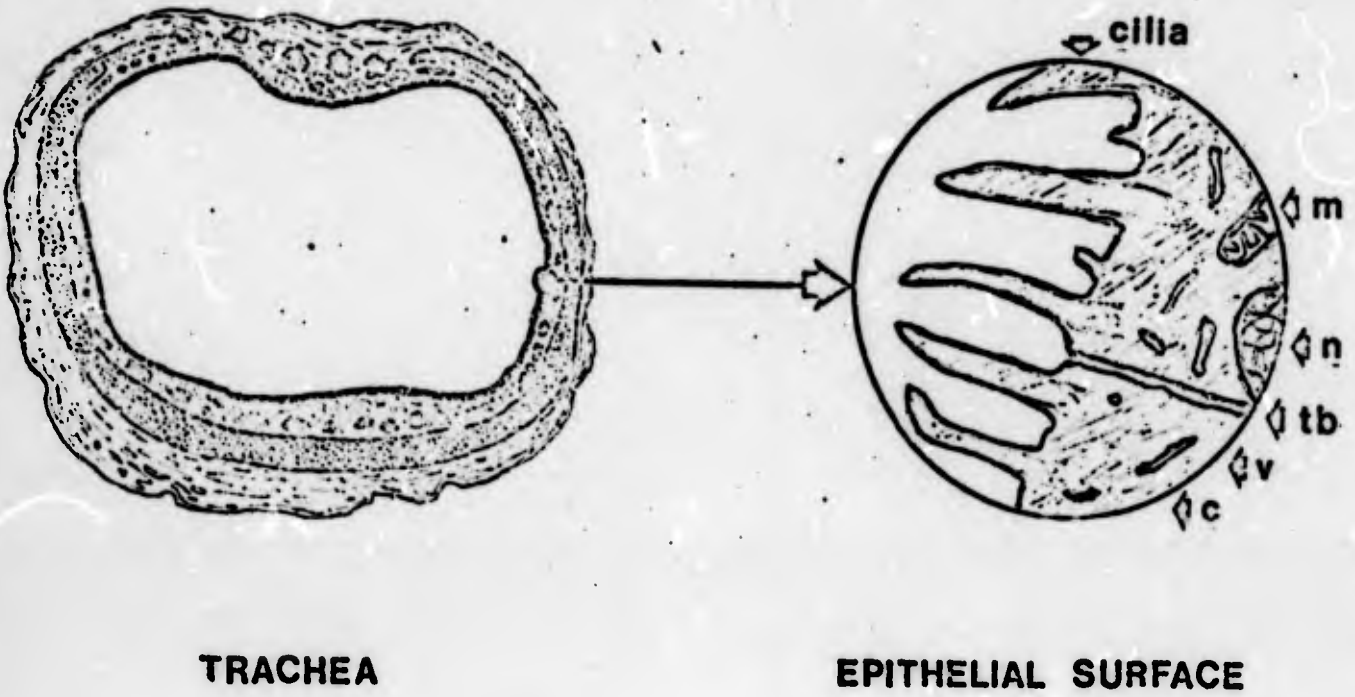

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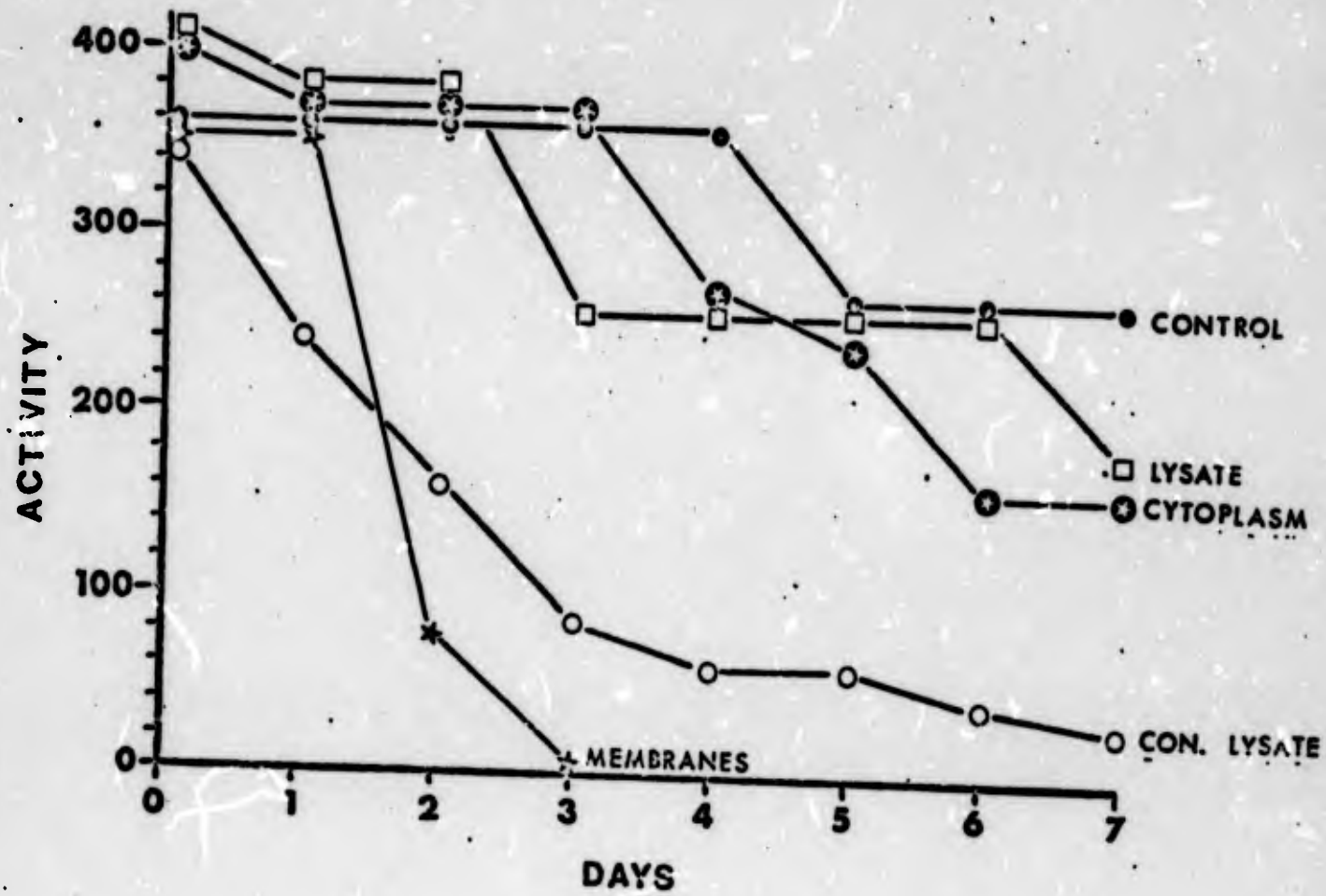

Fig. 4. Relative cytotoxic activity of viable M. pneumoniae cells, whole lysate, cytoplasm, and membranes.

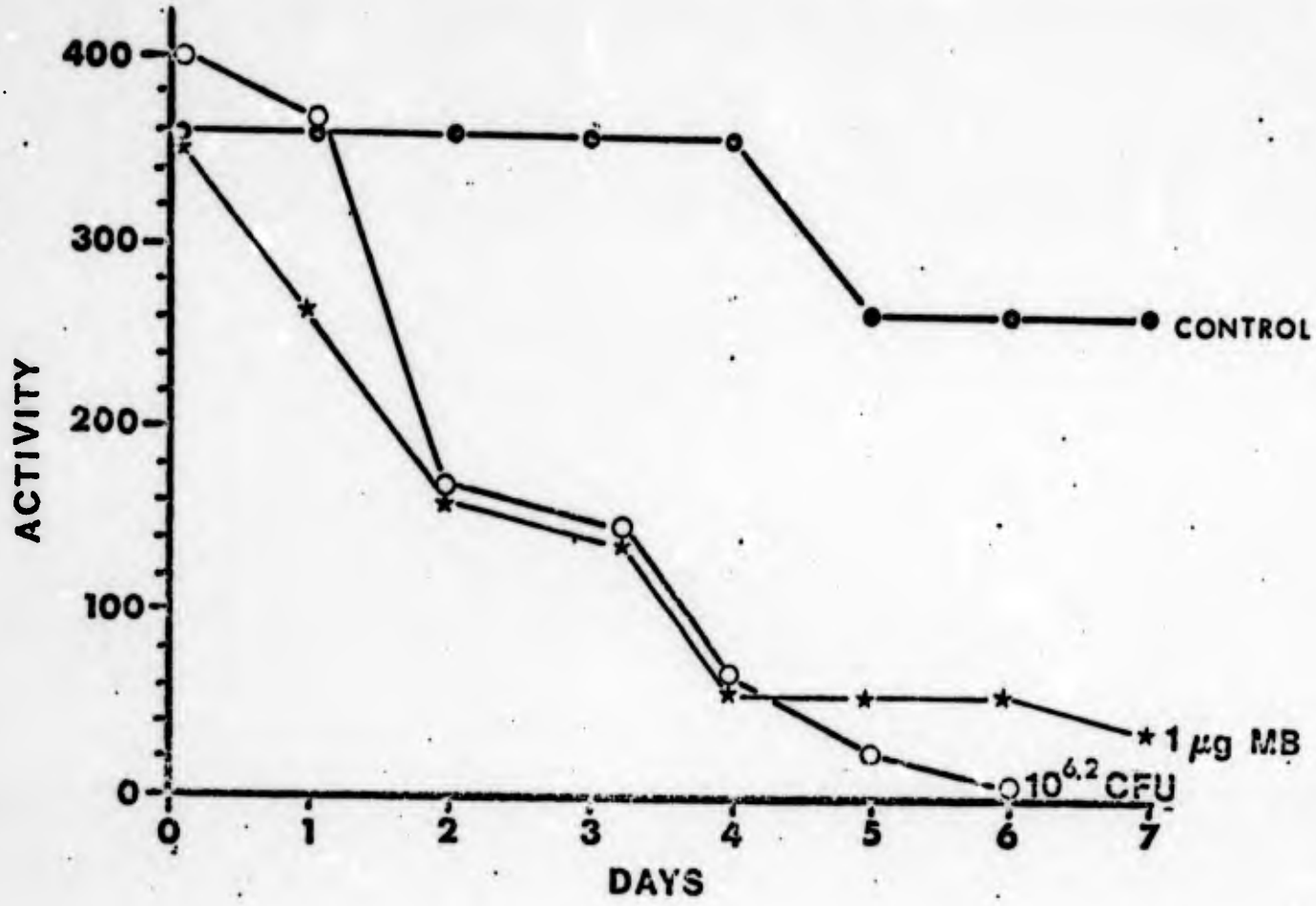

Fig. 5. Relative cytotoxic activity of M. pneumoniae cells (3×10^6 CFU/ml) and membranes (1 mg/ml).

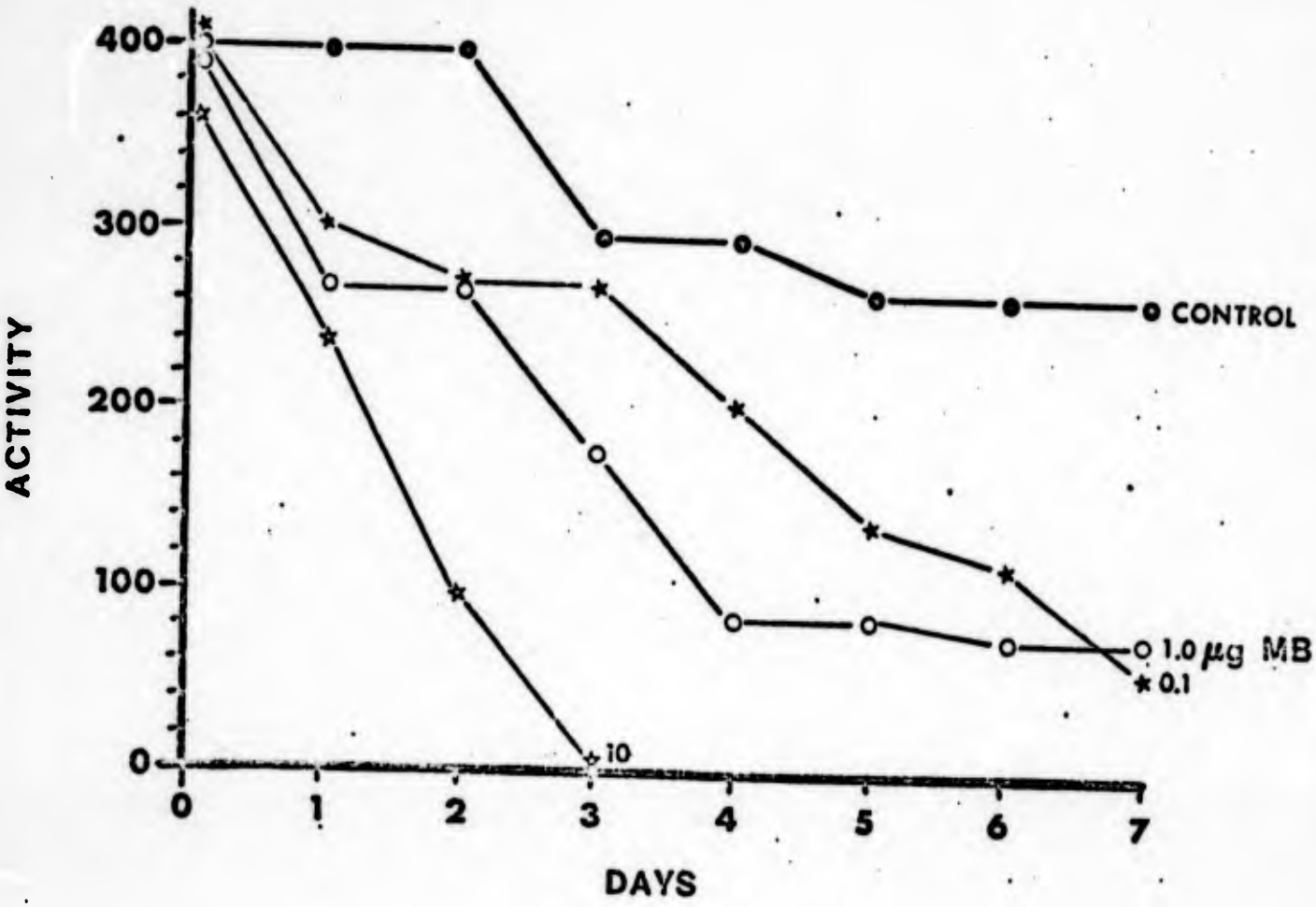

Fig. 6. Gradient of cytotoxic effects observed when various concentrations of M. pneumoniae membranes are tested in the hamster tracheal ring/organ culture system.

BIBLIOGRAPHY

1. Biberfeld, G. 1971. Antibodies to brain and other tissues in cases of Mycoplasma pneumoniae infection. Clin. Exptl. Immunol. 8: 319-333.

2. Butler, M. 1969. Isolation and growth of mycoplasma in human embryo trachea cultures. Nature 224: 605.

3. Chanock, R. M., H. H. Fox, D. James, R. R. Gutekienst, R. J. White, and L. B. Senterfit. Epidemiology of M. pneumoniae infection in military recruits. Ann N. Y. Acad. Sci. 143: 484-496.

4. Chanock, R. M., P. Steinberg, and R. H. Purcell. 1970. Mycoplasmas in human respiratory tract disease. In The role of mycoplasma and L forms of bacteria in disease, J. T. Sharp (ed.), Charles C. Thomas, Springfield, Illinois.

5. Clyde, W. A. 1968. An experimental model for human mycoplasma disease. Yale J. Biol. Med. 40: 437-443.

6. Clyde, W. A. 1971. Mycoplasma pneumoniae pneumonia. Military Med. 136: 20-22.

7. Cole, R. M., T. J. Popkin, B. Prescott, R. M. Chanock, and S. Razin. 1971. Electron microscopy of solubilized Acholeplasma laidlawii membrane proteins reaggregated with Mycoplasma pneumoniae glycolipids. Biochim. Biophys. Acta 233: 76-83.

8. Collier, A. M., and W. A. Clyde. 1971. Relationships between Mycoplasma pneumoniae and human respiratory epithelium. Infec. Immun. 3: 694-701.

9. Collier, A. M., W. A. Clyde, and F. W. Denny. 1969. Biological effects of Mycoplasma pneumoniae and other mycoplasmas from man on hamster tracheal organ culture. Soc. Exptl. Biol. Med. 132: 1153-1158.

10. Collier, A. M., W. A. Clyde, and F. W. Denny. 1971. Mycoplasma pneumonia in hamster tracheal organ culture: immunofluorescent and electron microscopic studies. Proc. Soc. Exptl. Biol. Med. 136: 569-573.

11. Denny, F. W., W. A. Clyde, and W. P. Glenzen. 1971. Mycoplasma pneumoniae disease: clinical spectrum, pathophysiology, epidemiology, and control. J. Infect. Dis. 123: 74-92.

12. Eaton, M. D., and I. E. Low. 1967. Propogation of Mycoplasma pneumoniae and other fastidious strains of PPLO. Ann. N. Y. Acad. Sci. 143: 375-383.

13. Foy, H. M., G. E. Kenney, R. McMahan, A. M. Mansy, and J. T. Grayson. 1970. Mycoplasma pneumoniae in an urban area. J.A.M.A. 214: 1666-1672.

14. Gabridge, M. G. 1971. Lethal toxicity of Mycoplasma fermentans for mice. Ph.D. Thesis, University of Michigan, Ann Arbor.

15. Gabridge, M. G. G. D. Abrams, and W. H. Murphy. 1971. Lethal toxicity of Mycoplasma fermentans for mice. Submitted to J. Infect. Dis.

16. Gabridge, M. G., and W. H. Murphy. 1971. Lethal toxicity for mice of Mycoplasma fermentans strains isolated from patients. Bacteriol. Proc.: 1971, p. 70.

17. Gabridge, M. G., and W. H. Murphy. 1971. Toxic membrane fractions from Mycoplasma fermentans. Submitted to Infec. Immun.

18. Griffin, J. P., and Y. E. Crawford. 1969. Association of Mycoplasma pneumonia infection with primary atypical pneumonia. Ann. Rev. Resp. Dis. 100: 206-212.

19. Ionno, J. A., and R. E. Westfall. 1970. Mycoplasma pneumoniae pneumonia: clinical course and complications. Military Med. 135: 459-463.

20. Jao, R. L., and M. Finland. 1967. Susceptibility of Mycoplasma pneumoniae to 21 antibiotics in vitro. Am. J. Med. Sci. 253: 639-649.

21. Lipman, R. P., and W. A. Clyde. 1969. The interrelationship of virulence, cytadsorption, and peroxide formation in Mycoplasma pneumoniae. Proc. Soc. Exptl. Biol. Med. 113: 1163-1167.

22. Lipman, R. P. W. A. Clyde, and F. W. Denny. 1969. Characteristics of virulent, attenuated, and avirulent Mycoplasma pneumoniae strains. J. Bacteriol. 100: 1037-1043.

23. Liu, C., P. Jayanetra, and D. W. Voth. 1970. Effect of combined Mycoplasma pneumoniae and pneumococcal infections in hamsters. Ann. N. Y. Acad. Sci. 174: 828-834.

24. Mogabgab, W. J. 1968. Protective effects of inactive Mycoplasma pneumoniae vaccines in military personnel, 1964-1966. Amer. Rev. Resp. Dis. 97: 345-358.

25. Nakao, T., M. Umetsu, N. Watanabe, and N. Kikuchi. 1970. An outbreak of Mycoplasma pneumoniae infection in a community. Tohoku J. Exptl. Med. 102: 23-31.

26. Piercy, D. W. 1970. Synovitis induced by intra-articular inoculation of inactivated Mycoplasma mycoides in calves. J. Comp. Path. 80: 549-558.

27. Pollack, J. D., N. L. Somerson, and L. B. Senterfit. 1970. Isolation, characterization, and immunogenicity of Mycoplasma pneumoniae membranes. Infec. Immun. 2: 326-339.

28. Prescott, B., O. Sobeslavsky, G. Caldes, and R. M. Channock. 1966. Isolation and characterization of fractions of Mycoplasma pneumoniae. I. Chemical and chromatographic separation. J. Bacteriol. 91: 2117-2125.

29. Purcell, R. H., J. R. Valdesuso, W. L. Cline, W. D. James, and R. M. Chanock. 1971. Cultivation of mycoplasmas on glass. Appl. Microbiol. 21: 288-294.

30. Razin, S., B. Prescott, and R. M. Chancock. 1970. Immunogenicity of Mycoplasma pneumoniae glycolipids: a novel approach to the production of antisera to membrane lipids. Proc. Nat. Acad. Sci. 67: 590-597.

31. Sieber, O. F., T. J. John, V. A. Fulginiti, E. C. Overholt. 1967. Stevens-Johnson sydrome associated with M. pneumoniae infection. J.A.M.A. 200: 79-81.

32. Sobeslavsky, O., B. Prescott, W. D. James, and R. M. Chanock. 1966. Isolation and characterization of Mycoplasma pneumoniae. II. Antigenicity and immunogenicity. J. Bacteriol. 91: 2126-2138.

33. Somerson, N. L., W. D. Janes, B. E. Walls, and R. M. Chanock. 1967. Growth of Mycoplasma pneumoniae on a glass surface. Ann. N. Y. Acad. Sci. 143: 384-393.

34. Taylor, M. J., G. N. Burrow, B. Strauch, and D. M. Horstmann. 1967. Meningoencephalitis associated with pneumonitis due to Mycoplasma pneumoniae. J.A.M.A. 199: 813-816.

35. Trisch, J. A., L. Shapiro, and R. A. Wulzer. 1970. Vesiculopustular eruption with Mycoplasma pneumoniae infection. J.A.M.A. 211: 1694-1697.

36. Williams, W. H., J. Brostoff, and I. M. Roitt. 1970. Possible role of Mycoplasma fermentans in pathogenesis of rheumatoid arthritis. Lancet, Aug., 1970: 277-280.

APPENDIX A

Patent disclosure from submitted to University of Illinois and U.S. Army Medical Research and Development Command in January, 1973. Patent rights were relinguished by both agencies and given to the principal investigator.

Item: Continuous Observation Diffusion Chamber