IMMUNE   RESPONSE OF THE RAT TO OUTER MEMBRANE

PROTEINS OF Legionella pneumophila

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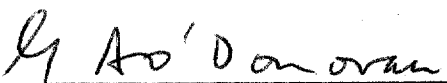
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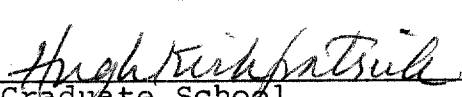
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IMMUNE RESPONSE OF THE RAT TO OUTER MEMBRANE

PROTEINS OF Legionella pneumophila

DISSERTATION

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Outer membrane proteins (OMPs) were recovered from

eleven strains (eight serogroups) of Legionella pneumophila

by sequential treatment with Tris buffer (pH 8), citrate

buffer (pH 2.75) and Tris buffer (pH 8). Transmission

electron microscopy revealed clearly the separation of the

outer membrane from the bacteria. The solubilized OMPs,

separated and visualized by discontinuous polyacrylamide

gel electrophoresis, contained a large variety of proteins

ranging from 10 to 79 Kd. A 24 Kd band was common to all

eight serogroups. A male New Zealand white rabbit infected

with 1.5×10^6 L. pneumophila (Chicago 8, serogroup 7) died

in nine days despite the administration of erythromycin.

Histopathology was particularly evident in the heart and

lungs. Both myocardial lesions and pneumonitis were evident.

Active immunity was produced in rats injected in the footpads

with the OMPs from Chicago 8 strain employing Freud's com-

plete adjuvant, a double emulsion adjuvant or saline. At

intervals, serum agglutinating titers were measured as were

blood leukocytes (total and differential).

The development of delayed hypersensitivity was also noted by measuring the area of erythema and induration produced by intradermal injections of the OMPs from Chicago 8 strain. The adjuvants enhanced greatly both active and cell-mediated immunity (CMI). Transient lymphocytopenia with a slight rise in neutrophils was noted in each of the immunized groups. Intraperitoneal challenge, seven days after the OMP booster, of one LD (1.5×10^6) of legionellae resulted in lymphocytopenia with elevated neutrophils. All immunized rats survived the challenge, although those in the saline-OMP group were clearly the sickest. Post-challenge, legionella antibody titers rose greatly and CMI was heightened. Passive immunization (homologous and heterologous) was found to protect the rats from a challenge of one LD. Actively-immunized rats retained their immunity for at least six months as determined by their resistance to a second challenge.

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CHAPTER I

INTRODUCTION

With the finding by Enders, Weller and Robbins (50)
in 1949 that poliovirus could be grown in vitro in non-
neural mammalian cell cultures, the past quarter of a
century has seen the isolation and identification of
hundreds of viral etiologic agents of animal/human disease.
On the other hand, few bacterial pathogens of human disease
have been discovered since the 1940s. Interestingly, the
last two bacterial agents of human infection cause pneu-
monia. The principal cause of primary atypical pneumonia
(PAP), Mycoplasma pneumoniae, had been considered for years
to be a virus since it defied cultivation in non-living
media and was filterable (50). In 1962 Chanuck, Hayflick
and Barile (39) cultivated this cell wall-less bacterium
in a cell-free medium and confirmed it as the etiologic
agent of PAP in human volunteers. Fifteen years later, a
major outbreak of a severe pneumonia erupted at a state
convention of the American Legion in Philadelphia,
Pennsylvania. Within six months, largely through the
efforts of researchers at the Center for Disease Control
(CDC) in Atlanta, Georgia, the etiologic agent of the

Philadelphia outbreak had been isolated in vitro. While
human volunteers were not employed in establishing cause
and effect, the agent was soon shown to cause a fatal
pneumonia and systemic infection in the guinea pig and to
infect embryonated eggs; it also reacted with antibodies
in human convalescent sera. This bacterium was named
Legionella pneumophila.

The nature of the disease, legionellosis, and of its
etiologic agents will comprise the thrust of this histori-
cal review. A topic of special consideration will be the
outer membrane proteins (OMPs) of L. pneumophila inasmuch
a significant portion of the research to be reported in
this dissertation was devoted to this subject.

Early Developments

Legionnaires' disease (LD) is a pneumonia produced by
Legionella pneumophila, a newly-recognized gram-negative
bacillus (78, 140). The term "Legionnaires' disease" was
originally used to describe an acute, febrile, respiratory
illness that affected members and visitors attending the
58th annual convention of the Pennsylvania American Legion
held in Philadelphia, Pennsylvania, July 21-24, 1976 (30).
Because the etiology of the disease was not immediately
apparent in the face of 182 cases, including 29 deaths, an
investigation of unprecedented magnitude was mounted. The
CDC of the Department of Health, Education and Welfare (now

the Department of Health and Human Services) spent many hours and millions of dollars in the pursuit of the cause of the disease. The Pennsylvania State Department of Health and other state and private laboratories also contributed to the gigantic effort to solve the mystery of this threatening illness. So great was public concern that U.S. Congressional hearings were held within four months after the eruption. The first hearing was called for the purpose of eliciting testimony from the experts concerning causative factors and to inquire into the handling of the investigation (45); in the second hearing, the experts focused mainly on the nature of LD in an effort to place the disease in its proper perspective (46).

Over four thousand legionnaires and their families attended the convention. While the convention ended on July 24 and the first death occurred on July 27, the relationship between attending the convention and acquiring a serious respiratory illness was not recognized until Friday, July 30, when Dr. Ernest Campbell of Bloomsburg, Pennsylvania, realized that he was treating three legionnaires for a similar febrile illness. At the same time, in a hospital in Chambersberg, Pennsylvania, an infection control nurse, Mrs. Geneva Baxter, noted a similar illness in three other legionnaires who had attended the convention. An attempt was made to report these findings to state

health officials; however, offices were closed for the
weekend (56). It was on Monday, August 2, that the news
of this serious illness reached the state and federal
public health officials. However, by this time, 18 of the
29 deaths associated with the outbreak had occurred, and
all but 16 of the 182 cases were symptomatic (131).

Five and one-half months after the Philadelphia out-
break, the CDC published a special issue of the Morbidity
and Mortality Weekly Report (32) in which it was announced
that Dr. Joseph McDade, a CDC microbiologist, had isolated
a gram-negative bacterium from lung tissues of fatal cases
of the disease employing a technique generally used to iso-
late riskettsial agents.

The techniques employed by McDade and co-workers were
those in regular use in CDC's Leprosy and Rickettsia Branch
(140). Adult male guinea pigs (weighing approximately
600 g) were inoculated intraperitoneally (i.p.) with 1 ml
of freshly-recovered autopsy tissues suspended in sucrose-
phosphate-glutarate buffer, pH 7.2. Animals were monitored
by daily rectal temperatures, and those that became ill were
killed on the second or third day after the onset of fever.
Pieces of spleen, liver and lung were ground with alundum
in a mortar and pestle, and 10 percent suspensions of each
were prepared in the buffer. Aliquots of these suspensions
were tested for the presence of bacteria by inoculation

onto trypticase soy agar (TSA) and sheep blood agar (SBA) plates and into tubes of thioglycollate broth. Portions of the tissue suspensions were inoculated into the yolk sac of six- to seven-day old embryonated hen's eggs from antibiotic-free flocks. Samples of the yolk sac from those embryos that had died after the third day were stained by the method of Gimenez (88) and examined microscopically. The stained yolk smears revealed slender, irregular rod-shaped bacteria.

A bacterium-like agent was found in the lungs of guinea pigs injected with suspensions of lung tissue col-lected at autopsy. Three isolates were from patients classified clinically and epidemiologically as having LD. In all successful isolation attempts, a febrile illness characterized by watery eyes and eventual prostration developed in the guinea pigs. Impression smears of guinea pig liver and spleen obtained on the second day of fever, stained by the Gimenez method, contained scattered bacilli. An exudate containing numerous bacilli was observed in the peritoneum.

Spleen, liver and lung tissue suspensions from the affected guinea pigs, when inoculated into embryonated eggs, caused death in four to seven days. Eggs injected with spleen tissue died before those injected with tissue from the liver or lungs suggesting that the spleen contained a

greater concentration of the agent than the other two organs.
The organism did not grow on trypticase-soy agar or sheep
blood agar or in thioglycollate broth; it did grow in
Mueller-Hinton agar (BBL) supplemented with hemoglobin and
"IsoVitaleX" (adenine, p-aminobenzoic acid, cocarboxylase,
L-cystine, glucose, nicotinamide adenine dinucleotide,
ferric nitrate, glutamine hydrochloride, guanine hydro-
chloride, thiamine hydrochloride, and vitamin B_{12}). Also,
it was discovered that for primary isolation a 10 percent
CO_2-air mixture was essential; the optimal temperature of
incubation was 35°C.

The isolate was confirmed as the causative agent by
demonstrating antibodies to it in sera from LD cases.
Investigators at the CDC soon recognized similarities
between the Philadelphia outbreak and two previous out-
breaks of febrile disease of unknown etiology. The cause
of these earlier outbreaks, occurring in 1965 at St.
Elizabeth's Hospital (Washington, D.C.) and in 1968 in a
local public health office building (Pontiac, Michigan),
had not been determined despite extensive investigation
(89, 192). However, by demonstrating antibodies against
the newly isolated LD agent in convalescent sera that had
been stored during those nine or twelve years, the cause
of these outbreaks was thus diagnosed, retrospectively
as LD (32, 140).

Because the etiology of LD was unknown at the time of
the outbreak in Philadelphia, the CDC used clinical and
epidemiologic criteria to define cases. Naturally,
attendance at the convention was included in the definition
(31). Initially, many members of the American Legion
opposed the use of the organization's name in referring
to the illness, feeling that it might discredit their
group. Eventually, the Pennsylvania American Legion voted
to accept the term, "Legionnaires' disease," when referring
to this illness. On April 8, 1977, the CDC referred to the
illness as Legionnaires' disease (33). In reports on sub-
sequent outbreaks, which were identified with increasing
frequency, it became the practice to include the name of
the city, state, or country in which the outbreak occurred,
e.g. Legionnaires' disease-Ohio (34).

Legionnaires' disease is now the commonly used name
of the infection caused by Legionella pneumophila, the
name proposed in 1979 by McDade (21) for the gram-negative
bacterium which he had isolated from lung tissues of vic-
tims of the Philadelphia outbreak two years earlier (32).
In subsequent years, other species of legionellae were
isolated. These will be described in the next section.

The Nature of the Legionella Bacteria

The etiologic agent of LD, Legionella pneumophila, was
found to be sufficiently unique to be classified in both a

new genus and a new family (Legionellaceae) (21, 131). It is a pleomorphic, obligately aerobic, gram-negative bacillus, the morphology of which varies considerably depending upon cultural conditions. In both human lung tissue and the egg yolk sac, the Legionnaires' disease bacterium (LDB) is predominantly bacillary, although coccobacilli and filamentous forms are commonly encounted. On bacteriologic media, early forms are bacillary, but with age become highly filamentous. By scanning electron microscopy, some individual organisms are found to have a slightly swollen appearance with rough, cigar-shaped ends (131); also, polar and bipolar flagella and pili of varied morphology have been observed. Multiplication is by transverse binary fission (175, 176, 193).

Morphologic identification of LDB in tissues is difficult because conventional histopathologic stains for microorganisms, including Brown-Brenn, Brown-Hopps, Giemsa, Gomori methenamine silver, Gridley's fungus stain, periodic acid-Schiff, and acid-fast methods have been negative or unreliable (37). On the other hand, cells in gram-stained smears of LDB from artificial culture media appear bright red when counterstained with carbol fuchsin or very faintly pink when counterstained with safranin-O.

The Gimenez stain, originally designed for the identification of rickettsiae, is very useful when applied to

crude preparations of tissue or to smears of exudates or to
infected yolk sacs as well as to suspensions of organisms
taken from agar plates. With this method, slide prepara-
tions are air dried, briefly heat-fixed, and then stained;
bacteria appear bright red against a bluish green back-
ground.

Even though LDB is a gram-negative bacterium, it stains
poorly by the conventional gram stain (37), particularly in
tissue sections, because it is readily decolorized and does
not take up much of the counterstain. However, the use of
a "half-gram" stain has produced much better visualization
of LDB (52). When terminating gram's procedure upon appli-
cation of the mordant (Lugol's iodine), the organisms will
appear as highly visible, purple-black bacilli in the
tissues. This procedure is recommended when no bacteria
are visualizable following the conventional gram stain.

One of the most sensitive methods for detecting LDB in
tissue sections is the Dieterle Silver Impregnation (DSI)
stain (14). With it, the bacteria are seen as short, pleo-
morphic, occasionally vacuolated, brown to black rods. The
most useful and reliable means of identifying LDB, espe-
cially in tissues or exudates, is the application of
fluorescently-labeled antibodies (FAB) prepared from
hyperimmune rabbit antisera (143).

Because of its unusual staining properties, LDB should
be suspected when appropriate tissues from pneumonia patients
stained by gram's method reveal no organisms but with a
"half-a-gram" or DSI stain show many pleomorphic bacilli.
More conclusive is the FAB procedure, although so many new
serotypes are being encounted that its value is diminishing
(145).

When grown on Mueller-Hinton agar supplemented with
iron and cysteine and observed under the electron micro-
scope, the LDB is found to have an ultrastructure typical
of gram-negative bacteria. The cell is surrounded by two
complete trilamellar membranes, each composed of two
electron-dense layers which are separated by an electron-
translucent layer (72). The two types of cell membranes
from intact cells and from sonicates of spheroplasts were
separated by sucrose density gradient sedimentation by De
Petris (53) into two fractions; one denser fraction was
found to contain a high concentration of 2-keto-3-deoxy-
octonate, a compound characteristic of endotoxins in the
outer membrane of gram-negative organisms, and a lighter
fraction which contained most of the succinic dehydro-
genase activity found principally in the cytoplasmic or
inner bacterial cell membranes. Also, De Petris found the
peptidoglycan to be similar in composition to that of most
gram-negative bacteria, except for the absence of

diaminopimelic acid (DAP), a usual constituent of the tetra-
peptide of gram-negative peptidoglycans. Thus, absence of
DAP in the peptidoglycan would appear to constitute a
significant departure from typical gram-negative peptido-
glycans. Later, however, DAP was isolated, in concentrations
typical of gram-negative behavior, from various strains of
L. pneumophila, all in serogroup 1 (94).

The nature of the lipids of the envelope of LDB has
received particular attention. Finnerty and his colleagues
(71) noted the following characteristics to be shared gen-
erally by a broad spectrum of LDB strains: (a) similar, if
not identical, simple and complex lipids; (b) high phos-
phatidylcholine content, usual among gram-negative bacteria;
(c) high total phospholipid content, indicative of intra-
cellular membrane development; (d) unusually fatty acid
composition dominated by branched-chain fatty acids; and,
(e) neutral lipid composition similar to other gram-
negative bacteria.

Others have characterized the total cellular fatty
acid content of legionellae and found it to contain a
high proportion of branch-chain fatty acids (151, 158).
These include iso- and anti-iso-monounsaturated and
saturated fatty acids as well as a C_{17} cyclopropane-
terminated fatty acid. Mono- and dihydroxy fatty acids
also have been detected by Mayberry (138) in legionellae;

they are likely to be bound to peptidoglycan. He suggested that these fatty acids are β-hydroxyisomyristic acid and β-hydroxyarachidic acid.

Branched chain fatty acids are generally restricted to gram-positive bacteria (90, 185) and are the predominant form of fatty acids in the Bacillaceae, Micrococeaceae and Actinomycetales. The occurrence of branched chain fatty acids as major fatty acids in gram-negative bacteria has been documented only in a few species, including the thermophilic bacteria Thermus flavis, Thermus aquaticus and Flavobacterium thermophilum (160). Desulfovibrio gigas, an anaerobic sulfate reducer, was also found to contain branched chain fatty acids (137). The presence, in major amounts, of branched-chain fatty acids, coupled with the absence of other fatty acids usually prominent in other gram-negative bacteria, results in a unique fatty acid profile for LDB (131).

Endotoxin, a universal and biologically-active constituent of the outer membrane of gram-negative bacteria is the lipopolysaccharide (LPS) constituent in phenol extracts (209). According to Wong (218), the "endotoxicity" detected in LDB by in vitro and in vivo biological assays seemed to differ in several respects from the classic endotoxicity generally associated with gram-negative organisms. For example, with endotoxins from Salmonella,

Klebsiella, Escherichia, and other gram-negative bacteria, the Limulus lysate test is some 10 to 20 times more sensitive than is the rabbit pyrogen test (219, 168). With LDB endotoxin, however, the Limulus lysate test is 1,000 times more sensitive than the rabbit pyrogen test. This difference in biological responses raises the possibility of a unique LDB endotoxin (218). Wong et. al. in 1973 (220) found that endotoxins of a low order of toxicity respond better to potentiation by dactinomycin than do the more highly active endotoxins. Also, it was shown in 1967 (170) that polymyxin B reduces the toxicity of endotoxin by complexing to the lipid A moiety. When LDB endotoxin was treated with the later antibiotic, its toxicity was not affected as measured by dactinomycin potentiation (218). Friedman et al. (85) reported that LDB endotoxin has greater adjuvant activity than that of E. coli. Also, as is typical of all endotoxins, he found LDB endotoxin to be relatively heat stable.

Moss and his colleagues (151) have shown cellular fractions of LDB to contain branch-chain fatty acids; no hydroxy-fatty acids, as associated with classical endotoxin, were found. Unlike the hydroxy-fatty acids of lipid A, LDB branched-chain fatty acids could be extracted with chloroform without prior saponification. In one study (86) of the effects of LDB endotoxin on leucocyte procoagulation activity, LDB-LPS, extracted by the Westphal (209)

procedure, was found in both the aqueous and phenolic phases of extraction, unlike endotoxin from other bacteria which are found only in the phenolic phase. The later study was directed at the matter of abnormal clotting time and thrombotic complications seen in legionellosis. The foregoing findings suggest that the endotoxin of LDB is unique, chemically and biologically.

Nevertheless, LDB endotoxin would seem to be a true endotoxin since it is pyrogenic, causes coagulation in the Limulus lysate assay, has procoagulating activity and is probably the substance responsible for vascular complications in legionella pneumonia patients. Thus, the legionellae possess most of the _in vivo_ and _in vitro_ biological properties associated with endotoxins found in typical gram-negative bacteria. Also, the morphological evidence of an outer membrane, as revealed by electron microscopy (122), would imply a functional endotoxin in LDB.

The formal classification of the legionellae was established in November 1978 at the First International Symposium on Legionnaires Disease (6). At that time the family name _Legionellaceae_, the genus _Legionella_, and the single species _Legionella pneumophila_, were introduced (21). As early as 1947, a bacterium resembling _L. pneumophila_, had been deposited unnamed in the American Type

Culture Collection (141). By 1979 four serogroups had been detected by direct immunoflourescence (DIF) (208). Since the International Symposium on Legionnaires' Disease held in 1979 a total of eight serogroups (12, 13) and ten species of Legionella pneumophila have been described (99, 151). Members of the genus Legionella characteristically are oxidase and catalase positive, hydrolyze hippurate, utilize α-ketoglutarate, pyruvate, and certain amino acids as sources of carbon, produce a hemolysin active upon guinea pig, horse, rabbit and dog erythrocytes and possess a distinctive pattern of predominantly branched-chain fatty acids as revealed by gas chromatography (152). DNA hybridization is the method by which speciation is unequivocal. Brenner (121) states, " . . . this DNA relatedness, as determined by hybridation, is the definitive means by which new Legionella species and serogroups are classified."

Legionella-like organisms (LLO) have been encounted with increasing frequency since 1979 and are still being found. The LLO are defined as gram negative bacteria that do not meet the definition of L. pneumophila but will grow on at least one agar medium designed for the growth of L. pneumophila but fail to grow on other commonly used laboratory media (22). These LLO include two isolates recovered many years before LD was known; L. bozemanii isolated in 1959 (16, 22) and L. micdadei isolated in 1943 (99, 190).

Others include the "Pittsburgh pneumonia agent," L.
dummoffii, isolated from soil on a creek bank (150), L.
longbeachae, isolated from human lung tissue and trans-
tracheal aspirates (144), L. jordanis (40) from natural
water, L. oakridgensis (158), also isolated from water but
not implicated clinically, L. wadsworthii, isolated from
a patient with pneumonia (60), and L. feeleii, isolated
from an oil-water cooling mixture in an automobile assembly
plant in Canada (102). Serological evidence implicated the
later strain as the causative agent of an outbreak of a
disease resembling Pontiac fever in that plant.

Recently, Brenner and associates (23) have isolated
and characterized ten new species of Legionella on the
basis of biochemical reactions, antigens, cellular fatty
acids, isoprenoid quinones, and deoxyribonucleic acid
relatedness. The genus Legionella is characterized as
gram-negative, rod-shaped, motile bacteria. All the spe-
cies exhibit certain biochemical reactions typical of
legionellae, e.g. growth on buffered cysteine-yeast extract
agar, but not on blood agar; requirement for cysteine;
nonfermentative; catalase positive; production of a brown
pigment on tyrosin-containing yeast extract agar; and
liquefaction of gelatin. All species have a comparable fatty
acid content and have major amounts of ubiquinones with
more than ten isoprene units in the side chain. Each of

new species characterized is serologically distinct from
previously described Legionella species.

Pathogenesis and Pathology of Legionellosis

How L. pneumophila actually enters the lungs is not
known with certainty, but direct inhalation is more likely
than aspiration (38). Adherance of L. pneumophila to
various sites in the respiratory tract has not been studied
in detail. Once the organism enters the lungs, the disease
occurs primarily in the alveoli and terminal respiratory
bronchioles (213). The inflammatory response is character-
ized by a varying mixture of intra-alveolar macrophages and
polymorphonuclear leukocytes, resulting in a consolidating
type of pneumonia (100, 212).

Two distinct patterns of illness caused by L. pneumo-
phila have been observed. Legionnaires' disease as it
occurred in Philadelphia in 1976 (51) and elsewhere (65),
is a multisystem illness involving the gastrointestinal
tract, kidney and central nervous system; however, pneumo-
nia is the most prominent feature in most cases. In
Philadelphia, the incubation period averaged five to six
days and the fatality rate was 15 percent. In sharp con-
trast is "Pontiac fever," reported in 1968 as a single
outbreak that affected some of the personnel working in the
building housing the Oakland County Health Department in

Pontiac, Michigan (89). Pontiac fever was characterized by fever, headache, myalgia, cough, diarrhea, vomiting and chest pain; mild sore throats were observed in some of the patients. No pneumonia was seen although one patient had a pleural friction (89). The incubation period averaged thirty-six hours; none of the cases was fatal.

The histopathology of pneumonia caused by L. pneumophila is characterized by a cytoclastic picture with necrosis of mononuclear and polymorphonuclear cells in the alveolar inflammatory exudate (4). The detailed pathogenesis of this lesion has not been elucidated. One possible mechanism suggested by Baine (4) could involve the release of bacterial cytotoxin acting on host cell membranes.

Baine and his coworkers (5) in 1979 reported that cultures of L. pneumophila lyse guinea pig, horse, rabbit and sheep erythrocytes in an agar medium, whereas human erythroctyes are less susceptible. In an earlier study, Turner (200) in 1958 showed that erythrocytes from guinea pigs are particularly sensitive to hemolytic substances because of a higher content of lecithin in the membranes than found in erythrocytes of other species. Baine also reported seven species of Legionella to possess cytolytic activity that can be detected using dog erythrocytes as the target cells. Furthermore, he discovered legionellae to produce a "cytolytic exotoxin" and a phospholipase C,

both of which he felt might contribute to the pulmonary
lesions seen in infected hosts. It has been suggested that
Pontiac fever resulted from the acquisition of a large dose
of non-toxigenic legionella; however, no differences of
obvious importance, including toxin production, have been
found in guinea pig susceptibility tests between strains of
L. pneumophila that cause classical Legionnaires' disease
and the strain responsible for Pontiac fever (110). Eickhoff
(65) has suggested that Pontiac fever may have resulted
from exposure to a large number of dead L. pneumophila.
The Pontiac outbreak was very likely airborne since a guinea
pig exposed in an air duct of the health department's
facility became sick. Its tissues were frozen and some
years later workers at CDC isolated a new legionella
organisms, subsequently identified as L. feeleii (131).

Laboratory studies have shown L. pneumophila to be an
intracellular parasite able to grow within a variety of
mononuclear cells (105). While neither polymorphonuclear
leukocytes (PML) nor monocytes kill L. pneumophila very
well in vitro (105), activated monocytes are more effec-
tive than PMLs; hence, cell-mediated immunity has been
implicated in host defenses to LD (106, 148). Patients
with dysfunctions of the cellular immune system, whether
from therapy to underlying disease, are at high risk of
developing LD (107). It has been reported that serum from

LD patients promotes phagocytosis by human monocytes in vitro (201). Horwitz and Silverstein (105) speculated that the promotion of phagocytosis in the absence of effective active immunity might promote growth of the bacterium and, hence, more severe disease. Recurrent LD has occurred in patients with pre-existing antibody, suggesting that LD-specific antibody is not protective (146), thus lending further weight to delayed hypersensitivity as the important immune response. Pathology from this disease, with rare exception, is confined to the lungs and pleura (213). In unsuccessfully treated cases, acute inflammatory consolidation of airspaces occurs (214). In most fatal cases there is extensive involvement of both lobes (214). The uniform distribution of the lesions throughout the lobes of the lung, from apex to base, is said to be compatible with an inhalation origin; this does not rule out aspiration as the mechanism (214). Lung tissue on autopsy has been found to be granular, friable and gray in color, reflecting extensive alveolar consolidation (213). Neutrophils, macrophases, proteinaceous debris and extensive deposits of fibrin have been reported to be the principal constituent of the infiltrate (214). Abcess formation, which is prominent in pneumonia due to gram-negative bacteria, is not always a feature of legionella pneumonia (164, 132).

Clinical Features of Legionnaires' Disease

Legionellosis is a general term reserved for all the clinical syndromes caused by organisms classified within the genus Legionella. Legionnaires' disease is the pneumonic form of legionellosis caused by L. pneumophila; in contrast, Pontiac fever is the self-limited nonpneumonic, respiratory illness caused by the same organism (11).

The incubation period of the more common pneumonic form is approximately two to ten days, but it may be longer if interrupted with antibiotic therapy, or it may be even shorter in immunosuppressed patients (148). The manifestation of disease ranges from asymptomatic seroconversion to mild pneumonia with multisystemic disease to severe pneumonia (78, 148). As described by Edelstein and associates (63), the onset of pneumonia is often gradual, but it may fulmmate, especially in immunosuppressed patients. A prodome of malaise, anoerxia, lethargy, weakness, and occasionally clumsiness occurs at first. Symptoms of upper respiratory tract involvement are usually absent at this stage (78, 148). Most patients have a dry, nonproductive cough which begins during the first few days of illness. In most patients, recurrent chills appear. If sputum is produced, it may be purulent in some and bloody in others. In one study, half of the patients exhibited a painless watery diarrhea, headache was common and about

95 percent of the patients produce a fever, sometimes as high as 104°F. The central nervous system may be involved as manifested by confusion, disorientation, agitation, hallucinations, stupor or other related symptoms (123).

Laboratory Diagnosis

In the evaluation of patients with infection, successful isolation of the causative agent is important because it provides a sound basis for diagnosis and thus for effective management. At the time of the Philadelphia epidemic, however, no diagnostic tools were available. Fortunately, two antibiotics, erythromycin and terramycin, were found by chance to be effective at that time (131).

Since its first appearance in 1976, considerable progress has been made in the laboratory diagnosis of LD (58, 61, 216). As already discussed in this historical review, one of the major advances has been the improvement of culture media for the isolation of LDB. Direct and indirect fluorescent antibody (FA) methods for detection of L. pneumophila in sputum or of antibodies in the serum have proven to be helpful in establishing an early diagnosis but still pose problems of both sensitivity and specificity. Detection of soluble protein antigens in urine has been reported to be diagnostically useful and may even supersede some of the other diagnostic procedures (62).

Taylor, et. al. (191) reported success in the diagnosis of LD with an indirect fluorescent antibody test using a formolized yolk sac antigen recovered from eggs injected with the Pontiac strain of L. pneumophila (now named L. feelei). More recently, Harrison and Taylor (97) described a semiautomated rapid microagglutination test. Others have reported that both tests have good specificity (191).

The initial successful effort to grow LDB on a solid medium was achieved with the commercially-produced mueller-Hinton medium (Difco Laboratories) supplemented with iso-VitaleX and hemoglobin (MHIM (131). This medium was improved by the replacement of hemoglobin with soluble ferric pyrophosphate and isoVitaleX with L-cysteine-HCL by Feeley and Gorman (F-G agar) (69).

The next improvement of a medium for the legionellae was the formulation of charcoal yeast extract (CYE) agar containing L-cysteine-HCL and soluble ferric pyrophosphate (70). In comparison with F-G agar, CYE agar reduced the time of incubation for the appearnce of visible colonies by some twelve hours; furthermore, CYE agar supported 100-fold more visible colonies than did F-G agar inoculated similarly (70). The development of the CYE medium (70) and, later, of the buffered CYE medium (BCYE) by Pasculle and his colleagues (162), has promoted both routine isolation of L. pneumophila from clinical specimens and laboratory studies on the legionellae. The addition of α-ketoglutarate

to BCYE medium (αKCBCYE) improved growth even more (59).
Also, the addition of certain antibiotics to BCYE medium
was found to enhance the selective isolation of L.
pneumophila and some other legionellae from sputum (121).

Two groups of investigators developed a chemically-
defined liquid medium for L. pneumophila (165). The
medium of Pine et. al. contains minerals, α-ketoglutarate,
pyruvate, L-cysteine, glutathione, and twenty amino acids.
Maximum growth in this medium was achieved in a 5 percent
carbon dioxide-air atmosphere. Pine concluded that LDB,
which is a strict aerobe, required carbon dioxide for meta-
bolic purposes as well as for its buffering action. The
other chemically-defined liquid medium, devised by Warren
and Miller (204) contains twenty-one amino acids and
inorganic salts, including ferric pyrophosphate, and could
be employed both as an agar or as a liquid; the requirement
for L-cysteine, L-serine and L-methionine was found to be
absolute.

A plethora of methods has been introduced for the
serological diagnosis of legionella infection. The indirect
FA method is still the reference method, as it is very well
standardized (67, 68, 96, 210). Rapid diagnosis of LD often
can be accomplished within a matter of hours by using the
direct FA technique for the detection of bacteria in clin-
ical specimens. Likewise, the rapid detection of soluble

LDB antigens in urine by either radioimmunoassay (RIA) (58, 216) or enzyme-labeled immunoassay (ELIA) (118, 181) has been successful. The tests for soluble antigen in urine are very specific, with about 80 percent sensitivity, approximating that of the direct immunofluorescent test (181). Direct immunofluorescent examination of sputum for L. pneumophila is very specific, but the likelihood of a successful isolation from sputum, even with αKGBCYE agar, is poor (118). Complicating the picture is the wide prevalence of LDB antibodies in the general population. The interpretation of a positive diagnostic test benefits from correlation with the occurrence of local outbreaks. Single, isolated cases are more difficult to diagnose.

By means of reactivity with antisera from rabbits (84), guinea pigs (84) or mice (84) injected with formalin-treated LDB, the legionellae have been placed in eight serogroups; however, strain differences exist within groups, sometimes leading to difficulty in allocation of isolates to a specific group. The introduction in 1975 by Kohler and Milstein (127) of cell fusion for the production of monoclonal antibodies of defined specificity has revolu-tionized antigenic analysis. The application of monoclonal antibody offers an alternative, one that is clear and reproducible, with the prospect of more reliable standards than presently exist (205). Thus, serogrouping of bacteria

(42) and viruses (128), previously indistinguishable with the usual polyclonal animal antisera, have been reported. Using monoclonal antibody techniques, Joly (117), has defined five subtypes of L. pneumophila serogroup 1.

In his study on the production of monoclonal antibody to L. pneumophila and its relationship to plasmids, Para et al. (161) coupled LDB-specific monoclonal antibodies which recognize subsets of L. pneumophila with analysis of several plasmids to provide markers for strains of serogroup 1. These markers should be useful in epidemiological studies of LD and in understanding environmental colonization by legionella (143).

Guillet et al. (95) recently developed an anti-legionella serotype 1 monoclonal antibody. Application of this antibody enabled the detection in clinical samples (bronchoscopic and urine) of the homologous antigen. It is to be hoped that the use of this monoclonal antibody would enable the diagnosis of legionellosis in the early steps of the disease. Once a battery of monoclonal antibodies is available, we may learn more about the specific LDB antigen responsible for the immune response.

Watkins and Tobin (205) have shown that monoclonal antibodies appear to be superior to whole sera for the accurate and reproducible serogrouping of legionella strains and should be useful for diagnosis and environmental

studies as well as for the analysis of the structure of LDB's cell envelope.

Epidemiology

In the 1976 seminal Philadelphia outbreak of LD, there was a high correlation of febrile respiratory disease and attendance at the American Legion Convention. The outbreak involved, in addition, a few others staying at the headquarters hotel, as well as about twenty persons who had walked within a block of the hotel but who did not enter it; however, the outbreak did not spread beyond the hotel site. With 182 cases and 29 deaths among the 4,400 conventioneers between July 27 and August 16, the occurrence can be considered to be an epidemic (78).

Infections caused by legionellae have been shown to be transmitted from contaminated aqueous sites, such as air conditioning cooling towers (55), evaporative condensers (80), shower heads (197), and water nebulizers (80). Also dust from the soil has been strongly implicated (76).

Common source outbreaks have been observed in four clinical-epidemiologic patterns. First are those of short-duration, with a low attack rate, high fatality rate, a relatively long incubation period and with clinical findings of fever and pneumonia. These are exemplified by the original outbreak in Philadelphia (78, 140) and by the

retrospectively identified one at Saint Elizabeth Hospital in Washington, D.C. (192). Second are those of short duration, with a high attack rate, a short incubation period, no pneumonia, and no fatalities, as encounted in Pontiac, Michigan (89) and in St. James, Virginia (80). Two patterns, less defined than the others, occur as intermittent common-source outbreaks of hyperendemic pneumonic or Pontiac-type disease, which at times may erupt into epidemic proportions in buildings, most commonly hospitals (10, 25, 123, 124).

The shape of the epidemic curve, with its rapid upswing and an incubation period of two to ten days, suggests a continuing common-source exposure. There is no evidence of spread from person to person. The mode of transmission of the organism was investigated extensively in the initial outbreaks. Epidemiologic evidence from the Philadelphia outbreak (78) suggests that the disease was not transmitted by ingestion of food or water nor by spread from person to person; rather, it was airborne. Similar findings were encountered by investigators of subsequent outbreaks of LD (47, 55, 89). The failure to implicate a common food served at the convention hotel, or sold by street vendors, or served at any of the local coffee shops or restaurants argues against a foodborne outbreak. The occurrence in the summer would be consistent with spread by an arthropod vector, but neither bites nor arthropods were found on the patients (131).

Airborne infection could explain the good correlation found between illness and the length of time spent in the hotel. Certainly an airborne agent could have affected the few non-legionnaire victims who stayed at the same hotel. Various agents were considered by the investigators of this outbreak; viruses, rickettsiae, bacteria, fungi and even toxic chemicals were candidates (131). The sudden outbreak in late July and its abrupt disappearance in mid-August lent weight to the airborne theory. Efforts were made to detect pneumonia-producing microorganisms in the local bird and rodent populations, as well as in samples of air, dust and soil (78).

From the earliest recognized outbreaks of legionellosis, strong epidemiological evidence has linked spread of the disease with certain types of water cooling systems which generate droplet clouds, and with domestic plumbing systems (170). Less than a year after the Philadelphia eruption, McDade et al. (140) had isolated the etiologic agent of LD from an autopsy lung specimen.

Inhalation as a mode of acquiring LD was supported by the work of Baskerville et al. (8, 9) who successfully induced experimental respiratory infection, similar to that of human legionellosis, in both guinea pigs and rhesus monkeys exposed to aerosols of LDB.

On three occasions (including the previous episode of Pontiac fever), common source outbreaks of legionellosis

have occurred in such a pattern as to suggest that they were caused by spread of droplet nuclei from aerosols generated from air conditioning cooling towers (35), evaporative condensers (36, 89), and respiratory devices (80). In one outbreak, LDB-laden aerosols were found in a chimney (7). The evidence that contaminating cooling towers may play a role in the transmission of legionellosis has raised serious concern in many circles over the environmental distribution of the organism and the feasibility of its eradication from certain kinds of equipment used in transporting domestic or fresh waters (41). Cooling towers known to transfer LDB should be disinfected routinely by a standard protocol (74). While there is reluctance to modify tried and true procedures of maintenance, surveillance of cooling waters by culturing them for legionellae is encouraged (74). However, some reports raise hope that chemical disinfection may solve this problem (77, 92, 129). Buildings equipped with cooling towers will continue to be an important source of this organism.

Studies of the distribution of legionellae in natural habitats have been confined primarily to fresh waters (75). On the other hand, Ortiz-Roque and Hazen (159) reported the isolation of L. pneumophila from rain forest areas of Puerto Rico and encounted legionella-like organisms in the Carribean Ocean. The distribution of legionellae in a

tropical habitat may well be different from that in the more temperate habitats thus far studied. Perhaps new serotypes or even new species of legionellae may be discovered in the tropics.

Therapy of Legionellosis

The efficacy of antimicrobial agents in the treatment of Legionnaires' disease has been assessed in various ways. Initially, retrospective analysis of the chemotherapy employed in several species of clinical cases was compared with the in vitro sensitivity of Legionella pneumophila to the antimicrobials administered. Clinical responses to antibiotic therapy and in vitro sensitivity patterns, however, were found by Fraser (79) to not always correlate. There are several reasons for this, including variable diffusion of the antibiotics in the test medium, the ongoing treatment of some of the patients with immunosuppressive agents, differences in the environment of the bacteria, LDB β-lactamase production, and failure of the antibiotic to reach the organisms in tissues in effective concentration (163).

Analyses of clinical cases have indicated that the drugs of choice for therapy of Legionnaires' disease are erythromycin and tetracyclines. Patients treated with these antibiotics have been shown to have a lower mortality

than those given other antibiotics (25, 127, 199). As

pointed out by Gibson, et al. (87), comparisons of dif-

ferent series of patients need to be interpreted with

caution since, inevitably, the groups are heterogeneous with

respect to age, sex, immunocompetence, concurrent disease,

strain of Legionella, duration of infection, severity of

lung lesions and nature of the therapeutic regimen.

Using the aerosol-infected guinea-pig model, Gibson and

associates (87) analyzed the efficacy of several antimicro-

bial agents in treating LD. Such a model provided informa-

tion not available from LDB-infected human cells or from

bacterial cultures in vitro. In these studies, the effects

of rifampicin, gentamicin and erythromycin on the number of

L. pneumophila in the lungs and on the nature of the

pulmonary lesions were tested. The above-mentioned anti-

biotics prevented death when administered after a low

infective dose (ILD_{50}); only rifampicin provided protection

against challenge with a high dose ($10LD_{50}$). Rifampicin

was also by far the most effective of the three drugs in

eliminating viable L. pneumophila from the lungs (87).

Results of therapy with tetracycline have been incon-

sistent, although most patients responded to it (78, 124).

The penicillins, aminoglycosides, vancomycin, clindamycin

and all the cephalosporins have no therapeutic value for LD

unless there is a mixed infection. In patients who are

critically ill, heavily immunosuppressed, or who have
pulmonary abcess due to Legionella, the use of rifampicin
in conjunction with erythromycin is recommended (63). How-
ever, those patients who are allergic to erythromycin
should be given the lipid-soluble tetracycline, deoxycy-
cline, along or with rifampicin. Other than relative cost,
a major objection to the use of rifampicin as the drug of
choice in LD is that one-step bacterial resistance to it
may develop, as has been shown to occur in Mycobacterium
tuberculosis cultures isolated from patients receiving this
drug (50). Also, at very high dosage, rifampicin has been
shown to be hepatotoxic and to have some immunosuppressive
effects (93).

Although Fraser and colleagues (79) reported that gen-
tamicin had no effect in preventing death of guinea pigs
infected intraperitoneally with L. pneumophila, Gibson and
his associates (87) reported that gentamicin cleared the
lungs of viable bacteria several days earlier than did
erythromycin.

Rodgers and Elliott (177) examined the effects of
ampicillin and erythromycin on the morphology and "regrowth-
ability" of L. pneumophila by means of thin-section negative
staining and scanning electron microscopy. Both antibiotics
caused cell lysis and induced the appearance of filamentous
forms along with surface defects. Viable cell counts showed

that ampicillin was more bactericidal than erythromycin; a
regrowth to a normal rate and restoration of normal mor-
phology occurred more rapidly in cells exposed to
erythromycin than in those exposed to ampicillin. To our
knowledge, ampicillin is not used in the treatment of LD.

The Gram-Negative Outer Membrane

All bacteria possess a cytoplasmic or plasma membrane
described by the well known fluid mosaic model (186) of a
lipid bilayer interspersed with proteins, mostly enzymes,
similar in structure and function to those of eucaryotic
cells but with some significant differences. The bacterial
cell membrane contains the system for active transport,
oxidative phosphorylation and the biosynthesis of such
unique bacterial macromolecules as peptidoglycan (20). All
gram-negative bacteria also contain the external or outer
membrane (OM) which, although morphologically similar to
the cytoplasmic membrane, contains a lower content of
phospholipids, fewer proteins and a unique lipopolysac-
charide (LPS) or endotoxin (48).

The nature and function of the outer membrane has been
reviewed extensively by Costerton (48), Inouye (114) and
DiRienzo (54). Essentially the outer membrane acts as a
diffusion barrier against various compounds, for example
antibiotics; it contains receptors for bacteriophages and

colicins; it is involved in the process of conjugation and also of cell division, or, more precisely, in septum formation; it contains various specific uptake systems for nutrients, such as iron, vitamins, and carbohydrates; and it contains specific and nonspecific passive diffusion pores of proteins ("porins") that allow the diffusion of low molecular weight substrates. The outer membrane also provides a protective environment for certain hydrolytic enzymes and binding proteins that reside in the periplasmic space--the region between the outer membrane and the thin layer of peptidoglycan--and participates, in conjunction with the peptidoglycan, in maintaining the structural integrity of the protoplast.

Since the outer membrane proteins (OMPs) serve diverse roles, and thus are crucial to membrane function and integrity, more information on the nature of these proteins is crucial to an understanding of outer membranes. Because of the relatively small number of OMPs, some of which are present in rather large quantities, they are easy to isolate for subsequent characterization (48).

Research on the synthesis and assembly of OMPs has been extensive since these proteins were first separated and identified in the late 1960s (20) and early 1970s (112). Thus, today, we have a fairly comprehensive picture of the nature of these OMPs. Investigation of the assembly of

proteins in the outer membrane reveal that some of them form oligomers with themselves or with other neighboring proteins, and are exposed on the surface of the cell while spanning the entire thickness of the outer membrane often forming micro-pores (54). Many of the minor and major OMPs have been identified as receptors for phages and bacteriocins. Most of them are now known to enable the permeation of specific nutritional substrates through the outer membrane; some of them have no known receptor function (18, 19).

In 1970, Schnaitman (183) recovered cell envelopes by passing Escherichia coli cells through a French pressure cell and separated them into two distinct particulate frac-tions by sucrose density gradient centrifugation. The outer membrane function was found to contain one major pro-tein with a molecular weight of 44,000 daltons which accounted for 70 percent of the total OMPs. Several years later, Inouye and Yee (112, 113), using 1 percent sodium dodecyl sulfate and various solubilization conditions, showed that Schnaitman's "major peak" (44Kd) was an arti-fact. Instead, they found several proteins migrating at different rates.

By now it has been established that Schnaitman's major OMP consists of at least four different components (20). In addition to these major OMPs of relatively high molecular weight, another major OMP, a lipoprotein, has been found to be present in E. coli (114).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (PAGE) is an extremely useful technique for the analysis of membrane proteins. Results must be interpreted cautiously since some of the protein bands may not be homogeneous. Furthermore, the composition and amount of OMPs may vary greatly depending on the bacterial strain and conditions of cultivation (111, 134, 184). The resolution and the order of migration of the major OMPs undergoing electrophoresis greatly depend on specifics of the sodium dodecyl gel system used, such as concentration of the acrylamide, extent of cross-linking, pH of the buffer, ionic strength, voltage and amperage and the way in which the sample is solubilized. In the outer membrane of E. coli are found some ten to twenty "minor proteins." The term minor protein is misleading since under certain growth conditions some of the minor proteins may be produced in major amounts.

Legionella pneumophila Outer Membrane

Interest in the cellular antigens of L. pneumophila has focused largely on endotoxin. Preliminary reports (73) have indicated that L. pneumophila OM material can be isolated by treatment of the organism with ethylenediaminotetracetic acid (EDTA)-lysozyme or by extraction with EDTA. Hindal and Iglewski (103) have suggested that the OM of L. pneumophila

presents a hydrophobic surface to the environment. In fact, recent studies on the crystal violet uptake and hexadecane affinity have shown that the surface of L. pneumophila is distinctly more hydrophobic than that of a wild-type Salmonella cholerasuis (15).

Recently, Hindal and Iglewski (103) and Ehret (64) reported the separation by PAGE of OMPs from a number of legionella species. One protein (29 ± 500Kd) was common to all of them. It was thought that such a protein could serve as a suitable antigen for a specific serological test in the diagnosis of legionellosis.

Statement of Problem

Since L. pneumophila is a gram-negative bacterium, it is presumed to contain OMPs; however, at the start of this study nothing was known of OMPs in legionellae. If they exist, no doubt the host's immune system would respond to them. While patients with LD reveal an active immune response to the eight endotoxins of this bacterium, cell-mediated immune responses to unidentified antigens also have been noted. The most obvious surface antigens in addition to endotoxin are the OMPs. Because so little is known about the immune response to L. pneumophila, because better diagnostic reagents are needed and because the surface proteins of L. pneumophila have not been investigated, the several objectives of this project were

1.   To separate and visualize by gel eletrophoresis the principal OMPs of L. pneumophila and to compare their distribution among the eight endotoxin serotypes.

2.   To assess in the rabbit the antigenicity of the OMPs from one of the serotypes of L. pneumophila.

3.   To assess by challenge with LDB the protective capacity of OMP antibodies through heterologous or homologous passive immunoprophylaxis in rats.

4.   To determine in the rat whether the OMPs of LDB play a role in cell mediated immunity.

CHAPTER II

MATERIALS AND METHODS

Study of Outer Membrane Proteins

Bacteria

This study was carried out with the eight serogroups of L. pneumophila which were kindly supplied by Dr. William Bibb of the Center for Disease Control, Atlanta, Georgia. These serotypes were represented by the following strains:

Serotype 1, Knoxville, Darby and Olda

Serotype 2, Togus

Serotype 3, Bloomington

Serotype 4, LA-1

Seroptye 5, DIAE

Serotype 6, Ottawa 3 (environmental)

and Chicago 2

Serotype 7, Chicago 8

Serotype 8, Concord 3

All these organisms were maintained by monthly transfer on slants of charcoal yeast extract (CYE) agar (131). Although elevated carbon dioxide is beneficial in the primary isolation of these bacteria, after prolonged laboratory propagation additional carbon dioxide becomes unnecessary.

Since the above strains were well adapted to the laboratory environment, we found them not to require elevated carbon dioxide. The incubation temperature employed was 35.5°C. The Chicago 8 strain (serogroup 7) was the only one used in the animal studies.

Growth Medium

Although several other media have been described (70, 172), our laboratory has consistently obtained excellent growth of legionnaires' disease bacteria (LDB) in CYE agar. To prepare the CYE agar, 10 g Difco yeast extract, 2.0 g activated charcoal (Sigma Chemical Co., St. Louis, MO) (washed with phosphoric and sulphuric acids) and 17 g Difco agar were added to 980 ml distilled water. After a short period of boiling, the mixture was autoclaved at 121°C for fifteen minutes. A solution of 0.40 g L-cysteine HCL in 10 ml of distilled water was filter-sterilized using a 0.22 μm membrane filter; it was added aseptically and gently mixed with the molten agar base. Also, a 2.5 percent solution of 0.25 g soluble ferric pyrophosphate (Sigma Chemical Co., St. Louis, MO) in 10 ml distilled water was filtered-sterilized and added aseptically to the agar base. The pH of the completed medium was adjusted to 6.9 by the addition of sterile 1N sodium hydroxide.

Preparation of Suspensions of
Legionella pneumophila

A fresh 24-hour culture on a CYE agar slant (after several successive transfers, 36 h apart) was used as the inoculum which was streaked on one or more plates of CYE agar. The plates were incubated at 35.5°C; when growth was abundant (generally within 36 to 72 hours), the cells were harvested by loop-transfer into a tube containing 10 ml of sterile 0.1 M Tris buffer, pH 8 and washed twice by centrifugation (Sorval superspeed RC2-B, Newton, Connecticut) in the buffer at 6,500 rpm for 10 minutes at 4°C.

Recovery of Outer Membrane from
Legionella pneumophila

After trying several methods (1, 104, 136) for releasing the outer membrane (OM) from L. pneumophila, the method of Irvin, et. al. (116) was adopted as it consistently yielded good preparations of OMPs.

The washed L. pneumophila cells were suspended in 10 ml of 0.1 M Tris buffer (pH 8), pelleted by centrifugation at 10,000 rpm for 10 minutes at 4°C, suspended in 10 ml of 0.01 M citrate buffer, pH 2.75, and then held for 30 minutes at room temperature. This was followed by centrifugation at 10,000 rpm for 10 minutes at 4°C and resuspension of the cells in 10 ml of 0.1 M Tris buffer pH 8 (room temperature). After a wait of 10 minutes at room temperature, the cells

were pelleted by centrifugation at 6,500 rpm for 20 minutes
at 4°C. The supernatant fluid, containing the OMPs, was
then centrifuged at a higher rpm (18,000) for 20 minutes
in order to remove any remaining cells, protoplasts or cell
fragments. The supernatant fluid was carefully decanted
into a sterile, chemically-clean, screw-capped test tube
and frozen at -20°C until needed. Proteins in the frozen
preparation remained stable for at least one month. The
protein concentration, as determined by the method of Lowry
et al. (133) employing bovine serum albumin as a standard,
was generally about 2 mg/ml.

Electron Microscopy

In an attempt to reveal that the treatment leading to
OMPs did not result in cell lysis but rather led to separa-
tion of the OM from the cells, the preparations at several
stages of the treatment procedure were examined by means of
electron microscopy, as recommended by Irvin et al. (115).
Cells from three phases (Tris, pH 8.0; citrate, pH 2.75;
and Tris, pH 8.0--after citrate) were fixed for 20 minutes
at room temperature in one-tenth volume of 5% (vol/vol)
glutaraldehyde in 67mM cacodylate buffer (Sigma Chemical
Co.), pH 6.7. The fixed cells were pelleted by centrifu-
gation (12,000 rpm for 10 minutes at 4°C), and resuspended
in the glutaraldehyde-cacodylate buffer at room temperature.
One and one-half hours later, the fixed cells were pelleted

as before by centrifugation and then suspended in one drop

of molten 2% Noble agar (Difco) at 45°C. Upon cooling at

room temperature, the congealed agar was washed five times

consecutively for 10 minutes each with the cacodylate buffer.

The samples were then postfixed with 2% osmium tetraoxide

(Polysciences, Inc., Warrington, PA) for two hours at room

temperature. After five washes (10 minutes each) in the

cacodylate buffer, the specimens were dehydrated in a series

of acetone concentrations (30 minutes each in 30, 50, 70, 90

and 100% acetone). To prepare these solutions, freshly-

distilled acetone was diluted to 30, 50 and 70% in 67mM

cacodylate buffer (pH 6.7); 90% acetone was made by diluting

100% acetone with distilled water. The specimens were then

washed twice (20 minutes each) at room temperature in 100%

propylene oxide (Polysciences, Inc.) and subsequently

embedded in a resin, "Vestopa W" (Polysciences, Inc.). Sec-

itons were cut using a Porter Blum ultramicrotone (MT-2,

Sorval) and placed on grids (Polysciences, Inc.).

The sections were stained with lead as described by

Reynolds et al. (169). Lead citrate was prepared by

placing 1.33 g Pb(NO$_3$)$_2$, 1.76 g sodium citrate and 30 ml

distilled water in a 50-ml volumetric flask. The resultant

suspension was shaken vigorously to insure complete conver-

sion of lead nitrate to lead citrate. After 30 minutes,

8 ml of NaOH was added and the suspension was diluted to the

50-ml mark with distilled water and mixed by inversion. The
lead citrate was thus dissolved and the solution, pH 12,
was ready for use.

Grids with their sections were stained by floatation
on a single drop of staining solution contained in a dental
wax-coated Petri dish. The specimens in the dish were
protected from the light during the 20-minute staining expo-
sure. Thereafter, the grids were washed sequentially in
jets of 0.02 N NaOH and distilled water from plastic wash
bottles and allowed to air-dry.

All samples were examined with a Siemens Elmiskop 101
microscope operating with an accelerating potential of
80KV. The use of this microscope, including vital help,
was kindly provided by Dr. Louise Higgins, Texas Woman's
University, Denton.

Gel Electrophoresis

Sodium dodecyl sulfate discontinuous polyacrylamide
gel electrophoresis (SDS-PAGE), as described by Neville and
Glossman (154), was utilized for fractionation of the OMPs
contained in the Tris buffer solution.

Preparation of gels.--The running gels were prepared
in a buffer of 0.0308 N HCl and 0.4244 M Tris, pH 9.18,
contained in a side arm flask. To 8.8 g acrylamide and
0.05 g bis-acrylamide was added sufficient buffer to bring

the volume to 100 ml; 0.15 ml of N',N',N',N'-tetramethyl
ethylenediamne (TEMED) was then added. Deaeration was
accomplished by attaching a rubber tubing from a side arm
flask to a vacuum pump. After deaeration (15 minutes),
0.05 g of ammonium persulfate was added. The solution was
dispensed into 10-cm electrophoresis tubes (Bio Rad Labs.,
Richmond, CA). Approximately one hour was required for
the gel to solidify at room temperature.

The stacking gel was prepared using a buffer of
0.0267 M H_2SO_4 and 0.0541 M Tris containing 3 g acrylamide
and 0.2 g bisacrylamide per 100 ml; 0.15 ml TEMED was
added. After deaeration (15 minutes), 0.05 g ammonium
persulfate was added and the solution was placed, by means
of a Pasteur pipette, on the top of the running gel.

The upper reservoir buffer (cathode) contained 0.04 M
boric acid, 0.041 M Tris and SDS (1%), pH 8.64. The lower
reservoir buffer (anode) was the same as running gel buffer.

Determination of molecular weights of proteins.-- To
0.5 ml (100 µg) of the OMP preparation in Tris (pH 8.0) was
added 10 mg sodium carbonate, 40 mg of SDS and 100 µl of
2-mercaptoethanol. This mixture, contained in a small
glass tube (5 mm diameter), was placed in a boiling water
bath for five minutes. The protein solution was applied to
the running gel tubes in various volumes, and the loaded
gels were then electrophoresed (Bio-Rad Labs., Richmond,

CA) with a constant current of 48 mA for approximately three hours. Gels were stained subsequently with coomassie brilliant (R250) blue (Sigma Chemical Co.) for one hour and destained with a solution of acetic acid (8%), ethanol (25%) and water (67%). Molecular weights were assigned as described by Weber and Osborn (206), utilizing a Dalton Mark IV, SDS molecular weight marker kit (Sigma Chemical Co.) containing the following proteins: bovine albumin (66,000 daltons), egg albumin (45,000), pepsin from porcine stomach mucosa (34,700), trypsinogen from bovine pancrease (24,000), lactoglobulin from bovine milk (18,000), and lysozyme from egg white (14,300). The extracted OMPs were electrophoresed side by side with the standard. The standard was employed at a level of 25 μg total protein. The relative mobilities of the standard proteins and of the OMPs were then measured. A plot of molecular weights versus relative mobilities of the standard was made on semilogarithmic graph paper; the molecular weights of the OMPs could then be ascertained from the straight line standard curve.

Animal Studies

Lethal Dose Determination

Following several closely spaced transfers on CYE agar slants, L. pneumophila, serogroup 7 (Chicago 8) was streaked

on Petri plates of CYE agar. When growth was abundant (36-48h at 35.5°C), the cells were suspended in sterile saline and serial decimal dilutions, from 10^{-1} to 10^{-10}, were prepared in tubes containing 9 ml sterile saline. The optical density of each test tube was measured at a wavelength of 620 nm in a Spectronic 20 spectrophotometer (Bausch and Lomb, Rochester, NY). The instrument was zeroed with sterile saline.

Spread plate counts, in triplicates, were prepared from each dilution by placing 0.1 ml on the surface of CYE agar plates. Without delay, the inoculum was spread over the agar surface with a sterile glass spreading rod and the plates were incubated at 35.5°C until colonies were clearly visible, and thus countable (generally three days). The plates were examined on a Quebec colony counter (American Optical Co., Buffalo, NY); the triplicate set with colonies numbering between 30 and 300 were counted. The colony count per ml was determined by multiplying the average number of colonies on the three countable plates by the reciprocal of the dilution they represented. The result obtained was referred to as the "colony forming units per ml" (cfu) (44, 66). Subsequently, a standard curve was constructed by plotting the spectrophotometer readings against cfus. Thereafter, turbidity readings could be converted directly to cfu.

From each dilution, one ml was injected intraperitoneally into each of five white rats. The rats were young male adults (200 to 300 g each); they were obtained from Dr. J. R. Lott's colony in the Department of Biological Sciences, North Texas State University. Before injection, the fur and skin were disinfected with alcohol. Five rats injected with sterile saline served as the controls. The rats were observed daily until no more deaths occurred. Most deaths occurred within three days. A few rats appeared sick for a couple of days but recovered. The highest dilution which killed all five animals was the end point. Since turbidity readings were made of each dilution and a standard turbidity/cfu plot had been constructed, the lethal end point could be converted to cfu.

Infection of a Rabbit with Legionella pneumophila Serogroup 7

A male New Zealand white rabbit, weighing approximately two kilograms, was injected intraperitoneally with approximately 1.5×10^6 Chicago-8 legionella cells freshly recovered in normal saline from the growth on CYE agar plates. Two days later the rabbit became lethargic and refused to eat. During the next five days, symptoms worsened and treatment with erythromycin was initiated. One hundred mg of erythromycin sulfate was injected intramuscularly twice a day (dose recommended by a local

veterinarian). The rabbit died two days later. An autopsy
was performed and samples of tissues of the heart muscle,
lung, gastrointestinal tract, brain, liver, spleen and
kidney were excised aseptically and fixed in formalin.
Prior to initiation of antibiotic treatment, 1 ml of
blood was recovered from the marginal ear vein; at autopsy,
one ml of blood was recovered aseptically from the heart.

Histology.--Tissues obtained from the rabbit were
fixed in 10% formalin for 24 hours and then placed directly
into 70% alcohol. The histologic methods employed were
those of Ann-Preece (3). For embedding, the tissue was
placed in an L-shaped plastic container and warm paraffin
poured, about midway, into the container. With a warm for-
ceps, the tissue was oriented in the bottom of the container
in the correct plane for sectioning. The mold was trans-
ferred to a chilled enamel tray in order to anchor the
tissue firmly in place and held flat with the forceps until
it maintained its position. The remainder of the paraffin
was poured on top of the mold and the mold was transferred
to the refrigerator to hasten hardening of the paraffin.

The paraffin-embedded sections were cut on a rotary
microtome (American Optical Co., Buffalo, NY). The tissue
blocks were chilled on a block of ice and mounted in the
vise of the microtome. The block was carefully adjusted to
the microtome so that it remained behind the knife edge.

The block holder was firmly set and the feed mechanism adjusted. The knife was fixed in its proper position and the indicator, which regulates the thickness of the sections, was set between 60 and 90 μm.

The paraffin sections were placed in dishes containing xylene and deparaffinization was carried out in two changes of xylene, two minutes each, followed by two changes of absolute alcohol, two minutes each and one change of 95% alcohol for 30 seconds. The deparaffinized sections were rinsed with 80% alcohol, washed in tap water and then stained with the hematoxylin solution for 4.5 minutes. After staining, the sections were rinsed with tap water, decolorized with acid alcohol (0.5% HCl in 95% ethyl alcohol) and rinsed again with tap water. Ammonium hydroxide was applied for blueing the sections; it was rinsed away after a few minutes with tap water.

The sections were counterstained with a solution of eosin (1.0% in alcohol) and then dehydrated by two changes of 95% alcohol and three changes of absolute alcohol. Three changes of xylene helped to clear the sections. The sections were preserved by fixing them to glass coverslips with Permount (Fisher Scientific Co., Silver Springs, MD). The slides were examined with a Zeiss binocular microscope for signs of histopathology and photographs were taken of appropriate fields by means of an attached camera (M-35, Nikkon, Tokyo, Japan).

Within an hour after death of the rabbit or rat, attempts were made to recover legionellae and other bacteria from the various tissues before they were processed. Plates of CYE agar, blood agar, nutrient agar and eosin methylene blue agar were inoculated by smearing the specimens across the surface of the agar; they were incubated at 35.5°C and observed daily for ten days. Blood specimens were streaked by means of a sterile loop on the agar media.

Agglutinating Antibody Titration

Legionella outer membrane antibody titers were determined using the Widal agglutination test (27). Ten tubes were placed in a rack; 1.8 ml of saline was pipetted into the first tube; one ml was added to each of the other tubes. Rabbit antiserum (0.2 ml) to the outer membrane was placed in the first tube to give a 1:10 dilution; it was mixed gently and one ml of this mixture was pipetted into the second tube. This serial dilution procedure was continued through nine tubes. One ml was discarded from the last tube. The tenth tube contained only cells in saline, representing the antigen control. One ml of a normal saline suspension of L. pneumophila cells (1×10^9/ml) freshly harvested from CYE agar plates, was added to each tube and the contents were mixed by shaking the rack vigorously. After incubation at 37°C for 18 hours, the endpoint was determined

by gently flicking the bottom of each tube with a finger tip in order to resuspend the cells that had settled. The last serum dilution showing microscopically visible agglutinates was the endpoint. In contrast, the control tube showed uniform turbidity.

Active Immunity in Rats and
Its Persistence

Five male, Sprague-Dawley rats weighing about 200-250 g were injected with 100 µg of OMPs in Freunds complete adjuvant (FCA) into the foot pads. Control animals were injected similarly with 0.5 ml sterile saline. Fourteen days later, serum was obtained from the rats and, at the same time, a booster (100 µg OMPs) of L. pneumophila was injected into the foot pad of each rat. Ten days later a final booster of 100 µg protein was administered similarly. After seven more days, the rats were injected with a lethal dose (1.5 x 10^6 cells of L. pneumophila). At the same time, two ml of blood was recovered from the tail vein of each rat. The blood was allowed to coagulate and the serum was transferred to small sterile test tubes; it was frozen (-20°C) to await agglutination titration. The control animals died within 24 hours while the immunized animals survived the challenge. Six months later, serum was obtained from the surviving rats, and the animals were again challenged with a lethal dose of L. pneumophila.

Passive Immunization of Rats

Five rats were injected via the foot pad with 100 µg legionella OMPs in Freunds complete adjuvant. Fourteen days later, 100 µg OMPs were injected into the foot pads of each rat (91). Further 100 µg boosters of OMPs were administered every 14 days for two months. Serum was obtained from these rats via the tail vein five weeks after the last immunization. Each of five "virgin" rats were immunized with one ml of immune serum obtained from the former rats. Control animals (five) were given comparable doses of serum from a rat not exposed to L. pneumophila or its products. Twenty-four hours later, the immunized and control rats were challenged intraperitoneally (i.p.) with 1.5×10^6 L. pneumophila cells freshly harvested from CYE agar plates.

Heterologous Passive Immunity

One New Zealand white rabbit, weighing approximately two kg, was immunized by the intramuscular injection of 100 ug of an emulsion in FCA of the OMPs recovered from L. pneumophila (Chicago-8). Injections were made every 14 days for two months. Ten days after the last immunization, serum was obtained by bleeding the rabbit from the ear vein. The serum was frozen (-20°C) for two days before use. Each of five adult, male Sprague-Dawley rats were injected (i.p.) with one ml of the rabbit antiserum while

five others were injected with normal rabbit serum. Twenty-four hours after immunization, the rats were challenged (i.p.) with 1.5×10^6 Legionella cells freshly harvested from CYE agar plates.

<div align="center">

Effects of Suspending Vehicle on the
Development and Duration of Active
and Cell Mediated Immunity to
Legionella OMPs; Parallel
Leukocyte Responses

</div>

Adult Sprague-Dawley rats weighing between 200-250 grams each were used in this experiment. Forty rats were divided into four equal groups of ten. Animals in the three experimental groups were immunized by foot pad injections of OMPs of L. pneumophila prepared in Freund's complete adjuvant (FCA) (Group 1), a double emulsion adjuvant (DEA) (Group II), or in saline (Group III). Animals in the fourth group (Group IV), the controls, were injected with only saline.

Freund's complete adjuvant-antigen suspensions were made by emulsifying equal volumes of FCA (Difco Laboratories) and the buffered aqueous OMPs. The DEA-antigen was prepared by mixing equal volumes of aqueous OMPs, mineral oil, poly-oxyethylene sorbitan monooleate and mannide monooleate (91). The latter two ingredients served as emulsifiers. The mix-ture was sonicated (Sonifier Cell Disruptor, Heat System Ultrasonics, Inc., Plainview, L.I., NY) by 30-second pulses

every 30 seconds in an ice bath for 10 minutes, at which
time the water-in-oil-in-water emulsion was complete. The
homogeneity of the DEA emulsion was verified microscopically.
A third OM antigen was prepared in 0.9% sterile physiologic
saline. All three preparations of antigen contained one mg
OMP/ml.

Animals in the test groups (I, II and III) were sensi-
tized by injecting 0.1 ml (100 µg) of the OMPs into the
hind footpads. Fourteen days later, the animals received a
booster injection of 100 µg proteins. Seven days after the
latter injection, all rats were challenged with 1.5 x 10^6
cells of L. pneumophila freshly harvested from CYE agar
plates.

On day zero, just before the injection of OMPs, blood
and serum were obtained from the tail veins. Twice a week,
blood and serum were collected; just prior to injection and
post infection, the animals were bled again. The sera were
stored at -20°C until they could be tested serologically.

The methods of Mishell and Shiigi (149) were employed
for counting leukocytes. Total leukocyte counts were deter-
mined by mixing one ml of blood recovered from the tail
vein with nine ml of 3 percent acetic acid. Using a Pasteur
pipette, the cell suspension was allowed to flow under
the coverslip of the haemocytometer. Cells were counted
in each of the four large squares and the average

number per large square was determined, yielding the number
of cells per 10 ml.

Thus

Cells/ml = average number per large

square x 10 x 1/dilution

In preparation for the differential cell counts, the
blood was smeared on an alcohol-acetone-cleaned glass slide
and allowed to air dry. The slide was then submerged in a
hematoxylin-eosin staining solution (Camco Quik Stain II,
American Scientific Products, McGrew Park, IL) for 90
seconds. The specimen was allowed to air dry and the various
types of leukocytes (lymphoctyes, neutrophils, monocytes and
eosinophils) were enumerated. A total of 200 cells were
counted at random using the 4-mm objective lens and 10X
occular lens of the Zeiss light microscope.

Cellular Immune Response of Rats to OMPs of Legionella pneumophila

The development of delayed hypersensitivity was tested
in ten rats immunized according to the schedule outlined
for inducing active immunity. Two days after the final
booster, the immunized and control rats were injected with
10 μg OMPs (0.1 ml) intradermally into their shaved flanks.
The reactions were noted 24, 48 and 72 hours later and
measured (diameters of induration/erythema) at 48 hours,
the time of the peak response. Five days after challenge

injections (i.p.) of 1.5 x 10^6 L. pneumophila cells, 10 μg of OMPs (0.1 ml) were again injected (i.d.) into another site on the shaved flank of each rat. As before, the extent of induration and erythema was observed at 24, 48 and 72 hours, and measured at 48 hours.

CHAPTER III

RESULTS

Outer Membrane Proteins of Legionellae

To ascertain whether the citrate-Tris treatment of L.
pneumophila resulted in the release of the outer membrane
into the supernant, electron microscopy was performed at
different stages of the procedure.

Figure 1 reveals untreated cells, recovered directly
from CYE agar, to possess a typical gram-negative cell wall
morphology with an intact, adherent outer membrane. In
Figure 2, before the citrate-Tris treatment, the Tris-washed
cells are seen to be morphologically similar to the controls.
After exposure to citrate-Tris (Figures 3, 4), there are
clear indications of OM separation. In no case was cell
lysis detected by the photomicrographs. Thus, the proteins
recovered can be assumed to have arisen from the outer mem-
brane. The materials released by the citrate-Tris treatment
consistently yielded at least two milligrams of protein per
ml buffer.

Legionella Outer Membrane Profiles

Figure 5 represents sodium dodecyle sulfate gel elec-
trophoresis of standard proteins. Samples of the Dalton

59

60

Figure 1. Thin Sections of <u>Legionella</u> Cells, Recovered
Directly in Phospate-Buffered Saline (pH 8) from CYE Agar.

Notice the trilamellar inner and outer membrane charac-
teristic of gram-negative bacteria (50,000 X).
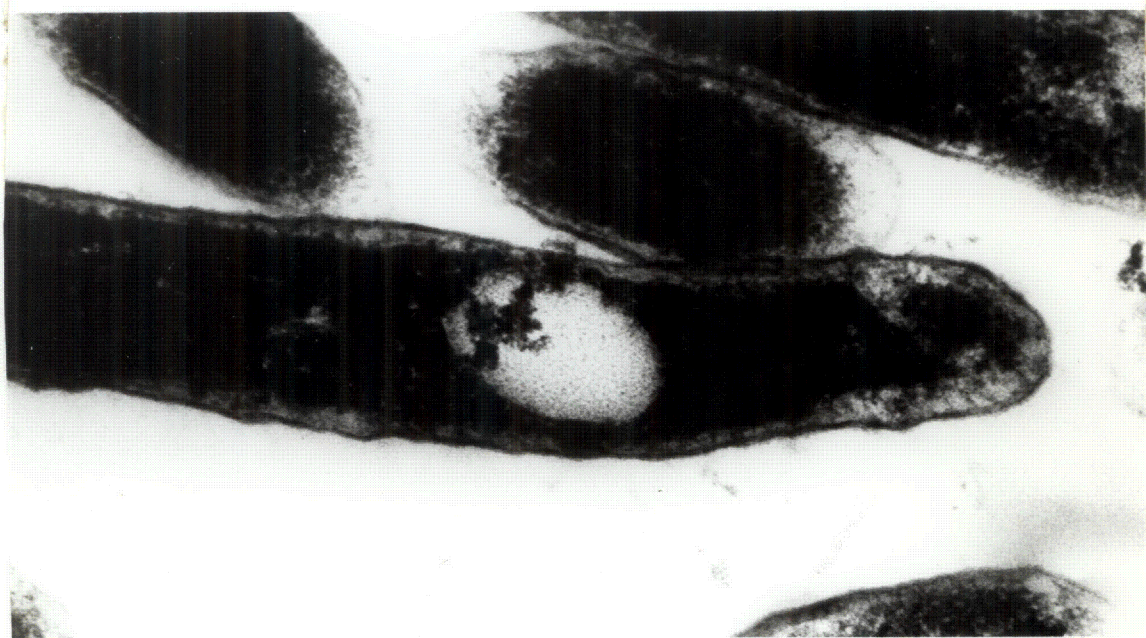
Figure 2. Thin Sections of Legionella Cells Suspended in Tris Buffer (pH 8) Prior to Citrate-Tris Treatment

Note the morphologic similarity with the freshly-washed cells seen in Figure 1.
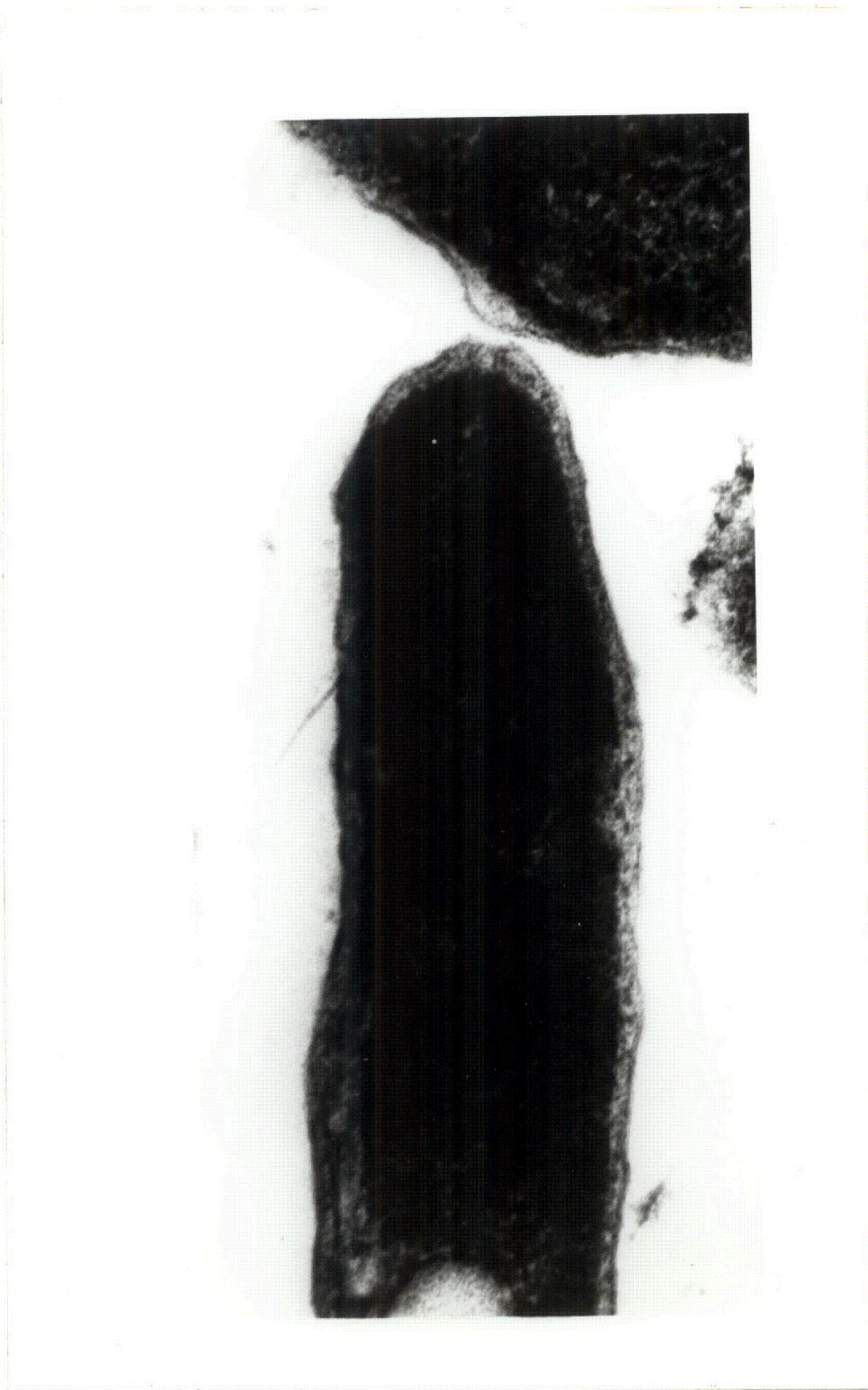
Figure 3. Thin Sections of Legionella Cells Exposed to
10mM Citrate Buffer (pH 2.75)

Note signs of separation of the outer membrane from the
cells.

Figure 4. Thin Sections of <u>Legionella</u> Cells Exposed
to 10mM Citrate Buffer (pH 2.75) and Resuspended in 100 mM
Tris Buffer (pH 8)

Note that portions of the outer membrane are absent,
some being only marginally associated with the remaining cell
envelope.
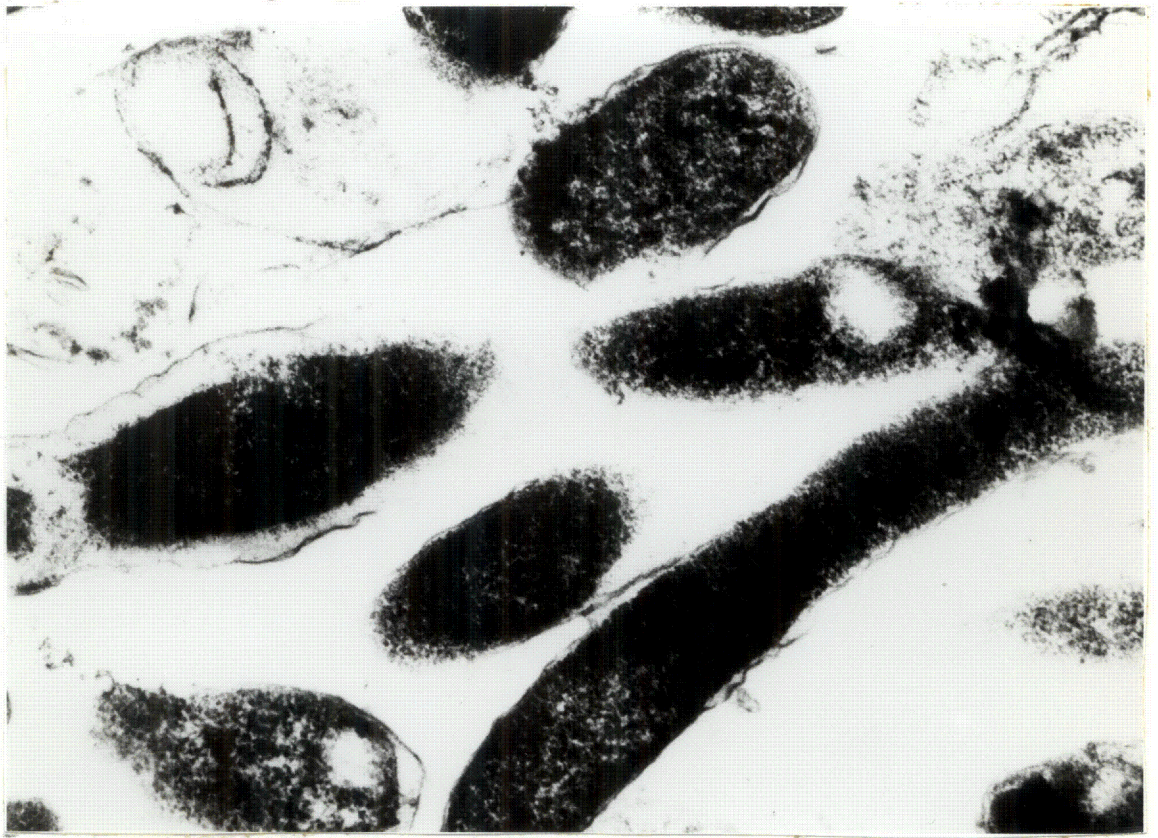
Figure 5. Determination of Molecular Weight of Standard Protein by Polyacrylamide Gel Electrophoresis

Twenty-five µg proteins of the standard protein mixture were applied to 8.8% polyacrylamide gel, eletrophoresed in a discontinuous buffer system, stained with Coomasie blue (R250) and relative mobilities calculated.
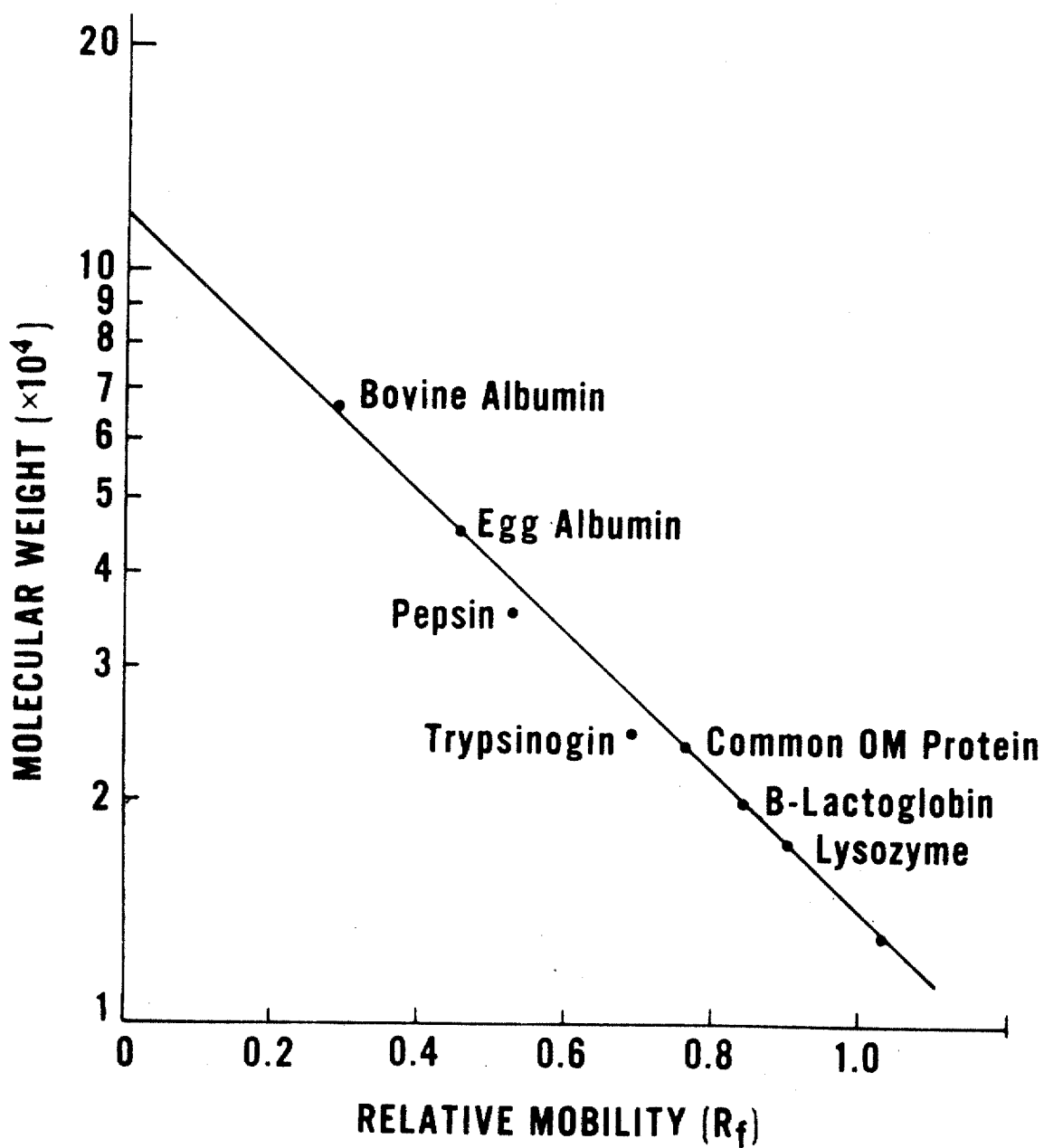
Mark IV molecular weight marker kit were loaded onto 8.8%
discontinuous SDS polyacrylamide gels and run as described
in the Methods section.

The protein profiles of the outer membranes generated
by the SDS-PAGE procedure demonstrated distinct strain and
serogroup differences, although the eight major serogroups
of L. pneumophila were found to share some of the proteins.

Close scrutiny of the photographs of the gels (Figure
6) reveals bands of varying resolution and intensity. They
can be conveniently called major and minor proteins based
upon their prominence in the stained gels. Thus the Knox-
ville strain of serogroup 1 revealed two major bands and at
least seven lesser bands. Another good example of this is
strain DAIE of serogroup 5 with five major and six minor
proteins.

Interpretation of the similarities and disparities of
the OMPs from the eleven strains is facilitated by the
tabulation in Table I. The tabulation indicates that all
three strains of serogroup 1 share proteins of 24, 20 and
19 Kd; the two strains of serogroup 6 share six proteins
(39, 26, 24, 19, 14 and 11 Kd), leaving nine proteins of
Ottawa-3 and five of Chicago-2 not shared. One major pro-
tein of 24,000 daltons (24 Kd) was found in common among all
eleven strains tested. Molecular weights of proteins shared
by four or more of the strains include 56, 47, 45, 39, 28,

Figure 6. SDS-PAGE of _Legionella_ _pneumophila_ Outer Membranes

Following treatment with 10 mM citrate buffer (pH 2.75), the cells were suspended in Tris buffer (pH 8) and the outer membrane proteins were solubilized for five minutes in SDS/ sodium carbonate at 100°C. Twenty-five μg of protein were applied to each tube. The gels were stained with Coomassie blue (R250) and decolorized with ethyl alcohol/acetic acid/ water. First lane (S) represents the standard proteins: bovine albumin (66Kd), egg albumin (45 Kd), pepsin (34.7 Kd), trypsinogen (24 Kd), lactoglobulin (18.4 Kd) and lysozyme (14.3 Kd). The remaining lanes represent proteins from the test strains, as follows: K = Knoxville, O_1 = OLDA, D_1 = Darby, T = Togus, B = Bloomington, L = Los Angeles, D_2 = DIAE, O_2 = Ottawa, Chicago-2, 7 = Chicago-8, and 8 = Concord-3.
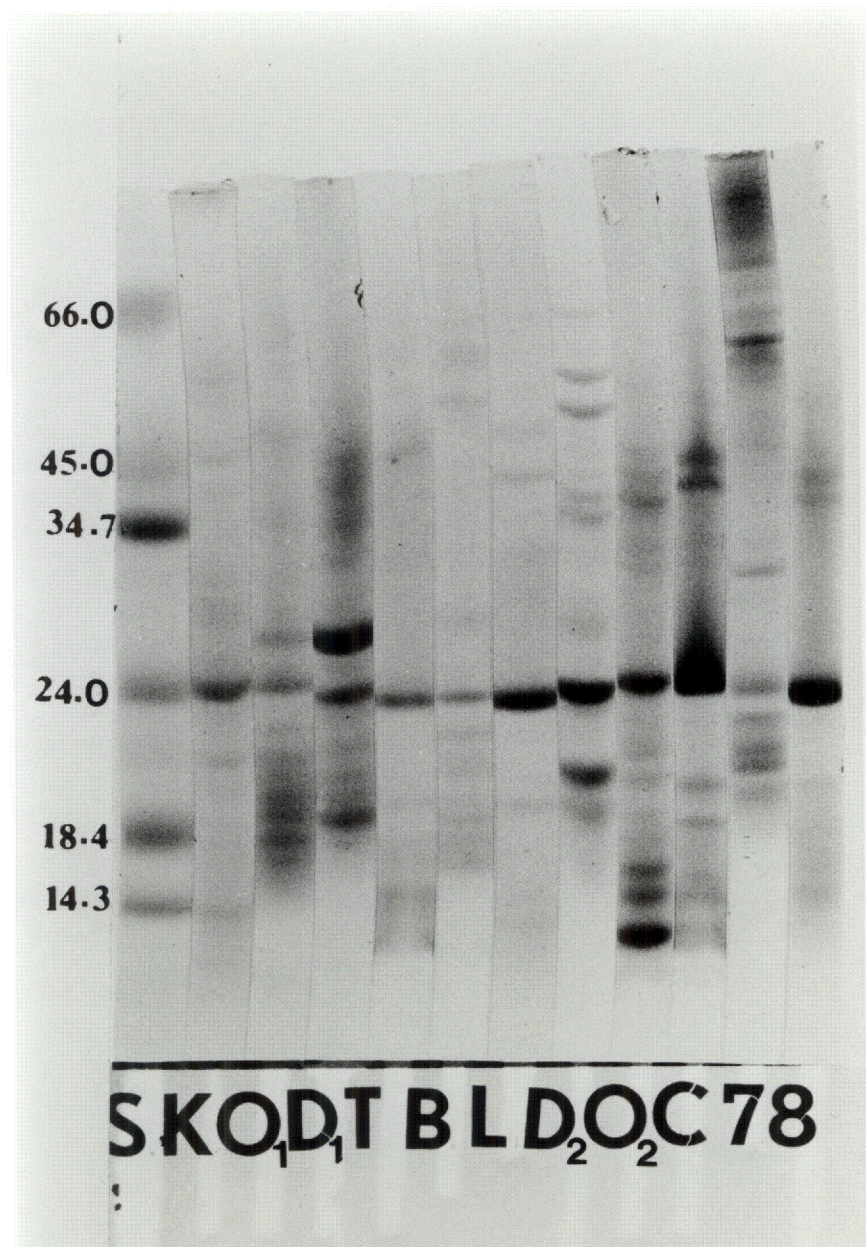
TABLE I

DISTRIBUTION BY MOLECULAR WEIGHT (Kd) OF PROTEINS IN POLYACRYLAMIDE
GEL ELECTROPHOROGRAMS OF _Legionella pneumophila_
OUTER MEMBRANE PROTEINS

Strain (Serogroup)	76	73	71	69	66	56	54	53	52	50	49	48	47
Knoxville (1)									XX		XX		
Olda (1)													XX
Darby (1)												XX	
Togus (2)							XX						
Bloomington (3)				XX		XX				XX			XX
LA-1 (4)			XX		XX	XX							XX
Daie (5)						XX		XX		XX			
OT-3 (6)											XX		
CHI-2 (6)		XX		XX		XX			XX	XX			
CHI-8 (7)	XX				XX								
Concord-3 (8)					XX				XX				XX

TABLE I--Continued

Strain (Serogroup)	28	29	31	33	34	36	37	38	39	41	42	43	45
Knoxville (1)	XX												XX
Olda (1)						XX				XX		XX	
Darby (1)	XX							XX					XX
Togus (2)	XX												XX
Bloomington (3)	XX	XX										XX	
LA-1 (4)				XX								XX	XX
Daie (5)		XX		XX			XX		XX		XX		
OT-3 (6)							XX		XX		XX		
CHI-2 (6)			XX		XX				XX				
CHI-8 (7)			XX										
Concord-3 (8)		XX					XX		XX				

TABLE I--Continued

Strain (Serogroup)	16	17	17.5	18	18.5	19	20	20.5	22	22.5	24	26	27
Knoxville (1)						XX	XX		XX		XX		XX
Olda (1)	XX				XX	XX	XX				XX	XX	
Darby (1)		XX	XX		XX	XX	XX			XX	XX		
Togus (2)		XX		XX				XX			XX		
Bloomington (3)	XX			XX				XX	XX	XX	XX		
LA-1 (4)				XX							XX		
Daie (5)	XX	XX		XX		XX	XX				XX		XX
OT-3 (6)						XX		XX	XX		XX	XX	XX
CHI-2 (6)					XX	XX		XX			XX	XX	
CHI-8 (7)		XX			XX	XX					XX		
Concord-3 (8)		XX				XX	XX				XX		

TABLE I--Continued

Strain (Serogroup)	14	13	12	11.5	11	10
Knoxville (1)			XX			
Olda (1)	XX		XX			
Darby (1)	XX					
Togus (2)	XX			XX		XX
Bloomington (3)			XX			XX
LA-1 (4)			XX			XX
Daie (5)						
OT-3 (6)	XX				XX	
CHI-2 (6)	XX				XX	
CHI-8 (7)					XX	
Concord-3 (8)	XX		XX			

24, 20.5, 20, 19, 18.5, 18, 17, 14 and 12 Kd. In serogroups
1 and 6, which are represented by more than one strain,
there appear to be some common OMPs. This is visually
apparent in the gels (Figures 7, 8).

Animal Studies

Investigations of host response to L. pneumophila were
confined to the Chicago-8 strain of serogroup 7.

Determination of Lethal Dose (LD)

Surface plates inoculated with 0.1 ml of the serial
dilutions of the suspension of L. pneumophila were incu-
bated at 35.5°C for 48 hours, at which time colonial
development was maximal. The dilution 10^7 yielded the most
countable triplicate plates of which there appeared 150,
135 and 165 colonies, an average of 150; thus the undiluted
population was approximately 15 x 10^9 cfu/ml. Since all
rats injected with one ml of dilutions of 10^{-4} or less died
and those injected with dilutions of 10^{-5} or higher lived
(Table II), the end point was 10^{-4} (OD approximately 0.106).
In Figure 9 it can be seen that a turbidity of 0.106
equated as 1.5 x 10^6 cfu/ml, the lethal dose (LD) for the
rat under the conditions employed.

Infection of a Rabbit with
Legionella pneumophila

Within 48 hours after an intraperitoneal (i.p.) injec-
tion of a rat LD (1.5 x 10^6 cfu) of L. pneumophila, the

Figure 7. SDS-PAGE of _Legionella pneumophila_ Outer Membrane Fractions from Serogroup 1 [Strains Knoxville (K), Olda (O_1) and Darby (D_1)]

See Figure 6 for description of treatment and preparation.
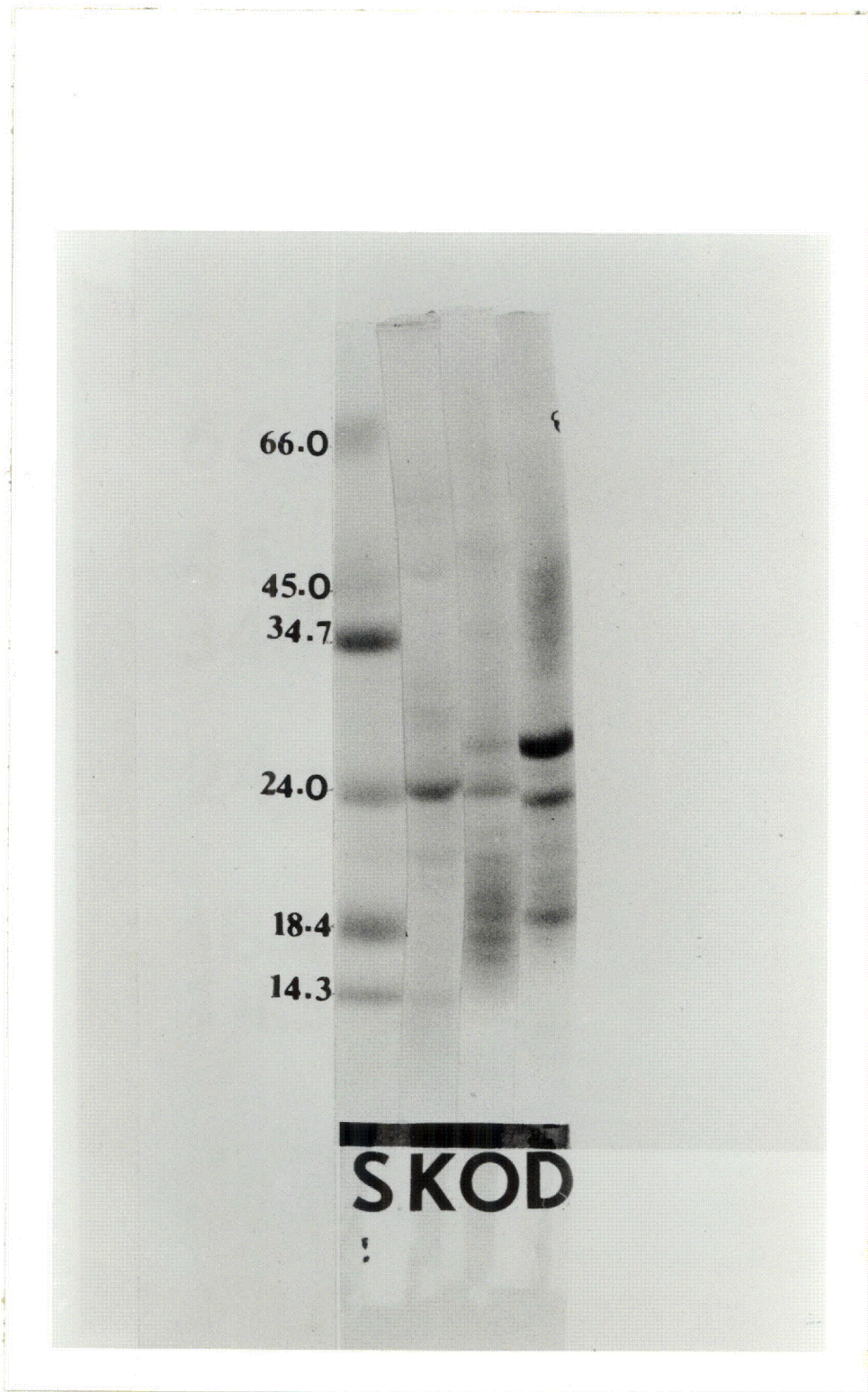
Figure 8.   SDS-PAGE of <u>Legionella</u> <u>pneumophila</u> Outer
Membrane Fractions from Serogroup 6 [Strains Ottawa-3 (O)
and Chicago-2 (6)]

See Figure 6 for description of treatment and prepara-
tion.
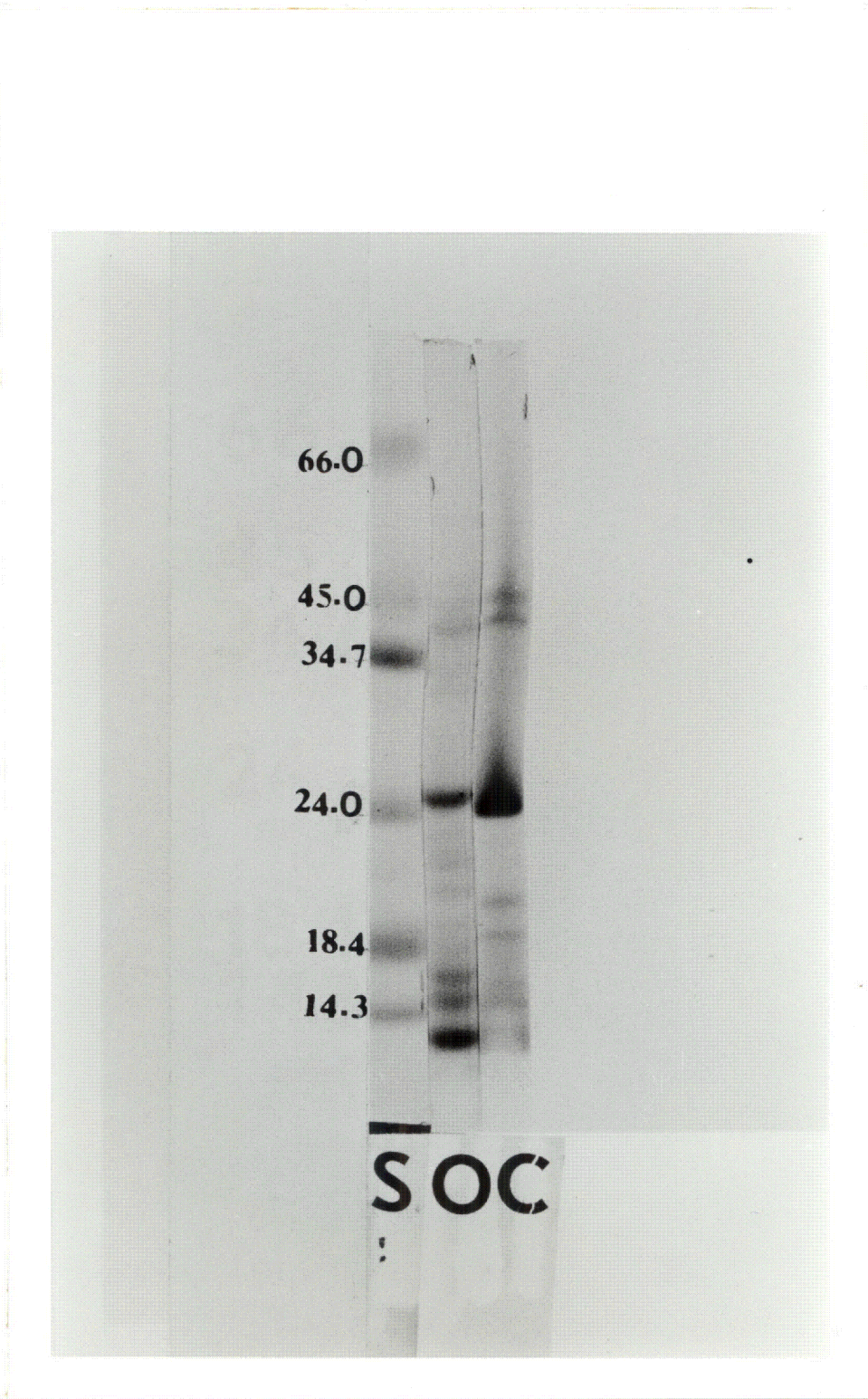
TABLE II

TITRATION OF THE LETHAL DOSE OF
Legionella pneumophila
SEROGROUP 7

OD_{620}	Dilution	Survivors/death*
0.279	10^{-1}	0/5
0.201	10^{-2}	0/5
0.119	10^{-3}	0/5
0.106	10^{-4}	0/5
0.087	10^{-5}	5/0
0.037	10^{-6}	5/0
0.024	10^{-7}	5/0
0.019	10^{-8}	5/0
0.009	10^{-9}	5/0

*0.1 ml of each dilution was injected i.p. (five rats per group).

Figure 9. Densitometer Calibration of Legionella

Suspension Used for Determination of Lethal Dose

One ml injected i.p. into each of five rats and the

lethal effect noted. The optical density of each dilution

was determined.
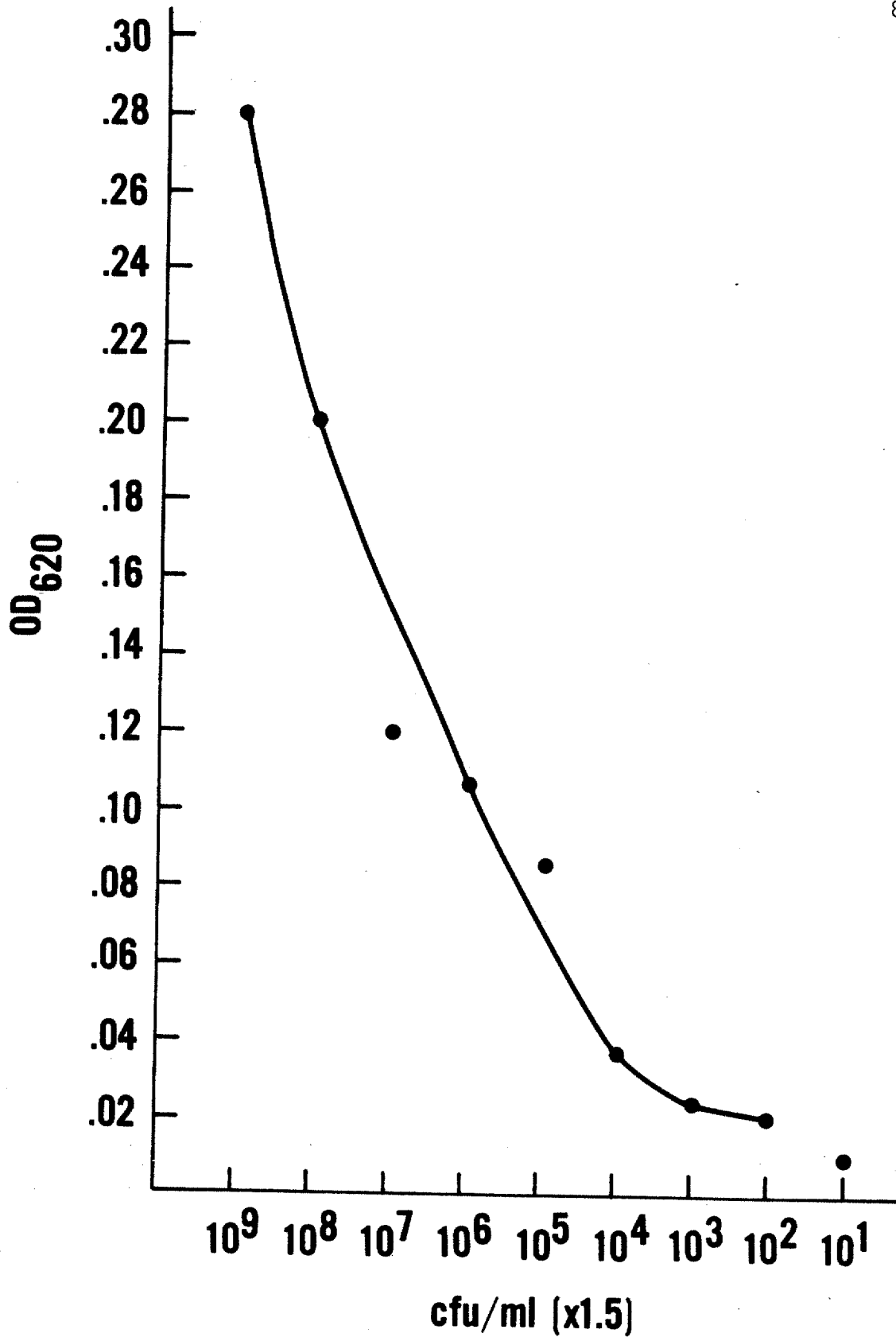
rabbit became listless and stopped eating. By the sixth day the rabbit could no longer stand, its breathing was labored and its eyes and nose were inflamed. Administration at that time of the antibiotic erythromycin was not helpful, and the animal was nearly moribund 48 hours later, following four injections of the antibiotic, whereupon the rabbit was anesthetized with ether and autopsied. Blood and tissues were recovered.

Pathology

Heart.--Multiple sections of the myocardium revealed scattered areas of degeneration in which the normal fibrous architecture was replaced by small spindeloid cells together with some round cells (monocytes and lymphocytes), neutrophils and eosinophils; however, inflammatory cells (giant cells and fibroblasts) were relatively uncommon. The remainder of the myocardium was composed of small, uniform muscle cells with no obvious abnormalities (Figure 10).

Lungs.--Multiple sections of lung tissue (Figure 11) revealed patchy areas of atelectasis and a marked diffuse interstitial pneumonitis (as found in humans with Legionella pneumonia) characterized by severely thickened alveolar walls with many neutrophils and eosinophils as well as a few plasma cells and lymphocytes.

Figure 10. Photomicrographs of Heart Tissue from a
Rabbit Infected with Legionella pneumophila and from a Control
Rabbit

The tissues were preserved in formaldehyde, embedded in
paraffin, sectioned, dehydrated and stained with hemtoxylin-
eosine. They were examined by means of a 10 X ocular lens and
a 4 mm (40 X) objective lens. Top photomicrograph is of
normal heart; bottom is from the Legionella-infected animal.
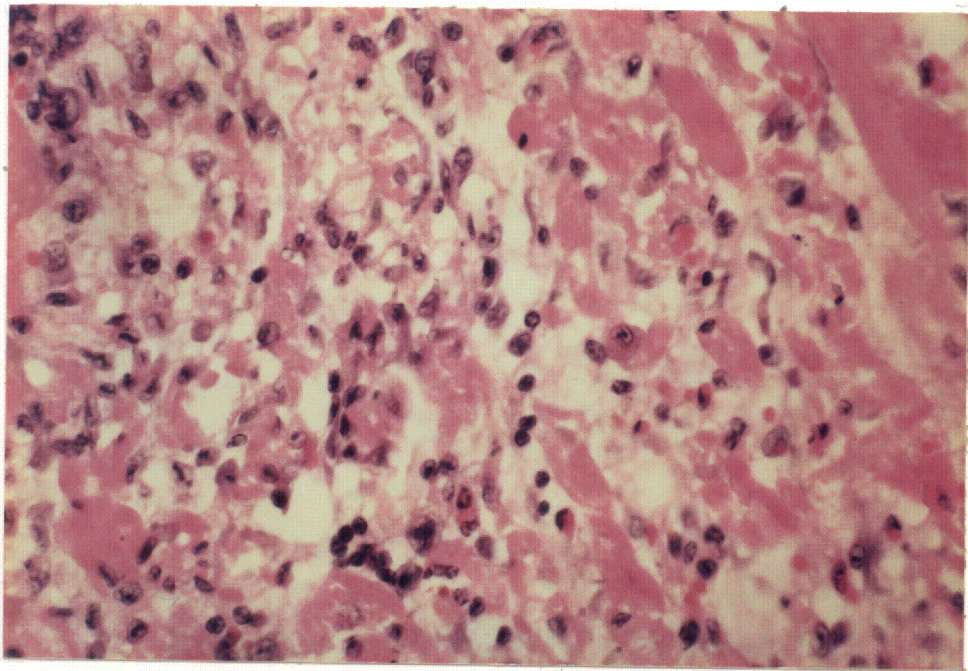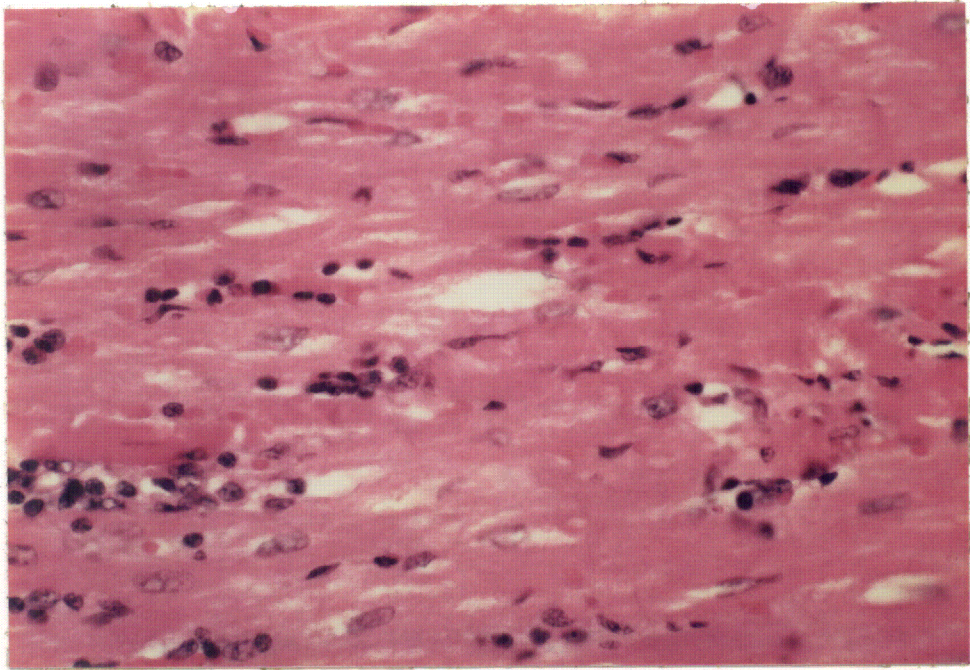
Figure 11.   Photomicrograph of Lung Tissue from Rabbit Infected with <u>Legionella</u> <u>pneumophila</u>.   See Figure 10 for description of methods.

Top photomicrograph of normal lung shows thin alveolar cells.

Bottom photomicrograph shows thickened alveolar cells, indicating pneumonitis.
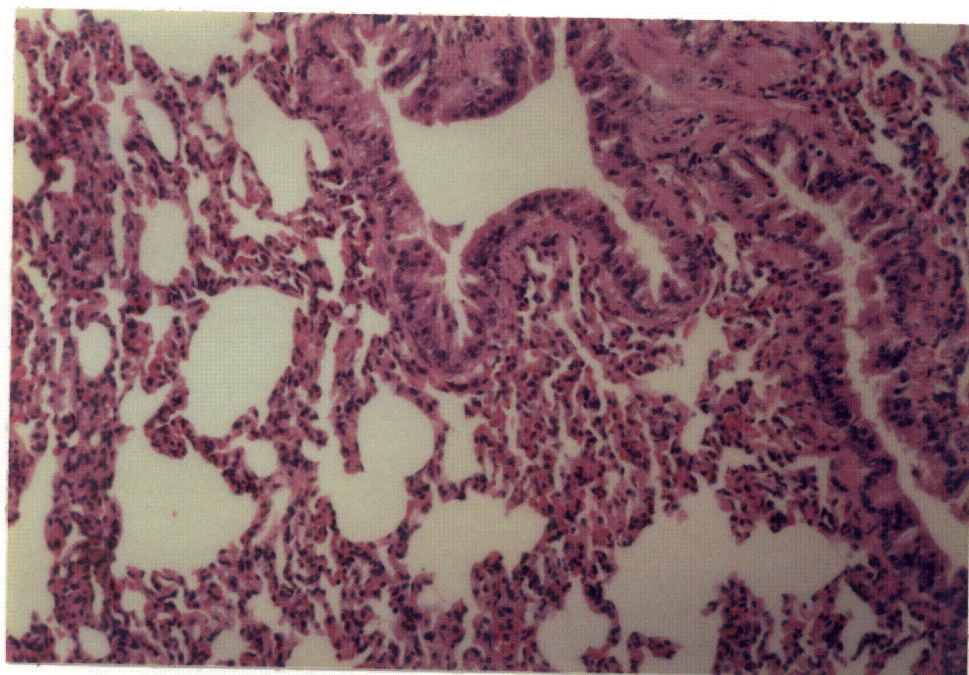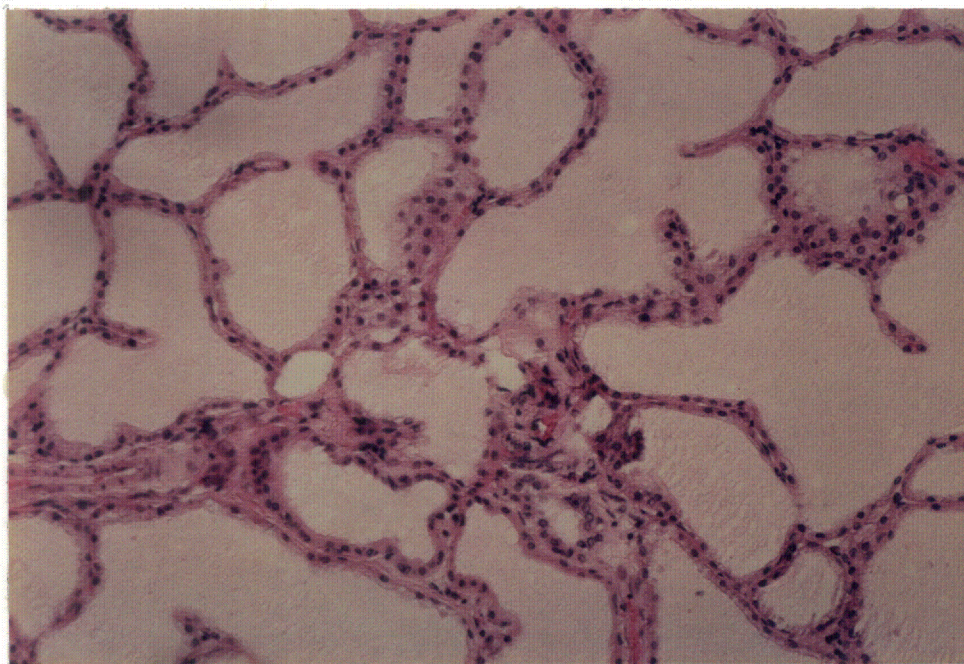
Gastrointestinal tract.--Sections of the stomach, small bowel and colon revealed early superficial autolytic changes of the mucosa. No inflammation or other histopathology was evident.

Brain.--The histologic picture of the brain appeared normal, showing an orderly arrangement of acini lined by a uniform, cubocolumnar epithelium with no inflammation.

Liver.--The lobular architecture was intact throughout the liver sections (Figure 12). Mild congestion was evident. Scattered at random throughout the parenchyma, predominantly within the sinusoids, were clusters of Kupffer cells together with some lymphocytes. Some of the clusters were relatively large and contained swollen Kupffer cells, distinctly epithelioid in appearance. These so-called "granulomas," which did not contain multinucleated cells, are characteristically seen with a wide variety of systematic diseases to which the liver responds; these include chemical and mechanical inflammation, neoplasia and immune disorders as well as infection (174). A few of the heptocytes were found to have enlarged hyperchromatic nuclei probably resulting from a nonspecific reaction. There were no indications of necrosis, reactive inflammation, metastatic tumor or cirrhosis.

Spleen.--Figure 13 reveals the spleen to be markedly congested; the red pulp contained clumps of dark, golden-

Figure 12.   Photomicrograph of Liver Tissue from Rabbit

Infected with <u>Legionella</u> <u>pneumophila</u>

See Figure 10 for description of methods.   Note granu-
lomas and mild congestion with clustering of Kupffer cells.
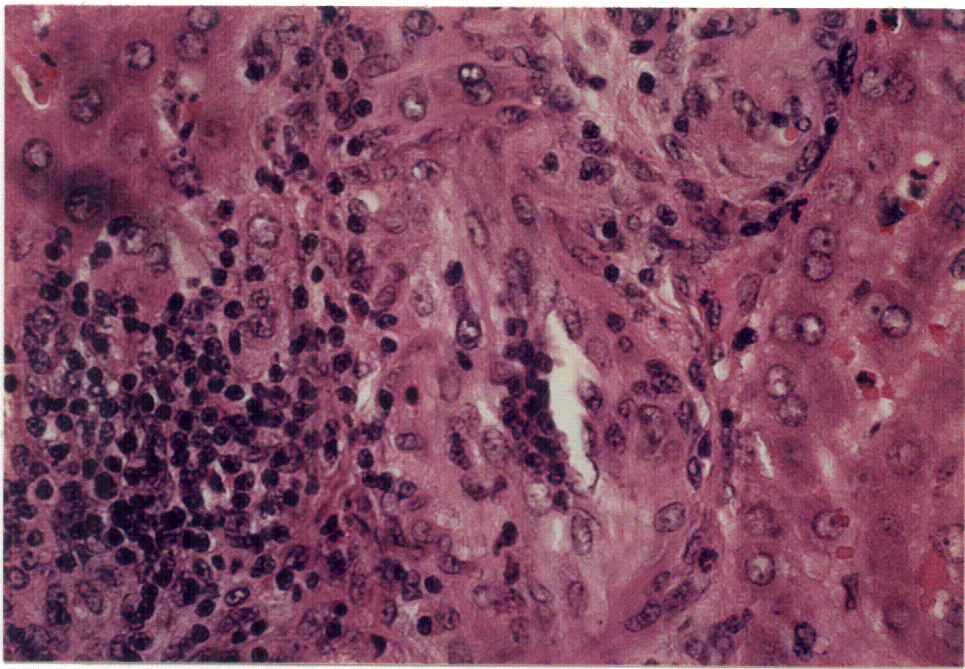
Figure 13.   Photomicrograph of Spleen Tissue from Rabbit

Infected with <u>Legionella</u> <u>pneumophila</u>

See Figure 10 for description of methods.   Notice the

pigmented macrophages.
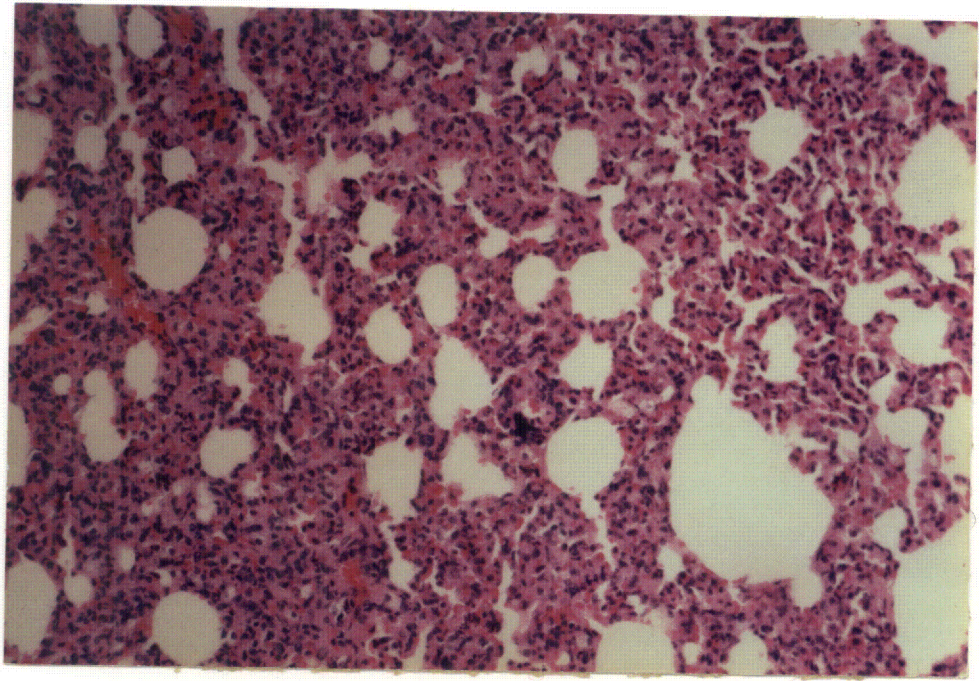
brown pigmented areas with numerous erythrocytes. A few
pigmented macrophages were evident. No histopathologic
signs of malignancy can be discerned.

Kidney.--Sections of the kidney revealed a mild hyper-
emia. Small foci of calcification were noted in the
tubules, although the tubular and glomerular architecture
was generally intact. No inflammation or other histo-
pathologic changes were evident.

Altogether, the foregoing histopatholgic findings indi-
cate that the rabbit most likely suffered from septcicemia
and respiratory collapse.

Bacteriological Findings

The various tissue specimens were inoculated on dif-
ferent bacteriologic media, including nutrient agar, eosin
methylene blue agar, blood agar and CYE-agar. After several
days there appeared on some of the media a few non-legionel-
lae; they probably arose from external contamination. The
CYE-agar inoculated with blood and lung smears yielded
colonies, not seen in the other bacteriologic media and
characteristics of L. pneumophila. The consistency of the
colonies was sticky, but they were not difficult to sub-
transfer to CYE-agar. These isolates, stained by the
Gimenez method (88), revealed bright red bacteria against a
light-blue background. In these stained smears were seen

slender rods, short to medium in length, and occasional filamentous forms. Later, these isolates were found to agglutinate when added to a sample of blood serum from the infected rabbit. Of all the specimens examined, only the lungs and blood yielded L. pneumophila. The dead rabbit's serum agglutinating antibody titer, employing a suspension of the homologous stock Chicago-8 strain, was determined to be 1:80.

Rat Immunization

The pooled agglutinating antibody titer of rats immunized with OMP was measured at intervals employing serum recovered from the tail vein. There was a good antibody response to the primary and booster injections of the OMP as seen by a final agglutinating titer of 1:1280 on the day of challenge. These data are seen in Table III.

This initial study indicates that OMP admixed with immunoadjuvant induced active immunity sufficient to protect the rats upon challenge with the homologous strain of L. pneumophila (Table IV). The single death in the immunized group occurred few minutes after infection due to unknown causes.

That this protection was at least partly mediated by humoral factors was shown in experiments in which transfer of serum from actively immunized animals (Table V) to non-immuned syngeneic rats conferred protection to infection

TABLE III

ANTIBODY TITERS OF RATS INJECTED WITH OUTER MEMBRANE
PROTEINS OF Legionella pneumophila*

Days Post Injection	Preparation Injected	
	FCA** Plus OMP	Saline
0	0	0
4	10***	0
7	40	0
11	160	0
14	80	0
18	320	0
24	640	0
31	1280	0

*Each group contained five rats. Blood sera were
pooled prior to the Widal test. The outer membrane proteins
were mixed with adjuvant; 0.1 ml (100 µg OMP) was injected
into the footpads. Control animals receivd 0.1 ml sterile
saline. A booster was administered on the 14th day, and
the rats were challenged with 1.5 x 10^6 Legionella pneu-
phila cells on the 31st day.

**FCA = Freunds Complete Adjuvant.

***Reciprocal of agglutinating endpoint.

TABLE IV

PROTECTION AGAINST THE LETHALITY OF Legionella
pneumophila IN RATS ACTIVELY IMMUNIZED
WITH OUTER MEMBRANE PROTEIN*

Treatment	Number of Rats	
	Alive	Dead
Saline (Control)	0	5
FCA + OMP	4**	1

*The rats had been immunized with outer membrane
protein from Legionella pneumophila and were challenged
with 1 LD of the same strain 31 days later.

**One rat died during manipulations at the start of
this test.

TABLE V

ANTIBODY TITERS OF RATS INJECTED WITH OUTER MEMBRANE
PROTEINS OF Legionella pneumophila (ANTISERA
USED FOR PASSIVE IMMUNIZATION)*

Days Post Injection	Preparation Injected	
	FCA + OMP	Saline
0	0	0
14	80**	0
28	160	0
42	640	0
56	1280	0
91	640	0

*Each group contained five rats. Blood sera were
pooled prior to the Widal test. The outer membrane pro-
teins (OMP) were mixed with the adjuvant; 0.1 ml (100 µg
OMP) was injected into the footpads. Control animals
received 0.1 ml sterile saline. A booster was adminis-
tered on the 14th day and subsequent injections made every
14 days for two months. The rats were challenged with
1.5×10^6 Legionella pneumophila cells on the 91st day.

**Reciprocal of agglutinating endpoint.

with L. pneumophila, Chicago 8 (Table VII). Rats could

TABLE VII

HOMOLOGOUS AND HETEROLOGOUS PASSIVE PROTECTION
OF RATS AGAINST A LETHAL DOSE OF Legionella
pneumophila USING ANTISERA AGAINST OUTER
MEMBRANE PROTEIN FROM THE SAME STRAIN

| Treatment* | Number of Rats | |
	Alive**	Dead***
Normal Rat Serum	0	5
Rat Immune Serum	5	0
Normal Rabbit Serum	0	5
Rabbit Immune Serum	5	0

*Five male rats injected intramuscularly with 1 ml
of serum from synegeneic donor or from rabbit immediately
before challenge of 1.5 x 10^6 cells.

**Survived without signs of infection for several
months.

***Died within 24 hours.

also be protected with heteroantisera. Serum from rabbits

that had been repeatedly immunized with OMP belonging to

Chicago 8 (Table VI) protected against the lethal effect of

L. pneumophila, Chicago 8 in rats (Table VII). The results

indicate that slight signs of illness were noted during the

first 24 hours after challenge. However, both homologous

and heterologous sera were protective.

TABLE VI

AGGLUTINATION TITERS OF RABBITS IMMUNIZED WITH
OUTER MEMBRANE PROTEINS FROM Legionella
pneumophila SEROGROUP 7*

Day of Immunization	Reciprocal of Aggl. Titer	Control**
0	0	0
14	640	0
28	ND***	ND
42	ND	ND
56	ND	ND
70	ND	ND
80	1280	0

*Antigenic preparation: OMP in FCA.

**No antibody was detected in the control rabbit.

***ND = Not Done.

Duration of Active Immunity

Six months after surviving a lethal dose of L. pneumo-phila, the four animals which had received FCA + OMP were once again challenged with 1.5×10^6 cells, freshly har-vested. Although the animals looked sick for 24 hours, all survived, thereby demonstrating persistence of active immunity for at least six months. No antibody titers were

obtained from the rats following the second challenge.
While low, the titers at the time of the second challenge
indicated that a residual protective immunity toward these
proteins remained. These results can be seen in Table VIII.

TABLE VIII

PERSISTENCE OF OMP-INDUCED ACTIVE IMMUNITY TOWARD
Legionella pneumophila

Treatment	Agglutination Titer* (Reciprocal)	Effect**
FCA + OMP	80	4/0
Saline (control)	0	0/5

*180 days after first challenge (titer at that time
was 1:1280).

**Number survivors/number dead.

Antibody and Cell-Mediated Immunity

Forty adult Sprague-Dawley rats were divided into four
groups of ten animals each. Each animal in the first two
groups was immunized by an injection in the hind footpad of
100 μg of Legionella OMP contained in an immunoadjuvant,
either FCA (Group A) or a DEA (Group B); animals in Group C
were similarly injected with 100 μg of OMP in saline. Rats
in Group D served as saline-injected controls. Fourteen
days later all procedures were repeated. Seven days after
the booster immunization all rats were challenged with 1.5
x 10^6 L. pneumophila cells injected intraperitoneally.

Throughout the course of this experiment the following experimental parameters were measured: total leukocyte count (Figure 14); differential leukocyte count (Figure 15); agglutinating antibody titer to L. pneumophila (Figure 16); and the abiity to survive the challenge of a lethal dose (Table XII).

Total and Differential Leukocyte Counts

All animals had total leukocyte and differential counts in the expected range before immunization with OMP. Four days after immunization, total leukocyte counts (Table IX and Figure 14) had increased in all three test groups, peaking at day seven and returning to near the pre-immunization levels by day 14. After the booster immunization on day 14, this pattern recurred. Upon challenge with L. pneumophila on day 21, the leukocyte counts of all the sensitized animals sharply increased; by the fifth day post-challenge, the leukocyte counts, as seen in Table IX, had reached: 24.1 ± 2.8 (Group A), 28.5 ± 8.2 (Group B), and 42.9 ± 7.4 (Group C) x 10^6/ml. Leukocyte counts of the control rats remained fairly constant throughout the experiment. Within 24 hours after the challenge, all the control animals had died.

The differential leukocyte counts are summarized in Table X and Figure 15. The animals immunized with OMP in either adjuvant demonstrated a decrease in the percentage

TABLE IX

TOTAL LEUKOCYTE COUNT (x 10^6/ml)

Day of Experiment	Group A	Group B	Group C	Group D
0	7.63 ± 1.3	7.79 ± 1.5	6.85 ± 0.9	6.15 ± 1.1
4	9.02 ± 1.0	9.81 ± 1.0	9.35 ± 1.3	6.16 ± 1.4
7	12.88 ± 2.4	11.85 ± 1.1	10.58 ± 1.3	6.22 ± 0.6
11	11.89 ± 2.5	9.97 ± 1.0	9.84 ± 1.2	5.82 ± 0.8
14	8.56 ± 1.7	8.61 ± 1.1	8.92 ± 1.2	5.95 ± 0.6
18	10.33 ± 3.0	11.33 ± 1.6	10.69 ± 1.7	6.05 ± 0.6
21	11.95 ± 2.6	12.36 ± 1.3	14.53 ± 2.5	· ·
26	24.14 ± 2.8	28.53 ± 8.2	42.98 ± 7.4	· ·

See legend for Figure 14.

Figure 14. Total Leukocyte Response to Immunization with <u>Legionella</u> <u>pneumophila</u> Outer Membrane Proteins Followed by Challenge

Each rat was injected with 100 µg OMPs contained in FCA (A), DEA (B), or saline (C). Group D animals were injected with sterile saline. The animals were challenged with 1.5 x 10^6 freshly-harvested <u>L</u>. <u>pneumophila</u> cells. All control rats died within 24 hours. The leukocyte counts represent the average of 10 rats ± S.D. It should be noted that on day 16, 10 µg OMPs in saline were injected intradermally in order to check for delayed hypersensitivity (see Figure 16).
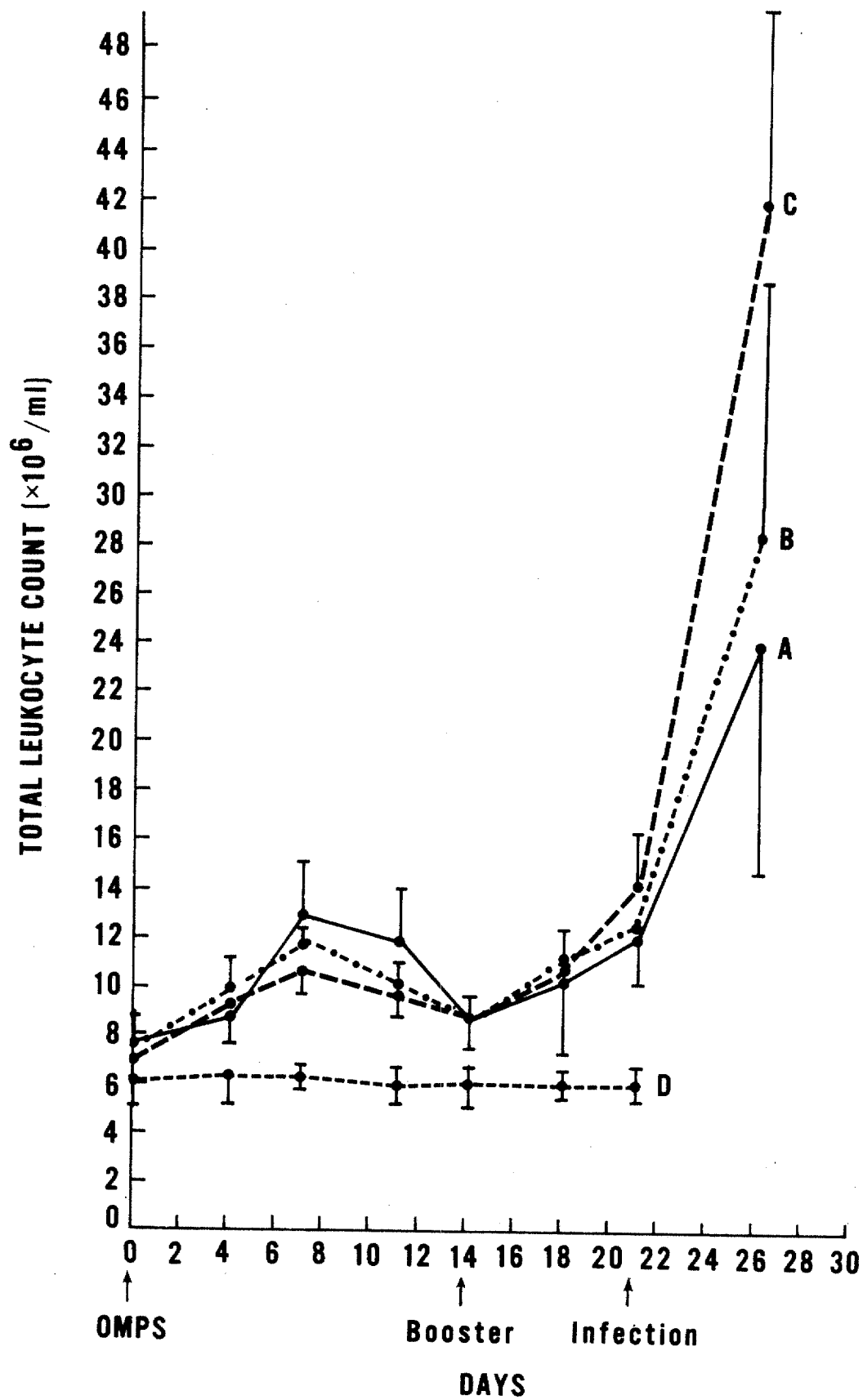
TABLE X

DIFFERENTIAL TOTAL (% OF TOTAL)

Group	Day 0				Day 4			
	L	N	M	E	L	N	M	E
A	78.8 ± 26.2	15.1 ± 5.0	3.6 ± 1.2	2.5 ± 0.8	62.7 ± 20.9	21.6 ± 7.2	5.9 ± 1.9	4.8 ± 1.5
B	77.9 ± 25.9	16.3 ± 5.4	3.0 ± 1.0	2.8 ± 0.9	69.2 ± 23.0	19.2 ± 6.4	6.5 ± 2.2	5.1 ± 1.6
C	81.9 ± 27.3	12.6 ± 4.3	4.2 ± 1.3	1.3 ± 0.4	73.4 ± 24.5	14.6 ± 4.9	6.9 ± 2.3	5.1 ± 1.7
D	80.6 ± 26.7	13.4 ± 4.4	4.1 ± 1.8	1.9 ± 0.6	79.8 ± 26.4	14.5 ± 4.8	3.4 ± 1.1	2.3 ± 0.7

TABLE X--Continued

Group	Day 7				Day 11				Day 14			
	L	N	M	E	L	N	M	E	L	N	M	E
A	59.5 ±19.8	24.9 ±8.3	8.5 ±2.8	7.1 ±2.3	71.5 ±24.5	17.5 ±5.8	5.6 ±1.8	3.4 ±1.1	78.2 ±26.0	15.4 ±5.1	3.9 ±1.3	2.5 ±0.8
B	62.5 ±20.8	22.5 ±70.5	8.1 ±2.5	6.9 ±2.3	78.4 ±26.1	19.6 ±6.5	5.4 ±1.7	4.6 ±1.5	75.1 ±25.0	16.5 ±5.4	6.3 ±2.2	2.1 ±0.7
C	69.9 ±23.2	19.1 ±6.4	6.1 ±2.2	4.9 ±1.6	77.8 ±25.9	13.1 ±4.4	5.8 ±1.9	3.3 ±1.1	79.7 ±20.5	12.4 ±4.1	7.5 ±2.5	2.9 ±0.8
D	82.0 ±27.6	13.1 ±4.0	3.3 ±1.1	1.6 ±.6	78.8 ±26.2	16.5 ±5.4	2.7 ±.9	1.8 ±.6	79.1 ±26.0	13.8 ±4.6	4.0 ±1.3	3.1 ±1.0

TABLE X--Continued*

Group	Day 18				Day 21				Day 26			
	L	N	M	E	L	N	M	E	L	N	M	E
A	80.3 ± 26.7	11.0 ± 3.6	5.5 ± 1.8	2.9 ± 0.9	77.1 ± 25.7	12.9 ± 4.3	6.2 ± 2.0	3.8 ± 1.2	31.7 ± 10.5	50.3 ± 16.7	11.6 ± 3.8	6.4 ± 2.1
B	79.4 ± 26.4	16.6 ± 5.4	4.4 ± 1.5	1.6 ± 0.6	78.5 ± 26.2	14.7 ± 4.9	4.1 ± 1.3	2.5 ± 0.9	35.5 ± 11.8	4.5 ± 14.9	14.3 ± 4.7	5.2 ± 1.3
C	82.7 ± 27.5	10.8 ± 3.6	3.1 ± 1.0	3.4 ± 1.1	81.7 ± 27.1	12.5 ± 4.1	3.6 ± 1.2	2.2 ± 0.7	47.5 ± 15.8	46.2 ± 15.3	3.8 ± 1.2	2.5 ± 0.8
D	79.4 ± 26.1	14.3 ± 4.7	4.2 ± 1.3	2.1 ± 0.7	.	.	.	.	.	.	.	.

*L=lymphocytes; N=neutrophils; M=monocytes; E=eosinophils
A=group injected with OMP in FCA; B=group injected with OMP in DEA; C=group injected
with OMP in saline; D=group injected with saline (control)

Figure 15. Differential Leukocyte Response to Immuniza-
tion with <u>Legionella</u> <u>pneumophila</u> Outer Membrane Proteins
Followed by Challenge

See legend of Table X.
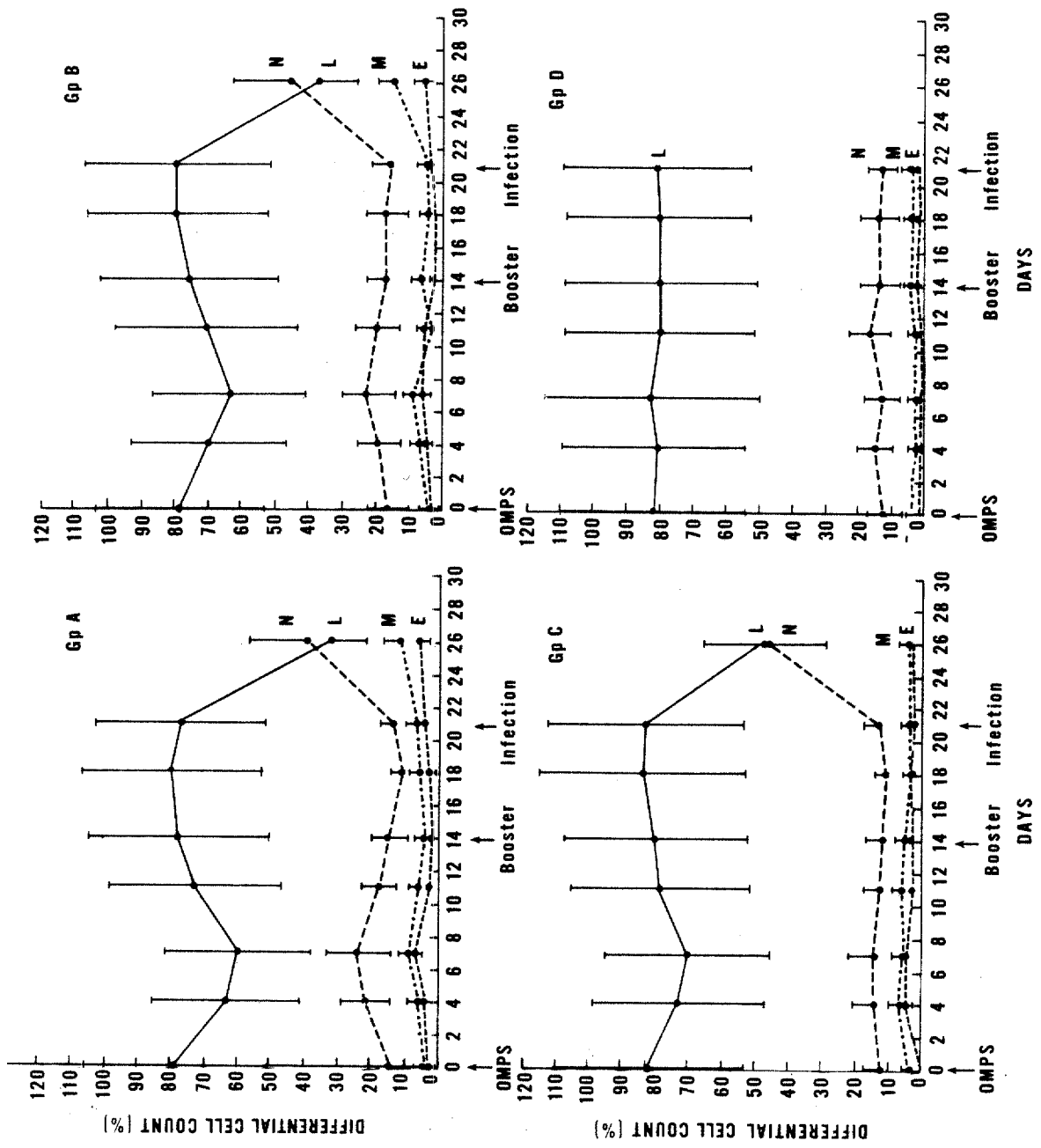
of lymphocytes concomitant with an increase in the neutrophil response after the primary dose; however, the lymphocyte response of animals in Group C (OMP in saline) was hardly apparent. By day 14, the differentials of all three groups of animals had returned to the values of the saline control animals. The booster injection had little effect on the differential counts of any of the rats. After challenge with L. pneumophila (day 21), a dramatic reduction in the percentage of lymphocytes and a marked increase in the percentage of neutrophils was seen; by day 5 post-challenge, the lymphocyte/neutrophil ratio had reversed itself.

Antibodies with specificity for the immunizing strain of L. pneumophila could be detected in immunized rats. The data in Table XI and Figure 16 indicate that primary sensitization with OMP emulsified in either FCA or DEA resulted in the production of antibody titers substantially greater than those induced by the injection of OMP in saline. This was most apparent after the booster immunization which resulted in titers of 1:1280 for Groups A and B and 1:640 for Group C. After challenge, the antibody titers of animals immunized with either FCA-OMP or DEA-OMP rose rapidly, reaching 1:2560 five days later. Animals immunized with the saline-OMP were less responsive than those receiving the adjuvant preparations, revealing a post-challenge titer of 1:1280.

TABLE XI

ANTIBODY RESPONSE OF RATS IMMUNIZED WITH
OUTER MEMBRANE PROTEINS FROM
Legionella pneumophila

Day	A*	B*	C*	D*
0	0	0	0	0
4	10	10	10	0
7	40	40	20	0
11	80	80	40	0
14	320	160	160	0
18	1280	1280	640	0
21	640	640	320	0
26	2560	2560	1280	0**

*Ten rats per group immunized with FCA-OMP (A), DEA-OMP (B), OMP-saline (C) and saline only (D). Reciprocal pooled agglutination titer by group

**All animals died within 24 hours

Figure 16. Antibody Response of Rats Immunized with

Legionella pneumophila Outer Membrane Proteins and Subse-

quently Challenged with the Homologous Strain

Protection After Challenge

Unprotected control rats demonstrated signs of illness
less than 12 hours after injection of a lethal dose of L.
pneumophila and died within 24 hours. Rats immunized with
OMP in saline evidenced signs of weakness, lethargy and
lassitude approximately 24 hours after challenge. Their
fur appeared ruffled and a discharge from the eyes and nose
was evident; however, they gradually recovered and all sur-
vived. Animals immunized with either FCA or DEA plus OMP
demonstrated no outward signs of illness and survived (see
Table XII).

TABLE XII

PROTECTION AGAINST A LETHAL DOSE OF Legionella
pneumophila IN RATS IMMUNIZED WITH
LEGIONELLA OUTER MEMBRANE
PROTEINS

Preparation Administered	Challenge Dose	No. Survivors/ Total
OMP + FCA	1.5×10^6	10/10
OMP + DEA	1.5×10^6	10/10*
OMP + Saline	1.5×10^6	10/10**
Saline	1.5×10^6	0/10***

*No clinical signs of illness.

**Severe clinical signs of illness with survival.

***All died within 24 hours.

Skin Test

Table XIII and Figure 17 show the extent of the dermal

response to the OMP of L. pneumophila injected intradermally

into animals already immunized with OMP emulsified in FCA or

DEA, or mixed with saline. All immunized animals exhibited

an area of erythema and subcutaneous swelling (induration)

within one to three hours after the shocking dose of an

intradermal injection of 10 µg OMP in 0.1 ml normal saline.

At 24 hours, the intensity and area of edema had increased;

TABLE XIII

CELL-MEDIATED IMMUNE RESPONSE OF RATS SENSITIZED
TO LEGIONELLA OUTER MEMBRANE PROTEINS

Sensitizing Antigen	Skin Reaction (mm)*	
	Erythema	Induration
FCA + OMP	7.9 (5.3-10.5) ± 2.6	4.5 (3.0- 6.0) ± 1.5
DEA + OMP	12.7 (8.5-16.9) ± 4.2	8.6 (5.8-11.4) ± 2.8
Saline + OMP	6.5 (4.4- 8.6) ± 2.1	4.2 (2.8- 5.6) ± 1.4
Saline	2.1 (1.4- 2.8) ± 0.7	0.6 (0.3- 0.9) ± 0.2

*Intradermal injection of 10 µg outer membrane protein
in 0.1 ml normal saline; reactions measured at 48 hours.
Diameters of erythema and induration measured in mm; expressed
as mean (range) ± standard deviation. Ten rats per group.

slight hemorrhage and necrosis were also observed. The peak

response was seen at 48 hours. The skin test responses in

animals immunized with OMP in either FCA or saline were

Figure 17. Detection of Delayed Hypersensitivity in Rats Injected with Legionella pneumophila Outer Membrane Proteins and Subsequently Challenged with Homologous Strain

The animals represented here are the same ones in which leukocyte counts (Figures 14 and 15) and OMP antibody titers (Figure 16) were measured. On day 16 each rat was injected intradermally with 10 μg OMPs; the responses were measured 48 hours later (day 18). The range of responses reported is the average of 10 rats ± S.D.
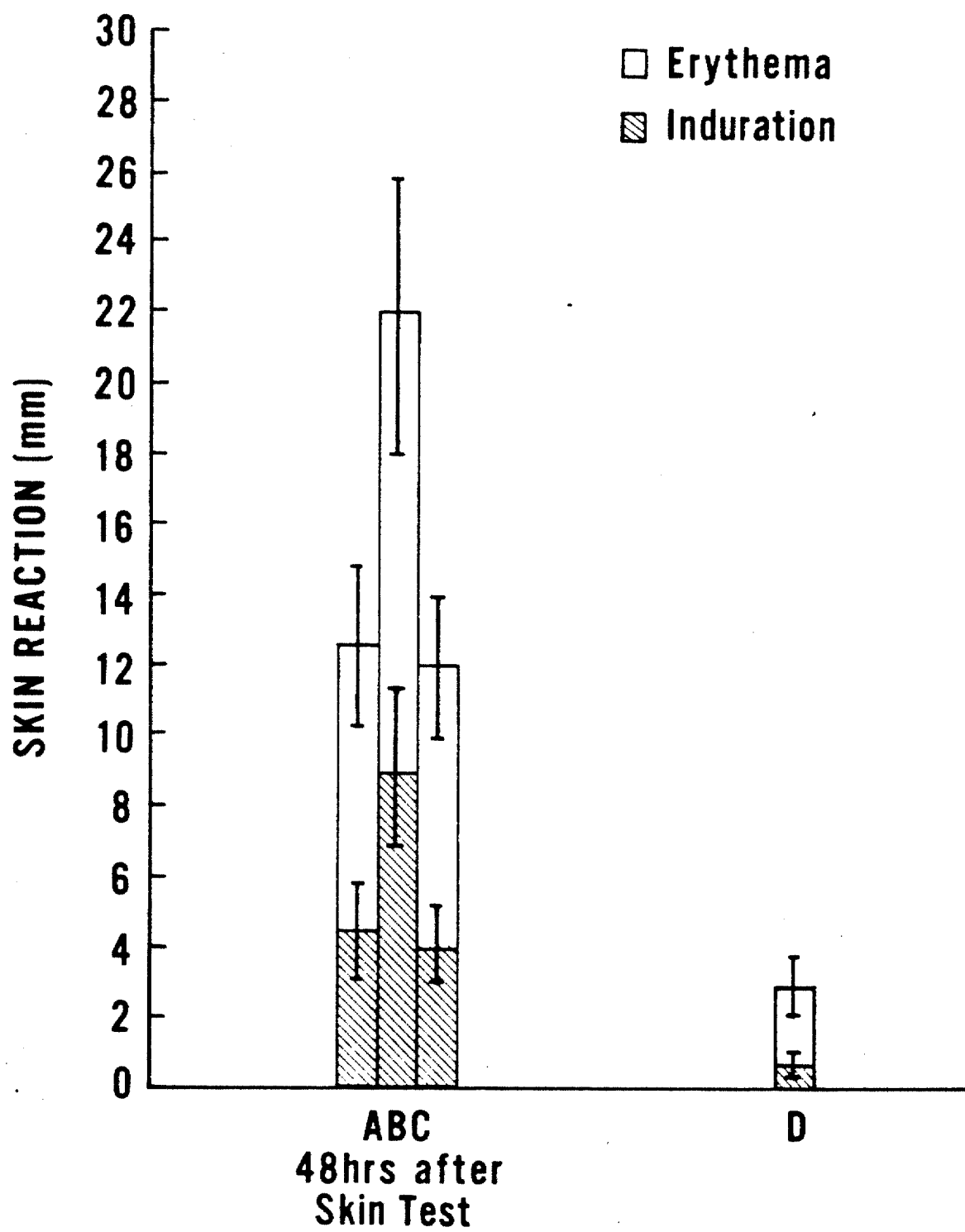
similar; the responses from DEA/OMP, however, were substantially greater (erythema in Group B animals was 61 and 78 percent greater than in Group A and C animals, respectively; similarly, induration was 91 and 98 percent greater.) A slight area of swelling and erythema did appear after six hours in the nonsensitized animals (Group D); however, this response waned before 48 hours had elapsed.

After an intraperitoneal injection of 1 LD of L. pneumophila, the animals which survived the challenge were skin-tested as described in the Methods section. Once again, all animals that were immunized exhibited an area of erythema and soft subcutaneous swelling. Within 24 hours there was a rapid increase in intensity and size of the subcutaenous swelling. Skin reactions were seen in all test animals and were measured at 48 hours, the time of the peak response. Results are given in Table XIV and Figure 18.

Skin reactions were induced in rats sensitized with OMPs and LD bacteria (Chicago 8) by intradermal injection of OMPs (Figure 19). The time sequence of skin reactions resembled that for a delayed type reaction. Induration was maximal between 24 and 48 hours after injection of the skin test agents (OMPs) and diminished slowly.

TABLE XIV

SKIN REACTION TO LEGIONELLA OUTER MEMBRANE
PROTEINS AFTER CHALLENGE WITH
LIVING LEGIONELLAE

Sensitizing Antigen	Skin Reaction (mm)*	
	Erythema	Induration
FCA + OMP	10.3 (6.9-13.7) ± 3.4	6.8 (4.6- 9.0) ± 2.2
DEA + OMP	14.9 (10.0-19.8) ± 4.9	11.1 (7.4-14.8) ± 3.7
Saline + OMP	10.1 (6.5-13.7) ± 3.6	7.3 (4.9- 9.7) ± 2.4
Saline (Control)	. . . .	. . . .

*Animals received intradermal injections of 10 μg outer
membrane protein in 0.1 ml normal saline; reactions were
measured at 48 hours. Reactions are expressed as mean (range)
± standard deviation. Ten rats per group were employed.

Figure 18. Delayed Hypersensitivity Reaction after Challenge with Viable Legionellae

Five days after challenge (1.5×10^6 cells) the rats were injected intradermally with 10 µg OMPs; the responses were measured 48 hours later (day 28). The range of responses reported is the average of 10 rats ± S.D.

Compare with Figure 17.
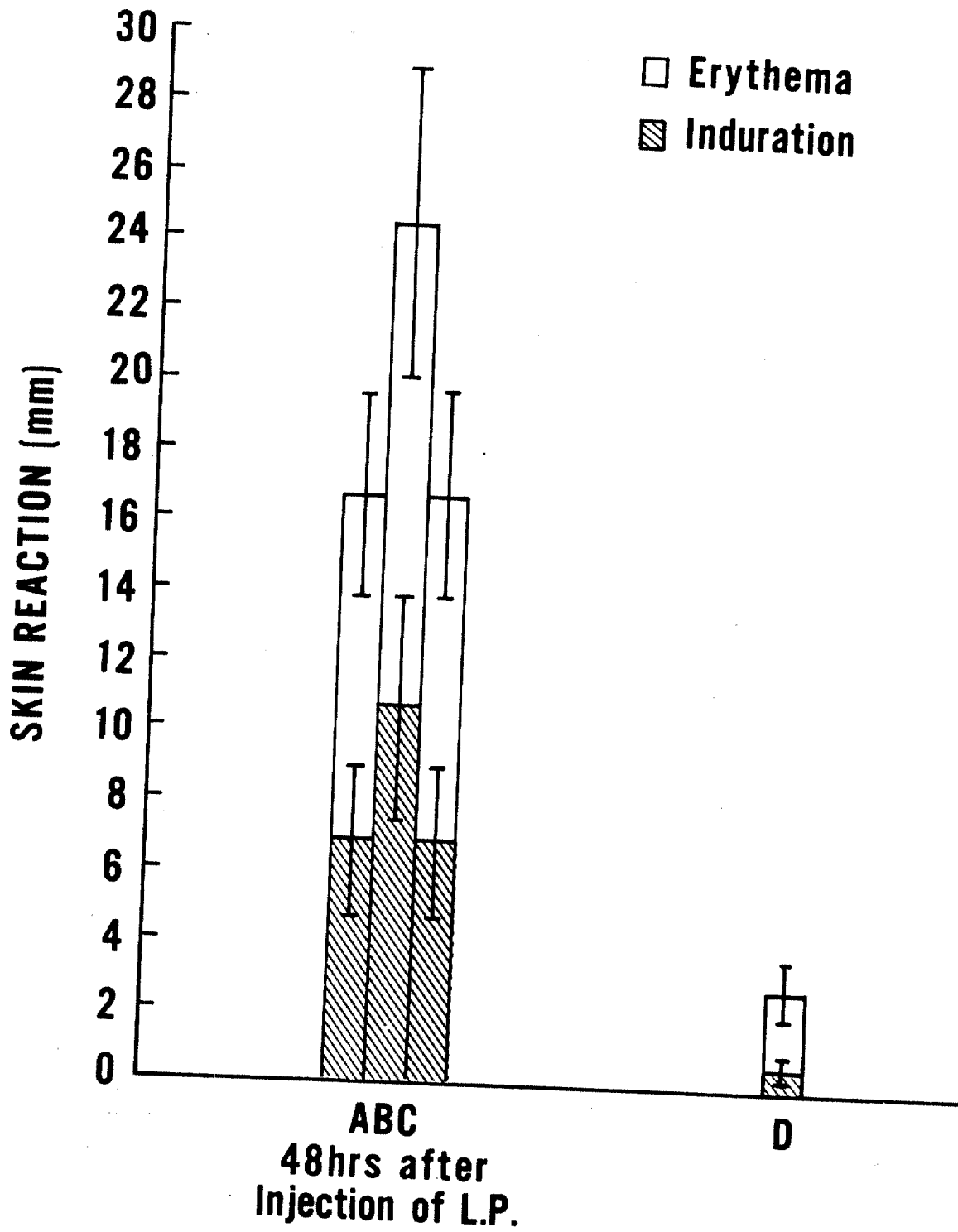
Figure 19. Skin Test Response to Outer Membrane Proteins of <u>Legionella</u> <u>pneumophila</u>

This rat was from Group B. The smaller dermal reaction was read on day 18 of the study. The greater reaction, revealing hemorrhage and necrosis, occurred after the animal had survived an infectious challenge.

CHAPTER IV

DISCUSSION

The method employed for the isolation and recovery of proteins from cellular structures is critical. However, the method we adopted for releasing the outer membranes from L. pneumophila is the one of Irvin, et al. (116) employed on Escherichia coli, a much different bacterium than L. pneumophila, although both are gram-negative bacilli and thus possess outer membranes. The key step in the process is the exposure of washed cells to 10 mM citrate buffer (pH 2.75) for 30 minutes, causing the separation of at least portions of the outer membrane to be released from the cell envelope as blebs or fragments. [It is of interest to note that L. pneumophila produces OM blebs or invaginations (72).] The blebs appeared to enlarge as more outer membrane dissociated in a linear fashion from the cell envelope, leading to a "flap," rather than a vesicle. These laminar fragments of outer membrane broke away and were recovered subsequently by high-speed centrifugation. The proteins they contained were then solubilized in a buffer at pH 8 wherein they remained stable in the refrigerator for at least a month. We feel confident that these preparations contained most, if not all, of the OMPs which constituted the thrust of this study.

The mechanism of citrate-Tris-mediated release of outer membrane from the cell of the envelope of L. pneumophila is unclear, but the reagent clearly weakens, at pH 2.75, the integrity of this membrane and its association with lipoproteins, which are covalently linked with peptidoglycan (mureine) (17) and/or with peptidoglycan-associated proteins (135, 179, 225, 182), thereby causing it to separate from the cell envelope. Similarly, the buffer very likely disturbs the structural relationship between porins and other constituents of the outer membrane. It seems likely that the interactions between OM constituents and murine are essentially ionic in character and can be disrupted by the intensification of positive charges within the outer membrane, as suggested by Schindler and Tenber (182). Since citrate is a good chelating agent, capable of binding such divalent cations as Mg^{2+} and Ca^{2+}, important in cell wall structure of Gram-negative bacteria (157), it will perturbate the macromolecular architecture of the cell wall by tying up such cations.

Analysis of the outer membrane eluates by SDS-PAGE revealed a great variety of proteins with varied molecular weights (ranging from 10 to 76 Kd), values of which are in some agreement with those reported by Hindahl and Iglewski (103). They used a French pressure cell to break up L. pneumophila; employing sucrose gradient centrifugation, they

were able to separate five distinct bands of cellular sub-
units, two of which originated from the OM. The latter
were recovered and their proteins were characterized by a
somewhat different SDS-PAGE method than we employed, namely
the method of Laemmli (130).

Proteins forming transmembrane pores (porins) in outer
membranes are generally abundant and within the molecular
size range of 30,000 to 45,000 daltons (156). In our work,
a 24 Kd major protein was found to be common in all sero-
group of L. pneumophila; often it appeared to be the most
abundant OMP. OMPs with molecular sizes of 30 Kd and 43 Kd
have been identified in Brucella abortus (202) and a 29 Kd
protein has been reported to be a major OMP in Rickettsia
prowazekii (188). Since L. pneumophila, like brucellae and
rickettsiae, is an intracellular pathogen (154), it is pos-
sible that the OM class of proteins may play an important
role in intracellular parasitism (155).

Ehret and his associates (64) have shown by means of
PAGE that all the twenty-one strains of legionellae (seven
serogroups of L. pneumophila and six other legionella
species) they studied contained a major OMP of 29 ± 500 Kd.
This protein was particularly predominant in formaldehyde-
inactivated preparations and could be detected only with a
7.5 percent gel or a 4 to 30 percent gradient polyacryl-
amide gel. The 29 Kd protein was not found in clinical

isolates of a number of non-Legionella species of gram-negative rods; thus, they assumed that the 29 Kd protein is a distinguishing feature of L. pneumophila. Ehret, et al. (64) found the 29 Kd protein only after heating the cells to 80°C in the presence of 2-mecaptoethanol; a lower temperature or the omission of the reducing agent prevented its appearance.

More recently, Hindahl and Iglewski (103) demonstrated two major OMPs L. pneumophila, viz., 29 Kd and 33 Kd. They found the 29 Kd protein to be recoverable from SDS-insoluble material (i.e., crude peptidoglycan) at temperatures greater than 60°C, a property characteristic of porins from E. coli (135). They also discovered that extraction from crude peptidoglycan from L. pneumophila with high salt, which normally releases porins from the peptidoglycan of Salmonella typhimurium (198) and Pseudomonas aeruginosa (224), failed to release the 29 Kd protein. The 33 Kd protein increased in apparent molecular size to 45 Kd upon application of moderate heat of less than 60°C, regardless of the presence of a reducing agent. Only be treatment at 100°C was it fully recoverable as a 33 Kd protein. In contrast, in the absence of heating and of 2-mecaptoethanol, the 29 Kd protein band did not appear to enter the gel as a recognizable band, thereby indicating that both heat and reducing agent are required to release the 29 Kd protein in its monomeric form.

While our methods resulted in the appearance of a 29 Kd protein in three strains and a 24 Kd in all eleven strains tested, it is possible that the methods used for processing of OMP preparation for PAGE by Hindahl (103) and Ehret (64) might have affected the 24 Kd protein, particularly in view of the preliminary application of high temperature and a reducing agent to the intact cells by Ehret, et al. In our hands, the omission of 2 mecapto-ethanol from the gel processing had no visible effect upon the specific bands resolved. It is likely that the 29 kd protein (and others) found by these other workers was derived from the 24 Kd protein by such denaturing agents as heat and formalin. Since Hindahl and Iglewski claimed that the 33 Kd protein could complex into a 45 Kd protein, by the same token it is conceivable that our 24 Kd protein could have been modified by unknown factors to result in the 29 Kd protein which they found. Once the OMPs were recovered, we found that treatment at 100°C for five minutes in the presence of the sulfhydryl reagent neither affected the 24 Kd protein nor caused the 29 Kd form to appear upon PAGE analysis. The 29 Kd protein we encountered in three strains is probably a different protein than the one reported by the other workers.

In view of finding mixed OMPs from serogroup 7 to induce both active and cellular immunity, the 24 Kd protein,

which is prominent in L. pneumophila, Chicago 8 (serogroup
7) and is common to all eleven Legionella strains we tested,
is a good subunit vaccine candidate; also, it might be
useful diagnostically for a precipitin or skin test for
legionellosis. Furthermore, antisera to this and other OMPs
of the legionellae might be useful therapeutically or pro-
phylactically.

Legionellosis in humans usually manifests itself as
pneumonia, often involving other organs such as the liver,
kidney, intestines and the central nervous system (147).
It also has been encountered in conjunctivitis (81). The
infection is invariably airborne and has not been shown to
be contagious (119). Multi-organ involvement is the conse-
quence of dissemination of the bacteria or their products
from the lung. In the experiments carried out under this
study the bacteria were introduced by intraperitoneal injec-
tion and found to rapidly metastasize to the blood and lungs
of the rabbit and the rat. Others (101, 142, 221) have also
injected L. pneumophila into rats and other rodents, intra-
peritoneally as well as by intranasal installation, and
reported infection of the lungs, liver, spleen and blood.

Quite accidentally, we obtained evidence that the
Legionella bacterium can be transmitted orally. Following
the death of the first rabbit that had been infected intra-
peritoneally with a suspension of L. pneumophila, a rabbit

in an adjacent cage became ill, showing symptoms identical
to those of the experimental animal. It was killed by
ether anesthesia and L. pneumophila was readily isolated on
CYE agar from blood and lung tissue. Before thoroughly
scrubbing and disinfecting the animal room, efforts were
made to isolate the bacterium from swabs of the cages and
environmental surfaces and from samples from the water
bottles. Legionellae were subsequently recovered, but
only from two samples of water from the deceased rabbit's
water bottle. The other three rabbits in the lower bank of
cages remained healthy and showed no clinical signs of
legionellosis. We assume that the water bottle from the
cage of the rabbit deliberately infected was exchanged with
the one from the adjacent cage. The second rabbit thus
consumed water laden with L. pneumophila and acquired the
disease. Katz and Matus (120) raised the question of the
likelihood of transmission of legionellae in experimental
animals via the ingestion of contaminated water.

The pathogenic mechanisms responsible for the devas-
tating effects of LDB in some species of experimental
animals are not clear. However, the fact that the patho-
logical picture, especially in lungs and liver of the rats
and rabbits we studied, was dominated by vascular changes
and necrosis, suggests that endotoxin and possibly other
bacterial toxins were, at least in part, responsible for
the histopathology seen.

Bacteria produce disease by adherence to cells, intra-
and intercellular invasion, elaboration of exotoxin, release
of cell wall endotoxin, triggering certain immunologic
mechanisms and by a combination of these factors (207).
Local tissue invasion does not appear to be a predominant
factor in the pathogenesis of LD. In lung tissue, LDB are
located in alveolar spaces, both within macrophages and
leukocytes and extracellularly (37). Tsai and his asso-
ciates (199) have shown that pulmonary, gastrointestinal,
renal and central nervous system abnormalities occur fre-
quently in human legionellosis. However, legionellae have
been isolated only from lung tissue, lung exudates and blood
(29, 57), thus suggesting that the pathologic effects seen
in the other tissues results from the elaboration of toxins
by LDB.

A number of toxins have been isolated from L. pneumo-
phila, including a hemolysin, cytotoxins, endotoxins (5,
82, 218) and a number of extracellular enzymes such as
lipase, proteases, phospholipases, DNAses, and an enzyme
with chemotrypsin-like activity (195, 196). Whether any
or all of these substances are responsible for tissue
damage is not proven; to date little in vivo work on these
"toxins" has been attempted. Friedman (82) provided evidence
that some L. pneumophila toxins inhibit certain biochemical
functions of polymorphonuclear cells, thus possibly affecting

their capacity to deal with the bacteria. Horwitz (199) has demonstrated that L. pneumophila prevents some lysosome-phagosome fusion in macrophages, hence preventing exposure of the bacteria to the lysosomal enzymes.

At present it is not known whether damage to lung tissue is caused solely by viable L. pneumophila organisms and their toxins or whether the bacteria, living or dead, release endotoxin and other cytotoxic factors. Ward (203), in reviewing the immunology and immunopatholy of LDB, found little evidence to support the operation of immunopathologic processes in legionellosis.

Rats that we immunized with legionella OMPs by footpad injection developed active immunity which was demonstrated to protect them against a lethal dose of the homologous strain. Moreover, we found that resistance could be acquired passively by the injection of serum from rats or rabbits immunized with OMPs. Specific Legionella antibodies, therefore, would seem to protect the host against infection by this bacterium; this has been found by others (28, 187). However, our results are the first to demonstrate an immune response to Legionella OMPs and to reveal that they lead to protective antibodies.

The role of cell-mediated immunity (CMI) in legionellosis is poorly understood. The bacteria have been shown to invade and grow in cultured monkey (125) and guinea pig

(126) macrophages and human peripheral monocytes (106).
Protection against Legionella could result from the release
of lymphokines from antigen-sensitized T-lymphocytes thereby
activating macrophages with enhanced microbicidal activity.

Initial studies by Wong et al. (222) have shown that
guinea pigs developed marked cutaneous hypersensitivity
after sensitization with heat-inactivated cells, purified
serotypic antigen or cross-reacting antigens of L. pneumo-
phila; these skin reactions appeared within two to four
weeks after immunization. Likewise, Wong and Feeley (223)
found that this hypersensitivity could be induced by sero-
group antigens mixed with cross-reacting antigens isolated
from L. pneumophila.

Although humoral immunity by itself resulted in sig-
nificant protection in our rat model, the participation of
cellular immunity in defense against L. pneumophila, as
postulated by several authorities (83, 107), cannot be
ignored. The legionellae join a long list of pathogens
from bacteria (e.g., Mycobacterium tuberculosis, Nocardia
spp., Salmonella typhi, Brucella spp., and Treponema
pallidum) to many fungi and viruses, which produce intra-
cellular infection, leading to cell-mediated immunity
(delayed hypersensitivity) in the infected host (189), often
detected by antigenic preparations injected intradermally.
Legionella pneumophila invades and multiplies within phago-
cytic cells, especially in the alveolar macrophages of

lungs (147); thus, it is well sheltered from interacting with the cognate antibodies known to arise in the host (which then may enlist a T-cell response).

If CMI responses do take part in the defense of the host against the legionellae, our result showing the DEA-OMP preparation to induce an excellent CMI response takes on added significance.

Patients with Legionnaires' disease develop both humoral and cell mediated immunity to L. pneumophila (107). Moreover, under some circumstances, antibody may be counter-productive by enhancing the uptake of L. pneumophila by mononuclear phagocytes in which the bacteria multiply (106). In contrast, CMI would appear to play a major role in host defenses against L. pneumophila. Thus, mononuclear phago-cytes are activated and inhibit the intracellular multipli-cation of L. pneumophila (43). Daisy and Rinaldo (49, 171) have shown that legionellae can invade and grow intra-cellularly in tissue culture cells which are not phagocytes. Upon recovery from LD, patients are found to have peripheral blood mononuclear cells sensitized to L. pneumophila anti-gens (166). Remarkably, such cells are present for many months and in one case, persisted for nearly two years after full recovery (167).

Although the guinea pig has been used as the animal model in most work with L. pneumophila, less is known about

the rat; thus, it was used as the animal model in this study. The rat would seem to be a most appropriate model for further studies in the development of experimental Legionella vaccine since it has been shown to be more resistant to Legionella infection than certain other experimental animals and also responds immunologically to legionellae. The rat more closely mimics the relative resistance of humans to L. pneumophila than the guinea pig, which is considered to be highly susceptible (178).

Our results agree with, and extend, previous observations concerning immune responses, especially delayed hypersensitivity, to L. pneumophila. As L. pneumophila is an intracellular parasite, and thus would be expected to induce delayed hypersensitivity, the diagnosis of clinical or subclinical legionellosis could be facilitated by an identification test using such antigens as OMPs, as we demonstrated in the rat. Currently, the diagnosis is often delayed because of the difficulty in isolating the organism from patients, and because of the lag time (ten days) required to demonstrate a significant rise in antibody titer between acute and convalescent sera by immunofluorescense (211). As L. pneumophila is widespread in the natural environment (80), a skin test could be useful in epidemologic studies designed to assess the prevalence of legionellosis in the population, an important aspect of the disease which remains to be elucidated (180).

The study presented here should enhance interest in understanding the pathogenesis and development of protective immunity to L. pneumophila. This interest concerns both basic and applied research, leading eventually to the development of a protective vaccine. The results of our study have revealed that the double emulsion adjuvant (DEA) is superior to the well-known Freund's complete adjuvant (FCA), which is not acceptable for use in human medicine, in inducing both active and cell-mediated immunity toward the OMPs of L. pneumophila. This is a highly significant finding since DEA might be acceptable to the Food and Drug Administration for use in a human vaccine--FCA is not acceptable.

Conclusion

It can be concluded from this investigation that: (a) many proteins exist in the outer membrane of L. pneumophila, regardless of the serogroup, a common one (24 Kd) being shared by all eleven strains tested; (b) the OMP of L. pneumophila serogroup 7 are immunogenic in the rabbit and rat, leading both to active and cell-mediated immunity; (c) parenteral administration of OMPs of the legionellae leads to the production of protective antibody; (d) immunoadjuvants, particularly a double emulsion type, enhance the immune responses to legionella OMPs; and (e) immunization

with legionella OMPs protects the rat from an otherwise lethal dose of L. pneumophila.

Further research is needed on the identity of those OMPs, and other specific Legionella antigens to which the immune system responds. Also, it would be important to investigate the existence of common interspecific Legionella antigens which lead to protective antibodies and which, also, would establish a common marker for identification of Legionella species.

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