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Cytopathologic alterations induced by the agent

of ovine chlamydial polyarthritis

by

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INTRODUCTION

Ovine chlamydial polyarthritis (OCP) is one of a number of arthridities affecting lambs. Recognition and description of the disease and its cause occurred within the last decade (Mendlowski and Segre, 1960; Mendlowski et al., 1960; Storz et al., 1963; Shupe and Storz, 1964; Livingston et al., 1965; Pierson, 1967). Earlier recognition of the causal agent was undoubtedly hampered by the technical difficulties encountered in isolation and identification of agents of the genus Chlamydia (Psittacosis-lymphogranuloma agents). Ovine chlamydial polyarthritis is an acute febrile disease of lambs which occurs in epizootic proportions in the western and southwestern United States. That found in other states has been limited to lambs originating in the West. It is characterized clinically by fever, listlessness, conjunctivitis, and severe lameness which may meliorate in 2-3 weeks or become chronic. Descriptions of pathologic changes differ somewhat but basically consist of inflammation of synovial and subsynovial structures of most freely movable joints of the limbs. In chronic cases, there is fibrous thickening of the periarticular structures. A systematic study of the cytopathic effects of this agent by either light or electron microscopy has not been described, nor have the ultrastructure or replication of the causal agent been defined.

This study is an extension of the first reported outbreak

of OCP in Iowa (Page and Cutlip, 1968). In the fall of 1966, a flock of feedlot lambs in northern Iowa developed signs and lesions typical of those reported for OCP. An organism was isolated in embryonated chicken eggs from affected joints and the disease experimentally reproduced. The agent was identified as a member of the genus Chlamydia by characteristic growth in embryonated eggs and by antigenic analysis. It was further characterized as belonging to the species Chlamydia psittaci by resistance to the growth inhibitive effects of sodium sulfadiazine and failure to produce iodine-stainable carbohydrates during growth. Difficulties encountered during this outbreak in attempting to demonstrate the agent in tissues by histopathologic and electron microscopic techniques and the aforementioned undetermined factors of the disease prompted additional studies.

This project was designed to determine the ultrastructure and replicative cycle of the OCP agent, to further evaluate pathogenesis and cytopathology of the articular lesion, and to appraise microscopic methods of detecting the organism. Toward these ends, several techniques were employed, including histopathologic, histochemic, immunohistochemic, and electron microscopic methods on infected cell cultures and ovine synovial tissues.

REVIEW OF LITERATURE

Ovine Chlamydial Polyarthritis

Ovine chlamydial polyarthritis was described for the
first time as a distinct entity in western lambs shipped to
a Wisconsin feedlot during the fall and winter of 2 successive
years, 1957 and 1958, (Mendlowski and Segre, 1960; Mendlowski
et al., 1960). There were 3% and 7% mortalities in the respec-
tive years. The disease was characterized by an insidious on-
set with the highest losses approximately one month after the
lambs were received. The most notable sign was lameness with
a particular rigidity of the affected limbs. Fever, depres-
sion, loss of weight, and anorexia were constant, but complete
anorexia occurred only in the most severely affected lambs.
Most lambs eventually recovered but a few were permanently
lame. Their major necropsy findings were serous to fibrinous
synovitis with periarticular edema and petechiation. Micro-
scopically, the synovium was edematous, infiltrated with mono-
nuclear cells and overlaid with a fibrinocellular exudate.
Synovial intimal cells were swollen and in various stages of
degeneration. Changes were not observed in the articular car-
tilages or in adjacent bones. Numerous minute coccoid bodies,
which they interpreted as chlamydial elementary bodies, were
seen with Giemsa and Macchiavello stains in synovial inflammatory
cells and free in the exudate of the joints. Minor inflamma-

tory changes were observed in the liver and lungs. They cultivated the chlamydial agent in yolk sacs of chicken embryos and experimentally reproduced the disease. Guinea pigs were shown to be susceptible; whereas, mice were resistant to experimental infection. Articular lesions in experimentally infected lambs were the same as in naturally infected lambs; however, changes in other tissues were more extensive. There were interstitial pneumonia, hyperplasia of the reticular cells of the spleen, proliferation of Kupffer cells, and degeneration of hepatocytes and renal epithelial cells. With Giemsa and Macchiavello stains, they found elementary bodies in impression smears and sections of these parenchymal organs as well as in the articular tissues and exudate.

Storz et al. (1963) and Shupe and Storz (1964) reported the disease, known locally as "stiff lamb disease," to be widespread among range lambs in the intermountain region of the West. Most lambs affected weighed from 55-105 pounds; larger lambs were more frequently involved than smaller ones. Morbidity varied among flocks from 2-75%. Mortality was reported as low. Penicillin was effective during the acute stage of the disease but later was ineffective. Such chronically affected lambs did not recover to make satisfactory weight gains. Signs and lesions were similar to those described by Mendlowski and Segre (1960). However, Shupe and Storz (1964) reported changes in the articular cartilage of some lambs. These were slight erosion of the surface of

articular cartilages and marginal compensatory changes (lip-
ping). The round ligament of the coxofemoral joint was
shredded and fibrillated in some lambs. They also found
meningoencephalitis, characterized by gliosis, focal accumu-
lations of inflammatory cells and mild vasculitis. Intra-
cellular elementary bodies were found in smears of the choroid
plexus. They characterized the disease as a "polyarthritis,
tendovaginitis (tendosynovitis), tendofascitis and periar-
ticular myositis" and stated the "most striking change ap-
peared to be articular and periarticular reactive fibroblast-
like proliferation." The synovial changes were considered
to be a "granulomatous, inflammatory, hyperplastic reaction."

The pathogenesis of chlamydial polyarthritis was studied
by Storz et al. (1965) in experimentally infected lambs.
Lambs were exposed by either intravenous, intramuscular, or
intraarticular injection of chicken-egg-yolk-sac-propagated
agent and killed 3, 7, and 21 days postinoculation. Intra-
articularly inoculated lambs were lame in those joints exposed
directly 12 hours postexposure and manifested lameness in
other limbs 6 days after inoculation. The intravenously and
intramuscularly inoculated lambs developed stiffness 3 days
after inoculation and were obviously lame within 5 days of
exposure. The authors were able to reisolate the agent from
all lambs (joints and various internal organs) at each of the
3 termination periods, regardless of the route of administra-
tion. Thus, the agent did not remain localized in the joints

of lambs inoculated intraarticularly. Articular lesions were
minimal (increased fluid, petechiae and hyperemia) at 3 days
postexposure. Lesions were extensive and similar, 7 and 21
days after inoculation, regardless of the route. They found
an increased amount of fibrin-containing synovial fluid,
hyperemia and petechiation of the synovial membranes and
tendon sheaths, swollen synovial cells of irregular size, and
accumulations of mononuclear cells in the subsynovial tissues
which were regarded as granulomatous in nature. Polymorpho-
nuclear cells were scattered among the synovial cells. The
kidneys, hearts, livers, lungs, lymph nodes and ependymal
tissues were hyperemic, edematous, and infiltrated with lym-
phocytes and monocytes.

In 1965, Livingston et al. reported the disease to have
been enzootic in sheep raising areas of Texas for 15 years.
Lambs 2-4 months of age were affected most frequently with
an average morbidity of 80% in infected flocks. Deaths were
infrequent. Complement fixation tests were considered useful
as a diagnostic aid in older lambs but not in young lambs
which were believed to be incapable of producing detectable
complement fixing antibodies.

Pierson (1967) studied OCP in a large number of feeder
lambs in Colorado. He stated the disease had probably existed
in Colorado for 25 years and that it was one of the most common
nonfatal diseases of feedlot lambs in that state. The average
morbidity rate of epizootics involving 80,000 lambs since 1951

was less than 10%, but in some feedlots approached 100%. Most
outbreaks were recorded during October and November, 2-4 weeks
after transporting the lambs. Signs and lesions were similar
to those reported by others. Tylosin, oxytetracycline, chloro-
tetracycline, and penicillin were reported to be effective
therapeutic drugs when used early in the course of the disease.
He considered tylosin and oxytetracycline to be the most effi-
cacious of the antibiotics tested.

An outbreak of OCP in Iowa (Page and Cutlip, 1968) in-
volved 2% of a flock of 2,000 feeder lambs which had origi-
nated in Idaho and South Dakota. A tentative diagnosis was
based on signs, lesions and serologic evidence and confirmed
by isolation of the organisms in embryonated chicken eggs.
The disease was reproduced in 3-month old lambs by the intra-
venous and intraarticular routes.

In addition to confirmed reports of OCP, Shupe and Storz
(1964) suspected chlamydiae as the cause of lameness in lambs
reported by Griner (1959) and by Biberstein et al. (1959).
Griner (1959) described a number of outbreaks of polyarthritis
of unknown etiology in feeder lambs in Colorado. The lambs
had been shipped to Colorado feedlots from several western
and southwestern states including Wyoming, Montana, Colorado,
Texas, and New Mexico. Lameness appeared 3-4 weeks after
shipment. He described a fibrinopurulent polyarthritis char-
acterized by thickened, edematous synovial membranes contain-
ing fibrin, neutrophils, lymphocytes, and macrophages, with

fibroblastic proliferation along the luminal surface, myositis at the myotendon junction and an increased volume of synovial fluid containing fibrin and neutrophils. He attempted to transmit the disease to mice, guinea pigs, and normal lambs with synovial fluid from arthritic lambs but was unsuccessful. Biberstein et al. (1959) reported a similar disease of unknown cause in nursing and feeder lambs in California.

Marsh (1947) reported a nonsuppurative arthritis of lambs in several areas of the western United States from which Corynebacterium ovis was isolated and believed to be the cause. He later (Marsh, 1965) suggested this may have been OCP.

The mode of transmission of the OCP agent is uncertain but has been assumed to be via inhalation or consumption of infectious particles shed in feces or urine. Separate chlamydial isolates from the feces and joints of arthritic lambs have been shown by serum neutralization to be antigenically similar (Storz, 1966). Conjunctival infection has also been proposed as a route of entry for the polyarthritis agent (Storz et al., 1967).

Structure and Replication of Chlamydial Agents

The mode of replication of members of the genus Chlamydia has been studied for more than 60 years. As early as 1907, Halberstaedler and von Prowazek, as cited by Weiss (1949, p. 125), observed intracytoplasmic inclusions of the trachoma agent in conjunctival smears stained with Giemsa. They de-

scribed blue inclusions containing red granules and believed
the red granules represented the trachoma agent and the blue
matrix was of cellular origin. Linder, in 1909 and 1910, as
mentioned by Weiss (1949, p. 125), observed that trachoma
inclusions in tissue sections were composed of intracytoplas-
mic granules of different size and staining characteristics;
the larger blue granules he termed "initial bodies" and con-
cluded they divided and eventually formed smaller red granules
or elementary bodies.

The first systematic investigation of the growth cycle
of a member of the genus Chlamydia was done by Bedson and
and Bland in 1932. Similar investigations using light micros-
copy and conventional staining of infected tissues and cell
cultures have been done with various chlamydiae, including
the agents of psittacosis (Bedson, 1933; Bedson and Bland,
1934; Bland and Canti, 1935; Levinthal, 1935; Yanamura and
Meyer, 1941; Bedson and Gostling, 1954; Officer and Brown,
1960), trachoma (Gordon et al., 1960), lymphogranuloma
venereum (Rake and Jones, 1942), meningopneumonitis (Pinkerton
and Moragues, 1942), mouse pneumonitis (Karr, 1943), and
ovine enzootic abortion (Stamp, 1951). Except for minor
variations, these authors agreed that elementary bodies devel-
oped within the cytoplasm of cells into larger initial bodies
after a latent period of 5-20 hours. The initial body was
considered the vegetative form and division was regarded to
be by repeated binary fission, thus giving rise to new ele-

mentary bodies. Lysis of the cell was believed to release the newly formed elementary bodies.

Weiss (1949) claimed extracellular growth of the agents of mouse pneumonitis, feline pneumonitis, and meningopneumonitis occurred in the lungs of mice. The same agents grew only within the cell cytoplasm in embryonated eggs.

A principal difference existed over the nature of the intracytoplasmic inclusion formed. Inclusions were described as dense plaques or diffuse vesicular structures. In their initial report, Bedson and Bland (1932) described a dense inclusion which they called a "plaque" and considered it a true viral matrix; but, they later (Bedson and Bland, 1934) regarded it as closely packed elementary bodies. Some (Bland and Canti, 1935) believed the plaque essential for development of the agents while others (Levinthal, 1935; Yanamura and Meyer, 1941) regarded it as unnecessary. Levinthal (1935) considered the type of inclusion formed related to the degree of resistance offered by the cell. He believed compact inclusions represented comparatively greater cellular resistance. More recent work comparing the growth of various chlamydial agents has shown the density of the inclusion to depend on the particular host-agent system involved (Officer and Brown, 1960; Weiss (1955) stated that the environment influenced the type of inclusion formed; however, some of the strain characteristics of the agent were maintained regardless of the host cell type.

The resolving power of the light microscope was not adequate to settle the question of the appearance of the agents during the latent period (lag phase); i.e., from the time of inoculation until initial bodies appeared in the cell cytoplasm approximately 20 hours later. Many investigators have found a reduced infectivity during the lag phase of the growth cycle (Bedson and Bland, 1932 and 1934; Bland and Canti, 1935; Bedson and Gostling, 1954; Litwin, 1959). Bedson and coworkers were unable to demonstrate the psittacosis agent during the lag phase which they attributed to the size of the agent and limited resolving power of the light microscope. They believed the reduced infectivity of the psittacosis agent resulted during the change-over of the small elementary body form to the large initial body form within the cell. Bedson and Gostling (1954) demonstrated that suspensions with a high content of large forms were less virulent than preparations containing small forms.

Sigel et al. (1951) and Girardi et al. (1952) interpreted the rapid decline of infectivity of meningopneumonitis in chorioallantoic ectoderm of chicken embryos as a noninfectious phase of development. They suggested a true viral development and compared it with that of a bacteriophage. Extensive cytochemical investigations into the growth cycle of the psittacosis and trachoma agents were conducted by Pollard and coworkers (Pollard and Starr, 1962; Pollard and Tanami, 1962; Pollard et al., 1960). Infected cell cultures were studied

with the light microscope after acridine orange and immuno-
fluorescent staining. The findings were correlated with those
obtained after treatment of infected cells with various anti-
metabolites. They interpreted results of these studies as
direct proof of a true viral eclipse during the lag phase
of the growth cycle and synthesis by the cell of components
of the agent.

Observations with the light microscope and one-step
growth experiments left many questions unanswered about the
developmental cycle of chlamydiae (Armstrong et al., 1963).
Of particular interest was the nature of the agents during
the lag phase in which the infectivity had repeatedly been
shown to be decreased; the significance of which was contro-
versial (Armstrong and Reed, 1964). With the advent of the
electron microscope, the means became available by which dis-
crete structure of the agents during the entire developmental
cycle could be determined. However, early ultrastructural
studies were confusing because of artifacts produced during
the processing of tissues.

For technical reasons, the first use of the electron
microscope for studying chlamydiae was limited to examination
of air-dried or freeze-dried preparations shadowed with heavy
metals. Little concrete information was added to the knowl-
edge of the reproductive cycle gained by light microscopy.
Air-dried preparations of the agents of psittacosis (Swain,
1955; Heinmets and Golub, 1948; Rake et al., 1946), trachoma

(Collier and Sowa, 1958; Ito et al., 1951), feline pneumonitis
(Moulder and Weiss, 1951), and murine pneumonitis (Gogolak,
1953) revealed their mutual pleomorphism. Particles from
500 to 1000 nm in diameter were described. Most of the larg-
est particles consisted of a central electron-dense elevation
surrounded by a flat periphery of less density, assumed to
be membrane. Smaller particles appeared as rough-surfaced
spheres. Elongated particles with central constrictions were
occasionally seen. Freeze-drying revealed the flat periphery
and rough surface to be artifacts produced by drying in air
and established the spherical nature of the meningopneumonitis
agent (Crocker and Williams, 1955). Ito et al. (1951) and
Swain (1955) interpreted the difference in size and shape of
particles of the trachoma and psittacosis agents to represent
different stages of development and concluded fission was the
basic mode of replication. Heinmets and Golub (1948) studied
silica replicas of psittacosis-infected chorioallantoic mem-
branes of chicks and demonstrated entrance of the agent into
cells and intracellular particles which suggested division
by fission. However, they stated this did not exclude the
possibility of incorporation of the agent into normal cellular
constituents which then produced new infectious particles.

Thin section electron microscopy has been used by numer-
ous investigators to study the ultrastructure and develop-
mental cycle of different chlamydiae, including the agents
of meningopneumonitis (Gaylord, 1954; Tajima et al., 1957;

Litwin et al., 1961; Higashi et al., 1962), psittacosis
(Tajima et al., 1957; Litwin et al., 1961; Kajima et al.,
1964; Higashi, 1965), feline pneumonitis (Litwin, 1959),
trachoma (Mitsui and Suzuki, 1956; Mitsui et al., 1957, 1958,
1962 and 1964; Litwin, 1962; Bernkopf et al., 1962; Armstrong
et al., 1963; Higashi, 1965), lymphogranuloma venereum (Arm-
strong and Reed, 1964), and inclusion conjunctivitis (Mitsui
et al., 1967). Structure at different stages during the
growth cycle was easily determined; however, elucidation of
the structure of transitional forms proved more difficult.

Two structurally distinct types of particles have been
described; each type of particle was regarded as indistin-
guishable regardless of the chlamydial agent examined (Litwin
et al., 1961; Higashi, 1964, 1965). The smaller of the two
particles (small particle) was 200-300 nm in diameter and
corresponded to the elementary body seen by light microscopy.
It consisted of a central dense area, generally regarded as
the nucleoid, and a peripheral area of less density. Some
authors (Mitsui et al., 1964) reported an eccentric nucleoid.
The larger of the two particles (large particle) was 500-1000
nm in diameter and corresponded to the initial body seen with
the light microscope. It was much less dense than the small
particle and had a uniform internal structure of fine granules
and filamentous strands. Similar filaments have been observed
in the nucleoid of small particles and are believed to repre-
sent strands of deoxyribonucleic acid (Armstrong et al., 1963;

Mitsui et al., 1964; Erlandson and Allen, 1964). Intermediate
size particles (intermediate particle) have been described;
their structure is similar to the large particle except they
are smaller and contain a small central nucleoid (Mitsui et
al., 1964; Kajima et al., 1964; Anderson et al., 1965). There
is general agreement that large particles are most numerous
approximately 20 hours after infection; intermediate particles
then become apparent and finally at termination of the cycle
(30-72 hours after infection), small particles predominate.

Most electron microscopists have reported a single limit-
ing membrane surrounding the various size particles; others
(Mitsui et al., 1962, 1964; Gaylord, 1954; Anderson et al.,
1965) have found two membranes investing the large and inter-
mediate particles. Armstrong and Reed (1964) considered the
two membranes investing the large particles reminiscent of
the combined cell wall and cytoplasmic membranes of bacterial
cells. Internal membranes have been found in large particles
by several investigators, most of whom have attributed their
presence to various forms of replication including endosporu-
lation (Gaylord, 1954; Bernkopf et al., 1962; Higashi et al.,
1962). Other membranous-like structures ("whorls"), found
primarily in large particles, are believed similar to meso-
somes of bacteria (Erlandson and Allen, 1964).

Chlamydial replication was first examined in ultrathin
sections by Gaylord in 1954. Large and small particles of
the meningopneumonitis agent were described; some large par-

ticles had constricted centers and others had multiple dense internal granules. The different particle configurations were considered to signify replication by binary fission and multiple endosporulation. Small particles were believed to represent a spore-like stage. An amorphous substance found within the infected cells was considered of cellular origin and not directly related to reproduction of the agent. Bernkopf et al. (1962) also observed structures within large particles of the trachoma agent which they interpreted to represent a form of endosporulation.

Mitsui and coworkers (Mitsui and Suzuki, 1956; Mitsui et al., 1958, 1962) examined the trachoma agent and concluded replication proceeded by formation of polygonal structures from an amorphous matrix. These polygonal structures and other large particles were thought to divide by "polygonal fission," "sporogenous fission," or binary fission. Also, they found structural evidence suggesting the "possibility that the early forms of the agents are formed from mitochondria."

In 1957, Tajima et al. studied the growth of psittacosis and meningopneumonitis agents and concluded reproduction was of a viral nature. Following a complete morphologic eclipse, they found large amorphous nonmembrane-bound inclusions within the cytoplasm of infected cells. These inclusions, similar to those described by Mitsui et al. (1958, 1962), were regarded as viral precursor matrices. Membranes progressively

enclosed portions of the matrices and thus formed large par-
ticles which divided into equal or unequal parts by con-
striction of their limiting membrane. Small particles were
believed to be formed by condensation of large particles.

Following the viral-type reproduction proposal by Tajima
et al. (1957), Litwin and coworkers (Litwin, 1959, 1962;
Litwin et al., 1961) reported finding no morphologic eclipse
or viral matrix in cells infected with the agents of feline
pneumonitis, psittacosis, meningopneumonitis, or trachoma.
Instead, they found large particles of all the agents studied
which indicated a fission process. They were unable to deter-
mine if the fission was equal or unequal and suggested the
process may be too rudimentary to classify.

Entrance of chlamydial particles into cells was not ob-
served in thin sections until 1962 when Higashi et al. demon-
strated phagocytosis of the meningopneumonitis agent by strain
L cells. The remainder of the growth cycle was considered
similar to that reported by Tajima et al. (1957) except large
particles were found not to divide after formation from a
viral matrix.

In 1963 and 1964 three additional studies were reported
(Armstrong et al., 1963; Kajima et al., 1964; Mitsui et al.,
1964) which supported the observations of Tajima et al. (1957)
and Higashi et al. (1962). The agents of trachoma and psitta-
cosis were found to become unidentifiable subsequent to phago-
cytosis by cells. Approximately 20 hours later, reticulated

viral precursor was seen from which large particles were formed. The authors believed that after repeated fission, the large particles condensed to form small particles. Armstrong et al. (1963) found evidence, later supported by Anderson et al. (1965) that small particles were also capable of dividing.

After additional studies, 3 investigators refuted their earlier hypothesis of a viral type replication (Armstrong and Reed, 1964; Higashi, 1964, 1965; Mitsui et al., 1967). They concluded that a morphologic eclipse did not occur and that division was entirely by binary fission after enlargement of the phagocytized particle to the large particle stage. The fission process was believed comparable to the "pinching off" of gram-negative bacteria (Armstrong and Reed, 1964). The amorphous substance, originally believed to be viral precursor, was shown to be an artifact resulting from rupture of large particles which had been damaged by the fixatives (osmium tetroxide and potassium permanganate) and by polymerization of the embedment (merthacrylate monomers) (Armstrong and Reed, 1964; Higashi, 1964).

Electron microscopic detection of the OCP agent in lamb synovial tissues was compared with its isolation in chicken embryos by Norton and Storz (1967). Correlation was excellent. The agent was reported to be recognizable throughout the synovial membrane 7 and 14 days postinoculation. Isolated particles were found in synovial lining cells, in endothelial

cells, and in fibroblasts; numerous mononuclear cells contained inclusions filled with mature particles. After 36 days, the organism could not be demonstrated in synovial tissues by either culture or electron microscopy.

Structure and Function of the Synovial Membrane

Hamerman and Schubert (1962) listed the components of the synovial or diarthrodial joint as the capsule and synovial membrane, ligaments, fibrocartilages, synovial fluid, articular cartilages, subchondral bones and occasionally tendons. According to these authors, the synovial membrane is the glistening, white inner surface of the joint capsule and lines the joint cavity except over the articular cartilages. They reported a gradual transition from the thick bundles of collagen and large blood vessels of the capsule into a more delicate areolar tissue of the synovial membrane, with its fine collagen fibers, capillaries and many cells. The cells on the inner surface of the joint capsule were called synovial cells, intimal cells, lining cells, and surface cells.

Key, as quoted by Ham (1965, p. 470), distinguished 3 morphologic variations of the synovial membrane, based on whether the inner lining cells of the joint capsule rested directly on the fibrous capsule or was separated from it by a layer of areolar or adipose tissue. He considered the surface cells to be grouped close together in the areolar and adipose membranes and widely separated from one another

in the fibrous membrane. In sections stained with hema-
toxylin and eosin, surface cells of the fibrous membrane
were difficult to distinguish from subjacent fibroblasts.

Hamerman et al. (1961) found the tetrazolium technique
for demonstrating oxidative enzymes particularly useful in
revealing morphology of the lining cells. With this proce-
dure, they found cytoplasmic processes which anastomosed
at the surface of the synovial membrane to form a limiting
margin.

The synovial lining cells, in contrast to subjacent
cells of the synovium and joint capsule, were reported (Hamer-
man et al., 1961) to stain intensely for lactic dehydrogenase,
reduced diphosphopyridine nucleotide diaphorase and reduced
triphosphopyridine nucleotide diaphorase and with less inten-
sity for succinic dehydrogenase, isocitric dehydrogenase and
acid phosphatase. Glucose-6-phosphate dehydrogenase activity
was not found in the lining cells.

Ultrastructure of the normal synovial membrane has been
studied in man (Lever and Ford, 1958; Barland et al., 1962;
Coulter, 1962), the rabbit (Lever and Ford, 1958; Ghadially
and Roy, 1966; Adam, 1966), the pig (Roberts et al., 1969),
the rat (Roy and Ghadially, 1967), the calf (Langer and Huth,
1960), the guinea pig (Langer and Huth, 1960; Wyllie et al.,
1964), the dog (Langer and Huth, 1960), the cat (Lever and
Ford, 1958) and the chicken (Luckenbill and Cohen, 1967).

Barland et al. (1962) described 2 cell types (or 2 states

of activity of a single cell type) in the synovial lining
based on their cytoplasmic contents. Type A cell was more
numerous and contained a prominent Golgi apparatus, numerous
single-membrane-bound vacuoles (0.4-1.5 μ in diameter) con-
taining varying amounts of a dense granular material, many
filopodia (finger-like surface projections), mitochondria,
intracellular fibrils, and micropinocytotic-like vesicles.
Type B cell contained large amounts of granular endoplasmic
reticulum, fewer large vacuoles, micropinocytotic-like ves-
icles and mitochondria. They found long cytoplasmic proc-
esses of these cells which were usually directed toward the
membrane surface where they overlapped and intertwined. The
lining cells formed a discontinuous outer margin, 2-3 cells
in depth, and gradually merged with the underlying connective
tissue where fewer cells, with typical appearance of fibro-
blasts, were found. The intercellular matrix of the lining
cells was an amorphous material similar to that observed in
cytoplasmic vacuoles of the type A cell. They found numerous
thin nonperiodic filaments in the intercellular matrix but
no collagen fibers were present. Langer and Huth (1960) found
a distinct basement membrane separating the lining cells from
the underlying collagenous tissue in calves, guinea pigs and
dogs. In contrast, Coulter (1962) and Roberts et al. (1969)
demonstrated fibers of collagen extending to the synovial
surface in man and swine respectively. Roy and Ghadially
(1967) stated that "in contrast to the cells of the synovial

membrane of other species, the cells of rat synovium form
a more or less continuous layer facing the joint cavity. The
adjacent cell walls show many invaginations and also struc-
tures resembling tight junctions and desmosomes."

Roberts et al. (1969) described 4 types of cells within
the synovial membrane of swine. They found mast cells char-
acterized by large (134 nm by 400 nm) irregular shaped cyto-
plasmic granules, cells with regular outline and well devel-
oped granular endoplasmic reticulum, cells with a regular
outline and a minimal number of cytoplasmic organelles, and
cells with an irregular outline and numerous large vacuoles
and pinocytotic vesicles. Acid phosphatase activity was found
in the large vacuoles of the latter type cells. Mast cells
were reported (Asboe-Hansen, 1950) to be common in the syno-
vium of man and were considered as a possible source of hy-
aluronate.

Luckenbill and Cohen (1967) examined the synovial mem-
brane of the chicken and found cells similar to those reported
by previous workers. They regarded the lining cells as a
single type; variation in the organelle content was believed
to be a function of their distribution within the cytoplasm
and plane of the section.

In 1929, Key, as cited by Ball et al. (1964) observed
that synovial lining cells ingested particulate matter in-
jected into the synovial cavity. The next year, Franceschini,
as cited by Adam (1966), suggested that synovial membranes

should be considered part of the reticuloendothelial system.
The high acid phosphatase activity of the lining cells was
interpreted by Hamerman et al. (1961) as evidence for abundant
lysosomes, thus indicating these cells may absorb and hydro-
lyze material in synovial fluid. This view was supported by
a suggestion by Barland et al. (1962), based on ultrastructure,
that the type A synovial cell may have phagocytic properties.

Ball et al. (1964) found when iron dextran was injected
into the joints of rabbits, the vacuolar (type A) cells rapid-
ly took up the iron. Iron was rarely found in the endoplasmic
reticulum-containing (type B) cells. They found the iron
eventually was concentrated in macrophages just beneath the
lining layer of cells and suggested that the macrophages may
have arisen from vacuolar lining cells. On the other hand,
Adam (1966) found intraarticularly injected colloidal carbon
was removed from the synovial cavity primarily by invading
macrophages. He stated that these cells could be regarded
as synovial cells only in the sense of location. The type A
and B cells of the synovium were found to ingest very little
of the carbon particles. He suggested that the type B cell,
with its extensive Golgi apparatus, was important in the pro-
duction of mucopolysaccharide.

Luckenbill and Cohen (1967) studied the phagocytic prop-
erties of synovial lining cells of the chicken and found that
a colloidal suspension of carbon injected into the joint cavi-
ty was removed by synovial cells which exhibited both vacuoles

and synthetic organelles such as endoplasmic reticulum and
Golgi apparatus. Carbon was found only in surface cells in
the early stages (15 minutes and 2 hours) and in later stages
(3 and 7 days) deeper within the synovial membrane. They
interpreted this as evidence that the synoviocyte removed
material from the joint fluid and transported it to the sub-
synovial region.

Hyaluronate has been shown to be synthesized by synovial
membrane slices (Yielding et al., 1957) and by synovial mem-
brane cell cultures (Castor, 1957; Hedberg and Moritz, 1958)
but the exact cell involved was uncertain. The finding of a
higher level of oxidative enzymes in the lining cells than in
the other connective tissue cells of the synovial membrane,
the location of the lining cells on the outermost part of the
synovial membrane, and the presence in some synovial membranes
of a hyaluronidase-susceptible, metachromatically staining
substance in apparently clear spaces enclosed by lining cell
processes, led to the suggestion by Hamerman et al. (1961)
that the lining cells constitute the major site of synthesis
of hyaluronate.

Wyllie et al. (1964) described 2 types of microbodies
associated with the large Golgi apparatus of the type A lining
cell. They hypothesized that these microbodies represented
a storage form of hyaluronate synthesized by the cell. They
further suggested that the type B cell may be involved in
protein synthesis and secretion. Barland et al. (1962) had

also associated granular endoplasmic reticulum with protein

synthesis but said it remained to be determined if type B

cells synthesize and secrete protein. Previous to this specu-

lation of protein synthesis by synovial cells, protein of

synovial fluid was assumed to originate by diffusion from

the plasma (Schmid and MacNair, 1958). Sandson and Hamerman

(1962) reported isolating a hyaluronateprotein complex from

normal human synovial fluid. Protein accounted for approxi-

mately 2% of the complex. The function and origin of the

protein were not known but they suggested it may participate

in synthesis of hyaluronate by synovial cells or could be

added at the cell membrane and play a role in the passage

of hyaluronate to the synovial fluid. They (Hamerman and

Sandson, 1963) found hyaluronateprotein isolated from human

joints with rheumatoid arthritis or gout contained about 10%

protein and later (Sandson and Hamerman, 1964) demonstrated

by immunoelectrophoresis that the protein of the complex was

an alpha-globulin. Blau et al. (1965) have shown by immuno-

fluorescence that hyaluronateprotein is localized in the lining

cells and their intercellular spaces. They concluded that the

protein moiety of hyaluronateprotein is not a serum protein,

but is probably synthesized with the hyaluronate in synovial

lining cells. Williamson et al. (1966) found with immuno-

electrophoresis that a protein of alpha-globulin mobility,

antigenically related to serum $alpha_2$-macroglobulin and other

proteins antigenically related to serum proteins were syn-

thesized by cultured synovial cells.

Barland et al. (1962) summarized the function of the synovial membrane as follows: "It appears likely that the lining cells are the major source of hyaluronate, that they play a role in the turnover of the components of synovial fluid, and that their secretory products in the matrix influence the passage of molecules between blood and joint cavity."

MATERIALS AND METHODS

A general outline of the procedures followed during this study is presented in Table 1.
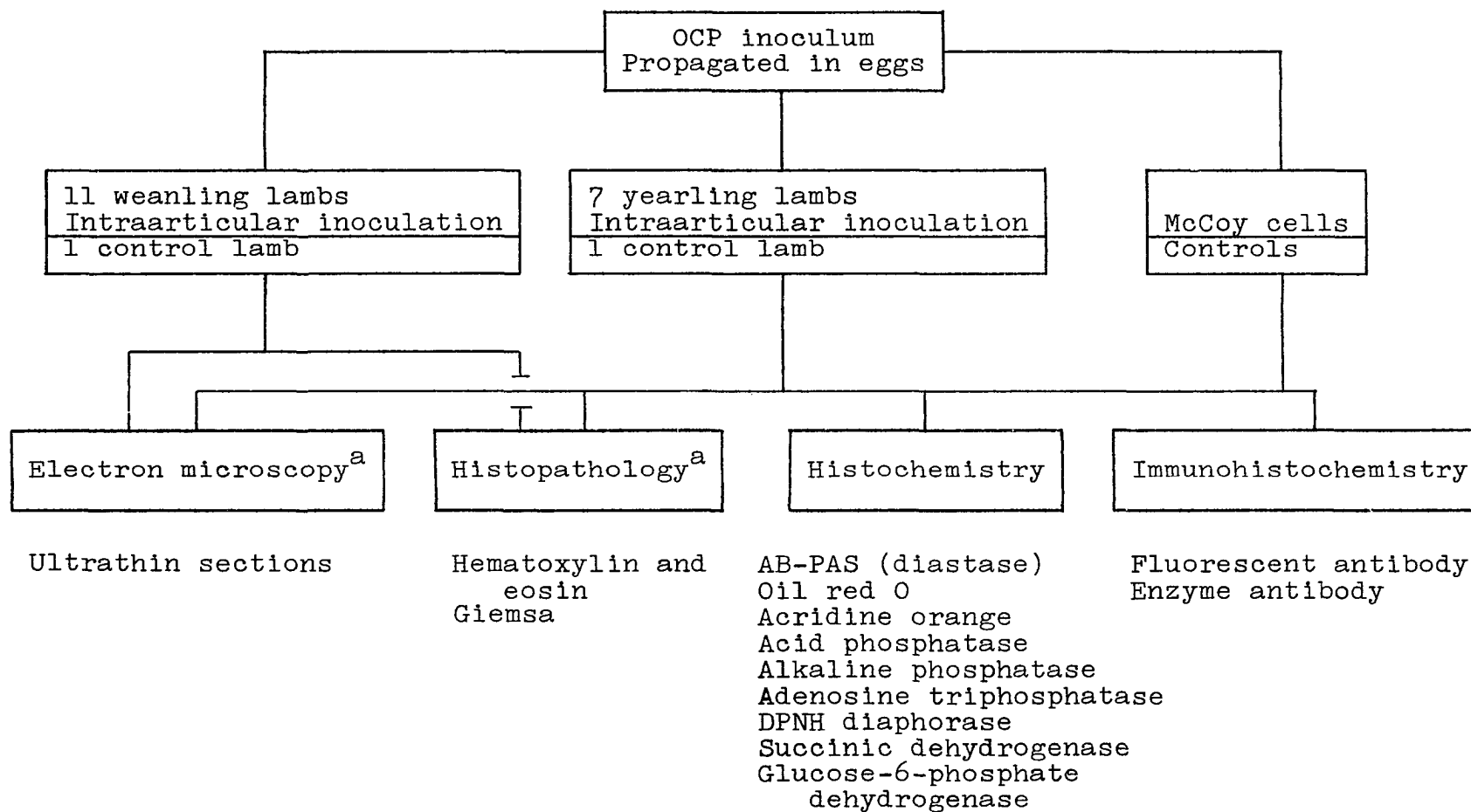
Agent

The organism was isolated from joint exudate of a lamb affected with OCP and identified as a strain of Chlamydia psittaci (Page and Cutlip, 1968). An inoculum of the OCP agent (OCP inoculum) for exposure of cell cultures and lambs was grown in yolk sacs of 7-day chicken embryos. Yolk sacs of embryos dying between 6 to 8 days after inoculation were harvested, homogenized in equal amounts of brain-heart infusion broth (Virtis "45" Homogenizer, The Virtis Company, Inc., Gardiner, New York), and centrifuged in the cold (4° C) for 10 minutes at 3,800 X g. The supernatant was decanted and centrifuged for 20 minutes at 27,600 X g and the sediment thus formed was resuspended in BHI broth and stored at -50° C until used. The chicken embryo LD_{50} of this material after freezing was 0.2 ml of a $10^{-5.6}$ dilution (Reed and Muench, 1938).

Noninfected egg yolk sac was prepared for inoculation of controls. The yolk sacs of 10 to 15-day incubated eggs were harvested and subjected to the same preparative procedures as the OCP inoculum.

All eggs used were verified to be free of bacterial con-

Table 1. Outline of experimental procedure


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                          ┌─────────────────────┐
                          │    OCP inoculum     │
                          │ Propagated in eggs  │
                          └─────────────────────┘
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11 weanling lambs Intraarticular inoculation	7 yearling lambs Intraarticular inoculation	McCoy cells
1 control lamb	1 control lamb	Controls

Electron microscopy[a]	Histopathology[a]	Histochemistry	Immunohistochemistry

| Ultrathin sections | Hematoxylin and
eosin
Giemsa | AB-PAS (diastase)
Oil red O
Acridine orange
Acid phosphatase
Alkaline phosphatase
Adenosine triphosphatase
DPNH diaphorase
Succinic dehydrogenase
Glucose-6-phosphate
dehydrogenase | Fluorescent antibody
Enzyme antibody |

[a]Articular tissues of 4 naturally infected and 2 intravenously inoculated lambs (1 control) were also examined.

taminants by incubation on blood agar at 37° C under aerobic

and anaerobic conditions.[1]

Cells

McCoy cells[2] were grown and maintained throughout the

study at 37° C in Medium 199 containing 10% fetal bovine

serum, 5% lactalbumin hydrolysate, and dihydrostreptomycin

and kanamycin each at 100 μg/ml. Cell monolayers for inocu-

lation were grown in roller tubes and in Leighton tubes con-

taining glass coverslips. When the cells were nearly con-

fluent, 0.2 ml of the OCP inoculum was added; after 1 hour

of incubation, the excess inoculum was removed and new medium

added. After incubation for 2, 8, 24, 48, 96, and 240 hours,

the cells were removed from the culture tubes and frozen at

-50° C or fixed as required for the various techniques. Cells

were removed at additional postinoculation intervals for elec-

tron microscopy. Cells from at least 2 tubes at each post-

inoculation time were examined by each procedure (Table 1).

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[1]Eggs were examined and found to be free of <u>Mycoplasmata</u>
by Dr. K. R. Rhoades of the National Animal Disease Laboratory,
Ames, Iowa.

[2]Monolayers of McCoy cells, ovine brain fibroblasts
(Oa-1B), embryonic ovine testicle cells, and guinea pig peri-
toneal macrophages (Allison and Mallucci, 1965) were inocu-
lated to determine which cell would best support growth of
the OCP agent. McCoy cells were found, by immunofluorescent
staining, to be relatively more susceptible and were used for
the remainder of this study.

Controls for all procedures included noninoculated cells and cells inoculated with noninfected egg yolk sac. Control cells were removed from the tubes after 24 and 96 hours of incubation.

Animals

Two groups of lambs from a commercial source were used and maintained in isolation throughout the study. One group consisted of 12, 6 to 7-week-old Suffolk and Shropshire lambs (weanling lambs) and the other consisted of 8, 10 to 12-month-old Suffolk lambs (yearling lambs). All were serologically negative for antichlamydial antibodies by the complement fixation test (Meyer and Eddie, 1964).[1]

Except for one control from each group, each lamb was exposed to the OCP agent by intraarticular inoculation. The right femorotibial and humeroradial joints of the weanling lambs and the right femorotibial joints of the yearling lambs were each injected with 0.5 ml of the OCP inoculum. The left femorotibial and humeroradial joints of 7 weanling lambs, including the control lamb, and the left femorotibial joints of 5 yearling lambs, including the control lamb, were each injected with 0.5 ml of noninfected egg yolk sac. An additional yearling lamb was inoculated with the OCP agent but did not become lame and was not included in this study.

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[1]Diagnostic antigen and known positive and negative serum controls were supplied by Dr. L. A. Page of the National Animal Disease Laboratory, Ames, Iowa.

Lambs were observed daily for signs of lameness and at predetermined intervals after inoculation they were killed with phenobarbital and necropsied immediately. Weanling lambs were killed 2, 4, 8, 14, 24, 48, and 72 hours and 6, 10, 12, and 24 days postinoculation. Yearling lambs were killed 8, 24, 48, 72, and 96 hours and 10 and 21 days postinoculation. The controls were killed after 24 hours (weanling lamb) and 10 days (yearling lamb).

Also included in this study were articular tissues from 4 naturally infected lambs, from 2 intravenously infected lambs, killed 11 days postinoculation, and from one noninoculated control lamb of the same origin as the lambs intravenously exposed.[1]

Synovial and subsynovial tissues were taken from several of the most inflamed areas of each femorotibial and humeroradial joint of the weanling lambs and each femorotibial joint of the yearling lambs. Smears of synovial fluid from the same joints were made on glass slides. Specimens from the yearling lambs were divided and either frozen in a dry ice-alcohol mixture or fixed as required for the different histopathologic, histochemic, immunohistochemic, and electron microscopic techniques (Table 1). Specimens from the weanling lambs, naturally infected lambs, and intravenously infected lambs were divided and fixed in either formalin or glutar-

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[1]These tissues were supplied by Dr. L. A. Page.

aldehyde for histopathologic and electron microscopic examination.

Two additional weanling lambs were used for antichlamydial antibody production.

Histopathology and Histochemistry

The stains and procedures listed below were used to evaluate histopathologic alterations and to localize specific chemical substances and organisms in McCoy cell monolayers and in synovial tissues. Methods for determining enzymatic activities were followed as described by Barka and Anderson (1965).

1. Hematoxylin and eosin: Formalin-fixed cell monolayers, smears of synovial fluid, and paraffin sections of synovial membranes were stained with Harris' hematoxylin and counterstained with eosin Y (AFIP manual, 1960).

2. Giemsa: Formalin-fixed cells, smears of synovial fluid, and paraffin sections of synovial membranes were stained by the May-Grunwald-Giemsa technique (AFIP manual, 1960).

3. Alcian blue-periodic acid Schiff: Formalin-fixed specimens were treated with periodic acid, stained with Schiff's reagent and counterstained with Alcian blue (AFIP manual, 1960).

4. Oil red O: Staining was done on formalin-fixed cells and cryostat sections (Preece, 1965).

5. Acridine orange: Ribonuclease treated and nontreated cells and cryostat sections, fixed in an ethanol-acetic acid mixture, were stained with acridine orange (Pollard and Starr, 1962).

6. Acid phosphatase: Localization was by Gomori's lead method using β-glycerophosphate as the substrate at pH 5.

7. Alkaline phosphatase: Activity of this enzyme was determined by the Gomori-Takamatsu calcium method at pH 9.2 with β-glycerophosphate as the substrate.

8. Adenosine triphosphatase: Activity was determined according to the calcium method of Padykula and Herman with the substrate, adenosine phosphate, at pH 9.4.

9. Reduced diphosphopyridine nucleotide diaphorase: Nitro BT was used as the hydrogen acceptor according to the method of Scarpelli as modified by Barka and Anderson (1965).

10. Succinic dehydrogenase: Localization of activity was by the method of Nachlas, as modified by Barka and Anderson (1965), utilizing the tetrazolium salt, Nitro BT as the hydrogen acceptor and sodium succinate as the substrate.

11. Glucose-6-phosphate dehydrogenase: Activity was
    determined by the tetrazolium method of Nachlas
    as modified by Barka and Anderson (1965). Nitro
    BT was used as the hydrogen acceptor, with the
    substrate, glucose-6-phosphate.

Cell monolayers from each postinoculation interval and
synovial tissues from each lamb were stained with hematoxylin
and eosin, and Giemsa.[1] Synovial tissues of yearling lambs
and McCoy cells were also stained with Alcian blue-periodic
acid Schiff, oil red O, and acridine orange (Table 1).

All enzymatic activities were determined on unfixed
frozen specimens of synovial tissues from yearling lambs and
McCoy cells. Infected and noninfected specimens were stained
in the same solutions. Substrate-deficient control solutions
were used for each enzyme; selected specimens were incubated
in solutions in which the respective substrate had been
omitted.

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[1]Preliminary staining of infected cell cultures and egg
yolk sacs by Castaneda's method (Meyer and Eddie, 1964), by
Pinkerton's modification of Macchiavello's method (AFIP manual,
1960), and by Giménez's method (1964) proved inferior to
Giemsa for detection of organisms and evaluation of cellular
changes. Organisms were easiest to find in eggs where they
occurred as compact intracytoplasmic inclusions. Similar
trials indicated phase microscopy was not satisfactory for
detection of organisms in cell cultures because intracyto-
plasmic granules were present in cells inoculated with either
the OCP agent or egg yolk sac. Such granules probably were
phagocytized egg yolk material. Similar results were reported
by Officer and Brown (1960) with the agent of psittacosis.

Ultraviolet light for examination of acridine orange stained slides was from a Leitz mercury vapor lamp equipped with a blue exciter filter (BG-12) and a blue absorption filter.

Immunohistochemistry

McCoy cells representing each postinoculation interval and synovial tissues from yearling lambs were stained by fluorescent antibody and enzyme antibody techniques. Synovial tissue from only one lamb was stained by the enzyme antibody technique.

Sheep antichlamydial antibody was prepared by exposing 2 lambs to the virulent organism, followed in 2 weeks by 3 biweekly intramuscular injections of a mixture of equal parts of the OCP inoculum and Freund's complete adjuvant (Difco, Inc., Detroit, Michigan).[1] The lambs were killed 2 weeks after the last injection and the antibody titer determined by serum neutralization (Cunningham, 1966). The serum with the highest titer (Neutralization Index = 4.4) was used for immunohistochemic procedures.

Fluorescent antibody techniques were, with minor modifi-

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[1]In an effort to obtain heterogeneous systems for use with immunohistochemical procedures, an unsuccessful attempt was made to establish persistent infection of the OCP agent in McCoy cells. After 4 serial passages, cell monolayers were noninfected as determined by fluorescent antibody staining.

cations, according to the methods of Coons (1958). The hyper-immunized lamb was killed, the serum harvested, and the gamma-globulins precipitated with equal parts of saturated ammonium sulfate. Ammonium sulfate was removed from the precipitate by dialyzing with 0.85% sodium chloride. The gamma-globulins were conjugated with fluorescein isothiocyanate, dialyzed, and stored at -50° C in 2 ml aliquots.

For enzyme antibody staining, the peroxidase method of Nakane and Pierce (1967) was employed. Horseradish peroxidase (Nutritional Biochemical Corporation, Cleveland, Ohio) was conjugated with ammonium sulfate precipitated gamma-globulins using p,p'-difluoro-m-m'-dinitrodiphenyl sulfone (General Biochemicals, Chagrin Falls, Ohio). The conjugate was divided into one ml amounts and stored at -50° C until used.

Before use, both conjugates were absorbed with dried, acetone-extracted rabbit-liver and egg-yolk-sac powders pre-pared according to the method of Coons (1958). Cells on coverslips and cryostat synovial sections were fixed in ace-tone,[1] air dried, flooded with rabbit anti-egg-yolk-sac serum and incubated at 37° C for 30 minutes before staining. The anti-egg-yolk-sac serum was prepared in rabbits by 3 biweekly intramuscular injections of noninfected egg yolk sac in Freund's complete adjuvant and the serum harvested 2 weeks

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[1]Antigenic specificity of the OCP agent as determined by fluorescent antibody staining was not retained after fix-ation in 95% ethanol, 10% formalin, or 2.5% glutaraldehyde.

after the last injection. After removal of the rabbit serum
by washing in 0.85% sodium chloride, the specimens were
flooded with the respective antichlamydial conjugates and
incubated at 37° C for 30 minutes.

Controls, in addition to noninfected specimens, consisted
of selected specimens incubated with nonconjugated antichla-
mydial globulins prior to application of each conjugate.

Specimens stained with the fluorescein conjugate were
observed immediately after staining using a Leitz mercury
vapor light source with a UV-UG1 exciter filter and a UV
absorption filter.

Sections of synovium to be stained with the conjugates
were selected by staining adjacent sections with hematoxylin
and eosin. Sections containing excess fat or fibrinous exu-
date were discarded. Subsequent to fluorescent antibody
staining, coverslips were removed from all specimens and
they were stained with Giemsa for further evaluation of
fluorescent particles.

Electron Microscopy

Infected cells were examined after 1, 2, 4, 8, 16, 24,
48, 72 and 96 hours of incubation and noninfected cells after
24 and 96 hours of incubation. Cells were removed from roller
tubes with 0.25% trypsin in 0.85% saline, washed in sodium
cacodylate buffer (0.2M, pH 7.4), fixed in 2.5% glutaralde-
hyde, embedded in agar, postfixed in 1% osmium tetroxide,

dehydrated in a graded series of ethanol, and embedded in epoxy resin (Epon 812: Shell Chemical Company, San Francisco, California) according to the method of Luft (1961).[1]

Synovial tissues and fluid from all lambs, except 2 naturally infected lambs, were examined by electron microscopy. Synovial fluid was fixed in glutaraldehyde and processed in the same manner as cell cultures. The synovial membranes were cut in 1 to 2 mm squares and processed likewise, except the agar embedment was omitted. For orientation with the light microscope, one micron sections of synovial membranes were cut and stained with paragon (Paragon C. and C. Company, Inc., New York, New York) according to the method of Martin et al. (1966).

Ultrathin sections were stained with uranyl acetate and lead citrate (Venable and Coggeshall, 1965), then examined with a Philips model 200 electron microscope.

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[1]Selected cell cultures infected with the OCP agent were also processed by the enzyme antibody method of Nakane and Pierce (1967) for electron microscopy. Acetone was used as the fixative. This procedure was not satisfactory; particles of the agent in enzyme antibody stained and unstained sections were indistinguishable.

RESULTS

McCoy Cell Cultures

Cytopathology

Hematoxylin and eosin   Monolayers of infected cells,
stained with hematoxylin and eosin, appeared unaffected after
48 hours of incubation. At 96 hours postinoculation, multiple
foci from which cells had detached from the glass were present
and at 240 hours, most cells were floating free in the medium.

Giemsa   Giemsa staining revealed a few cells in in-
fected monolayers with blue intracytoplasmic granules of the
OCP agent (Figure 1). None were observed prior to 48 hours
postinoculation, at which time only a few organisms were found.
After 96 hours the organisms were more numerous and were usu-
ally diffusely distributed in the cytoplasm. Less frequently,
dense aggregates of organisms were found. Similar inclusions
were not found in control monolayers.

Cytochemistry

Acridine orange   With acridine orange staining of
infected cell monolayers, many intracytoplasmic foci of red-
dish-orange fluorescence were present after 24 hours of incu-
bation (Figure 2). These fluorescent foci were smallest in
24-hour cultures and gradually increased in size with an in-
crease in incubation time. After 96 hours, the entire cyto-
plasm of many cells fluoresced a bright reddish-orange while

Figure 1.   Giemsa-stained-McCoy-cell monolayers 96 hours
            after inoculation with the OCP agent. (a)
            Organisms diffusely scattered in the cytoplasm
            of a cell and (b) in a compact intracytoplasmic
            inclusion in a mitotic cell. X 500

Figure 2.   McCoy-cell monolayers stained with acridine
            orange, (a) 24 hours, (b) 48 hours, and (c) 96
            hours after inoculation with the OCP agent.
            Orange cytoplasmic inclusions were smallest
            at 24 hours, larger at 48 hours, and filled
            the cytoplasm at 96 hours postinoculation.
            Note the similarity of color, indicating ribo-
            nucleic acid, regardless of the postinoculation
            interval. X 500

Figure 3.   McCoy-cell monolayers stained with acridine
            orange subsequent to treatment with ribo-
            nuclease, (a) 24 hours, (b) 48 hours, and (c)
            96 hours after inoculation with the OCP agent.
            Green inclusions (arrows), indicating deoxyribo-
            nucleic acid, were similar in size and location
            to orange inclusions without ribonuclease treat-
            ment in Figure 2. X 500
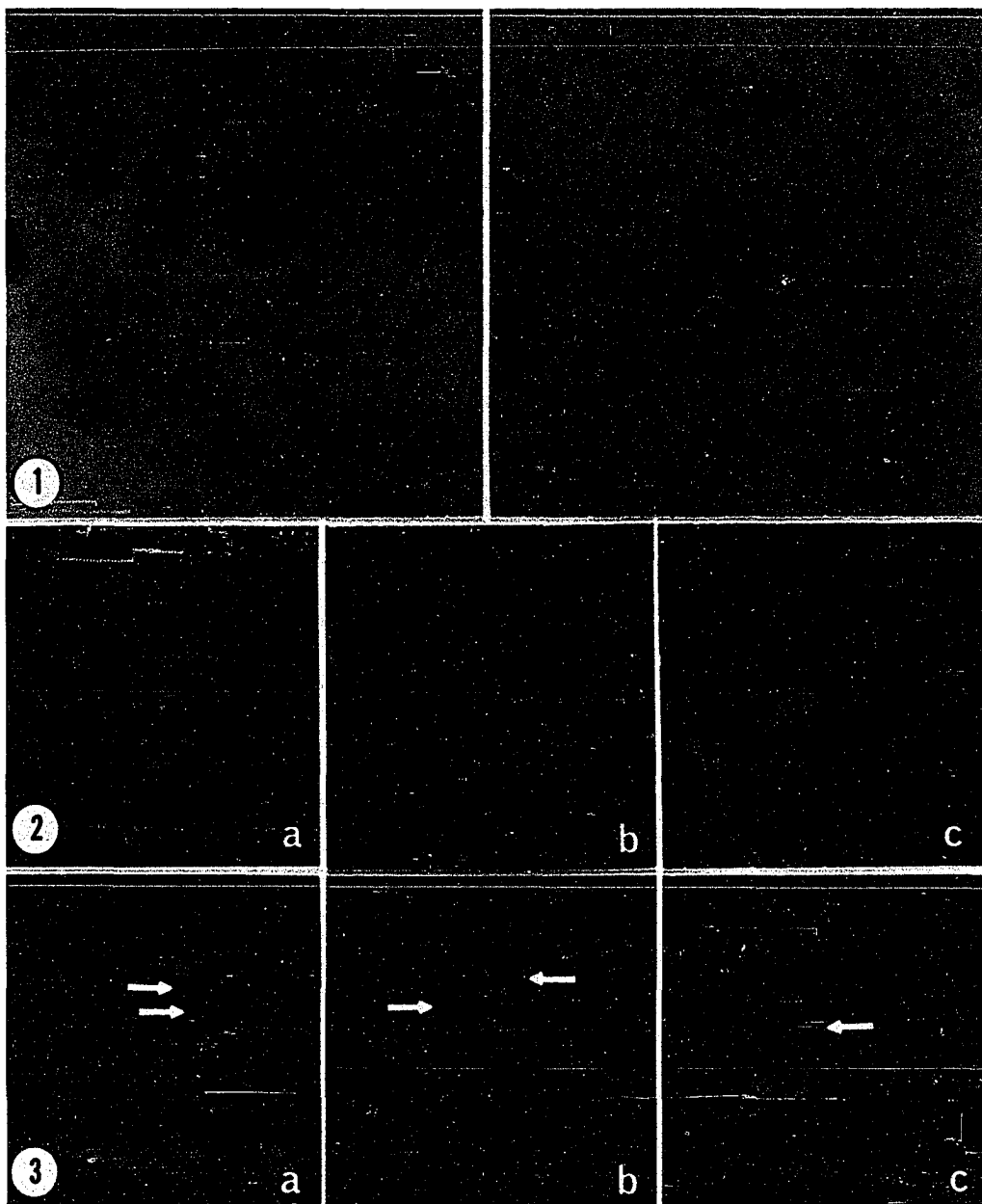
fluorescent foci similar in size and color to those in 24-hour
cultures were present in other cells. These foci appeared
as granular masses without definite borders, particularly
in older cultures, but were similar in color regardless of
age. Acridine orange staining of infected monolayers subse-
quent to treatment with ribonuclease caused green fluorescence
of cytoplasmic inclusions identical in size and distribution
to those without ribonuclease treatment (Figure 3). However,
green fluorescent inclusions were less distinct than orange
ones. Control monolayers did not fluoresce with the charac-
teristic inclusions of infected cells.

Alcian blue-periodic acid Schiff and oil red O   Dif-
ferences were not found between infected cells and cells to
which noninfected egg yolk sac had been added when stained
with oil red O or Alcian blue-periodic acid Schiff. Oil red
O staining material was found adhering to the surface of cells
and in small amounts within the cytoplasm. Noninoculated
cells did not stain with oil red O. The cytoplasm of all
cells, infected and controls, stained faintly with periodic
acid Schiff and contained multiple granules which stained
with Alcian blue. Staining was unaffected by treatment with
diastase.

Acid phosphatase   Inoculated and noninoculated cells
were similar in acid phosphatase activity. Cytoplasmic stain-
ing was diffuse, frequently with heavier concentrations at
one pole of the cell. Substrate-deficient controls were

negative.

Alkaline phosphatase   Following treatment to detect alkaline phosphatase activity, a fine precipitate was present on all infected and noninfected cells, and on the substrate-deficient controls. Specific alkaline phosphatase activity was not detected.

Adenosine triphosphatase   Cobalt sulfide precipitate, indicating adenosine triphosphatase activity, was diffuse throughout the cells. Intense nuclear staining was attributed to diffusion artifact. Difference was not detected in infected and noninfected cells. Substrate-deficient controls were negative.

DPNH diaphorase   Activity of this enzyme was confined to the cytoplasm of cells and was equal in infected and noninfected cells. Substrate-deficient controls were negative.

Succinic dehydrogenase   Formazan was deposited irregularly throughout the infected cell monolayers; however, because of similarity to controls, this could not be attributed to infection. Staining was light and localized in the cytoplasm.

Glucose-6-phosphate dehydrogenase   Cytoplasmic staining was diffuse in all cell monolayers to which the OCP inoculum or noninfected egg yolk sac had been added and occurred with less intensity in the cytoplasm of noninoculated cells. The difference was apparently related to the presence of egg yolk material.

Immunohistochemistry

Green fluorescent inclusions were present in the cytoplasm of OCP-infected cells incubated for 24, 48, 96, and 240 hours and stained by the fluorescent antibody technique (Figure 4). An estimated 10% of the cells contained fluorescent inclusions 24 and 48 hours after inoculation. At 96 hours, approximately 15% of the cells fluoresced and at 240 hours, most of the cells still adhering to the glass contained fluorescent inclusions. Minute fluorescent granules in cells prior to 24 hours of incubation were indistinguishable from controls. The inclusions, smallest at 24 hours postinoculation, increased in size to occupy most of the cell cytoplasm 96 and 140 hours after inoculation. They were composed of many small fluorescent particles. In addition to the large inclusions after 96 hours, many cells contained small inclusions similar to those at 24 hours postinoculation.

Staining of chlamydial inclusions with peroxidase-antibody conjugate was satisfactory only after 96 hours of incubation (Figure 5). The dark brown reaction product was identical in distribution to the large inclusions with fluorescent antibody staining. Smaller chlamydial inclusions, if present, were hidden by nonspecific stain precipitate.

Specificity of immunohistochemical staining was verified by blocking the reactions with nonconjugated-ovine-antichlamydial globulin and nonreactivity of noninfected cell monolayers. Substrate-deficient controls for the peroxidase

Figure 4. Chlamydial-infected McCoy cells (a) 24 hours
           and (b and c) 96 hours postinoculation.
           Stained with fluorescein-isothiocyanate-
           conjugated-antichlamydial antibody. Note
           the discrete cytoplasmic fluorescence at
           48 hours and diffuse cytoplasmic fluorescence
           at 96 hours. Arrows indicate the nuclei of
           the fluorescent cells. a. and c. X 500;
           b. X 125

Figure 5. Ninety-six-hour chlamydial-infected McCoy
           cells after staining with horseradish peroxi-
           dase-antibody conjugate. Dark brown to black
           cytoplasmic staining indicates the presence
           of organisms. a. X 50; b. X 320
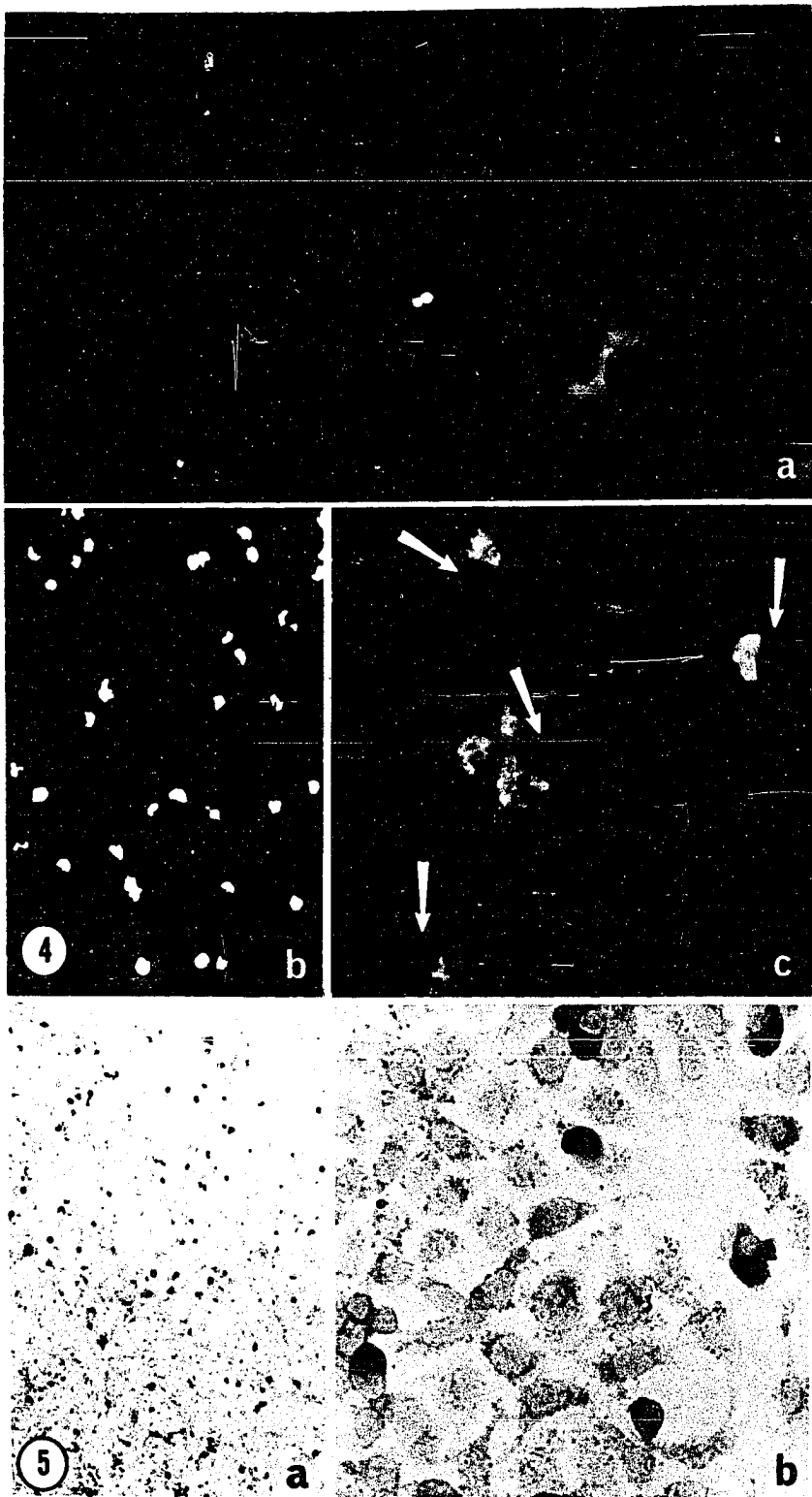
reaction were also negative.

Electron microscopy

Chlamydial organisms were found either in the extra-
cellular fluid or in the cell cytoplasm of all cultures inocu-
lated with the OCP agent. Structure of the agent varied from
small dense particles (small particles) with apparent rigid
cell walls to large granular particles (large particles) of
less rigidity and electron density. Small particles were
spherical, from 250-450 nm in diameter, with an eccentrically
placed electron-dense nucleoid and tightly invested by a multi-
laminated wall (Figure 6). The wall appeared to be composed
of 4 electron-dense laminas separated by electron-lucent
laminas; i.e., 2 unit membranes. This was more distinct
in organisms found in the extracellular fluid (Figure 7).
Particles (intermediate particles), intermediate in size and
structure to the small and large particles, were from 400-600
nm in diameter, had a centrally-placed nucleoid and were en-
veloped by either a rigid cell wall or by 2 unit membranes
(Figures 6 and 13). Large particles varied in size from 600-
1500 nm; however, most were from 800-1200 nm in diameter
(Figure 6). Internal structure consisted primarily of small
granules; many also contained electron-dense strands of deoxy-
ribonucleic acid. A few had internal structures which ap-
peared either as microtubules (Figure 8) or as unit membranes
which divided the particles into 2 or 3 portions (Figure 13).

Figure 6. Electron micrograph of a chlamydial inclusion
in the cytoplasm of a McCoy cell 48 hours after
inoculation. The various stages of the develop-
mental cycle are recognizable; small particle
(SP), intermediate particle (IP), and large
particle (LP). Respective sizes are 410, 630,
and 740 nm. Note the dumbbell shape of some
large particles, indicative of binary fission.
The multilaminated nature of the wall of the
small particles, double unit membrane surround-
ing the intermediate and large particles, and
fragments of the plasma membrane of the phago-
cytic vacuole (PVM) are visible. Strands of
deoxyribonucleic acid (arrows) are present in
large and intermediate particles. Uranyl acetate
and lead citrate. X 41,000

IP   - intermediate chlamydial particle
LG   - lipid globule
LP   - large chlamydial particle
PVM   - phagocytic vacuole membrane
SP   - small chlamydial particle
Arrows - strands of deoxyribonucleic acid
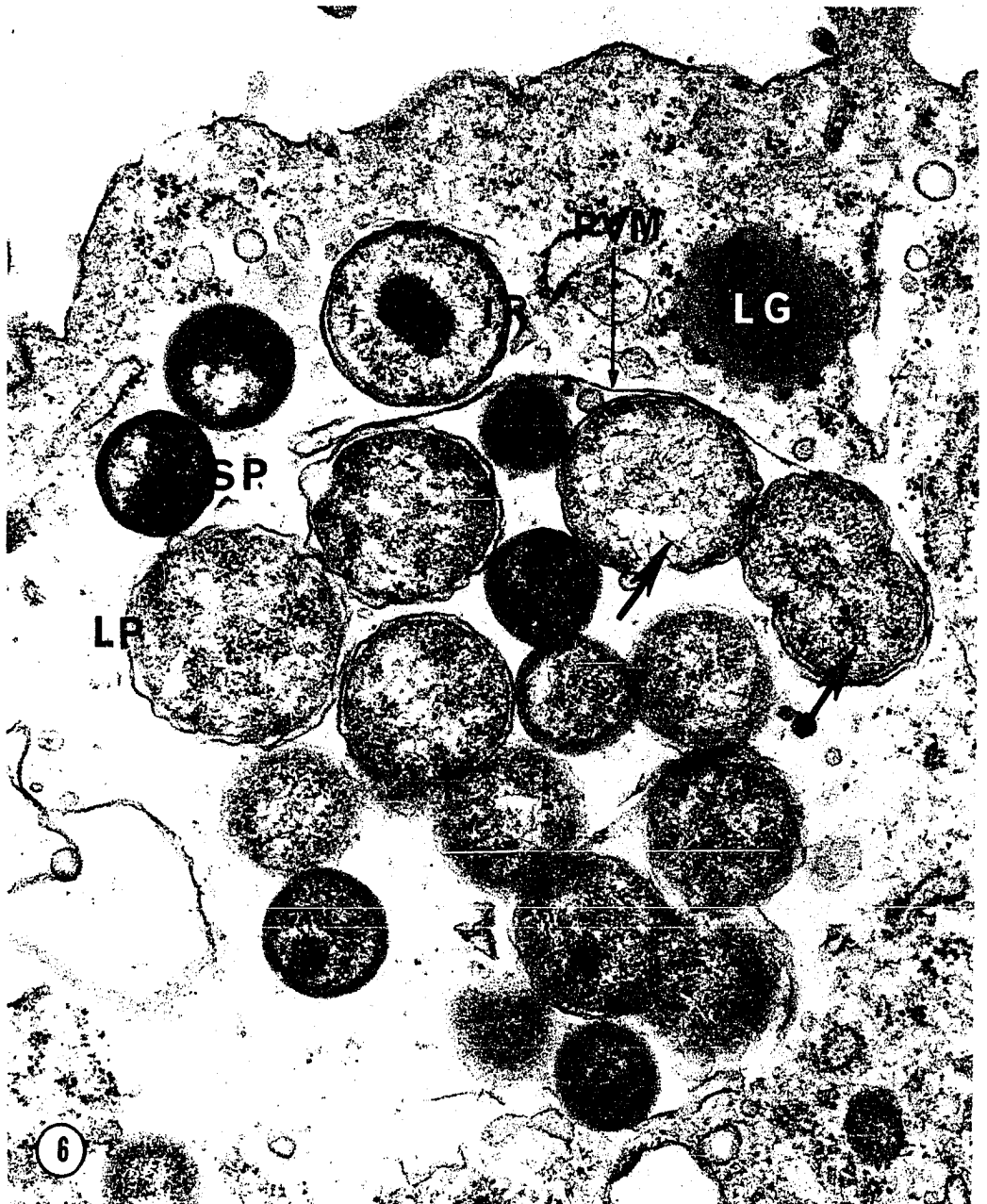
Figure 7.   Electron micrographs of chlamydial organisms
             found in the extracellular fluid of McCoy cell
             cultures. The walls appear to be composed of
             2 distinct unit membranes. Fibrous structures
             (arrows) are interpreted as deoxyribonucleic
             acid. Uranyl acetate and lead citrate.
             a. and b. X 92,000

Figure 8.   Electron micrographs of intracytoplasmic
             chlamydial particles containing microtubules
             (arrows). Uranyl acetate and lead citrate.
             a. X 24,000; b. X 41,000

Figure 9.   Electron micrographs of (a) one and (b) two-
             hour McCoy cell cultures containing extra-
             cellular chlamydial organisms (a) with and
             (b) without a visible nucleoid. The multi-
             laminated structure of the wall is apparent.
             Uranyl acetate and lead citrate.
             a. X 41,000; b. X 34,000

Figure 10.   Chlamydial organisms partially surrounded by
             cytoplasmic projections of McCoy cells after
             8 hours of incubation. Uranyl acetate and
             lead citrate.
             a. X 72,000; b. X 34,000
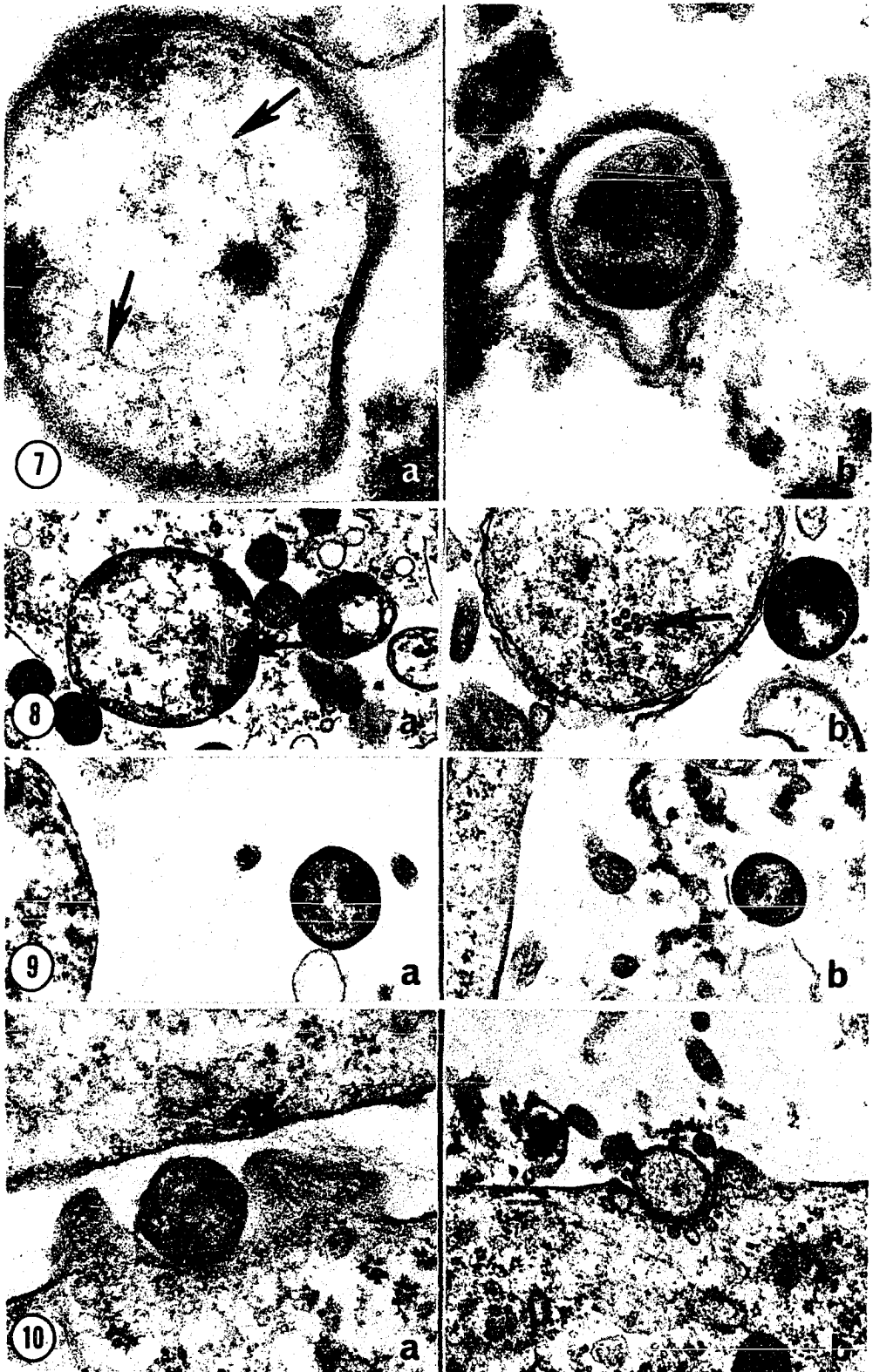
Large particles were surrounded by 2 unit membranes. All
chlamydial forms contained small (16-24 nm) unattached,
electron-opaque structures characteristic of ribosomes.

Prior to 8 hours of incubation, chlamydial organisms
were found only in extracellular fluid, either as discrete
small particles or as slightly larger particles without de-
tailed internal structure (Figure 9). These organisms were
structurally identical to organisms in the inoculum and were
partially enveloped by cell membrane in 8-hour cultures
(Figure 10). Intracellular organisms were found after 16
hours of incubation in phagocytic vacuoles or partially
surrounded by membranous projections (Figure 11). At 24
hours, small particles were seen in the process of being
engulfed by cells and large particles, either singular or
in clusters of 2-3, were scattered throughout the cytoplasm
(Figure 12). Intact or broken remnants of plasma membrane
of the phagocytic vacuoles surrounded the inclusions. After
48 hours of incubation, aggregates of organisms, predominantly
large particles, were found in many cells (Figure 13). Many
of the large particles were in various stages of division.
Small and intermediate particles were present in fewer num-
bers. The particles were free in the cytoplasm with only
remnants of the phagocytic-vacuole membrane remaining.
Seventy-two and 96 hours postinoculation, small particles
predominated, but many large and intermediate particles were
present (Figure 14). Rents in the plasma membrane with loss

Figure 11. Electron micrographs of McCoy cells containing
            chlamydial particles after 16 hours of incuba-
            tion. Small forms are present (a) in a plasma
            membrane invagination, and (b) in a cytoplasmic
            vacuole, and (c) a larger particle is present
            in the cytoplasm. Uranyl acetate and lead
            citrate.
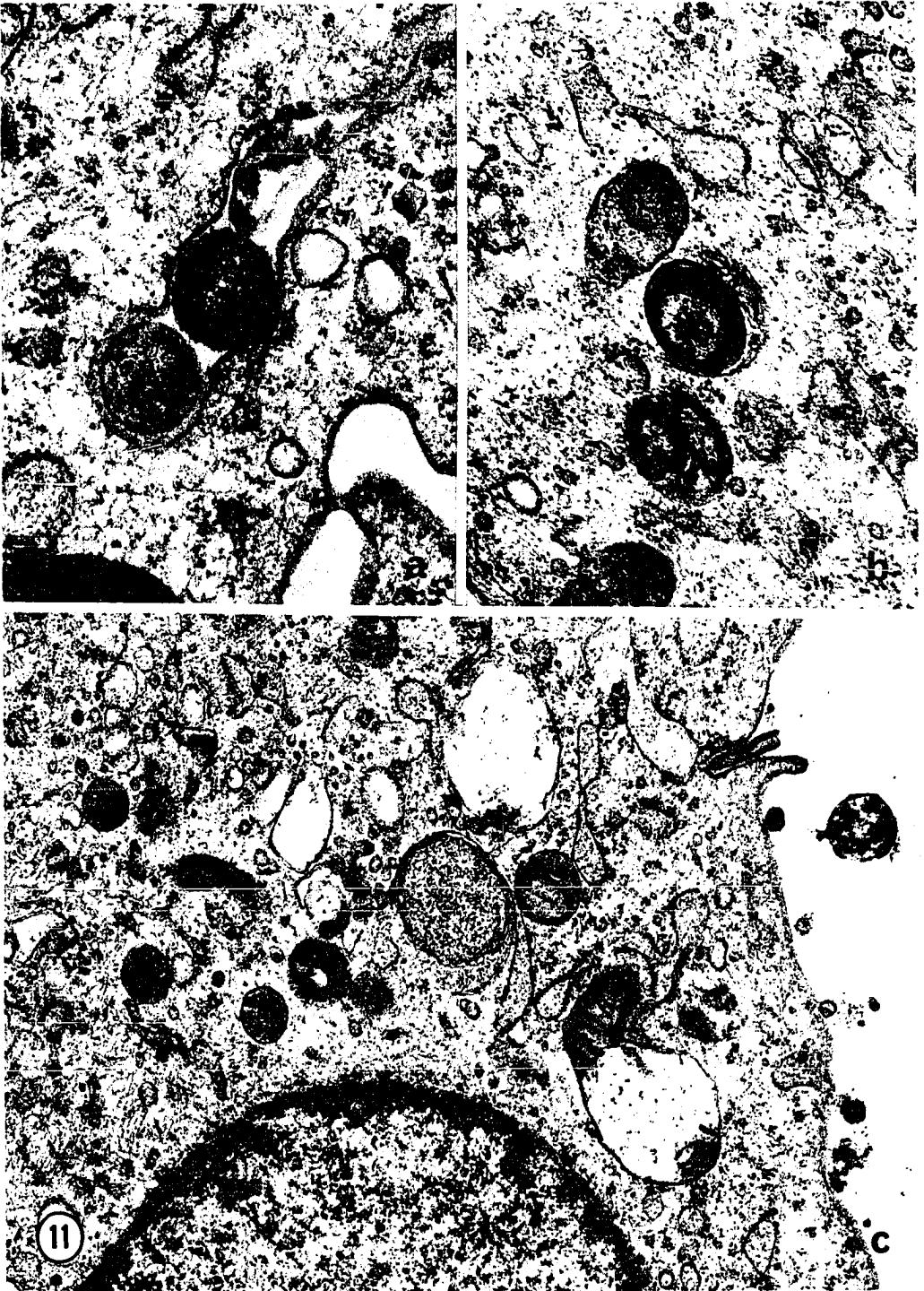   a. and b. X 41,000; c. X 18,000

Figure 12. Electron micrographs of McCoy cells 24 hours
            after inoculation with the OCP agent, (a) a
            small particle appears in the process of
            being phagocytized by a cell, (b) a large
            particle surrounded by the intact membrane
            of the phagocytic vacuole in the cytoplasm
            of a cell, and (c) 3 intracytoplasmic organisms
            surrounded by broken fragments of the phago-
            cytic-vacuole membrane (arrows). Uranyl
            acetate and lead citrate.
            a. X 34,000; b. and c. X 24,000
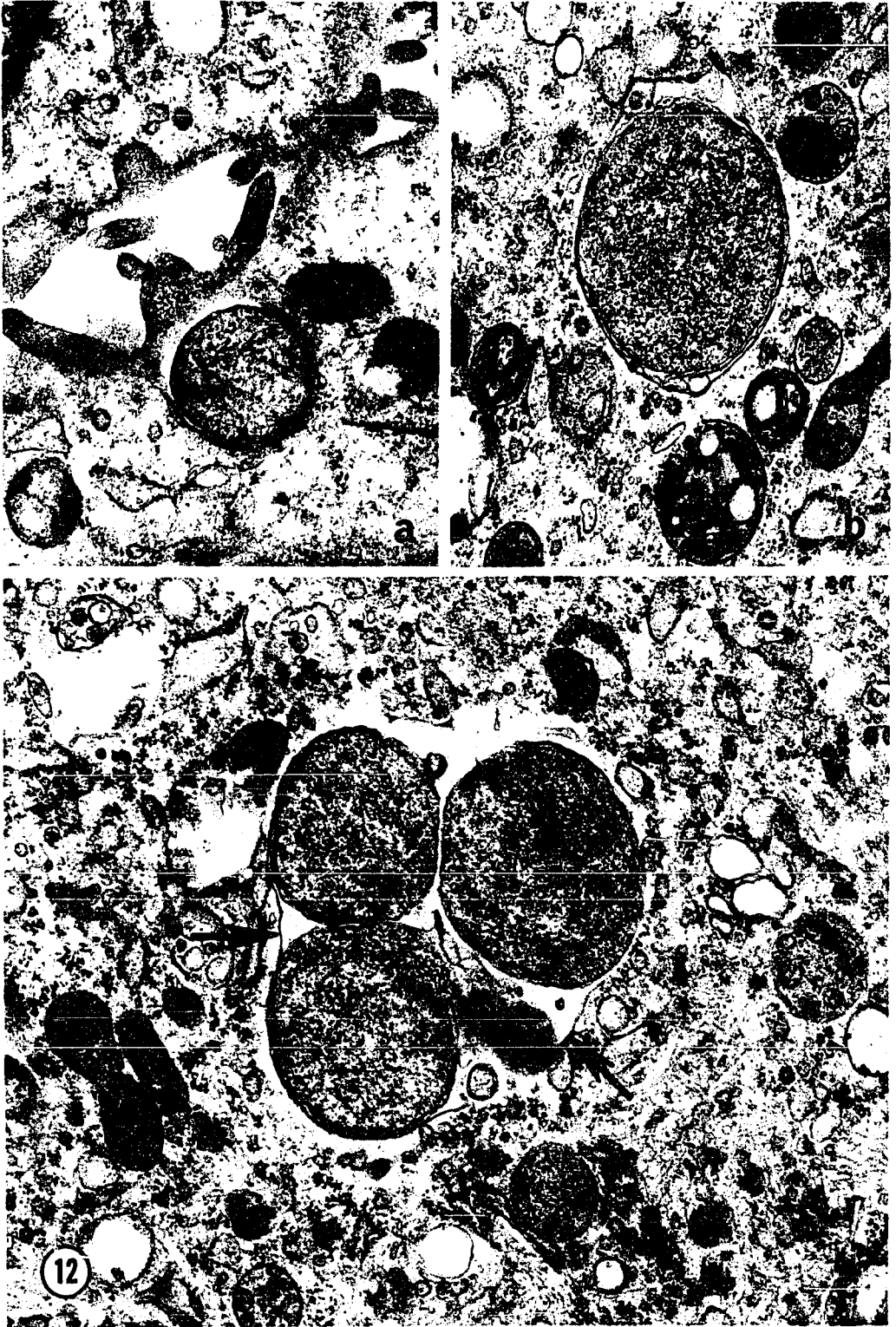
Figure 13. Cytoplasm of a McCoy cell containing various
            stages, predominantly large particles, of the
            OCP agent. Unit membranes divide several of
            the large particles into 2 or more portions.
            After 48 hours of incubation. Uranyl acetate
            and lead citrate.   X 11,000
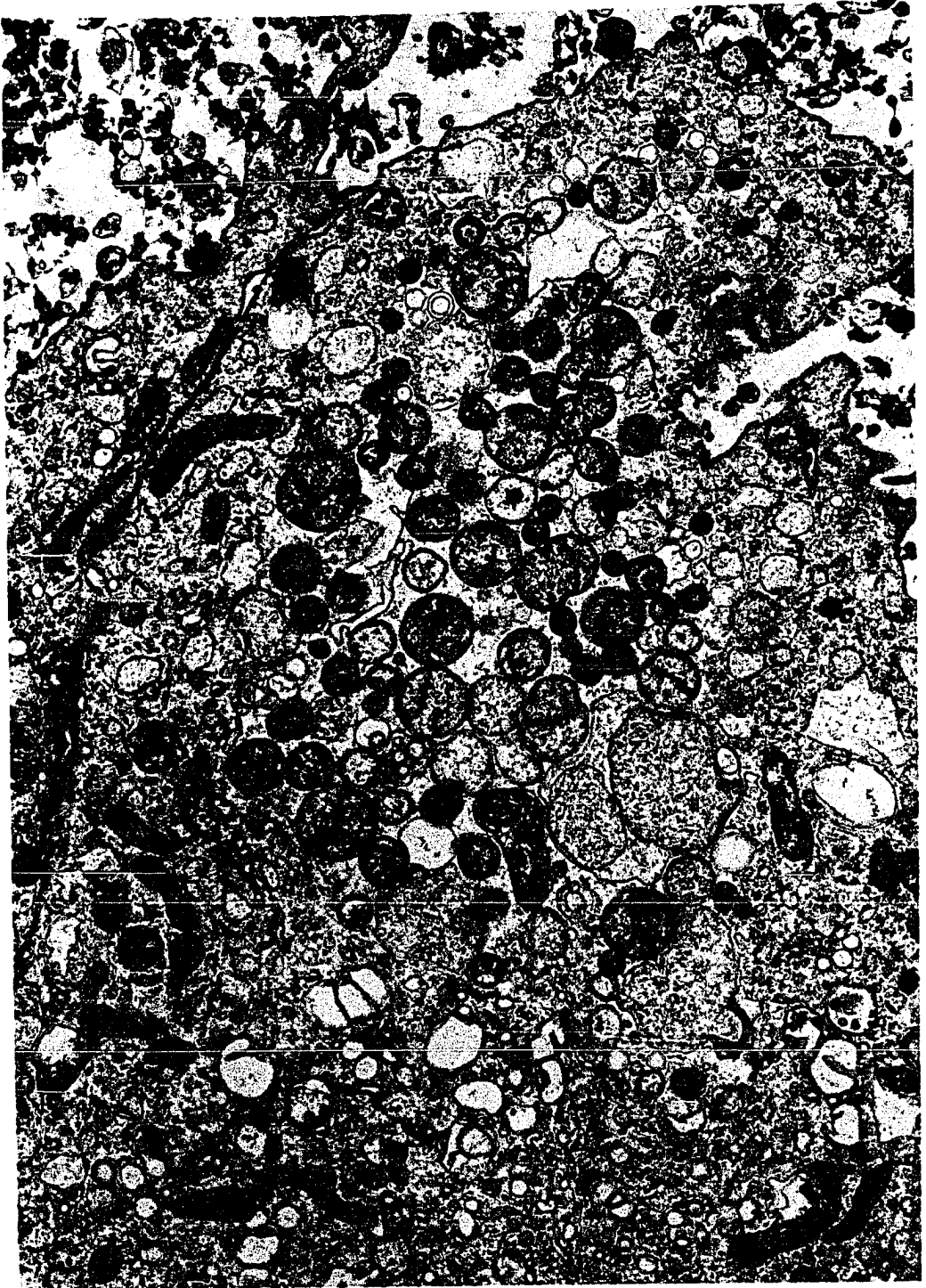
Figure 14.   Electron micrograph of a chlamydial-infected
             McCoy cell 72 hours after inoculation. Numerous
             organisms of various size, predominantly small
             particles, are scattered throughout the cyto-
             plasm. Several large particles are dumbbell
             shape, indicative of binary fission. Uranyl
             acetate and lead citrate. X 8,700
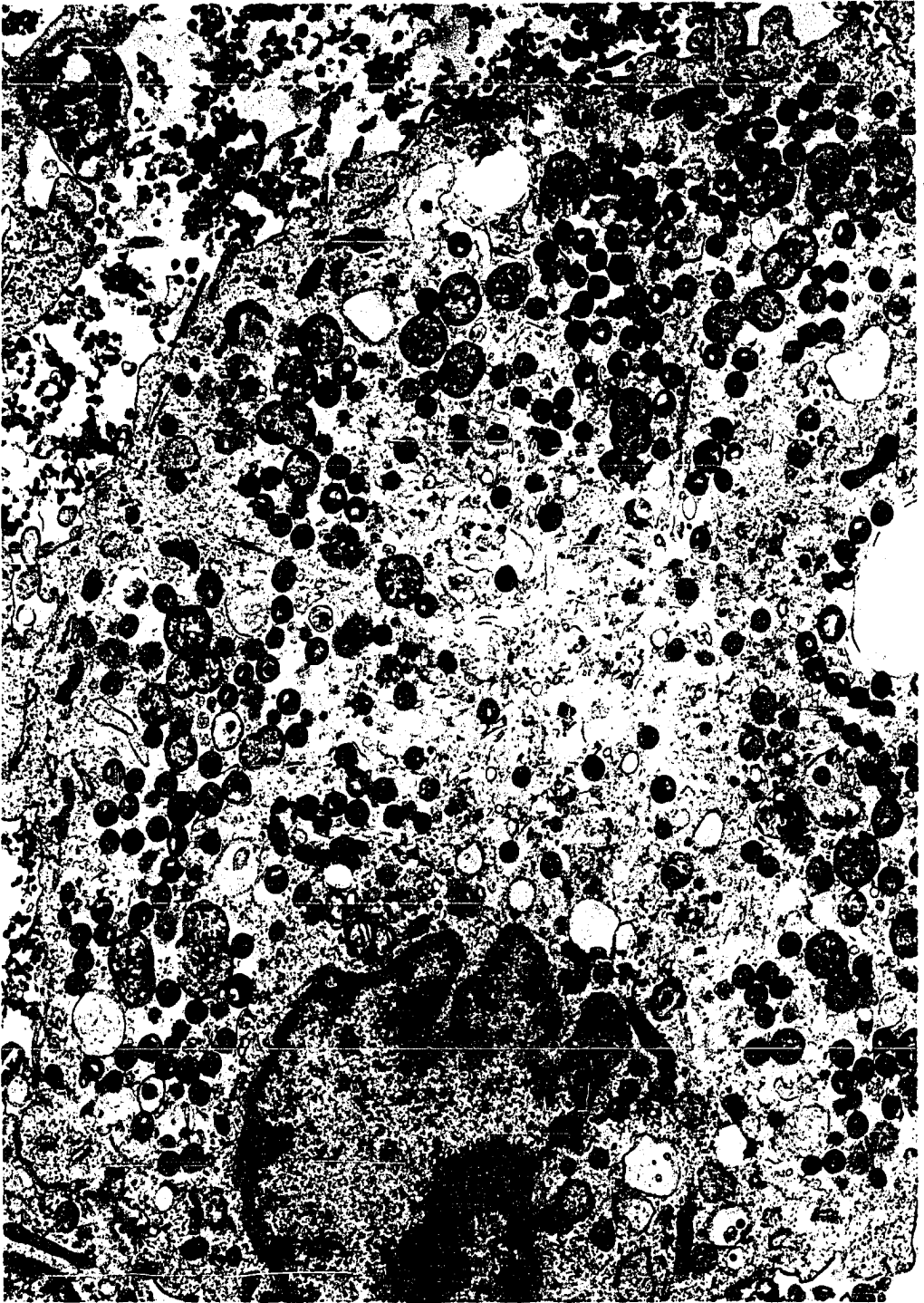
of cytoplasmic constituents and chlamydial organisms were evident in many cells (Figure 15). In a few cells incubated for 96 hours, small inclusions of large particles, similar to those in 24-hour cultures, were found.

In addition to chlamydiae, numerous viral particles typical of type C murine leukemia virions, as classified by Bernhard (1960), were found within and surrounding the cells in all McCoy cell cultures examined (Figure 16). The majority were located in the extracellular space. Intracytoplasmic virions were within membrane-lined vacuoles which may represent invaginations of the plasma membrane. An occasional cytoplasmic projection (virus bud) was present which contained a partially formed virus particle. These buds were found in both the membranes of cytoplasmic vacuoles and the external limiting membranes of cells.

The ultrastructure of the viral particles, except the budding form, was similar regardless of location. They consisted of a moderately electron-dense central nucleoid core surrounded by an electron-lucent zone and an envelope. The particles were from 90 to 100 nm in diameter.

Lamb Articulations

Clinical response

All lambs included in this study, which received the OCP inoculum intraarticularly, became severely lame within 24 hours and remained so until killed. No other signs of

Figure 15. A McCoy cell 96 hours after inoculation with
the agent of OCP. Ruptured cell membrane has
released the numerous intracytoplasmic organ-
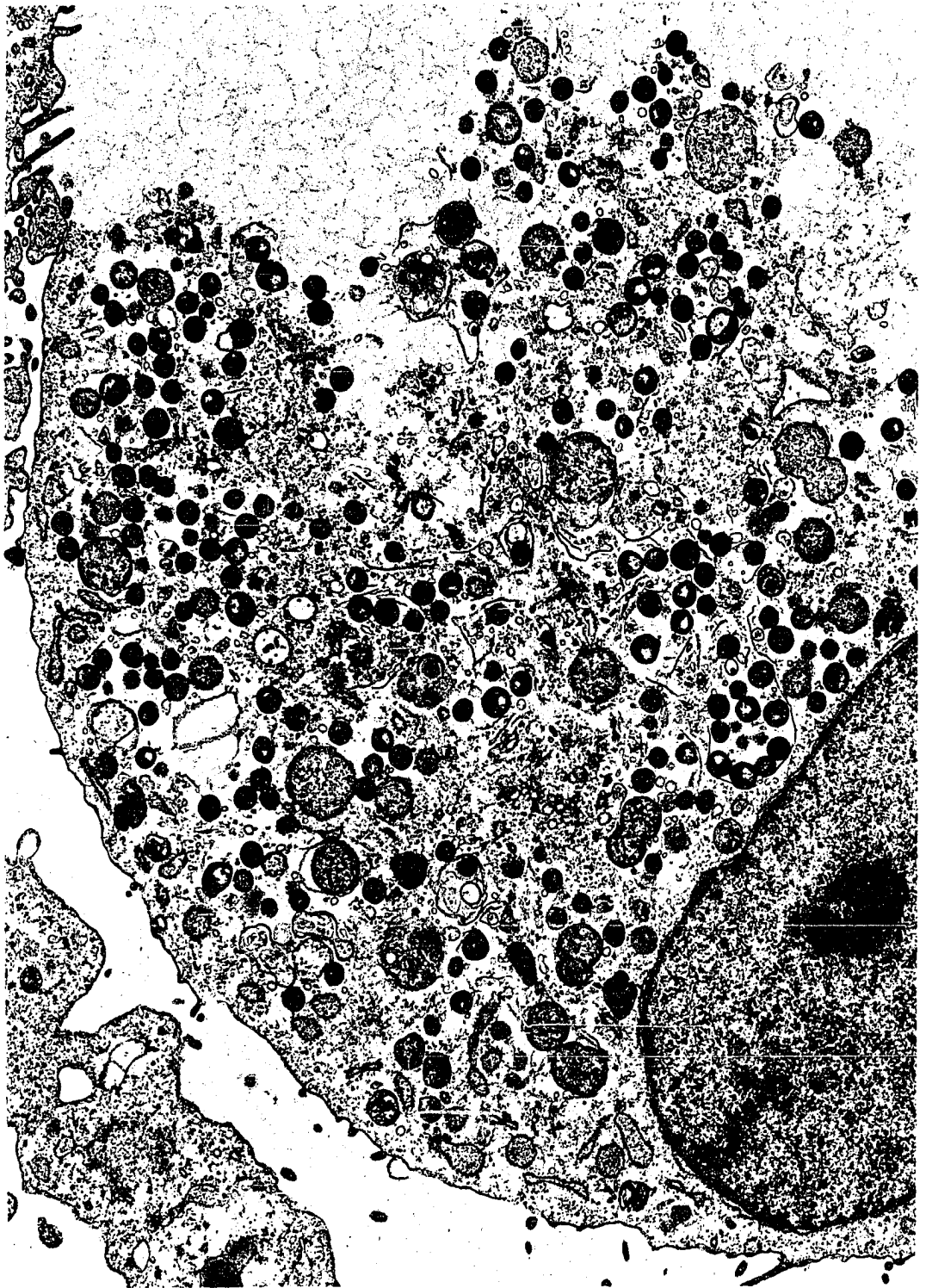isms. Uranyl acetate and lead citrate.
X 8,700

Figure 16. Electron micrographs of 8-day cultures of McCoy cells containing type C particles resembling murine leukemia virus. Virions are seen (a) in aggregates at the cell surface and incompletely formed in the plasma membrane as a viral bud (arrow), (b) attached to the cell surface, and (c) within vacuoles which probably represent invaginations of the plasma membrane. Uranyl acetate and lead citrate.
a. X 34,000; b. X 41,000; c. X 18,000
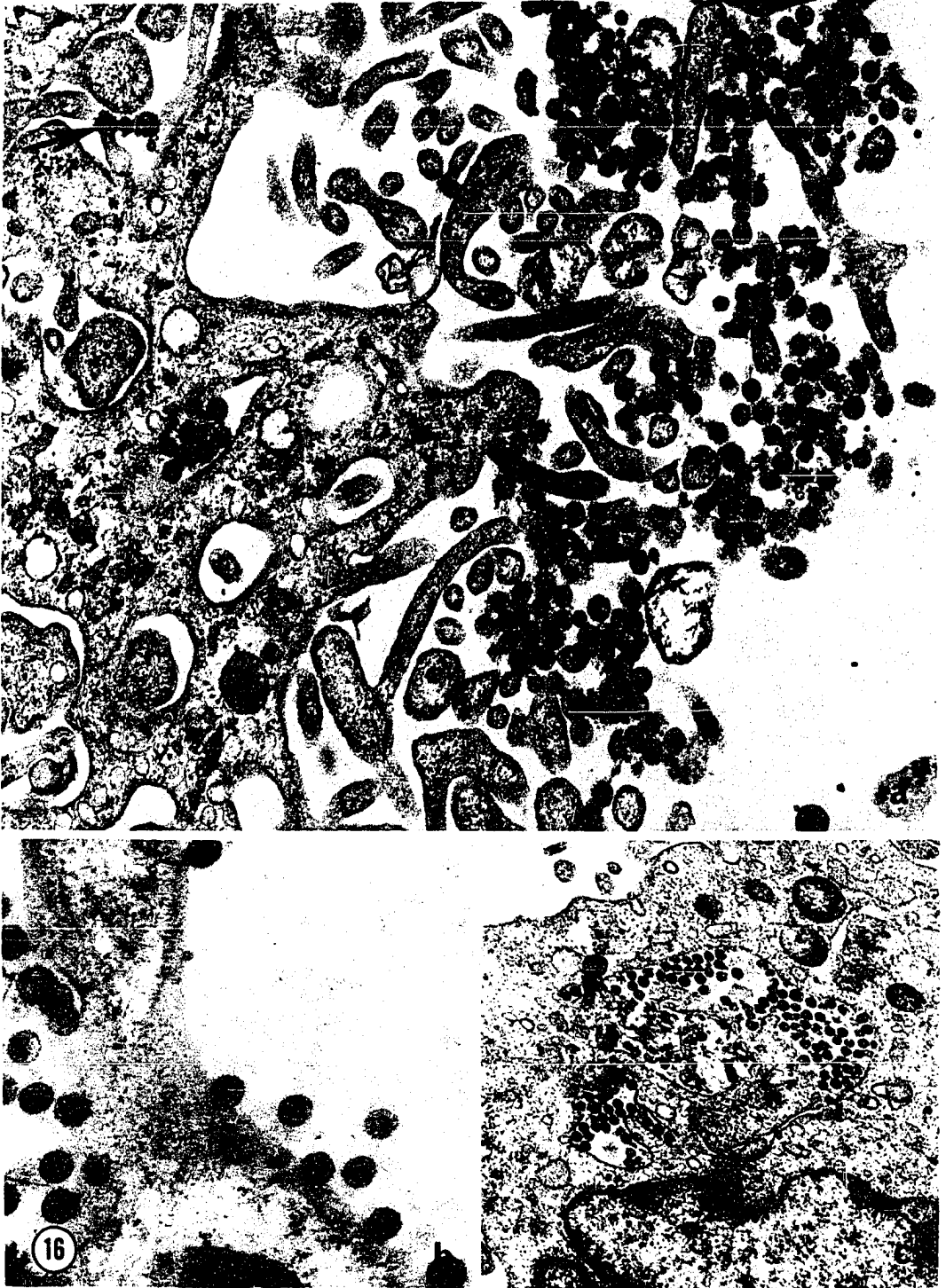
disease were seen. Lameness was not observed in noninoculated joints or in joints into which egg yolk sac had been injected.

Macroscopic lesions

A consistent difference was not apparent between articular lesions of weanling and yearling lambs; therefore, the 2 groups will be considered together. Neither was there a difference between the inflammatory reactions in the femorotibial and humeroradial joints of weanling lambs.

In joints of lambs killed 2-14 hours after injection of the OCP agent, there were hyperemic synovial membranes and excessive joint fluid. From 2-6 ml of a straw-colored fluid, containing an occasional strand of fibrin, were aspirated from each inflamed joint.

Infected joints of lambs killed after 24 hours contained excess fluid and large sheets of fibrin which covered most of the synovial surfaces. It was especially prevalent around tendons and under patellae. A few petechia were present in the synovial membranes.

The joints of lambs infected for 48, 72, 96, and 144 hours were similar to those infected for 24 hours; however, there was more fibrin (Figure 17) and the synovial fluid was grayish-yellow and of greater viscosity, thus resembling a mucopurulent exudate. Joints infected for 10 and 12 days were likewise affected, except the fibrin was firmly adhered to the underlying tissues. The femorotibial joint of one lamb, killed 12 days after inoculation, was slightly enlarged

Figure 17.   Femorotibial joint of a lamb killed 72 hours
              after being inoculated with the OCP agent.
              Large sheets of loose fibrin (F) cover much
              of the joint surface

Figure 18.   Femorotibial joint of a lamb killed 21 days
              after being inoculated with the OCP agent.
              Partially organized fibrin (OF) is firmly
              adhered to the underlying tissue

                        AC - articular cartilage
                        F - fibrin
                        OF - organized fibrin
                        SM - synovial membrane
                        FP - fat pad
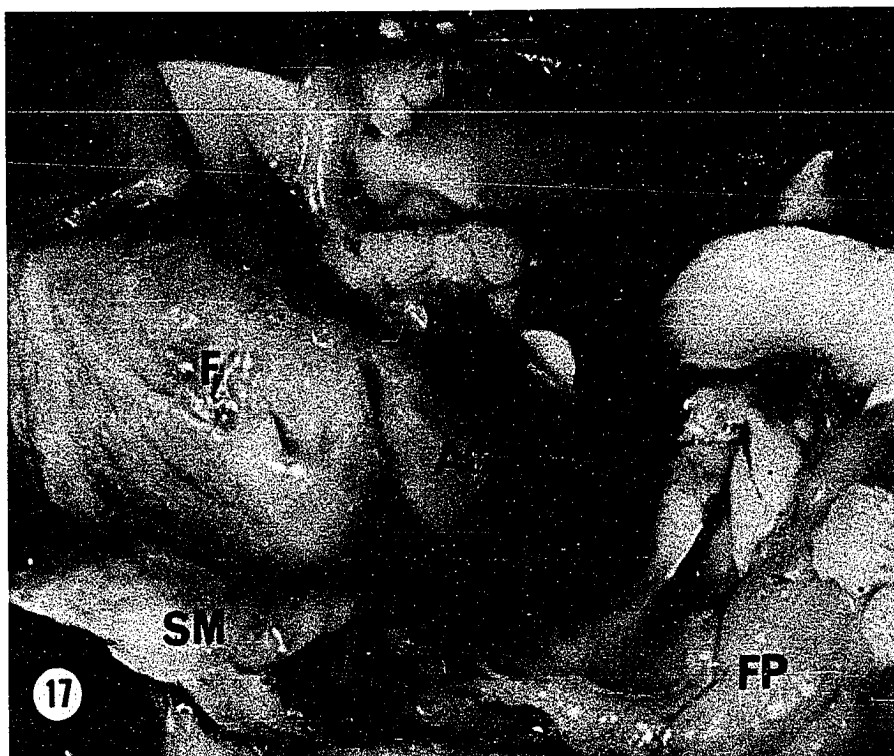
as a result of the articular exudate and periarticular edema.

Synovial membranes of lambs, killed 21 and 24 days after inoculation, were thickened by fibrous connective tissue and covered by partially organized fibrin (Figure 18). They were hyperemic and small hyperplastic villi projected into the joint spaces, frequently as flattened fronds extending over the articular cartilages. The synovial fluid was very viscous but only slightly excessive.

The noninoculated joints of the 2 control lambs, the noninoculated joints of lambs given the OCP agent and the joints examined 24 hours or more after egg yolk sac was injected were normal in gross appearance. Joints of lambs that had received egg yolk sac and examined no later than 24 hours postinoculation had excess normal appearing synovial fluid. Except in the joints examined at 2 hours postinoculation, the quantity of fluid was less than that in the contralateral joints infected with OCP.

Microscopic lesions

Histologic examination of hematoxylin and eosin stained sections of synovial and subsynovial tissues, myotendon junctions and patellae of the 2 groups of intraarticularly infected lambs revealed a consistent inflammatory reaction which gradually changed in character and increased in severity. The inflammatory reaction progressed from an initial serous reaction to a fibrinopurulent inflammation and finally to

proliferation of fibrous tissue.

At 2-14 hours postinoculation, synovial membranes were edematous, hyperemic and infiltrated with neutrophils. A variable amount of fibrin was present on the synovial surfaces (Figure 19). This reaction was slight at 2 hours but at 14 hours the synovium was infiltrated heavily with neutrophils and overlaid with considerable fibrin. Cellular infiltration and fibrin deposition were more extensive around tendons. Many neutrophils were associated with vessels, either in their lumens or in perivascular tissues.

After 24 and 48 hours of infection, the inflammatory process was similar to that at 14 hours but synovial involvement was more extensive. Areas of the synovial membranes under the fibrinopurulent exudate were necrotic and heavily infiltrated with neutrophils (Figure 20). In addition to neutrophils, the synovial and subsynovial tissues were infiltrated with several lymphocytes. They were edematous, hyperemic and had multiple hemorrhagic foci; the viable lining cells were hyperplastic and hypertrophic with an increased cytoplasmic basophilia (Figure 21).

Except for a small amount of fibroplasia and the presence of more lymphocytes and cells of the plasmacytic series, the reactions at 72 and 96 hours postinoculation were similar to that at 48 hours. A few macrophages were present in the necrotic debris and fibroblasts had proliferated at the junction of the fibrinous exudate and synovial tissue (Figure 22).

Figure 19. Acute serofibrinous synovitis 8 hours after
            intraarticular injection of the OCP agent.
            The edematous synovial membrane (SM) con-
            taining emigrating leukocytes (primarily
            neutrophils) is covered by small tags of
            fibrinous exudate (FE). Hematoxylin and
            eosin. X 125

Figure 20. Acute fibrinopurulent synovitis of an adipose
            membrane 24 hours after injection of the OCP
            agent. The synovial lining (SL) is necrotic,
            hemorrhagic, infiltrated with neutrophils,
            and overlaid with fibrinopurulent exudate
            (FPE). Hematoxylin and eosin. X 32

Figure 21. Synovium of a joint infected for 48 hours
            with OCP. The synovial lining (SL) and sub-
            lining (SS) layers are edematous, hyperemic,
            and infiltrated with neutrophils and lympho-
            cytes. Lining cells are hyperplastic and
            hypertrophic. Hematoxylin and eosin. X 32

Figure 22. Chronic fibrinopurulent inflammation and
            extensive necrosis of the synovium 96 hours
            after intraarticular injection of the OCP
            agent. Granulation tissue (GT) is subjacent
            to the fibrinopurulent exudate (FPE) and
            separated from it by a zone of neutrophils
            (N). Hematoxylin and eosin. X 50

                    FE - fibrinous exudate
                    FPE - fibrinopurulent exudate
                    GT - granulation tissue
                    N   - neutrophils
                    SL - synovial lining
                    SM - synovial membrane
                    SS - synovial sublining
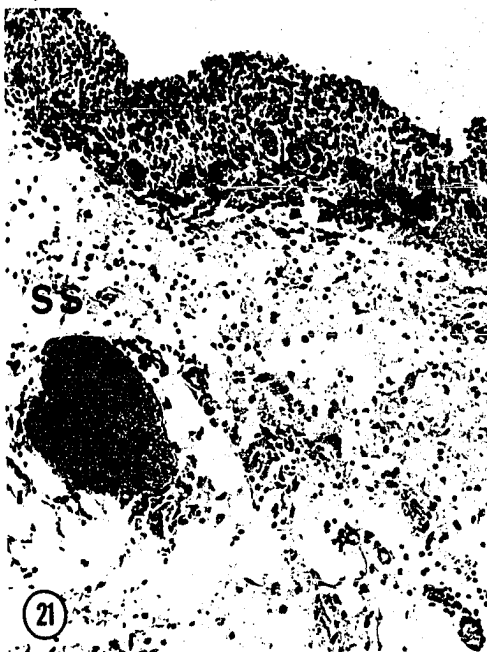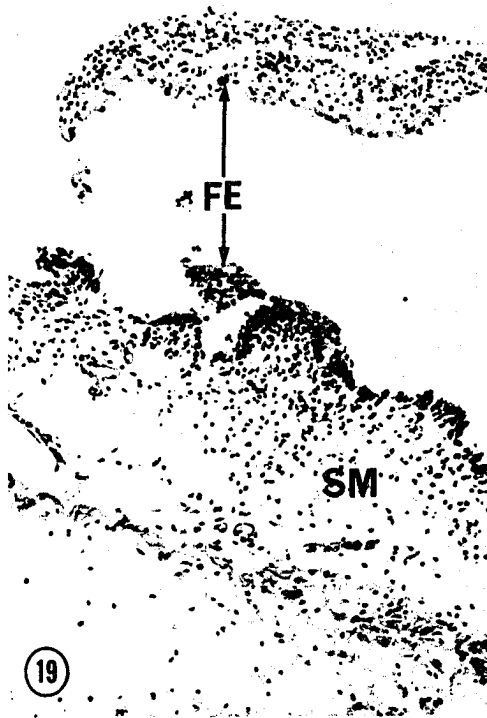
Focal hemorrhages were common in the areas of necrosis and the inflammatory cells extended to a greater degree into the periarticular tissues. Involvement of subsynovial connective tissue and muscle was limited primarily to perivascular accumulations of mononuclear cell (Figure 23). The reaction was similar 6 days postinoculation with the exception of additional fibrosis.

At 10 and 12 days after inoculation, fibrosis was more extensive with highly vascularized fibrous villi frequently projecting from the synovial surfaces (Figure 24). The newly formed fibrous tissue was hyperemic and infiltrated with inflammatory cells. These were primarily lymphocytes but also included a large number of mature and immature plasmacytes (Figure 25). The same type cells had accumulated in the subsynovial perivascular tissues. Far fewer neutrophils were present than in joints of lambs killed earlier; those present were largely confined to synovium still covered with fibrin and within the fibrin sheets. Large sheets of fibrin, firmly attached to the synovium by granulation tissue, remained over large areas of the synovial tissues (Figure 26).

After 21 and 24 days of infection, granulation tissue had replaced much of the fibrinopurulent exudate with the formation of large fibrous villi (Figure 27). Synovial surfaces were recovered by intact lining cells. The fibrous tissue contained numerous focal accumulations of lymphocytes with a diffuse distribution of plasmacytes (Figure 27).

Figure 23. Lymphocytes in periarticular muscle 72 hours
            after injection of the OCP agent. Hematoxylin
            and eosin. X 320

Figure 24. Fibrotic synovium of a lamb 10 days after re-
            ceiving the OCP inoculum. Vascularized fibrous
            villi, infiltrated with chronic inflammatory
            cells, project into the joint space. Hema-
            toxylin and eosin. X 32

Figure 25. Higher magnification of the synovium in
            Figure 24. Interspersed with the fibrous
            tissue are lymphocytes, plasmacytes, neutro-
            phils, and synovial cells. Hematoxylin and
            eosin. X 320

Figure 26. Partially organized fibrinous exudate in the
            joint of a lamb 10 days postinoculation.
            Fibrin (F) is separated from the granulation
            tissue (GT) by a few neutrophils (N). Hema-
            toxylin and eosin. X 50

                    F   - fibrin
                    GT - granulation tissue
                    H   - hemorrhage
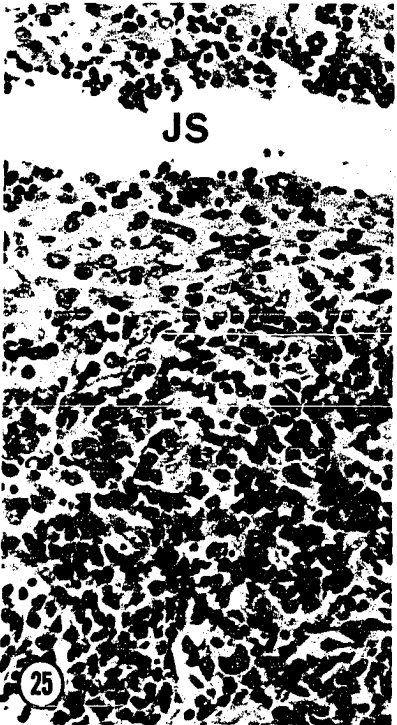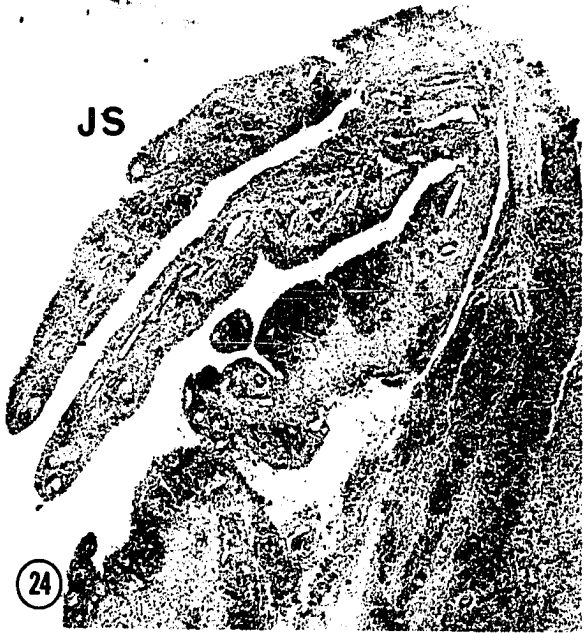   JS - joint space
                    N   - neutrophils
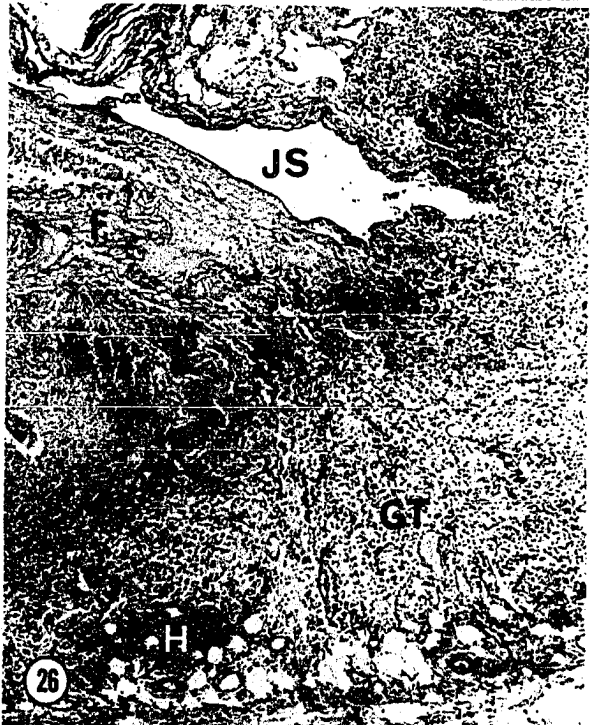
Figure 27. Chronic synovitis 21 days after intraarticular
            injection of the OCP agent. (a) Focal accumu-
            lations of lymphocytes and disperse plasmacyte
            infiltration of hyperplastic synovial villi.
            (b) Higher magnification of area indicated in
            (a) containing the edge of a lymphoid nodule
            (L) and surrounding plasmacytes (PC). Hema-
            toxylin and eosin. a. X 32; b. X 500

                 JS - joint space
                 L - lymphocytes
                 PC - plasmacytes

JS

a

PC

L

L

PC

PC

PC

27
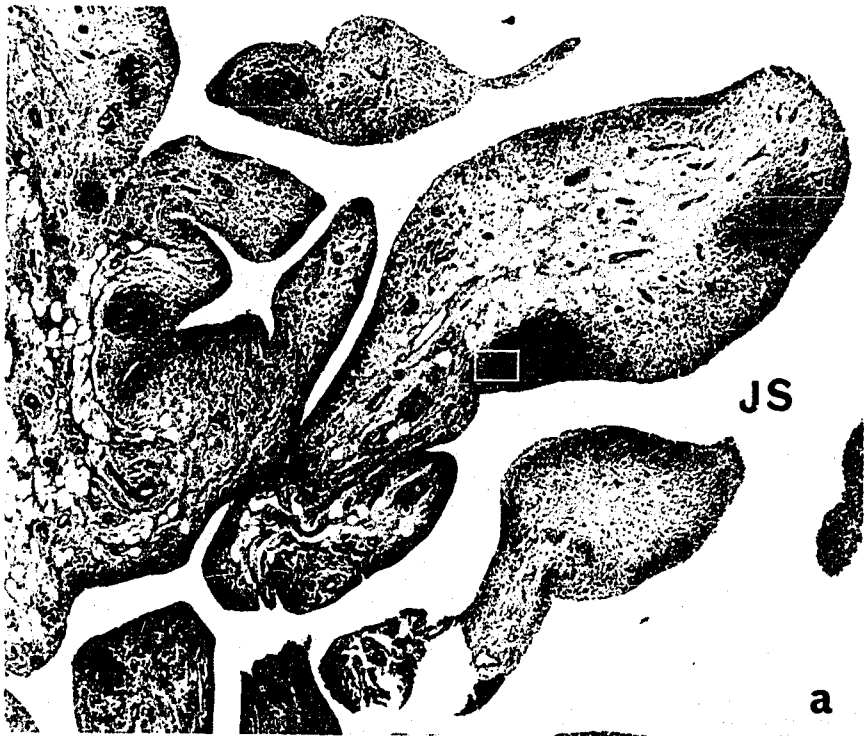
Noninoculated joints of all lambs were normal upon histologic examination. Joints of lambs inoculated with egg yolk sac and killed 24 hours or earlier after inoculation were edematous, hyperemic and infiltrated with a few neutrophils. After 48 hours, the egg-yolk-sac-inoculated joints appeared normal.

Smears of synovial fluid from infected joints contained numerous neutrophils and macrophages, the latter frequently with phagocytized material including degenerate neutrophils in their cytoplasm. Neutrophils predominated in most joints but were fewer in number after 6 days of infection. Smears from joints inoculated with egg yolk sac were similar but fewer cells were present. Few cells were present in smears from noninoculated joints.

With Giemsa staining, blue cytoplasmic inclusions were found in sections of synovial membranes and smears of synovial fluid from joints inoculated with either the OCP agent or egg yolk sac. Most were believed to represent phagocytized cellular debris; positive identification of chlamydial inclusions could not be made.

Articular changes in naturally and intravenously infected lambs

Pathologic changes in the joints of naturally infected lambs were similar to those found in joints inoculated intraarticularly at least 10 days previously. The femorotibial, tarsal, and carpal joints were most severely affected. Major changes were infiltration of the synovial tissues with plasma-

cytes, lymphocytes, and neutrophils and proliferation of
fibrous connective tissues. In 2 lambs, partially organized
fibrinopurulent exudate covered zones of necrotic synovial
tissue and in all lambs, the joint capsules were thickened
as a result of excessive fibrous tissue. A few small fibrous
fronds extended into the joint cavities.

The synovial membranes were hyperemic and edematous with
a few petechia. In some joints, the inflammatory reaction
extended into periarticular muscle and fascia. Copious syn-
ovia containing flakes of fibrin, neutrophils, and macrophages
were present in the joints. Gross and histologic examination
of articular cartilage and epiphyseal bone revealed no patho-
logic alteration.

Alterations in the joints of the 2 lambs infected intra-
venously were minimal; they were similar to those found in
the 2-hour intraarticularly infected lamb, except neutrophils
were fewer in number and the lining cells of one lamb were
hyperplastic. The synovial membranes were edematous, hyper-
emic and infiltrated with a few neutrophils and lymphoid
cells. Neutrophils and small flakes of fibrin were present
in the synovial fluid.

Histochemistry

Acridine orange   The nuclei of cells within the syn-
ovium of all lambs fluoresced yellowish-green (Figure 28).
Except for an increased number of cells in infected joints,

Figure 28. OCP infected synovial membranes of the femoro-
tibial joints of yearling lambs stained with
acridine orange. (a) After 8 hours of infection
only green nuclear fluorescence is visible;
whereas, (b) after 21 days of infection several
plasmacytes (PC) with orange cytoplasmic flu-
orescence are seen adjacent to small vessels
(Ve). a. X 125; b. X 320

        JS - joint space
   PC - plasmacytes
        Ve - vessels
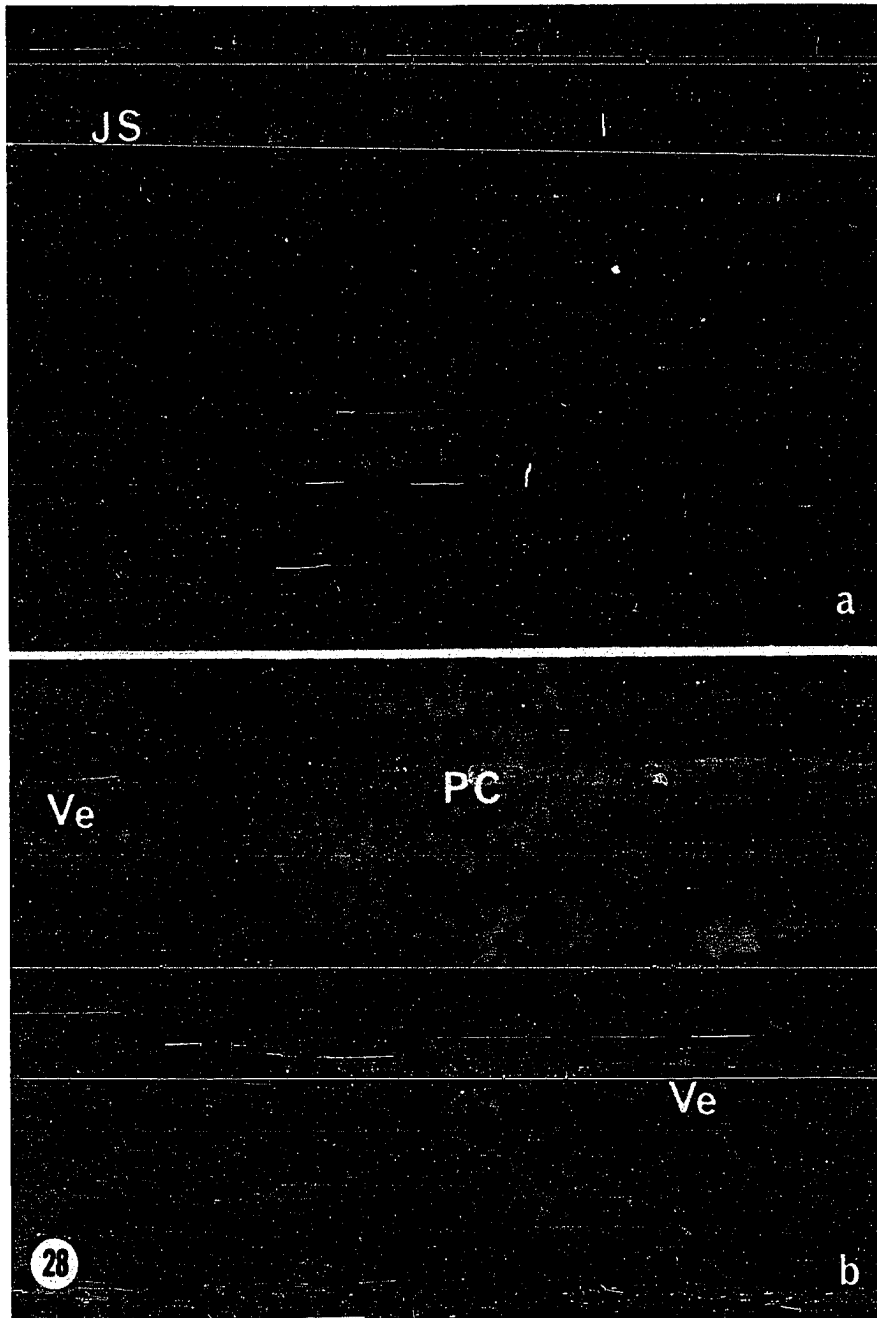
difference was not apparent between nuclear fluorescence of
infected and noninfected joints. Cytoplasmic fluorescence
was seen only in joints infected for 96 hours. A few cells
within the synovium of the lamb killed 96 hours after inocu-
lation had orange fluorescent foci in the cytoplasm. At 10-21
days postinoculation, the entire cytoplasm of numerous cells
in the synovial and subsynovial tissues fluoresced bright
orange (Figure 28). Cells of this type were widely distrib-
uted but most were in a perivascular location. Their plasma-
cyte morphology was confirmed by staining with Giemsa subse-
quent to acridine orange.

Alcian blue-periodic acid Schiff and oil red O   The
cytoplasm of normal synovial cells stained faintly with the
periodic acid-Schiff reagent and the intercellular matrix
of the synovial lining stained lightly with Alcian blue.
Periodic acid-Schiff staining outlined the limiting outer
margin of the synovium formed by cytoplasmic processes of
synovial lining cells. Except for disruption of the synovium
and the addition of inflammatory cells, the staining reaction
was not altered by infection. Diastase had no effect on
staining. Lipid, which stained with oil red O, was abundant
in all infected and control joints.

Acid phosphatase   In noninfected joints, there was
light acid phosphatase activity in synovial lining cells and
vascular endothelium; other cells stained only faintly or not
at all (Figure 29). In joints infected with the OCP agent,

Figure 29. Synovium of a normal lamb stained by Gomori's
            lead method for acid phosphatase activity.
            Light staining is present in the synovial
            lining cells and vessels. X 125

Figure 30. Synovium of a normal lamb stained by the
            Gomori-Takamatsu method for localizing alka-
            line phosphatase activity. Enzymatic activity
            is limited to vessels. X 125

Figure 31. Normal synovial membrane stained by the calcium
            method of Padykula and Herman for adenosine
            triphosphatase activity. Enzymatic activity
            is localized in lining cells and vessels.
            X 320

Figure 32. Normal synovium of a yearling lamb with forma-
            zan pigment, indicating activity of reduced
            diphosphopyridine nucleotide diaphorase in the
            synovial lining cells and vascular endothelium.
            Stained by the method of Scarpelli, as modified
            by Barka and Anderson, with the tetrazolium
            salt, Nitro BT. X 125

Figure 33. Light deposition of formazan pigment in the
            lining cells of noninfected synovium after
            staining for succinic dehydrogenase activity.
            Stained by the tetrazolium method of Nachlas,
            as modified by Barka and Anderson, utilizing
            Nitro BT as the hydrogen acceptor. X 125

Figure 34. Synovium of noninfected joint with glucose-6-
            phosphate dehydrogenase activity localized in
            the synovial lining cells and vascular endo-
            thelium. Stained by the tetrazolium method
            of Nachlas, as modified by Barka and Anderson,
            with Nitro BT as the hydrogen acceptor. X 125
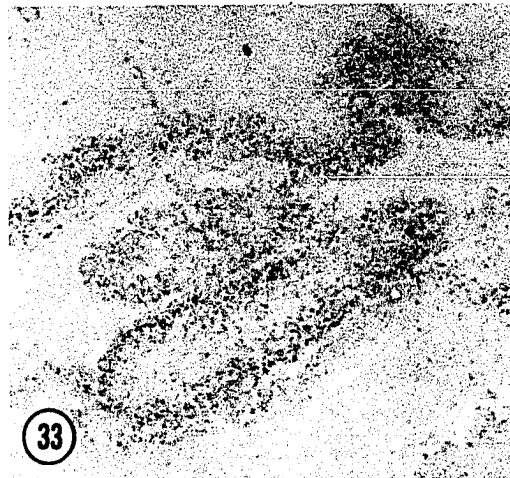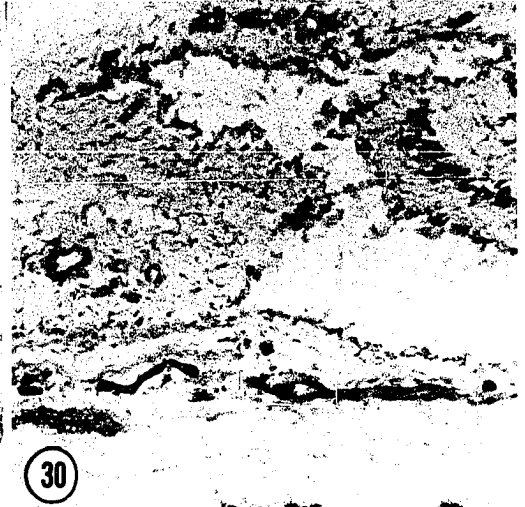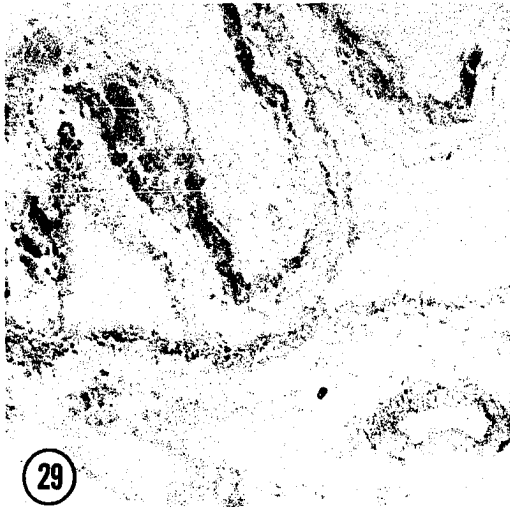
inflammatory cells (primarily neutrophils) in the exudate stained intensely in addition to the synovial structures. At 10 and 21 days postinoculation, new fibrous tissue cells were also moderately stained. Substrate-deficient controls were negative.

Alkaline phosphatase   This enzyme was limited to vessels and neutrophils. Staining of the synovium of infected joints paralleled the presence of neutrophils in the exudate; thus, staining was most intense at 48 to 96 hours postinoculation. A few neutrophils in the synovium of joints injected with the egg-yolk-sac inoculum and killed at 16 and 24 hours had enzymatic activity; otherwise, only vessels of the non-infected joints were stained (Figure 30). Precipitate was not present in the substrate-deficient controls.

Adenosine triphosphatase   In noninfected synovium, cells of the surface layer and vessels stained intensely; fibrocytes in the subsynovial tissues were lightly stained (Figure 31). In addition to normal structures, inflammatory cells and fibroblasts of infected joints contained adenosine triphosphatase activity.

Reduced diphosphopyridine nucleotide diaphorase
Activity of this enzyme was heaviest in the synovial lining cells with light activity in endothelial cells (Figure 32). Little difference was detected between infected and noninfected synovium, except the staining of more cells in the subsynovial tissue of infected joints. All staining appeared

to be confined to the cytoplasm of cell.

Succinic dehydrogenase   Succinic dehydrogenase activity was very light in noninfected synovium and confined to the synovial lining cells (Figure 33). Similar results were found in infected joints prior to 72 hours postinoculation. Subsynovial tissues of the lambs killed 72 hours postinoculation contained several cells, probably of the plasmacytic series, with enzymatic activity.

Glucose-6-phosphate dehydrogenase   Activity of this enzyme was confined to the synovial lining cells and vascular endothelium of noninfected joints (Figure 34) and of joints infected for less than 10 days. After 10 days, enzymatic activity was also found in fibroblasts. Intensity of staining varied greatly between individual lambs. Substrate-deficient controls were negative.

Immunohistochemistry

When stained by the fluorescent antibody technique, intra-cytoplasmic fluorescent granules were found in synovial membrane sections of all infected joints of the yearling lambs. Many infected joints had cells in the synovial fluid which contained fluorescent cytoplasmic granules. Prior to 72 hours postinoculation, only an occasional cell fluoresced; whereas, after 72 hours several cells in the synovial tissues and exudate fluoresced a bright green. Blocking the reaction with nonconjugated antichlamydial globulin did not inhibit all

fluorescence; but did appear to decrease the number of fluorescent cells. When the tissues were stained with Giemsa, subsequent to fluorescent antibody staining, many of the fluorescent cells were found to be eosinophils. Eosinophils were most numerous in lambs killed 72 and 96 hours postinoculation. Neither fluorescent cells or eosinophils were found in noninoculated joints.

With the enzyme antibody technique, there was diffuse staining of both infected and noninfected synovial tissue. This was true even without addition of the enzyme antibody conjugate. Tissue sections did not stain when benzidine was omitted from the incubating solution.

Electron microscopy[1]

Regional variations in the morphology of normal synovial membranes were recognized by electron microscopy. In some regions, which probably correspond to the fibrous membrane of light microscopy, the synovium was characterized by widely spaced cells indistinguishable from cells in the subsynovial fibrous tissue (Figures 35 and 36). Lining cells were stellate to slightly elongated; some had long cytoplasmic processes and surface folds. Elongated cells were orientated with their long axis parallel to the synovial surface. Nuclei had multiple indentations and diffuse clumps of chromatin.

---

[1]Terminology of ultrastructure is according to Fawcett (1966).

Figure 35. Electron micrograph of fibrous synovium from
the femorotibial joint of a normal lamb.
Lining cells resembling fibroblasts rest on
dense collagen (Co) and are surrounded by a
granular intercellular matrix (IM) containing
bundles of fine, 110 A, fibrils (FF). Note
the dense surface matrix (DSM). Uranyl acetate
and lead citrate. X 4,400

       Co - collagen
       DSM - dense surface matrix
       FF - fine fibrils
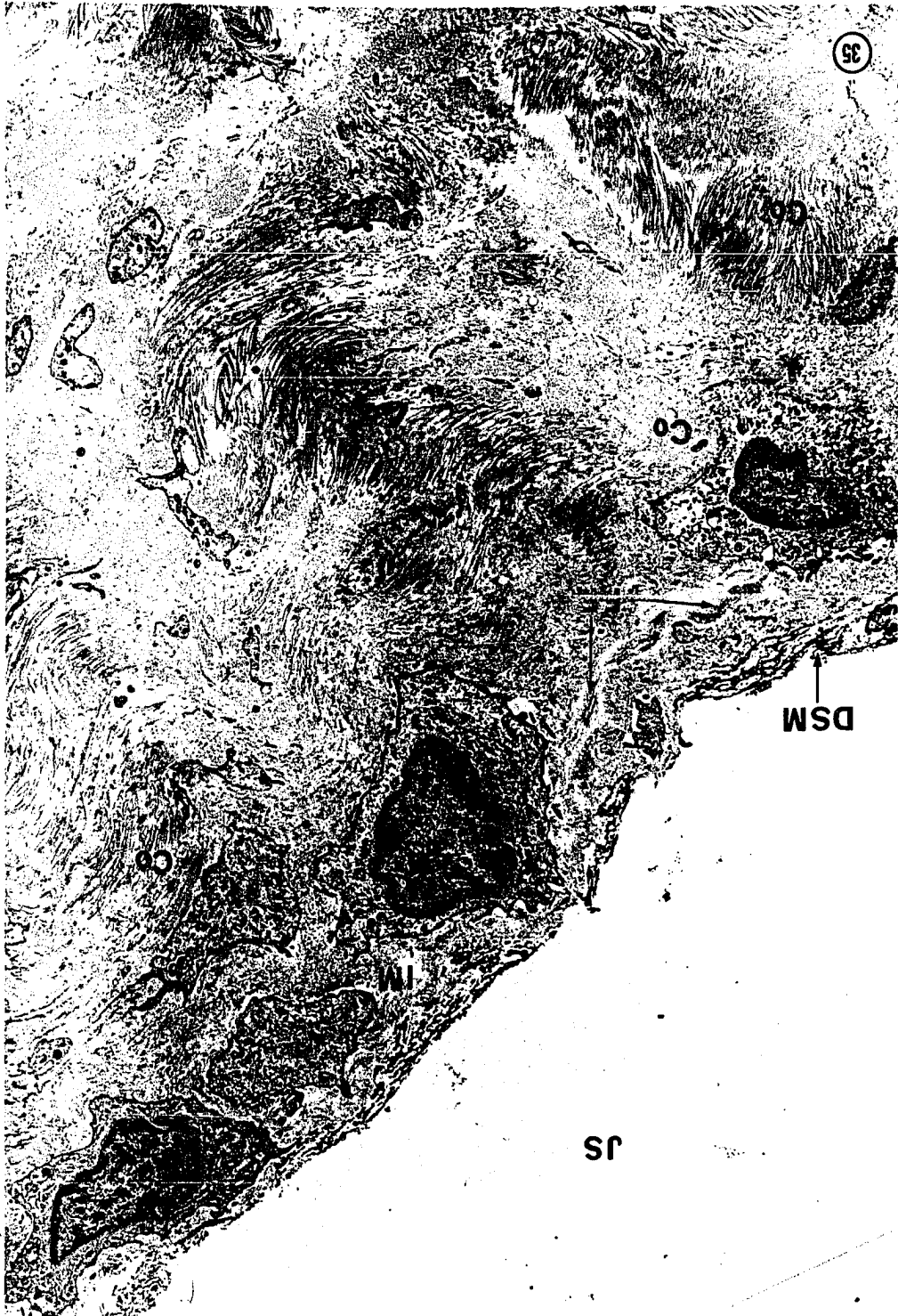   IM - intercellular matrix
       JS - joint space

Figure 36. Electron micrograph of a lining cell from
fibrous synovium of a normal lamb. The cell
is characterized by short saccules of granular
endoplasmic reticulum (GER), a Golgi apparatus
(GA), several small vesicles (SV), mitochon-
dria (M), numerous fine, 110 A, fibrils (FF)
especially in the perinuclear region, and
larger, 255 A, fibrils (arrows). Uranyl
acetate and lead citrate. X 18,000

                    Co - collagen
                    DSM - dense surface matrix
                    FF - fine fibrils, intracellular and
                            extracellular
                    GA - Golgi apparatus
                    GER - granular endoplasmic reticulum
                    IM - intercellular matrix
                    JS - joint space
                    M   - mitochondria
                    NP - nuclear pores
                    SV - small vesicles
                    Arrows - large intracellular fibrils

Cells of this type had a moderate amount of granular endo-plasmic reticulum in the form of short saccules which often were dilated. Several mitochondria were present and a Golgi apparatus was seen in a few of the cells. Numerous fine fibrils (100-125 A) were scattered through the cytoplasm with the heaviest concentration in the perinuclear zone. Larger fibrils (225-250 A) were seen in some cells. Several small vesicles were scattered throughout the cytoplasm. The intercellular matrix consisted of a fine granular substance containing fine fibrils similar to those in the cytoplasm of lining cells. The matrix usually completely surrounded the lining cells; in some areas the synovial surface was com-posed of a more opaque material than that surrounding the lining cells. The lining layer of synovium rested on dense collagenous tissue. Collagenic fibers occasionally extended into the intercellular matrix of the lining layer but were not observed to reach the synovial surface.

Other regions of synovial membranes were composed of cells similar in characteristics to Barland's A and B types. Mast cells were found rarely (Figure 37). Cell concentration varied from areas with large intercellular spaces to areas with 1-3 rows of closely approximated cells. In some areas, the luminal surface was made up of cytoplasmic processes and intercellular matrix (Figure 38); however, in most areas the cells were directly exposed to the joint space (Figure 39). Intercellular matrix was similar to that in the fibrous type

Figure 37. Mast cell from the synovial membrane of a
            normal lamb joint. Uranyl acetate and lead
            citrate. X 18,000

                 Co - collagen

Figure 38. Normal areolar synovium sparsely populated with
type A (A) and type B (B) lining cells. The
surface is covered by cytoplasmic processes (CP)
of lining cells and intercellular matrix (IM).
An arteriole, containing an erythrocyte (RBC),
is present at upper left. Uranyl acetate and
lead citrate. X 4,400

                    A   - type A synovial lining cell
                    B   - type B synovial lining cell
                    Co   - collagen
                    CP   - cytoplasmic processes of lining cells
                    EC   - endothelial cell
                    IM   - intercellular matrix
                    JS   - joint space
                    Mus - smooth muscle
                    P   - pericyte
                    RBC - erythrocyte
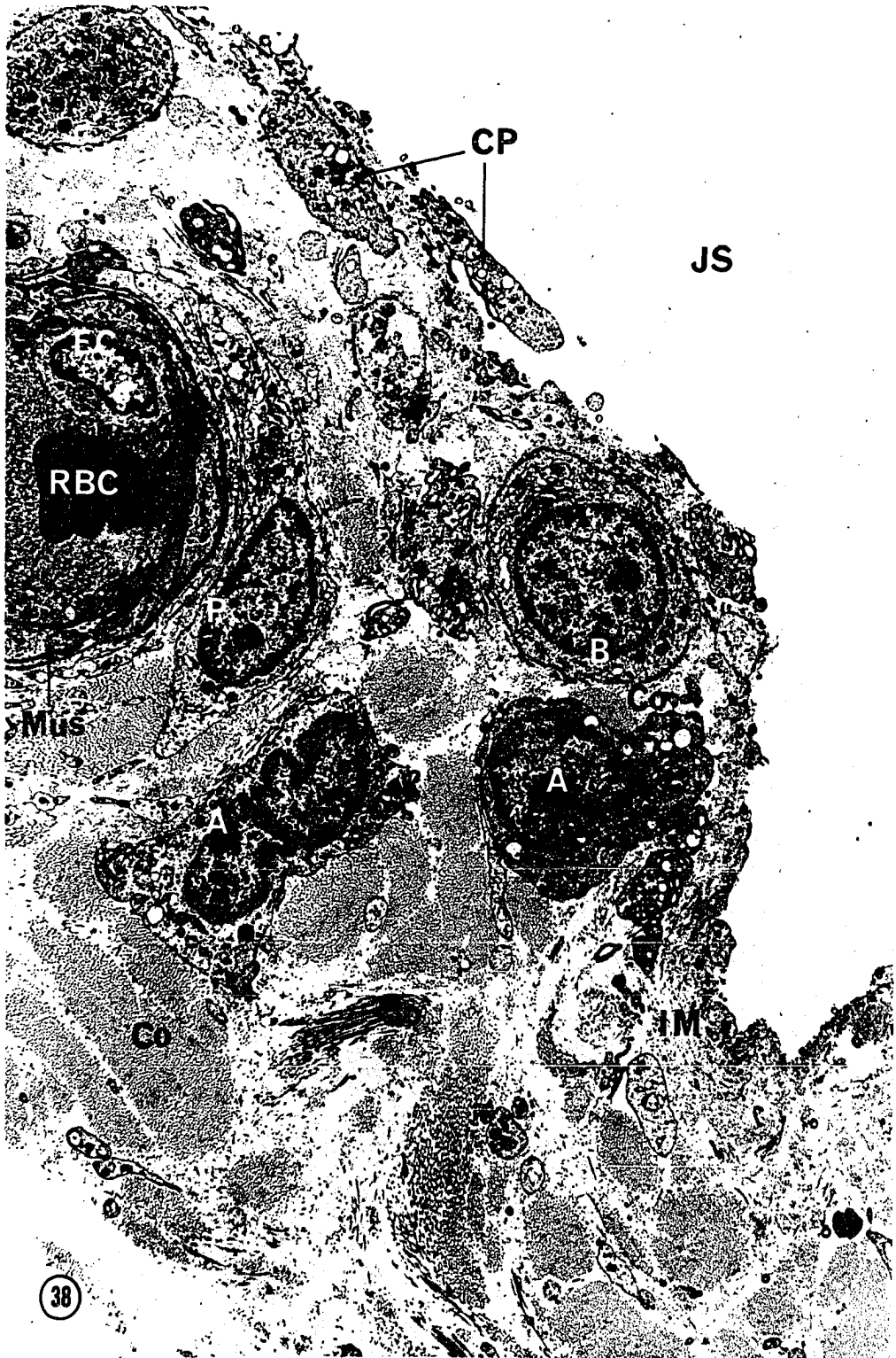
Figure 39. Normal areolar synovium composed of closely
            packed type A (A) and type B (B) lining cells.
            Uranyl acetate and lead citrate. X 4,400

                  A - type A synovial lining cells
                  B - type B synovial lining cells
                  Co - collagen
                  JS - joint space

JS

39
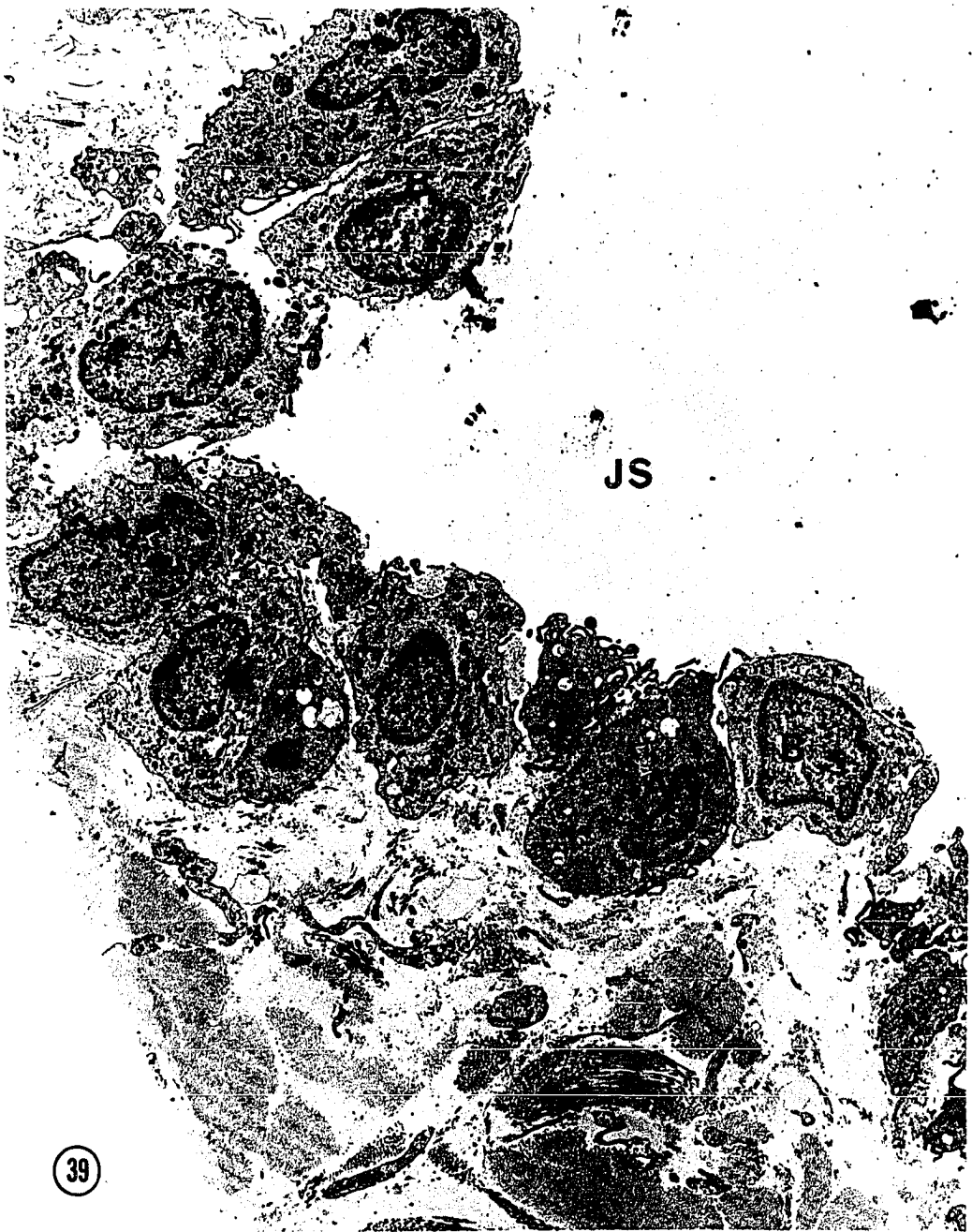
synovium. This variety of synovium was found over highly
vascular fibrous tissue and probably corresponds to the
areolar membrane of light microscopy.

Type A cells (Figures 38, 39 and 40) were characterized
by membrane-bound granules of variable electron density and
size (up to 1 micron in diameter), vesicles of similar size
which may represent pinocytotic vesicles, and numerous small
vesicles which resembled fuzzy and smooth type micropinocy-
totic vesicles. The small and large vesicles frequently con-
tained a moderately dense substance. Similar small vesicles
were present in the Golgi apparatus which was seen in many
type A cells. A few short saccules of granular endoplasmic
reticulum and many free ribosomes were present in most cells.
A few cells with free ribosomes but few or no granules, but
otherwise characteristic of the type A cell, were found.
Cell outlines were inconsistent, usually with long cytoplasmic
processes and numerous surface folds which occasionally ap-
peared to form pinocytotic vesicles. The nuclei were irregu-
lar in outline, frequently with marginated clumps of chro-
matin. Most cells of this type were located in the surface
layer of synovial lining cells.

Type B cells (Figures 38, 39 and 41) were characterized
by a large amount of granular endoplasmic reticulum which was
usually in the form of relatively short saccules of flattened
cisternae. A few large vesicles and electron-opaque granules,
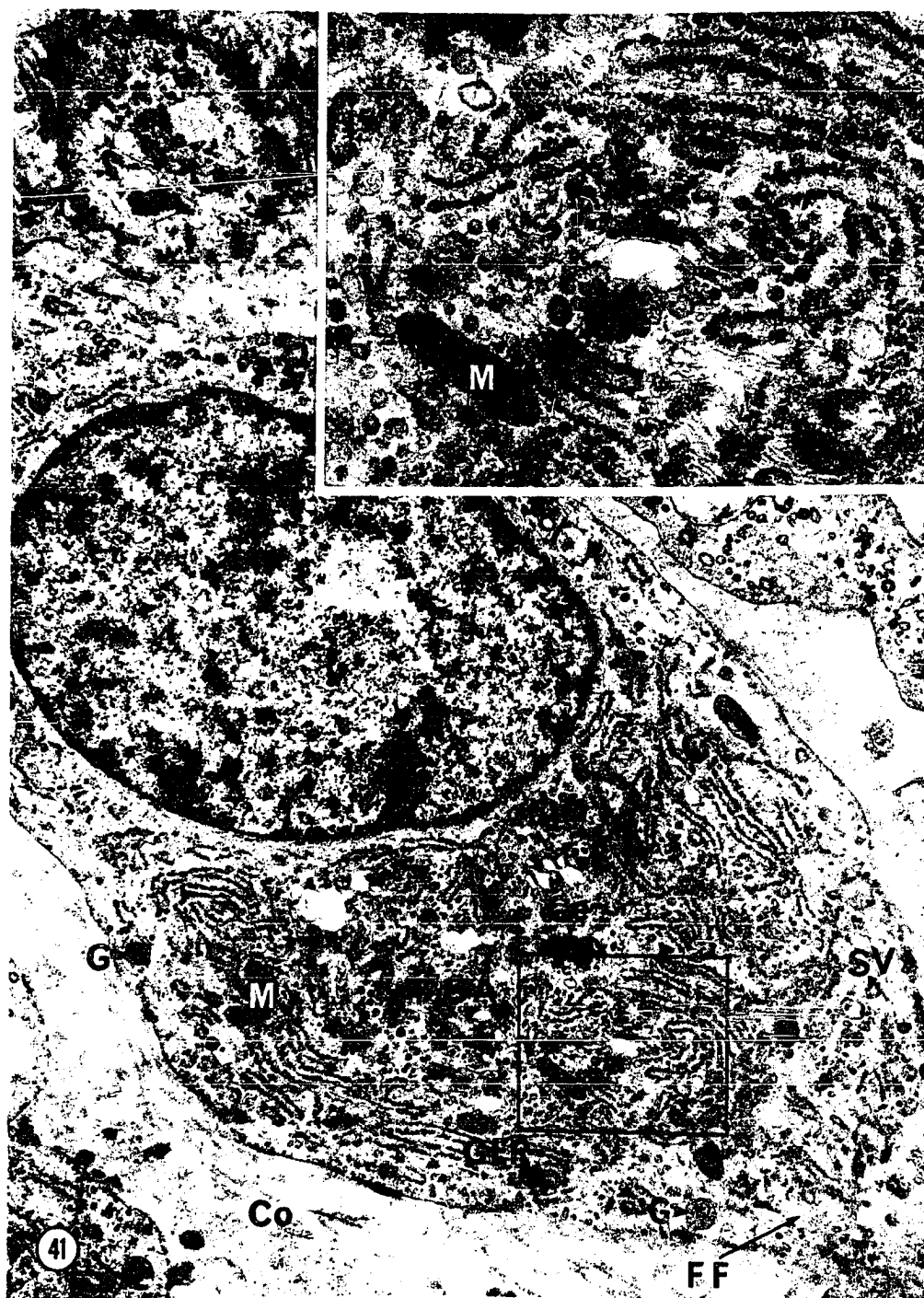similar to those in the type A cells were present. Many type

Figure 40.   Type A synovial lining cell from a normal joint.
             This type cell is characterized by large gran-
             ules (G), numerous small vesicles (SV) which
             may be either micropinocytotic vesicles or
             vesicles from the Golgi apparatus, and large
             vesicles (LV) which may represent pinocytotic
             vesicles formed by surface folds (SF) such as
             those at the left. Inset: higher magnifica-
             tion of area in the square. Note the limiting
             membrane (arrows) and varying internal struc-
             ture and density of the large granules. Uranyl
             acetate and lead citrate.
             X 14,000; inset X 41,000

                  Co - collagen
                  FF - fine fibrils
                  G - granules
                  LV - large vesicles
                  M - mitochondria
                  SF - surface folds
                  SV - small vesicles
                  Arrows - limiting membrane of granules

Figure 41. Electron micrograph of a type B synovial lining
            cell from a normal joint. The cell is charac-
            terized by regular outline, a network of granu-
            lar endoplasmic reticulum (GER), several small
            vesicles (SV), a few granules (G), and an ex-
            tensive Golgi apparatus (GA), a portion of which
            is shown in the inset of marked area. Golgi
            apparatus is composed of flattened and dilated
            saccules surrounded by numerous small vesicles
            many of which contain a moderately dense sub-
            stance. Uranyl acetate and lead citrate.
            X 14,000; inset X 41,000

                    Ce - centriole
                    Co - collagen
                    FF - fine fibrils
                    G   - granules
                    GA - Golgi apparatus
                    GER - granular endoplasmic reticulum
                    M   - mitochondria
                    SV - small vesicles

B cells had a relatively extensive Golgi apparatus consisting of flattened saccules and many small vesicles similar to those in the type A cells. Usually, some of the saccules of the Golgi complex were dilated. Nuclei were uniform in outline with chromatin clumps slightly more disperse than in the type A cell. Cellular outlines were more regular than type A cells; however, small cytoplasmic projections and surface folds were present on some cells. Both type A and B cells had fine intracytoplasmic fibrils similar to those in the cells of the fibrous membrane.

Electron microscopic examination of synovial membranes and synovial fluid of experimentally and naturally infected lambs revealed an inflammatory reaction which agreed with and complemented observations made with the light microscope. The synovial tissues were infiltrated with numerous inflammatory cells, of which the neutrophil was most common. Fibrin was abundant. In late infections, lymphocytes and plasma cells were prevalent along with fibroblasts.

In lambs killed before 14 hours of infection, synovial membranes were normal except for infiltration of inflammatory cells and localized degeneration of lining cells (Figure 42). After this time, there were large areas where the synovium was necrotic and disrupted or replaced by a fibrinocellular exudate (Figure 43).

By 96 hours postinoculation and afterward, the type B lining cells were increased in number and contained more

Figure 42. Acute serous synovitis of a lamb 8 hours after
            intraarticular injection of the OCP agent.
            Degenerate lining cells (DLC) and edema (upper)
            are adjacent to an area of normal type A (A)
            and type B (B) lining cells between which
            emigrating leukocytes (L, N) are found.
            Uranyl acetate and lead citrate. X 4,100

                A   - type A synovial lining cells
                B   - type B synovial lining cells
                DLC - degenerate lining cells
                JS - joint space
                L   - lymphocyte
                N   - neutrophils
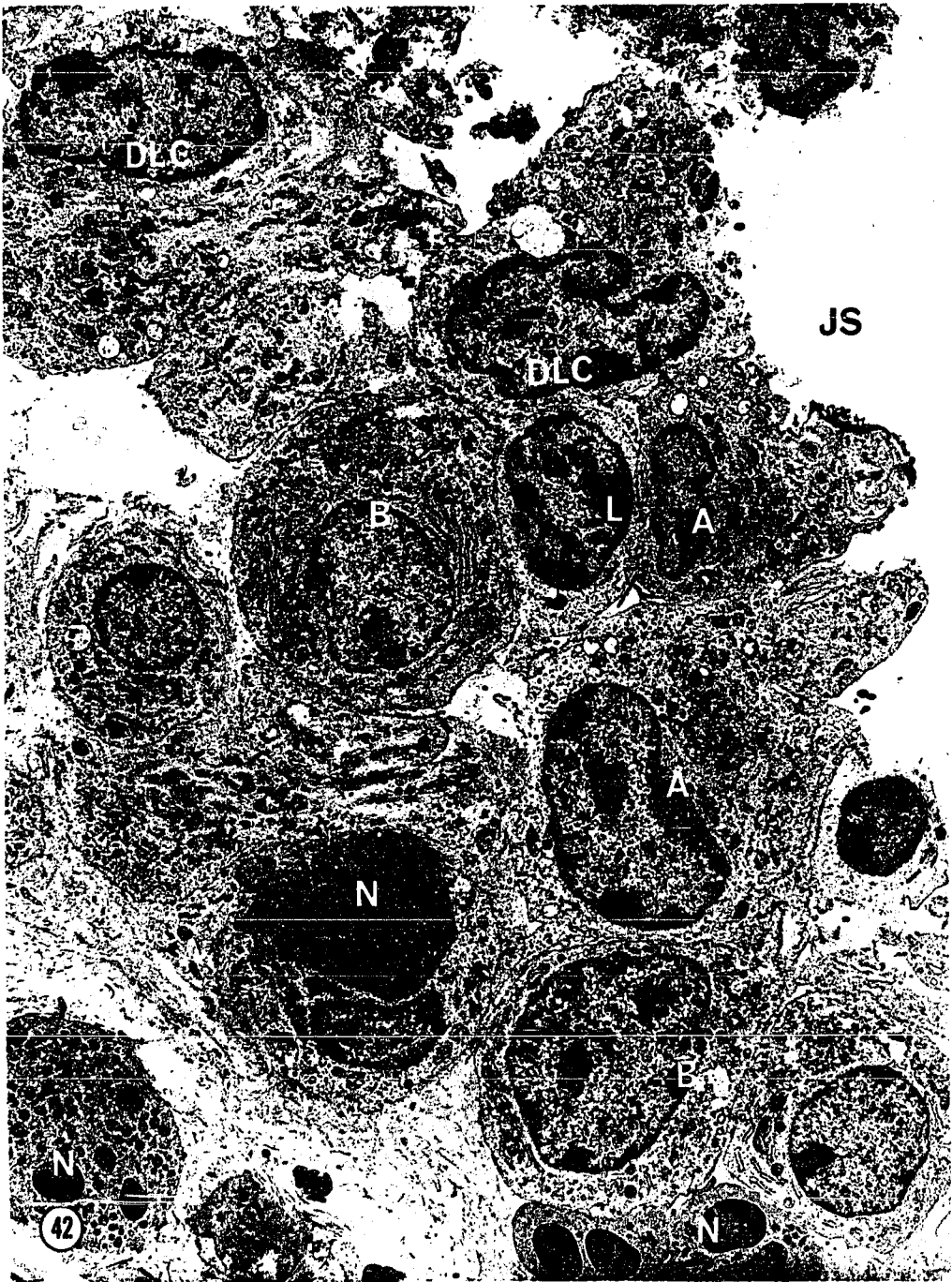
Figure 43.   Acute diffuse fibrinopurulent synovitis from
              the joint of a lamb 48 hours after injection
              of the OCP agent. Note the ruptured neutrophil
              with free lysosomal granules (arrow) and cells
              with the appearance of monocytes (Mon).
              Uranyl acetate and lead citrate. X 4,400

                   F   - fibrin
                   JS   - joint space
                   Mon - monocytes
                   N   - neutrophils
                   Arrow - lysosomal granules free in
                           exudate
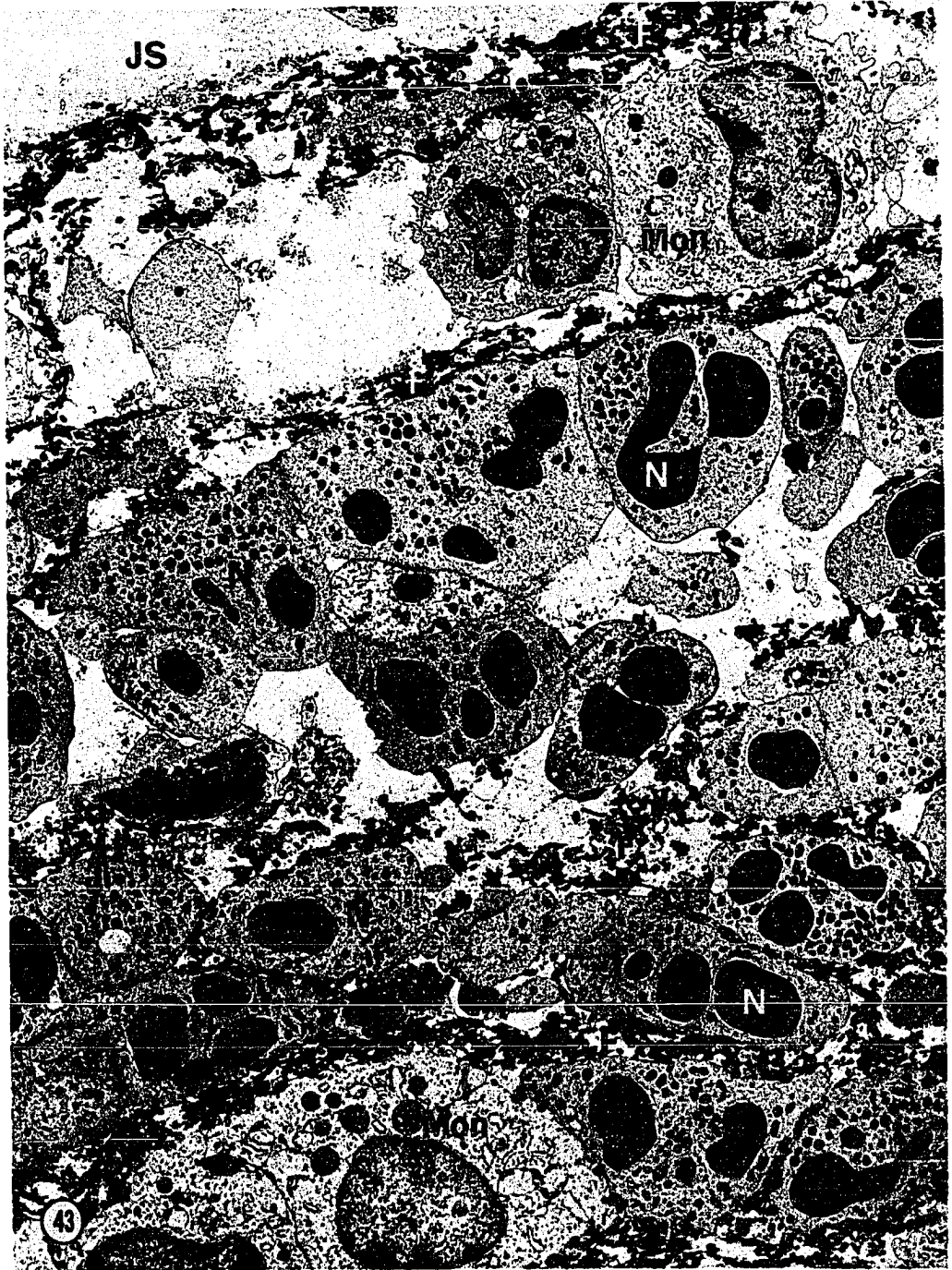
granular endoplasmic reticulum than cells of the same type
from normal joints (Figures 44 and 45). In some areas, this
was the only type of cells found in the synovial lining;
however, they were more frequently found in the subsurface
layer with type A cells and their cytoplasmic processes com-
prising the surface layer of the synovium. Other cells in
the surface layer possessed characteristics of both A and B
type cells; i.e., endoplasmic reticulum was concentrated in
the cytoplasm distal to the joint space; whereas, granules
and vacuoles were adjacent to the joint space (Figure 46).
This dual morphologic characteristic or polarization of
lining cells was more pronounced in joints infected for a
longer period of time.

By 21 days of infection, many elongated lining cells
were orientated with their long axis perpendicular to the
synovial surface. Several of these cells were polarized with
an extensive network of granular endoplasmic reticulum sur-
rounding the nucleus at the base and large granules and
vacuoles in the apex at or near the synovial surface (Figures
47 and 48). Such cells usually had a regular outline at the
base and many surface folds on the portion exposed to the
joint space. In addition to polarized cells, type A cells
were present at the synovial surface, many of which contained
membrane-bound granules up to 2.5 microns in diameter and of
variable electron density (Figure 49). Type B cells predom-
inated in the subsurface layer of lining cells.

Figure 44. Synovial lining 96 hours after injection of the
OCP agent. Hypertrophic type B cells (B) con-
taining extensive networks of rough endoplasmic
reticulum are surrounded by excessive inter-
cellular matrix (IM) in the subsurface layer
of the synovium. Irregular profiles of type A
cells (A) and their cytoplasmic processes (CP)
line the synovial surface. Uranyl acetate and
lead citrate. X 4,400

            A   - type A synovial lining cells
            B   - type B synovial lining cells
            CP   - cytoplasmic processes of type A cells
            IM   - intercellular matrix
            JS   - joint space
            N   - neutrophil
            RBC - erythrocyte
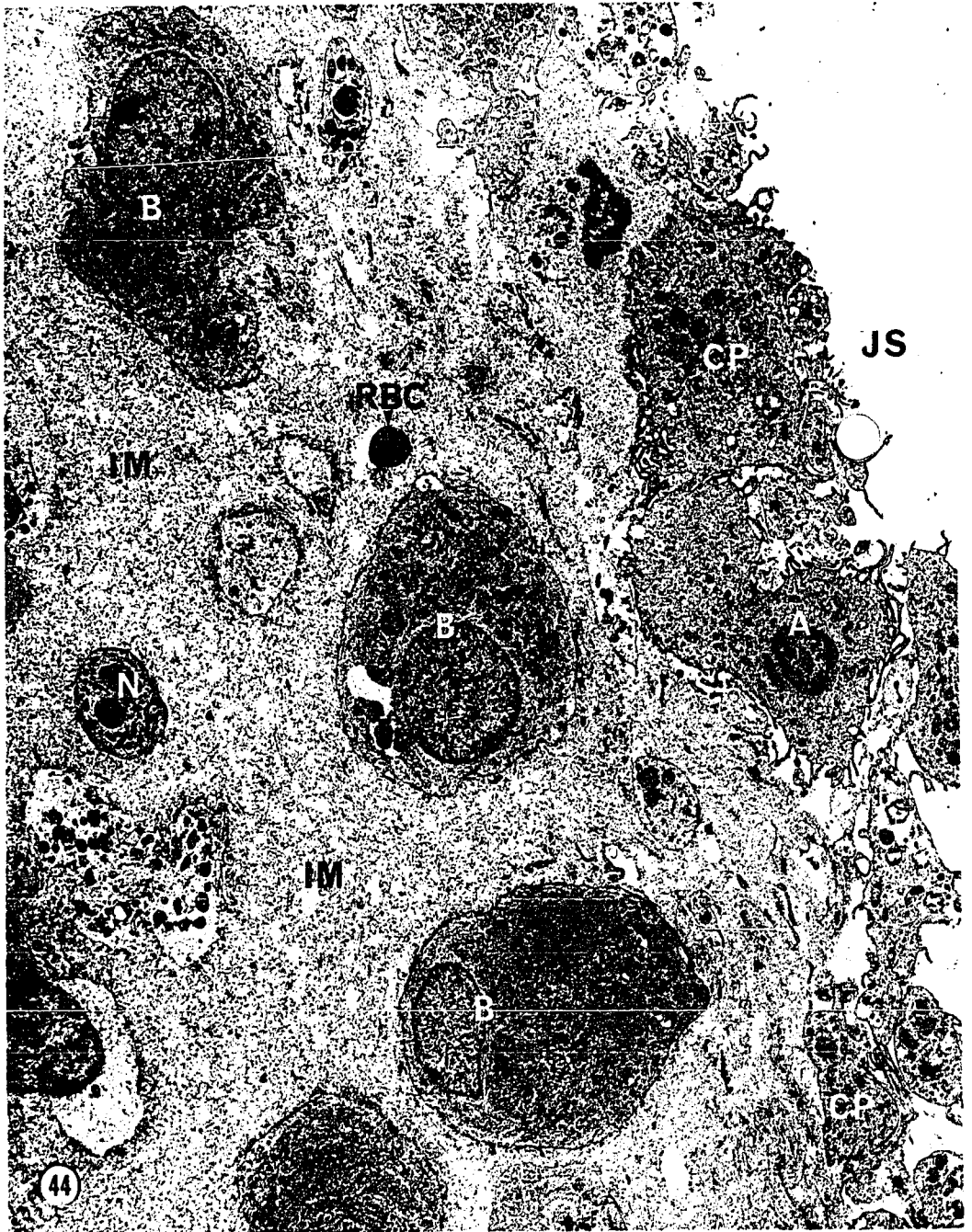
Figure 45. Hypertrophic type B synovial lining cell 96
            hours after intraarticular injection of the
            OCP agent. Note the extensive network of
            granular endoplasmic reticulum (GER) and Golgi
            apparatus (GA) composed of dilated saccules
            surrounded by numerous vesicles and granules
            of variable size and electron density. Larger
            granules (G) are scattered through the cyto-
            plasm. Uranyl acetate and lead citrate.
            X 24,000

                G   - large granules
                GA - Golgi apparatus
                GER - granular endoplasmic reticulum
   LG - lipid globules
                M   - mitochondria
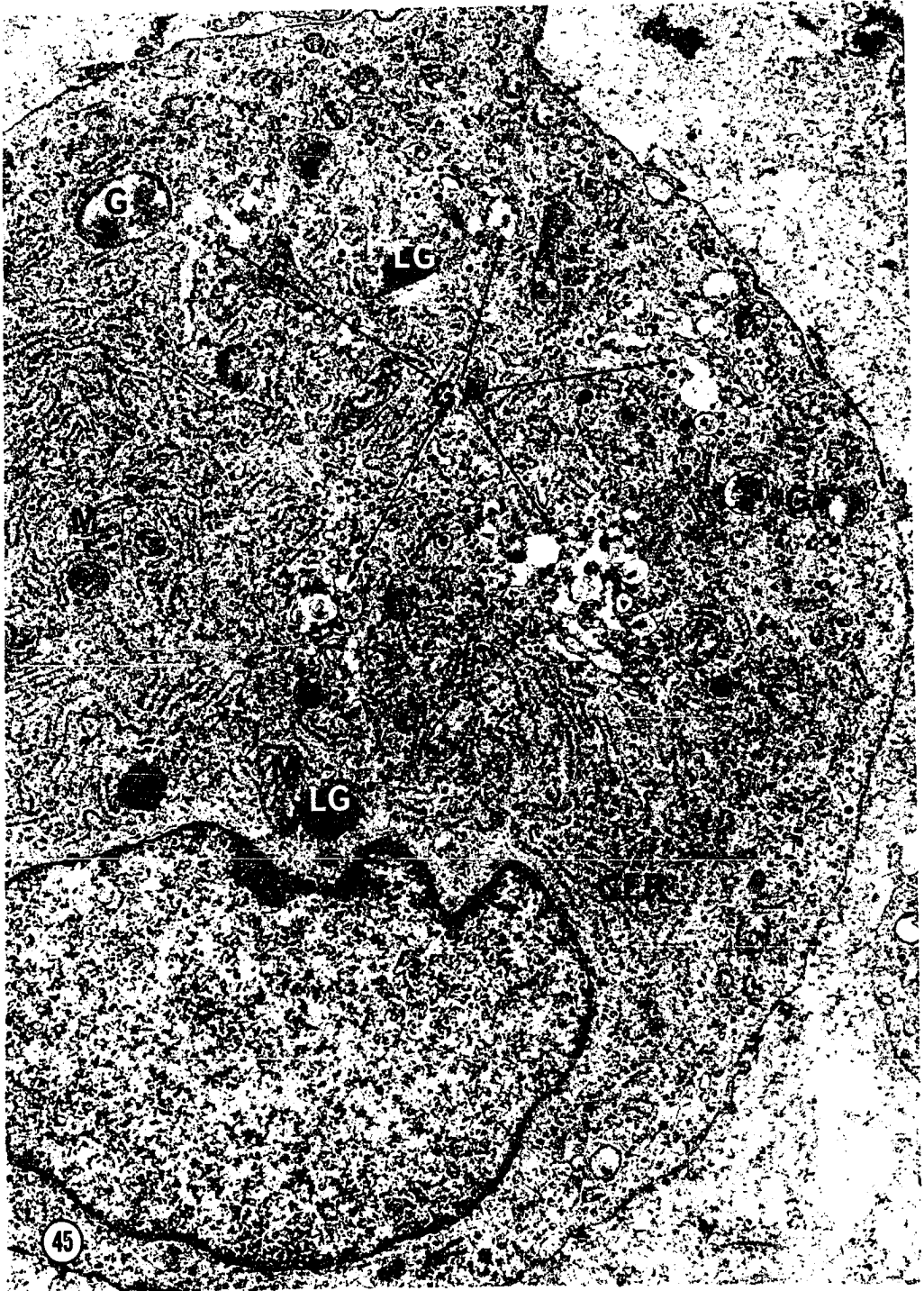
Figure 46. A synovial lining cell 96 hours after intra-
           articular injection of the OCP agent. Adjacent
           to the joint space (JS) are granules (G) and
           surface folds (SF), characteristic of type A
           lining cells; whereas, distal to the joint
           space is granular endoplasmic reticulum (GER),
           characteristic of type B lining cells. Uranyl
           acetate and lead citrate. X 9,600

                G   - granules
                GA - Golgi apparatus
                GER - granular endoplasmic reticulum
                JS - joint space
                SF - surface folds

Figure 47. Polarized lining cell after 21 days of infec-
            tion. Note the similarity to a type A lining
            cell at the apex and similarity to a type B
            lining cell at the base. X 14,000

                   G   - granules
                   GER - granular endoplasmic reticulum
                   JS - joint space
                   LV - large vesicles
                   M   - mitochondria
                   Nuc - nucleus
                   R   - free ribosomes
                   SV - small vesicles

Figure 48. Two polarized lining cells 21 days postinocu-
            lation. Each cell contains granules (G) in
            the apex and granular endoplasmic reticulum
            (GER) at or near the base. Note the dilated
            endoplasmic reticulum and greater electron
            density of cell at right and structure resem-
            bling a residual body (RB) in cell at left.
            Uranyl acetate and lead citrate. X 8,700

                    G   - granules
                    GA - Golgi apparatus
                    GER - granular endoplasmic reticulum
                    JS - joint space
                    M   - mitochondria
                    RB - residual body
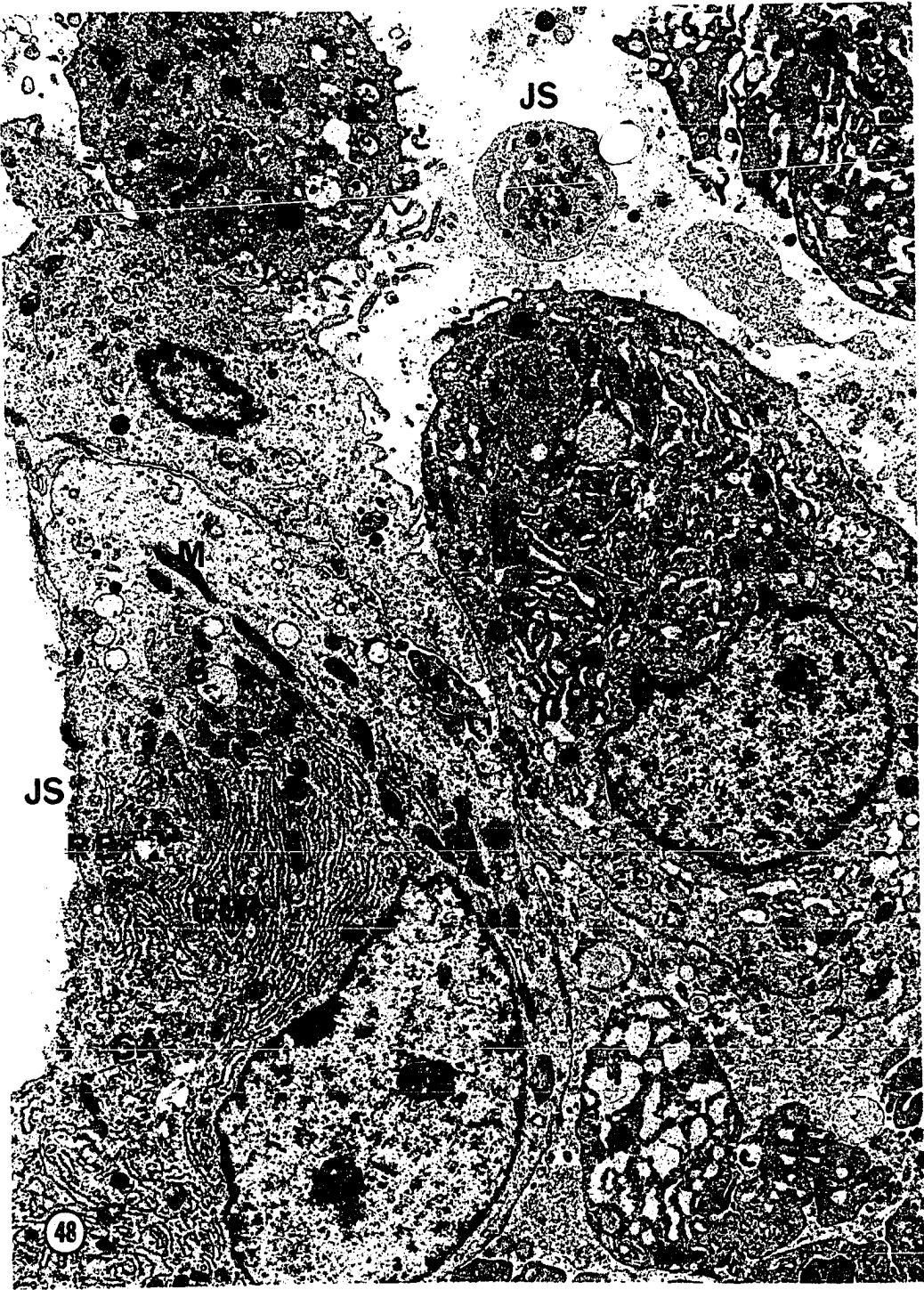
Figure 49. Type A synovial cells (A) containing cyto-
plasmic granules (G) up to 1.5 microns in
diameter, after 21 days of infection. The
edge of a type B cell (B) is seen at the upper
left. Intercellular vesicles (PRV) are inter-
preted as dilated endoplasmic reticulum from
ruptured plasmacytes. Uranyl acetate and
lead citrate. X 8,700

A   - type A synovial lining cells
B   - type B synovial lining cells
G   - granules
JS - joint space
PRV - plasmacyte-reticulum vesicles

Large cytoplasmic protrusions or blebs were attached
to a few polarized and type A cells and appeared in cross
section in the matrix surrounding the lining cells (Figure
50). They contained a fine granular substance similar to
the intercellular matrix of the synovial lining. Membranes
of some cytoplasmic protrusions were ruptured with an appar-
ent release of intracytoplasmic contents.

Neutrophils were the predominant inflammatory cells in
the synovium and synovia of infected joints (Figures 42 and
43). They were in greatest concentration at 48-96 hours
postinoculation, often interspersed with fibrin; most were
intact but some were fragmented with release of granules.
Eosinophils and mast cells were found occasionally.

Lymphoid cells were found in the synovium of infected
joints with an increasing frequency as the lesion progressed.
After 10 days of infection, they were seen in large numbers
mixed with numerous cells of the plasmacytic series (Figure
51). As classified by Leduc et al. (1968), these cells were
lymphocytes, hemocytoblasts, plasmablasts, immature plasma-
cytes, and mature plasmacytes. After 21 days, many plasma-
cytes were ruptured with cytoplasmic constituents in the
intercellular space.

Many macrophages were found in the synovium, subsynovium,
synovia, and exudate of infected joints (Figures 52, 53 and
54). They were recognized by the presence of large phagocytic
vacuoles (phagosomes) containing cellular debris and fibrin.

Figure 50. Synovium, 21 days postinoculation, with cyto-
plasmic protrusions (CP) of lining cells (LC)
extending into the (a) joint space (JS) and
(b) intercellular matrix (IM). (c) Cross
sections of the protrusions with light granular
contents similar to the extracellular matrix.
Note the ruptured plasma membrane of the cyto-
plasmic protrusions in micrographs a. and b.
Uranyl acetate and lead citrate. a. X 5,000;
b. X 6,200; c. X 14,000

    CP - cytoplasmic protrusions of lining
          cells
    IM - intercellular matrix
    JS - joint space
    LC - synovial lining cells
    PRV - plasmacyte-reticulum vesicles
    RBC - erythrocytes
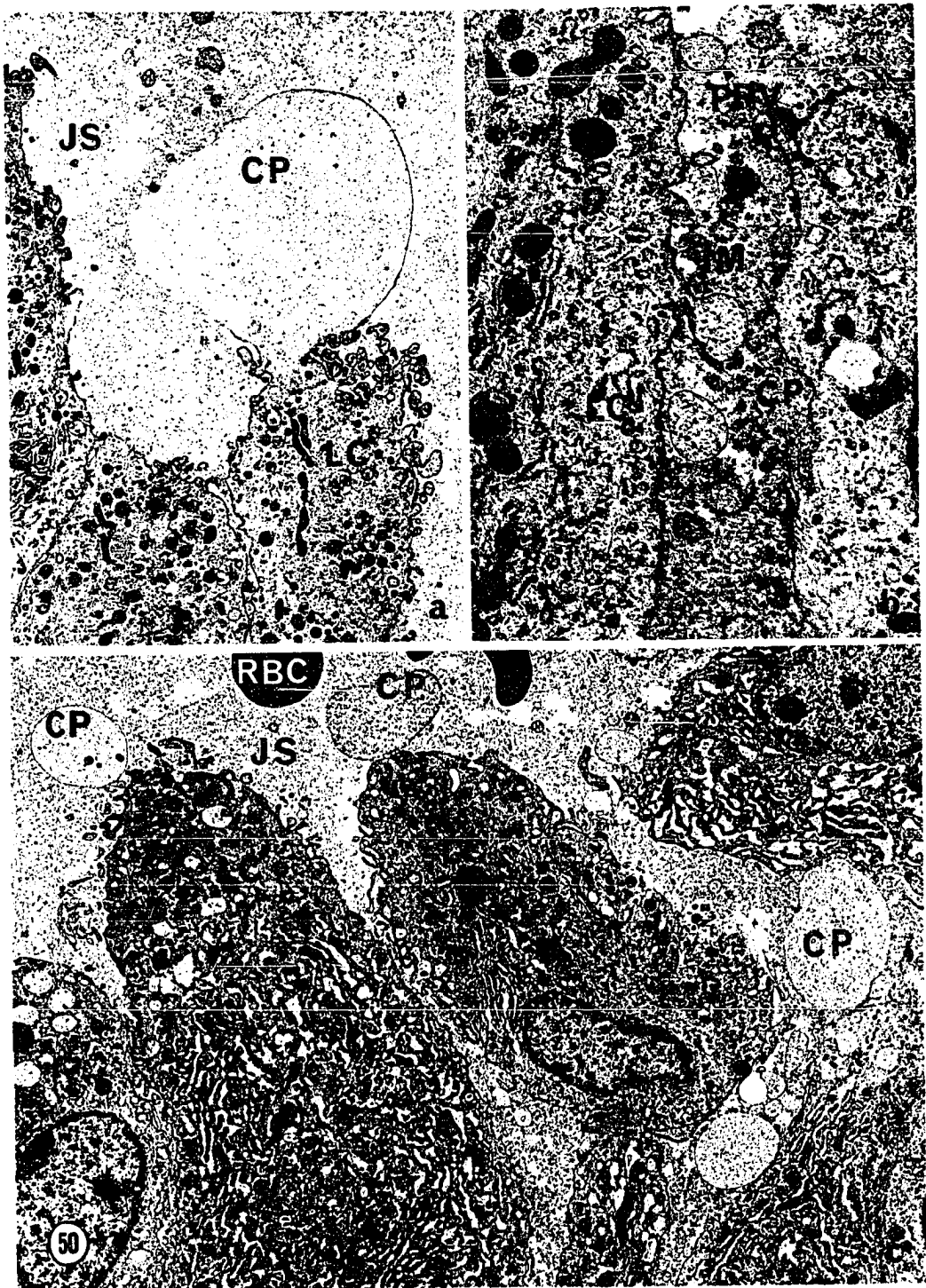
Figure 51. Chronic synovitis from lambs affected with
            OCP. (a) Twelve days and (b) 10 days post-
            inoculation, cells of the lymphoid and plasma-
            cytic series can be identified. (c) Twenty-
            one days postinoculation, degenerate plasma-
            cytes consisting of shrunken nuclei (Nuc)
            surrounded by vesicles (PRV) of granular
            endoplasmic reticulum. Uranyl acetate and
            lead citrate. X 4,400

                    Co - collagen
                    HB - hemocytoblast
                    IPC - immature plasmacyte
                    L   - lymphoid cell
                    MPC - mature plasmacyte with Russell
                          bodies
                    Nuc - nucleus
                    PB - plasmablast
                    PRV - plasmacyte-reticulum vesicles
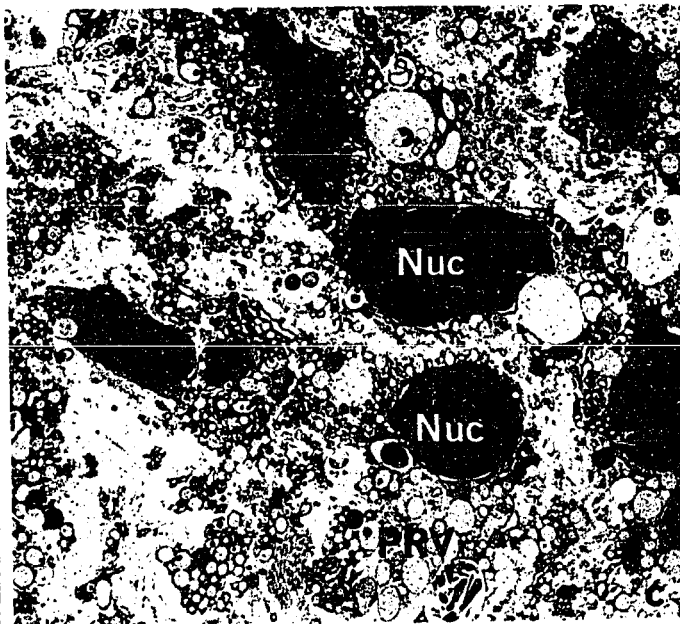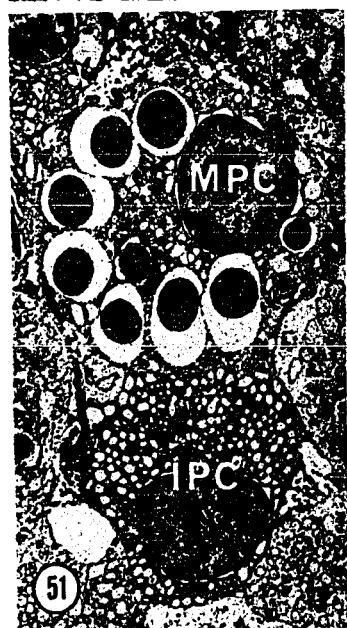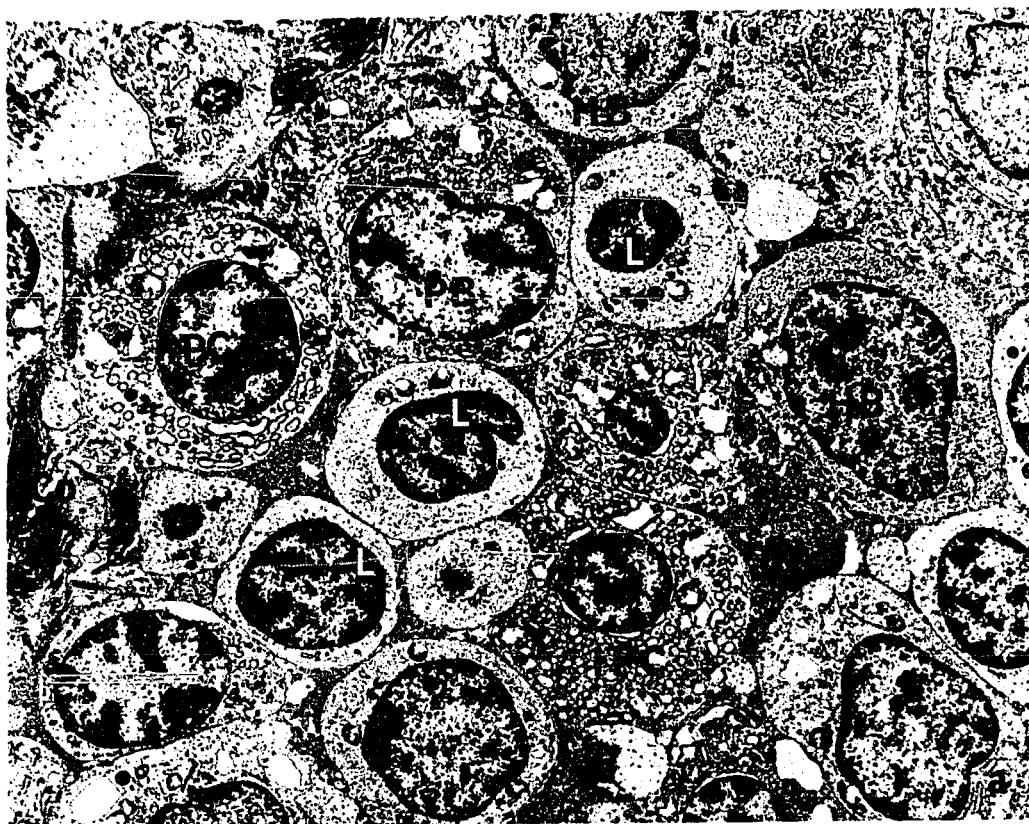
Figure 52. Phagocytic cell from the synovial fluid of an
            intravenously infected lamb. This cell has
            many characteristics of the type A lining cell,
            including large cytoplasmic granules (G), small
            vesicles (SV), and surface folds (SF) which
            appear to be forming pinocytotic vesicles. The
            large phagocytic vacuole (PV) contains apparent
            neutrophil granules. Uranyl acetate and lead
            citrate. X 14,000

            G - granules
            PV - phagocytic vacuole
            SF - surface folds
            SV - small vesicles

(52)
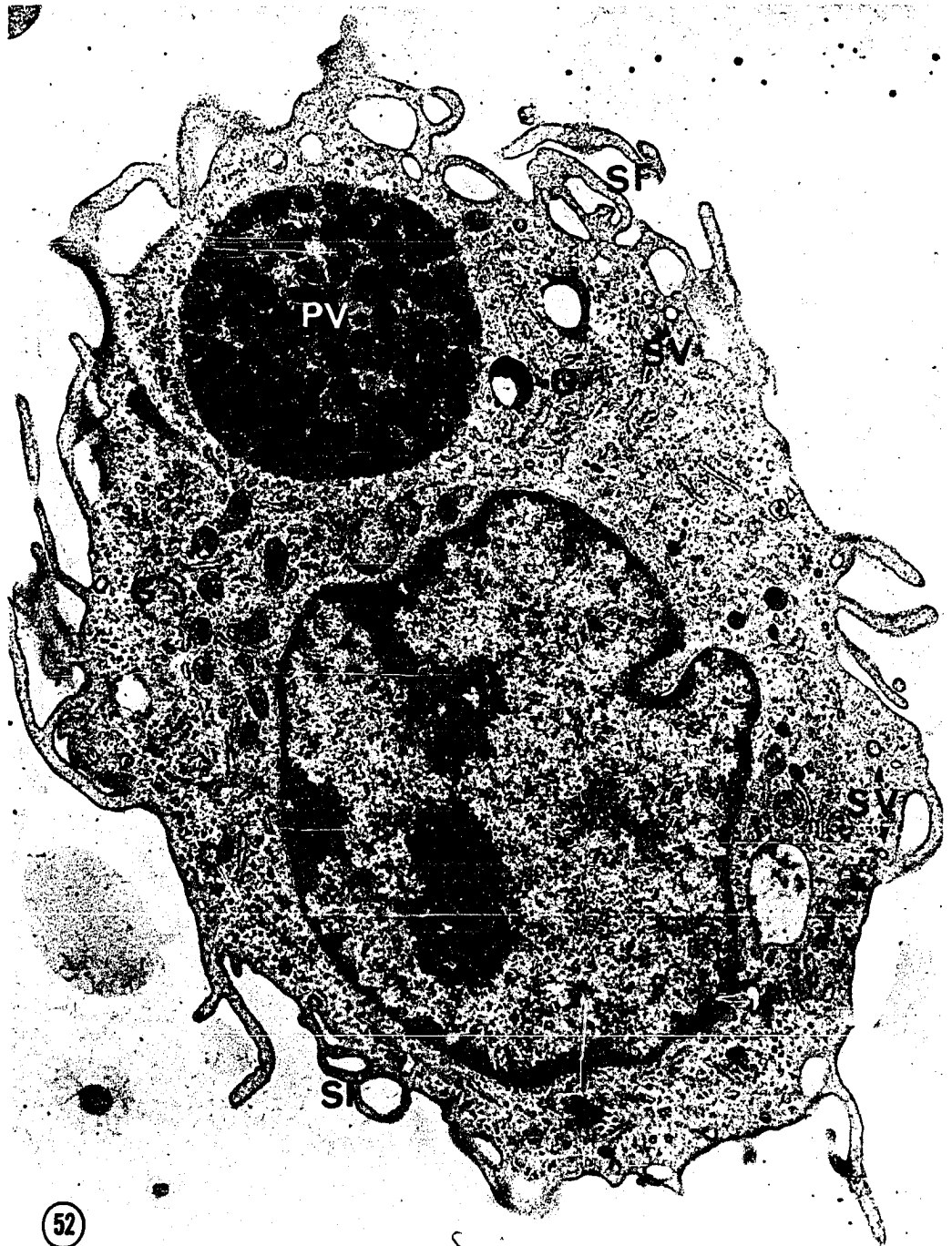
Figure 53. Phagocytic cell from the synovial fluid of a
lamb 12 days after intraarticular injection
of the OCP agent. This cell contains several
large phagocytic vacuoles (PV) and may repre-
sent a monocytic type cell. Uranyl acetate
and lead citrate. X 18,000

(53)

Figure 54.   Cells, (a) fibroblast and (b) macrophage, from
             an area of organizing fibrinous exudate in the
             synovium 21 days postinoculation.   Multiple
             phagocytic vacuoles (PV) which contain cellular
             debris and fibrin, and apparent lysosomes (Ly)
             are present in the macrophage. Collagen (Co)
             fibers surrounding the cells are presumably
             being produced by fibroblasts. Uranyl acetate
             and lead citrate. a. X 10,000; b. X 8,700

                  Co - collagen
                  F - fibrin
                  GA - Golgi apparatus
                  Ly - lysosomes
                  PV - phagocytic vacuoles
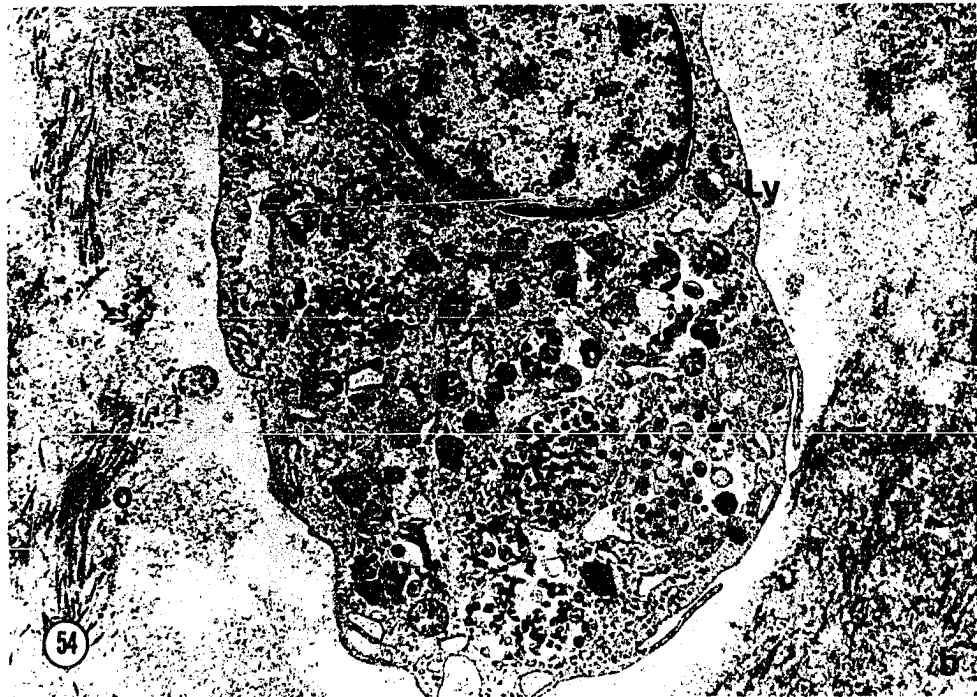
Similar cells, without phagosomes, were also widely distrib-
uted in the infected tissues (Figure 43). Whether these are
synovial cells or monocytes is uncertain; however, well dif-
ferentiated type A or B synovial cells were not found with
obvious phagosomes.

Fibroplasia was apparent in the synovium of lambs after
72 hours of infection. Many fibroblasts surrounded by col-
lagenic fibers were interspersed through the fibrinous exudate
in association with macrophages containing phagocytized fibrin
and cellular debris (Figure 54). Similar areas as well as
large areas of highly vascularized fibrous tissue were found
in the synovium of lambs killed later than 96 hours post-
inoculation.

Chlamydial organisms were encountered in 4 of the 29
joints that had been infected by the intraarticular route.
These were found in lambs killed 14, 24, and 48 hours and 12
days after inoculation. None were found in lambs with the
naturally occurring disease or in those given the agent
intravenously. Organisms found were either in extracellular
debris or within the cytoplasm of phagocytic cells, either
neutrophils or macrophages (Figures 55 and 56). Most were
of the small particle type and located within intracytoplasmic
vacuoles or phagosomes; often only remnants remained. Large
particles were found less frequently and their identity was
less definite. Numerous other structures were present in
the macrophages and synovial lining cells which at low magni-

fication were indistinguishable from chlamydiae; however,
at higher magnification, their morphologic detail was not
characteristic of chlamydial particles but were single-
membrane-bound granules.

Figure 55. Chlamydial organisms phagocytized by neutro-
phils in the synovium of a lamb killed 14
hours postinoculation. Small chlamydial
particles (SP) are present (a) within a phago-
cytic vacuole and (b) free in the cytoplasm
surrounded by what may be lysosomal material;
possible organisms (arrows) can be seen in a
phagocytic vacuole. These organisms appar-
ently were being destroyed by lysosomal en-
zymes. Uranyl acetate and lead citrate.
X 41,000

        F   - fibrin
        Ly - lysosomes
        Nuc - nucleus
        SP - small chlamydial particles
        Arrows - possible degenerate chlamydiae
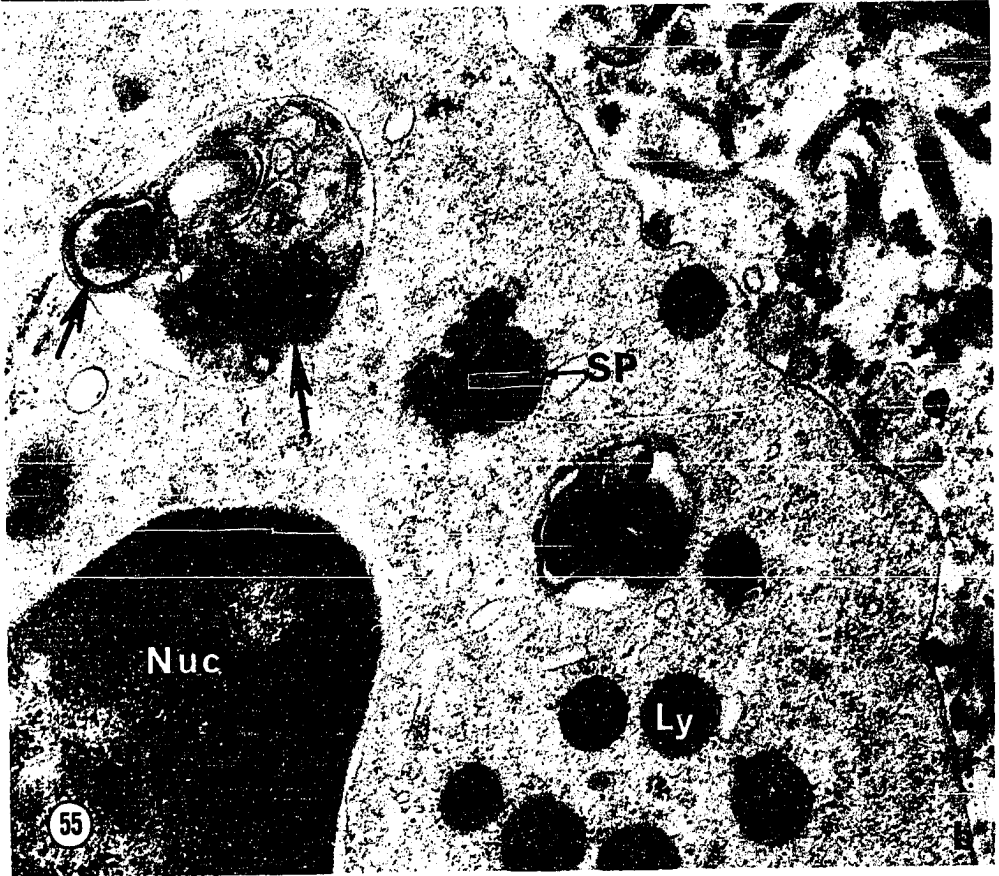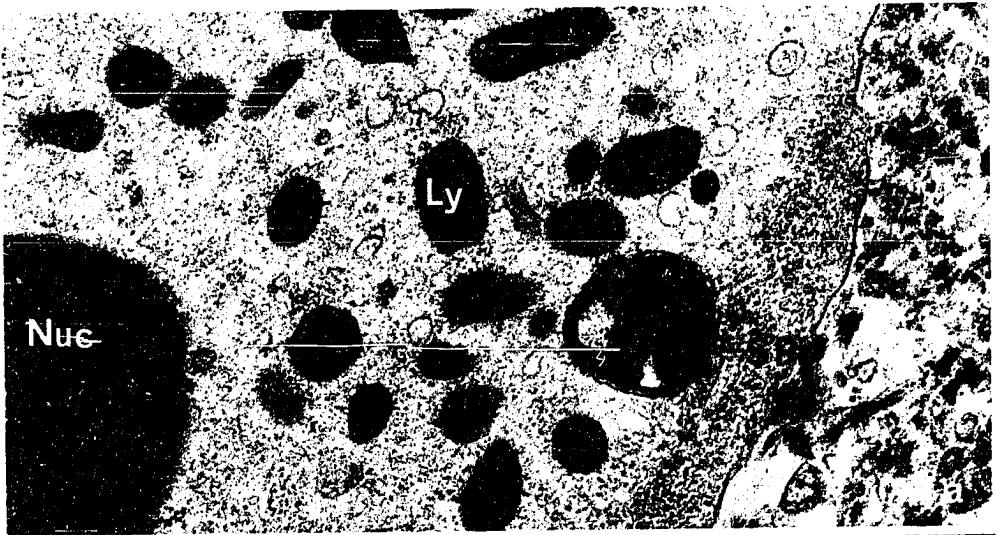
Figure 56.   Chlamydial particles in phagocytic vacuoles
             of macrophages from (a) the synovial membrane
             of a lamb killed 14 hours after inoculation
             and (b) the synovial fluid of a lamb killed
             48 hours after inoculation. The electron-
             dense material in the vacuole and extracellular
             space in micrograph a. is interpreted as egg
             yolk (EY). Uranyl acetate and lead citrate.
             a. X 41,000; b. X 24,000

                    EY   - egg yolk
                    F   - fibrin
                    GA   - Golgi apparatus
                    LG   - lipid globule
                    LP   - large chlamydial particle
                    M   - mitochondria
                    Nuc - nucleus
                    SP   - small chlamydial particle
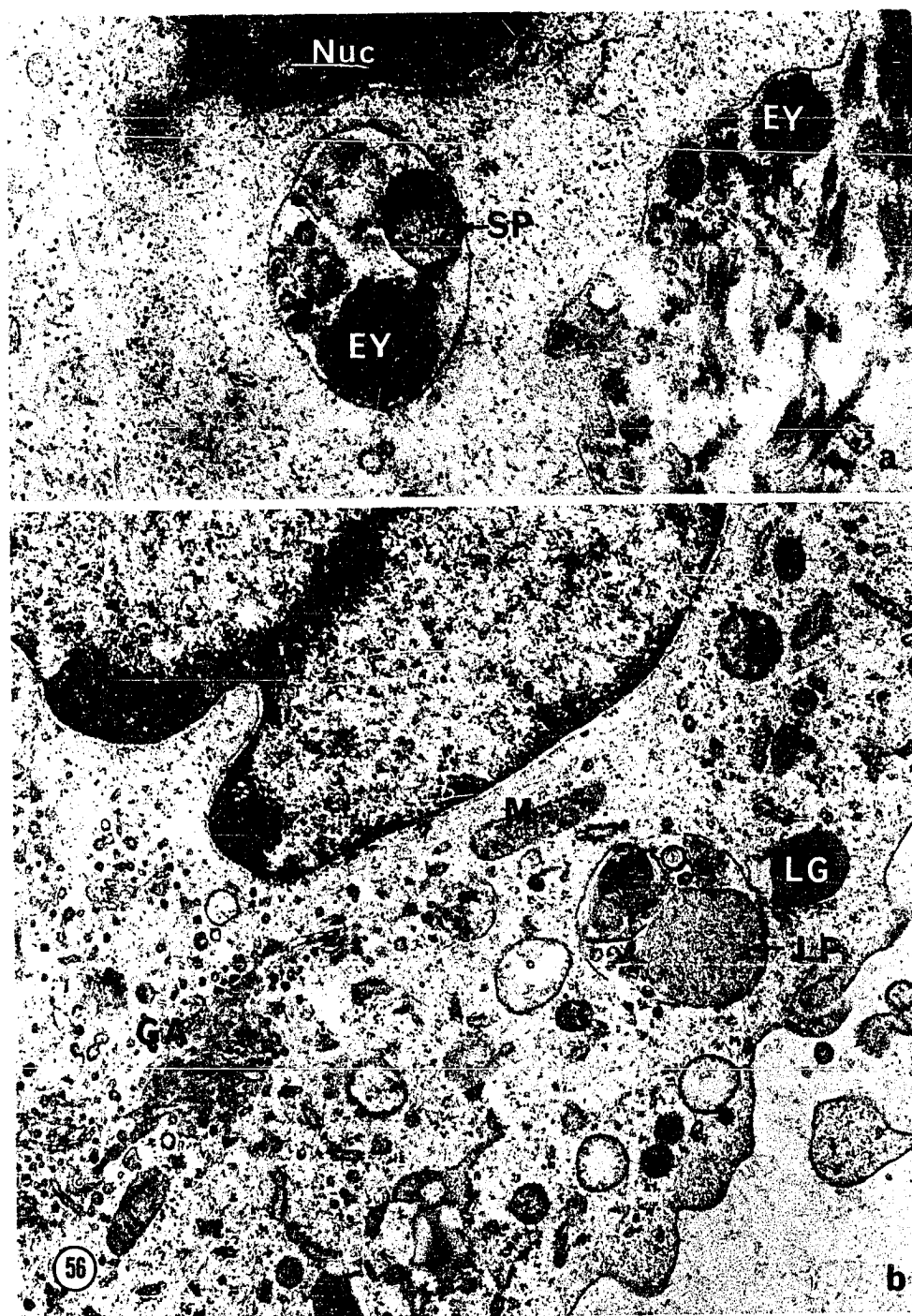
DISCUSSION

Morphology and replication of the OCP agent were studied in McCoy cell cultures by electron microscopy and will be considered together because of their intimate interrelationship. The replicative cycle could not be followed in synovial tissues since too few organisms were found. In cell cultures, growth of the organism was similar to other chlamydiae examined since 1964 (Armstrong and Reed, 1964; Higashi, 1964, 1965; Mitsui et al., 1967). Replication occurred in successive stages without loss of morphologic identity. Small particles were phagocytized by cells and developed within the cytoplasm into large particles which divided by binary fission; the daughter organisms either repeated the division or condensed to form new small particles which were released by rupture of the cell membrane. A schematic representation of this cycle is presented in Figure 57.

Phagocytosis occurred by cytoplasmic processes or pseudopodia of the cells surrounding and engulfing small particles of the organisms. Shortly after small particles were phagocytized by cells, or possibly upon contact with the cytoplasmic membrane, they began to enlarge and developed into large granular particles. Enlargement was associated with dispersal of the dense chromatin nucleoid, rearrangement of the multilaminated wall, and tremendous growth.

Fluorescent antibody staining during this phase of devel-

Figure 57. Scheme of replication of the OCP agent in
McCoy cells. Two and 4 hours postinoculation
small organisms are located extracellularly
and by 8 hours one is being engulfed by a
cell. Phagocytosis continues at 24 hours
plus large particles of the organism are
present within the cell cytoplasm. By 48
hours postinoculation the cycle is complete
with newly formed small particles inter-
spersed among large particles which continue
to divide by fission. Organisms are re-
leased by rupture of the cell 72 hours after
inoculation
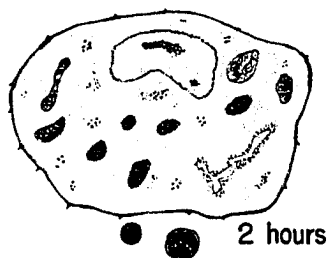
2 hours
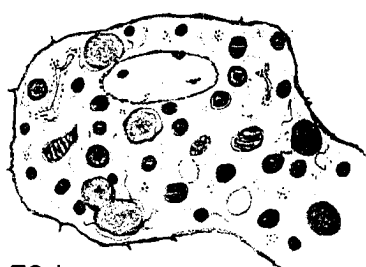
72 hours
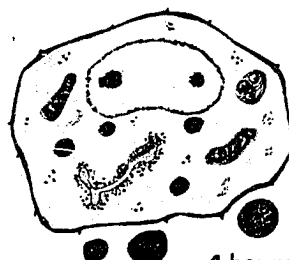
4 hours
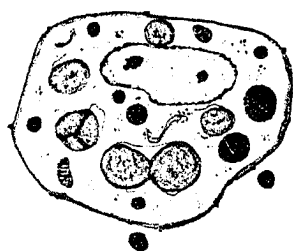
48 hours
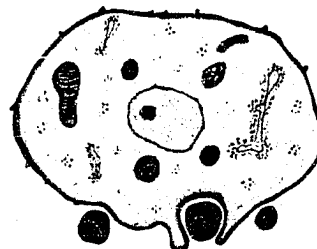
8 hours
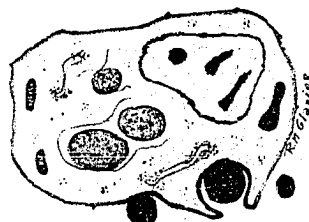
24 hours

opment was considered negative because of inability to differ-
entiate minute fluorescent foci from those present in control
cells. Probably inclusions of the OCP agent did stain but
were too small to be recognized since only single particles
were found by electron microscopy. Thus, failure of detect-
able staining cannot be interpreted to represent loss of anti-
genicity as the result of an eclipse as has been reported for
other chlamydiae (Pollard and Tanami, 1962). Antigens of the
6BC strain of the psittacosis agent have been shown to be
present throughout the developmental cycle (Buckley et al.,
1955).

Dispersal of the dense chromatin nucleoid of the small
particle was associated with appearance of strands of deoxy-
ribonucleic acid in the granular matrix of the large particle.
Ribosomes, present in all organisms, were typical of single
free ribosomes in other cells; these have been isolated from
the large particle form by Tamura (1967) and resemble ribo-
somes of other bacteria in buoyant density. The multilami-
nated wall of the small particle gradually changed into 2
trilaminar membranes surrounding the large particles. The
latter was also surrounded by the membrane of the phagocytic
vacuole which was ruptured soon after division began. Mem-
branes of large particles closely resembled the plasma mem-
brane and cell wall of other gram-negative bacteria as re-
ported by von Iterson (1965). Jenkin (1960) found the walls
of the meningopneumonitis agent chemically similar to those

of rickettsiae and gram-negative bacteria.

Division of the large particles was principally by simple
binary fission. Internal membranes found within some large
particles (Figure 13) may represent fission into 3 or more
daughter cells or faulty attempts at binary fission; however,
these forms were numerically insignificant when compared to
dumbbell shapes representing binary fission. Organisms with
internal membranes of this type but with the contents shrunken
during processing for examination, probably are similar to
those interpreted by others (Gaylord, 1954; Bernkopf et al.,
1962; Higashi et al., 1962) to represent endosporulation.
Endospores were not found in this study nor were particles
found which were believed to represent unequal fission as
proposed by Tajima et al. (1957) and by Litwin et al. (1961)
for several other chlamydiae. Unequal dumbbell profiles of
the OCP agent were interpreted as resulting from the plane
of the section and not from unequal fission.

After division, daughter particles either continued to
divide, apparently after enlargement, or condensed to form
mature small particles. This maturation process was accom-
plished by condensation of the chromatin, which at first was
located centrally, forming the intermediate particle, and
finally located at the periphery of the particle, and by
rearrangement or contraction of the limiting membranes into
an apparently rigid multilaminated wall. A similar process
of maturation has been suggested for several other chlamydial

organisms; however, Higashi (1964) reported that the trachoma
agent matured by forming a new membrane around a shrunken
central core with disappearance of the former external mem-
brane. Microtubules found in a few large particles are of
unknown significance but may be important in the maturation
process. Fawcett (1966) stated that microtubules found in
the cytoplasm of many cells are considered to be resilient
cytoskeletal elements that aid in maintaining cell shape by
imparting a certain stiffness to the cell. He also suggested
that microtubules probably have contractile properties and
may be involved in the internal movement of cells and altera-
tions of cell shape. If this assumption is correct, contrac-
tion of microtubules in large chlamydial particles may be a
significant means of size reduction during formation of the
mature small particle.

The mature small particles were generally composed of
an electron-opaque nucleoid in an eccentric location and a
fine granular matrix containing ribosomes. Small organisms
without a visible nucleoid are probably attributable to the
plane of the section. The multilaminated wall of the small
particle appeared to be composed of 2 trilaminar membranes
tightly apposed to the contents of the organism. This was
best seen in a few extracellular organisms where the membranes
had separated (Figure 7) and is in agreement with the specu-
lation of Anderson et al. (1965) that 2 membranes invest the
small particle of the lymphogranuloma venereum agent.

Division and maturation of intracellular particles continued until the plasma membrane of the cell ruptured and the organisms were released. As observed with the electron microscope, this cycle required 48 hours for formation of mature small particles and 72 hours for release of the organisms. The possibility of sampling errors and the use of nonpurified inoculum resulting in possible unsynchronized infection may have influenced the apparent replication time.

The results of acridine orange staining of chlamydial-infected cell cultures were interpreted by Pollard and Starr (1962) and Pollard and Tanami (1962) to indicate a true viral replication. They reported a change in the staining characteristics of the agents of psittacosis and trachoma at various stages of growth. In contrast, no change of color was detected during sequential development of the OCP agent; inclusions fluoresced bright orange before treatment with ribonuclease and green after treatment, indicating, according to Mayor (1962), the presence of ribonucleic acid and deoxyribonucleic acid in all stages of development. This is in general agreement with the observations of Furness and Csonka (1963) on the development of the agent of lymphogranuloma venereum. However, they found a gradual change in color of fluorescence from red, which was correlated with the presence of large particles, to yellow-green with the appearance of small particles. Green fluorescence, indicating pure deoxyribonucleic acid, was found only after treatment with ribo-

nuclease. Chemical analyses have shown that small particles of the meningopneumonitis agent are approximately equal in content to ribonucleic acid and deoxyribonucleic acid (Tamura and Higashi, 1967); whereas, large particles contain approximately 3 times more ribonucleic acid than deoxyribonucleic acid (Tamura et al., 1967). It is likely that orange fluorescence in late infection with the OCP agent was the result of many large particles in the cellular inclusions; even though the small particles predominated.

Enzyme histochemical procedures were uninformative other than indicating infection of McCoy cells with the OCP agent did not alter enzymatic activity to a degree that could be detected by the procedures used. Any difference in enzymatic activity of synovial cells in infected and noninfected joints was overshadowed by the intense activity in inflammatory cells. Similarly, oil red O staining lipid was not altered by infection.

Dissemination of the OCP agent to other joints following intraarticular injection was not detected as reported by Storz et al. (1965). Virulence of the respective organisms apparently was not a factor in this difference since their pathogenicity for 8 animal species was compared and found to be identical.[1] However, failure of the agent to spread to other

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[1]Page, L. A., National Animal Disease Laboratory, Ames, Iowa. Comparative pathogenicity of two isolates of the OCP agent. Personal communication. 1970.

joints may have been the result of a resistance in the lambs
which was not measured by the complement fixation test. Such
a resistance may explain the small number of organisms found
in synovial tissues. Apparently many organisms were destroyed
by phagocytic cells, as seen with the electron microscope,
soon after injection or after release from the few cells which
were infected. It is questionable if sufficient individual
cells were infected to cause the rapid and extensive articular
damage which resulted from intraarticular injection of the OCP
agent. Rather, it is likely that the organism contains an
inflammation-producing toxin as has been demonstrated in the
cell wall of other chlamydiae (Ross and Jenkin, 1962; Chris-
toffersen and Manire, 1969).

Sequential development of articular lesions can be di-
vided into 3 stages, serous inflammation from 2-14 hours
postinoculation, fibrinopurulent inflammation from 24-96
hours postinoculation, and resolution 6-24 days postinocula-
tion. Serous inflammation was characterized by excess syno-
vial fluid, edema of the synovium, and emigration of neutro-
phils. Destruction of synovial lining cells and fibrino-
purulent exudation rapidly followed. Intact synovial lining
cells were hyperplastic and hypertrophic with increased baso-
philic staining of the cytoplasm of many cells. Resolution
of the lesion, although not complete, was characterized by
extensive fibrosis, formation of lymphoid follicles, plasma-
cytosis, and recovering of the denuded synovial surface with

lining cells. Others (Shupe and Storz, 1964; Storz et al., 1965) considered accumulations of mononuclear cells as a granulomatous reaction. Foci of mononuclear cells in lambs of this study were not those of a granulomatous response but were lymphocytes and developmental forms of plasmacytes as outlined by Leduc et al. (1968). Such cells apparently function in local production of antibody. No changes were found in articular cartilages as reported by Shupe and Storz (1964).

It was necessary to first study the ultrastructure of normal synovium in order to evaluate changes caused by infection. Normal synovium was found to vary from region to region within the joint; one variety was believed to correspond to the fibrous membrane and the other to the areolar membrane of light microscopy. Lining cells of the fibrous membrane were indistinguishable from underlying fibroblasts; whereas, the areolar membrane was structurally similar to the synovium of man as described by Barland et al. (1962). There were no specialized areas of intercellular connections between the lining cells. Collagenous fibers were frequently located in the intercellular space of the lining cells but were found not to extend to the synovial surface as reported by some authors for other species (Coulter, 1962; Roberts et al., 1969).

Barland et al. (1962) first intimated a functional relationship between type A and B lining cells and considered

the possibility of a single cell type in different functional states in the synovial lining of man. This view was also held by Luckenbill and Cohen (1967) who considered only a single cell type to be present in the synovium of chickens. The synovial lining of lambs was found to be made up of 2 distinct cell types with a few cells characteristic of neither but a direct morphologic link between the two could not be established in normal synovium. However, in the synovium of lambs with OCP there was a morphologic transition between A and B cells during what appeared to be a maturation process. Following hypertrophy of the type B cells in the early stages of infection, they developed into polarized cells with large granules characteristic of type A cells in the apex and granular endoplasmic reticulum in the base similar to type B cells. The mature type A cell appeared to arise by formation of additional large granules accompanied by a gradual dissolution of the granular endoplasmic reticulum. In normal synovium the polarized cell apparently does not form; instead, the transition appears to be direct from B to A type cells by a less distinctive process of granule production and loss of endoplasmic reticulum.

The large granules are believed to be a secretory product possibly formed by coalescence of Golgi vesicles. Chemical composition of the granules is not known; however, the granular endoplasmic reticulum of the type B cell indicates protein production and the extensive Golgi apparatuses of both types

of cells may be active in the production of hyaluronate.
Involvement of the Golgi apparatus in the synthesis of com-
plex carbohydrates has been shown by the radioautographic
studies of Peterson and Leblond (1964). Thus granules of
the lining cells may consist of hyaluronateprotein. In addi-
tion to ultrastructural evidence, this suggestion is supported
by the demonstration by immunofluorescence of hyaluronate-
protein in lining cells (Blau et al., 1965) and by the finding
that several proteins, originally believed to be of plasma
origin, are produced by synovial lining cells (Williamson
et al., 1966). Hypertrophy, which included an increased
amount of granular endoplasmic reticulum, of type B lining
cells during infection with the OCP agent indicates increased
production of protein which may account for the increased
size of the secretory granules as compared to those of non-
infected synovium. A similar hypertrophy may also explain
the increased protein of the hyaluronateprotein complex found
by Hamerman and Sandson (1963) in some pathologic joint proc-
esses of man.

Several possible mechanisms could be involved in the
release of secretory products from the lining cells. One
possibility is an apocrine secretory process akin to that
demonstrated by Trier (1968) in intestinal crypt epithelium.
Cytoplasmic protrusions of the lining cells project into the
joint cavity and intercellular space, rupture, and release
their content (see Figure 50). The possibility exists that

such cytoplasmic protrusions do not represent active secre-
tion but rather are indicative of cellular degeneration. In
either event, the result would be the same, release of cyto-
plasmic content. A second possible mechanism of releasing
the secretory granules is sloughing of the mature type A cells
from their superficial location into the joint cavity and
their subsequent rupture. Such a mechanism does not explain
the presence of hyaluronateprotein in the intercellular areas
of the synovial lining as has been shown by Blau et al. (1965).
Another possibility is that secretion occurs by transport of
a product via small vesicles to the plasma membrane of the
cell with release of the contents by a process of reverse
pinocytosis.

However, most if not all of the small vesicles at the
cell membrane are believed to be micropinocytotic vesicles.
Pinocytosis is probably necessary to explain the uptake of
colloidal carbon and iron dextran by lining cells as has been
demonstrated in other species (Ball et al., 1964; Luckenbill
and Cohen, 1967). Also, the presence of lysosomes in synovial
lining cells of man, pigs (Hamerman et al., 1961; Roberts et
al., 1969), and lambs, as indicated by acid phosphatase activ-
ity, signify these cells are active in the uptake and hydrol-
ysis of some extracellular substance. Whether lining cells
actively phagocytize particulate matter, such as cellular frag-
ments, is questionable. Cells in the synovial fluid similar
to type A lining cells were found to contain large phagocytic

vacuoles; however, such vacuoles were not found in similar cells in the intact synovium. Phagocytic cells in disrupted areas of the synovium were believed to be more characteristic of monocytes than of lining cells; however, the morphologic differentiation of such cells is difficult.

Detection of the OCP agent in infected cell cultures was easiest with the fluorescent antibody technique; fluorescent inclusions were found in all infected cultures after 24 hours of incubation. Minute fluorescent foci, some of which probably were organisms, in cells incubated for less time could not be differentiated satisfactorily from similar foci in control cells. Giemsa staining was much less acceptable because of failure to stain many inclusions, especially small ones of early infection, and less distinct staining of others. These conclusions agree with those of Buckley et al. (1955) and Nichols et al. (1963) on the agents of psittacosis and trachoma respectively. Also, the nonspecificity of Giemsa stain is disadvantageous because of the difficulty in distinguishing chlamydiae from cytoplasmic and nuclear debris (Gikes et al., 1958). Acridine orange, although lacking the specificity and brilliance of staining, paralleled fluorescent antibody for detecting quantity of inclusions. Enzyme antibody staining was satisfactory only in 96-hour and 240-hour cultures. Excess stain precipitate occurred in all slides, making difficult the detection of smaller inclusions. However, this technique, with refinements, appears promising

for supplementing fluorescent antibody methods. It retains specificity for antigens, eliminates the need for an ultra-violet light source, results in permanent preparations, and has the potential for use in electron microscopy (Nakane and Pierce, 1967). Because of their distinctive ultrastructure, particles of the OCP agent were readily recognized in infected McCoy cell cultures with the electron microscope.

Few organisms were found in the joints of lambs either by light or electron microscopic techniques; even though the agent was injected intraarticularly in an attempt to infect a large percentage of cells. In contrast, Norton and Storz (1967) reported recognizing the OCP agent in infected joints with relative ease. However, illustrations of their intercellular particles do not have the characteristic ultrastructure interpreted as the OCP agent in this study or of other chlamydiae. Structures such as they illustrate were found in this study and were thought to represent necrosis or phagocytosis of cellular fragments. Failure to find many organisms with the electron microscope was believed not to be a sampling error since only a few organisms were found with fluorescent antibody staining. Results of staining with fluorescent antibody were confusing because of the large number of eosinophils in the tissues which fluoresced similar to chlamydial inclusions in McCoy cells. However, a few scattered cells with cytoplasmic fluorescence, found not to be eosinophils by restaining the sections with Giemsa,

were believed to contain the OCP agent. Giemsa stained organisms, if present, were overshadowed by intracytoplasmic cellular debris in cells either in smears or sections. Similarly, fluorescence of cellular ribonucleic acid in the inflammatory lesion obliterated any possible staining of organisms with acridine orange. Diffuse staining of synovial tissues with peroxidase labeled antibody was apparently caused by endogenous peroxidase or pseudoperoxidase.

Observations made during this study indicate that the OCP agent is a bacterium as suggested by Moulder (1966) for all chlamydiae. It was shown that the OCP agent contains both ribonucleic acid and deoxyribonucleic acid, divides by binary fission, and maintains a morphologic identity throughout the developmental cycle; any one of which is sufficient to separate this agent from viruses. Classification of members of the genus Chlamydia into 2 species based on morphologic and physiologic characteristics has been proposed by Page (1968). One criterion for classification was the presence or absence of glycogen associated with intracellular organisms. The faint periodic acid-Schiff reaction, failure of the reaction to be altered by diastase, and electron microscopic observations indicate the absence of glycogen in cells parasitized by the OCP agent. Another standard used to divide the genus was the type of intracellular colony. Intracellular organisms were reportedly found either in compact, membrane-limited microcolonies or diffusely distributed throughout

the cytoplasm. Electron microscopy revealed that the agent of OCP conformed to the latter division, the intracytoplasmic colonies being surrounded by membrane of the phagocytic vacuole only in early infections, later it was ruptured and organisms were scattered throughout the cytoplasm.

The noncytopathic virus contaminant found in McCoy cells during the course of this study structurally resembled type C murine leukemia virus as classified by Bernhard (1960). Virions of this type have been found in the tissues of leukemic mice, disease free conventional mice, and germfree mice (Bernhard, 1960; de Harven, 1964; Kajima and Pollard, 1965). Their significance in nonleukemic mice is undetermined but they may represent latent forms of leukemia virus. This observation of type C virions in McCoy cells, supplements previous reports (Dmochowski et al., 1967; Kajima et al., 1967) and tends to corroborate general contamination of McCoy cells. Dmochowski et al. (1967) reported that McCoy cells are not human synovial cells as originally characterized; instead, immunologic and karyotyping techniques indicate they are of murine origin. This supports the ultrastructural evidence that the virus is a murine leukemia or closely related virus. However, additional information on its biologic activity is needed to accurately determine its nature.

SUMMARY AND CONCLUSIONS

McCoy cell cultures and articular tissues of 24 lambs infected with the agent of ovine chlamydial polyarthritis were examined by light and electron microscopy in an attempt to determine the structure and mode of replication of the agent, to study the pathologic alterations, and to evaluate methods of detecting the organism. Synovial tissues were examined from 18 lambs exposed intraarticularly, 2 exposed intravenously and 4 with spontaneous disease.

As seen in thin sections of infected cell cultures, the organism exists either as small (250-450 nm) electron-dense particles with an eccentric nucleoid, ribosomes, and a multi-laminated cell wall or as large (600-1500 nm) granular particles containing ribosomes, strands of deoxyribonucleic acid, and enveloped by 2 unit membranes. Particles, intermediate in size and structure to the 2 major types, were also present. Replication, which occurred throughout the cytoplasm, was initiated by phagocytosis of small chlamydial particles. Upon entering a cell, they developed into large particles which divided by binary fission. Daughter particles either repeated the division or condensed to form new small particles. The replicative process was terminated by rupture of the plasma membrane of the cell. This cycle was complete with formation of new particles by 48 hours postinoculation. Failure to find sufficient organisms precluded a description

of the organism in ovine synovial tissues.

Pathologic changes in the joints of lambs following intraarticular injection of the OCP agent occurred in 3 stages; an initial serous synovitis and tenosynovitis 2-14 hours postinoculation, followed by fibrinopurulent inflammation 24-96 hours postinoculation, and finally early changes of resolution 6-24 days postinoculation. Serous inflammation was characterized by hyperemia and edema of the synovial tissues, emigration of neutrophils into the joint, and excessive synovial fluid containing a few flakes of fibrin. By 24 hours after inoculation areas of necrotic synovium were covered by thick layers of fibrinopurulent exudate and the synovial fluid contained numerous neutrophils and large flakes of fibrin. Areas of intact synovium had hyperplastic and hypertrophic lining cells. Early changes of resolution of the articular lesion were present in the lambs affected longest. These included organization of the fibrinopurulent exudate, recovering of the areas of denuded synovial surfaces with lining cells, and infiltration and proliferation of lymphocytes and plasmacytes. Changes in the joints of lambs infected intravenously were similar to the early changes in intraarticularly infected lambs; whereas, joints of naturally infected lambs were similar to those of lambs infected intraarticularly for the longest period of time.

Ultrastructure of normal synovial membranes was studied for comparison with infected membranes. Some areas of syno-

vium corresponded to the fibrous membrane of light microscopy where the lining cells rested on dense collagenous tissue and were indistinguishable from underlying fibroblasts. Other areas, corresponding to the areolar membrane of light microscopy, were characterized by lining cells similar to Barland's type A and B cells of man overlying loose fibrous tissue containing many vessels. Type A cells differed from type B cells by having large intracytoplasmic granules and an irregular outline; whereas, type B cells had a large amount of rough endoplasmic reticulum and a regular outline. In normal synovium, there was no apparent transition between cell types; however, in infected joints, changes in the lining cells were indicative of a gradual transition of type B cells into type A cells. Hypertrophic type B cells located in the subsurface layer of lining cells appeared to gradually change into polarized cells characteristic of type B cells at the base and of type A cells at the apex. Type A cells, containing large granules believed to be secretory product, then developed at the synovial surface. The composition of the granules is speculative; however, they may represent hyaluronateprotein. Structural evidence was found indicating release of cytoplasmic contents from type A cells by the formation and rupture of cytoplasmic protrusions. Whether this is a secretory or degenerative process is questionable. Numerous pinocytotic and micropinocytotic vesicles were found in lining cells, especially type A cells, indicating the uptake of some

extracellular substance; however, phagocytic vacuoles con-
taining particulate matter were not found in lining cells
of intact synovium. Most phagocytic cells in the synovial
membrane and synovial fluid were believed to be monocytes;
a few in the synovial fluid were morphologically similar to
lining cells. Other ultrastructural changes complemented
those seen with the light microscope.

Fluorescent antibody technique was the most satisfactory
of several light-microscopic methods used to detect the OCP
agent in McCoy cell cultures. However, in infected synovial
tissues the results of fluorescent antibody staining were
less satisfactory because of the small number of organisms
present and confusing autofluorescence of eosinophils. Organ-
isms were detected in infected cell cultures with Giemsa
staining but could not be identified in synovial tissues
because of inability to differentiate them from phagocytic
debris. Electron microscopy revealed numerous organisms in
infected cell cultures but very few in synovial tissues.
Organisms in synovial tissues were either extracellular or
within phagocytic cells where they apparently were being
destroyed by lysosomal enzymes. Rapid destruction of organ-
isms by such cells may account for the scarcity of organisms
in synovial tissues.

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