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Demonstration of the Phenomena of Microbial Persistence and Reversion with Bacterial L-Forms in Human Embryonic Kidney Cells

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A series of experiments was undertaken in which human embryonic kidney (HEK) fibroblasts were infected with either relatively stable or stable L-forms of *Streptococcus faecalis*. The infected cells were maintained by splitting over long periods (1 to 2 months) while samples were withdrawn for culture and electron microscopic studies. Relatively stable *S. faecalis* L-forms could be cultured from infected cells only during the first week after infection, although L-form-like material was frequently detected at later periods by electron microscopy. HEK cells continued to proliferate and showed no gross histopathology until reversion occurred. At reversion, electron microscopy revealed the presence of intracellular L-forms, transitional variants, and intra- and extracellular bacterial forms. Unlike relatively stable L-forms, stable L-forms were culturable throughout the experimental period. These experiments demonstrate the phenomena of persistence and reversion in vitro. Electron microscopy indicated that the L-forms entered the HEK cells, where they underwent morphological changes. Observations suggested that these altered L-forms (elementary bodies) persisted in the cell until reversion to the parent bacterial form was triggered. Infected cells, when treated with L-form antiserum and stained with fluorescein-conjugated goat anti-rabbit serum, were consistently positive regardless of cultural findings.

Although vast amounts of research have been directed toward cell wall-deficient bacteria since their discovery by Kleinberger-Nobel in 1934, the fundamental issue of the role of the L-form (both stable and unstable) in the human host in the absence of drug therapy remains unsolved. Because early studies indicated that L-forms were not pathogenic unless they reverted to their parent bacterial forms, they were regarded largely as laboratory curiosities of little clinical significance, and most investigations were directed toward in vitro aspects of L-form behavior (14).

In 1958, however, McDermott suggested that protoplasts might persist in the mammalian body (15), thus stimulating development of the concept of persistence in human tissue of a morphologically altered form of bacterium that was insensitive to most cell-inhibiting antibiotics, not culturable on media used in routine bacteriology, but capable of reverting to the parent bacterium (20).

Research in the last decade has centered around demonstration of occurrence and persistence of L-forms in vivo (20). Of particular interest were animal studies by Guze and Kalmanson (10) and Eastridge and Farrar (8) indicating persistence of L-forms (based on cultural findings) at various time intervals after cessation of penicillin treatment of experimentally induced pyelonephritis. In our laboratory, specific fluorescence of transitional-phase variants could be demonstrated for as long as 237 days postinfection in rats kept on penicillin therapy (7). Tissue culture studies of Hatten et al. (11, 12) and Schmitt-Slomska et al. (17) suggest that L-forms can persist within cells for various periods of time. These experimental findings, coupled with an ever-increasing number of well-documented clinical case reports of isolation of cell wall-deficient variants in cases of persistent and recurrent bacterial infection, dictate the necessity for critical assessment of the role of the L-form in persistence and relapse of infectious disease (6, 9).

The purpose of the present study was to determine whether one could demonstrate both culturally and visually (by electron microscopy) the presence, persistence, and reversion of bacterial L-forms in human embryonic kidney

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(HEK) cells and to compare the effects of stable and relatively stable L-forms on such cells in the absence of inducing antimicrobial agents.

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MATERIALS AND METHODS

Experimental design. This study involved a series of experiments in which HEK fibroblasts were removed from 32-oz (about 960-ml) stock culture bottles by use of trypsin, dispersed, and dispensed in equal portions into 4-oz (about 120 ml) Saniglass prescription bottles, where they were allowed to grow into a semiconfluent monolayer (approximately 10^6 cells).

Half of the bottles of cells were then infected in the following manner. Fluid medium was removed, and portions of L-form inocula dispersed in brain heart infusion broth (BHIB) (ranging from 0.5 to 2.0 ml in separate experiments) were introduced into all test bottles. The bottles were sealed and returned to the incubator (35 C) for 30 to 60 min during which time they were tilted every 5 to 10 min to assure complete coverage of the cells by the inoculum. After a minimum of 30 min, 10 ml of Eagle minimal essential medium supplemented with 10% fetal bovine serum was added to each bottle. Cells were not fed again until 48 to 72 h postinfection. The remaining control bottles of cells received identical treatment except that sterile BHIB was introduced in place of L-form inoculum. Tissue culture inoculum M-1 L-forms were grown for 48 or 72 h on brain heart infusion agar with 10% horse serum ($BHIAHS_{10}$) in Kolle flasks incubated at 35 C in a candle can. B-9 L-forms were grown for 48 or 72 h on *Streptococcus faecalis* agar with 7.5% bovine serum albumin ($SFABSA_{7.5}$) in Kolle flasks incubated aerobically at 35 C. Organisms were washed from agar surfaces with 3 to 5 ml of BHIB, pooled, and quantitated by duplicate plating of 10-fold dilutions on $BHIAHS_{10}$ and $SFABSA_{7.5}$, respectively. The remaining inoculum in an amount equivalent to that inoculated into each test system was incubated in BHIB to determine whether any classical bacteria were present at the outset of the experiment.

Both infected and control cells were then maintained over a 30- to 60-day period by splitting every 7 to 14 days by scraping cells from the glass with a rubber policeman. Separate samples for bacteriological culture, electron microscopy, hematoxylin and eosin staining, and immunofluorescent staining were withdrawn at weekly intervals. In each case, a sample consisted of one test and one control bottle of cells.

To culture all cells as well as the growth medium present in a given sample, brain heart infusion *S. faecalis* agar was melted and cooled (40 C), and 15 to 20 ml of the supplemented media ($BHIAHS_{10}$, $SFABSA_{7.5}$) were poured into both test and control bottles just before solidification of the agar. The samples were then returned to the incubator and examined daily for signs of bacterial growth. When growth did occur, organisms were examined by both phase-contrast (wet mount) and light (Gram stain) microscopy and submitted to standard bacteriological procedures for identification of the organisms. All experiments were carried out in the total absence of antibiotics.

Bacteriological media. The standard medium used for the production and propagation of the M-1 L-form was $BHIAHS_{10}$ as described by Roberts and Wittler (16). The medium used to propagate the B-9 L-form was 3% tryptic soy broth (Difco), 0.2 NaCl, 1% yeast extract, and $SFABSA_{7.5}$. Other media used during the course of this study included Eugon broth (BBL, Cockeysville, Md.), L-form agar (9), PPLO agar (Difco, Detroit, Mich.) enriched with 10% horse serum, mycoplasma agar base (BBL), and mycoplasma enrichment broth (BBL). The following tests were used for comparing reverted isolates and parent organisms: hemolysis on 5% sheep blood agar, catalase, optochin, and bacitracin susceptibilities, growth in *S. faecalis* medium (Difco), and the 20 biochemical test strip (Analytab Products, Inc., Carle Place, N.Y.).

Organisms. The bacterial form *S. faecalis* strain EM (SF-EM) was isolated in our laboratory from a patient with a urinary tract infection. The relatively stable L-form (M-1) was induced from the bacterial form (SF-EM) by the method of Roberts and Wittler (16) with the modification that organisms were grown in Eugon broth without agitation for 18 to 24 h before inoculation onto a gradient plate. Resulting L-form colonies were transferred to penicillin-free $BHIAHS_{10}$. L-forms were maintained by frequent passage on penicillin-free $BHIAHS_{10}$ for 8 to 12 months prior to use in these experiments. The resulting L-forms were relatively stable in the sense that they could be maintained in the L-state in the absence of inducing agent (penicillin) by passage to fresh media every 3 days. Although some confusion regarding definition of the term L-form exists in the literature (2), L-form in the present study refers to cell wall-deficient bacteria displaying fried-egg-type transferable colonies when grown on solid media (4).

The bacterial form *S. faecalis* strain GK as well as the stable L-form (B-9) derived from *S. faecalis* strain GK was made available to us by Harry Gooder (University of North Carolina, School of Medicine, Chapel Hill, N.C.).

Cells. HEK cells were derived from fetus T792, T825, or T828 (all at 7 months of gestation) by modification of the method of Hayflick and Moorehead (13). Cells were removed from penicillin-containing media and incubated in two to ten changes of non-penicillin-containing media before use. The cells were screened for mycoplasma by cultural and electron microscopic methods. Eagle minimal essential medium (Gibco) supplemented with 4 to 10% fetal bovine serum was used routinely for growing and maintaining cell cultures.

Vaccines and animal inoculations. M-1 and B-9 L-forms were washed from $BHIAHS_{10}$ and $SFABSA_{7.5}$ plates, respectively, with BHIB 72 h after initiation of the culture. The optical density of the M-1 L-form inoculum was 0.09 for the first set of injections and 0.31 for the second set at a wavelength of 560 nm on a

Bausch & Lomb Spectronic 20. Live and heat-killed vaccines were used. The optical density of the B-9 inoculum for the single set of injections was 1.0 at a wavelength of 560 nm. All B-9 inocula were injected live.

Classical parent bacteria M-1 were harvested from BHIB enriched with 10% horse serum 24 h after initiation of the culture and suspended in normal saline. The optical density of the inoculum was 1.0 at a wavelength of 560 nm. The inoculum was then killed by immersion in a 60-C water bath for 90 min.

New Zealand white male rabbits weighing 2 to 3 kg were given two sets of intravenous injections of the M-1 vaccine consisting of three injections per week for 3 weeks and a rest period of 4 weeks. The first two injections were 0.5 ml; all subsequent injections were 1 ml. Animals receiving live inocula were maintained on 300,000 U of penicillin per week to prevent reversion of the L-form to its classical parent bacterium. Immune sera to the B-9 L-form as well as to the bacterial form, SF-EM, were prepared by injecting rabbits intravenously three times per week for 3 weeks. The first two injections consisted of 0.5 ml; all subsequent injections were 1 ml.

All rabbits were bled, and serum was extracted from the blood, pooled, assayed by tube agglutination with the appropriate antigen, and frozen.

Indirect immunofluorescence staining techniques. HEK fibroblasts were stripped from test and control bottles with trypsin and dispensed into Leighton tubes in 1-ml portions. Monolayers appeared on the cover slips within 24 to 48 h. Cover slips were briefly rinsed with minimal essential medium free of bovine serum, fixed in acetone for 3 min, rinsed three times in either phosphate-buffered saline or FA buffer (5 min each), overlaid with rabbit immune serum (30 min), rinsed as before, overlaid with fluorescein isothiocyanate-conjugated (FITC) anti-rabbit gamma globulin (30 min) (Cappel Laboratories, Inc., Dowington, Pa.), rinsed as before, and inverted on slides in buffered albumin glycerine mounting fluid.

In certain experiments a 1:20 dilution of rabbit immune serum was used with a 1:60 dilution of FITC anti-rabbit gamma globulin, whereas in others undiluted rabbit immune serum was used with a 1:20 dilution of FITC antirabbit gamma globulin. Rabbit immune serum was absorbed for 30 min with HEK monolayers before use. All procedures were carried out at room temperature.

Samples were examined on a Leitz fluorescence microscope with a BG-12 exciter filter and a blue-absorbing barrier filter, using an Osram HBO 200-W mercury vapor bulb as the light source.

Electron microscopy. L-form samples for electron microscopic evaluation were either scraped from the agar surface or cut into 0.12-in (about 0.3-cm) agar blocks with intact L-form colonies on their surface, fixed for 2 to 4 h in 5% glutaraldehyde in 0.2 M collidine buffer with 10% sucrose added as an osmotic stabilizer, rinsed overnight in 0.2 M collidine buffer (pH 7.4 to 7.6) also containing 10% sucrose, and postfixed for 1 h in 1% osmium tetroxide in collidine. HEK fibroblasts were prepared in a similar manner with the following modifications. Cells were rinsed briefly in phosphate-buffered saline, fixed for 1 h in 5% glutaraldehyde in collidine buffer, scraped from the glass, sedimented by centrifugation for 5 to 10 min at 2,000 to 3,000 rpm, rinsed overnight in 0.2 M collidine (pH 7.4 to 7.6), and postfixed for 1 h in 1% osmium tetroxide in collidine. All the above procedures were carried out at 4 C. Samples were dehydrated through ascending concentrations of ethyl alcohol, transferred to propylene oxide, and embedded in Epon 812 epoxy resin (Ernest F. Fullam, Inc.). Sections were cut at 60 to 90 nm on a Sorvall ultramicrotome (MT2 or MT2-b) and mounted on 200-mesh copper grids. The sections were stained with uranyl acetate and lead citrate and examined with a JEM 100B electron microscope.

RESULTS

L-forms. Since its induction from the bacterial form SF-EM, the M-1 relatively stable L-form has displayed the fried-egg morphology typical of L-form growth on solid media (4). Colonies were clear and mucoid with a maximum size of 3.0 mm. In broth the M-1 L-form grew in fluffy balls approximately 1 mm in diameter. Sporadic reversion to classical bacterial forms occurred in specific portions of a low percent of the L-form colonies after 72 h of growth on solid media; other colonies reverted only after 6 to 10 days of incubation or not at all. Continuous subculture at 2- to 4-day intervals onto fresh brain heart infusion agar prevented reversion.

A heterogeneous population of small opaque and of large transparent vesiculated forms was observed when M-1 L-forms were viewed in wet mount by phase-contrast microscopy (Fig. 1). Electron microscopic observations of thin sections of M-1 L-forms confirmed the presence of both dense and transparent forms, the latter of which appeared to organize into vesicular and nuclear poles (Fig. 2). Dense bodies (ranging from 0.06 to 0.7 μm) as well as constricted elongate forms were observed consistently within the vesicles and on occasion were observed clustered loosely in cytoplasmic material.
rial.

The B-9 (stable) L-form exhibited fried-egg morphology on solid medium and diffuse growth in broth. Colonies on solid media were milky and attained a maximum size of 5.0 mm. Reversion to the bacterial form was not observed. Phase-contrast microscopy revealed dense forms of varying size. Electron microscopy confirmed the presence of large dense forms (averaging 2 μm in diameter), some of which contained empty vesicles, as well as numerous dense free-floating bodies (averaging 0.2 μm) (Fig. 3).

Both the B-9 and the M-1 forms (after 72 h of

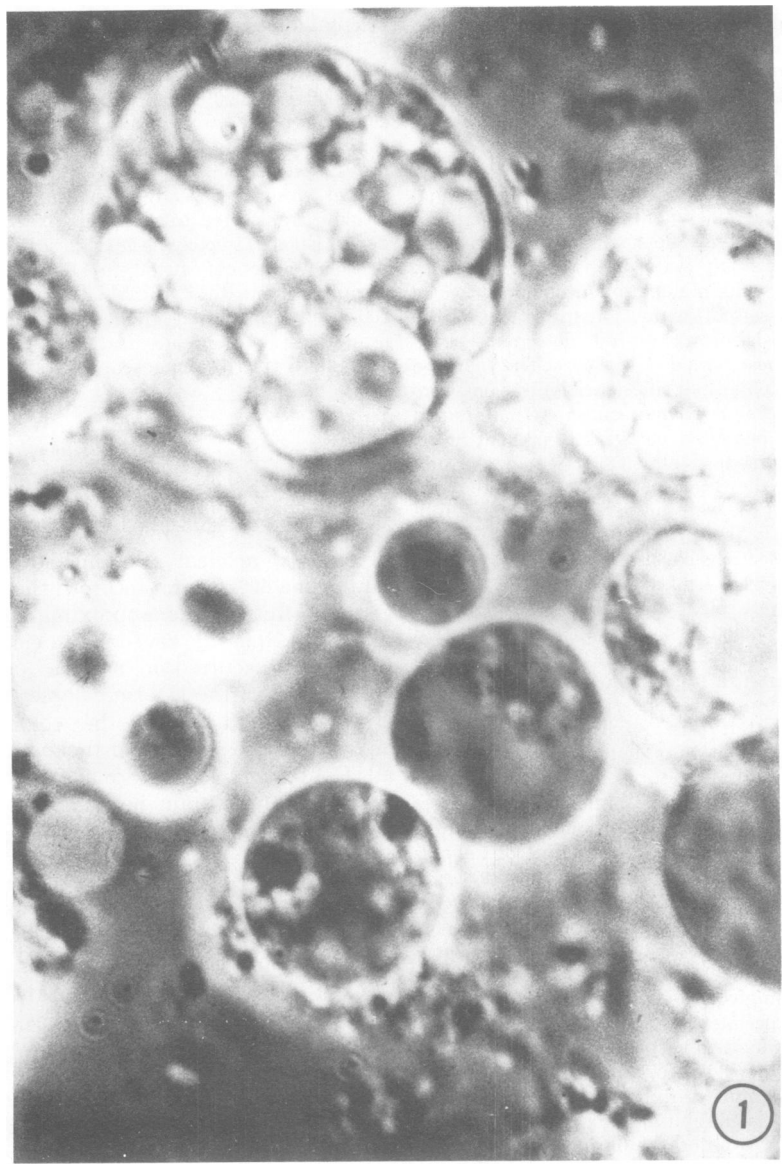


FIG. 1. *Phase photomicrograph of S. faecalis L-forms (72 h). Note extreme size range of organisms and presence of phase dense as well as vesiculated translucent forms. Some bulging vesicles appeared to contain dense material. ×6,000.*

growth on solid media) were diluted in broth and passed through 0.22-μm filters (Millipore Corp., Bedford, Mass.), resulting in heavy L-form growth in the B-9 filtrate and a complete absence of growth in the M-1 filtrate. Incubation in minimal essential medium supplemented with 10% fetal bovine serum in the absence of HEK cells resulted in a rapid decline in viable B-9 and M-1 L-form units.

Bacteriology of L-form-infected HEK cells. The major portion of this study concerning infection of HEK cells with *S. faecalis* L-forms consisted of 12 separate experiments ranging from 14 to 72 days in duration. The bacteriological findings of six experiments in which HEK cells were infected with the M-1 L-form are reported in Table 1. Ability to culture organisms after 48 h corresponded to an increase in

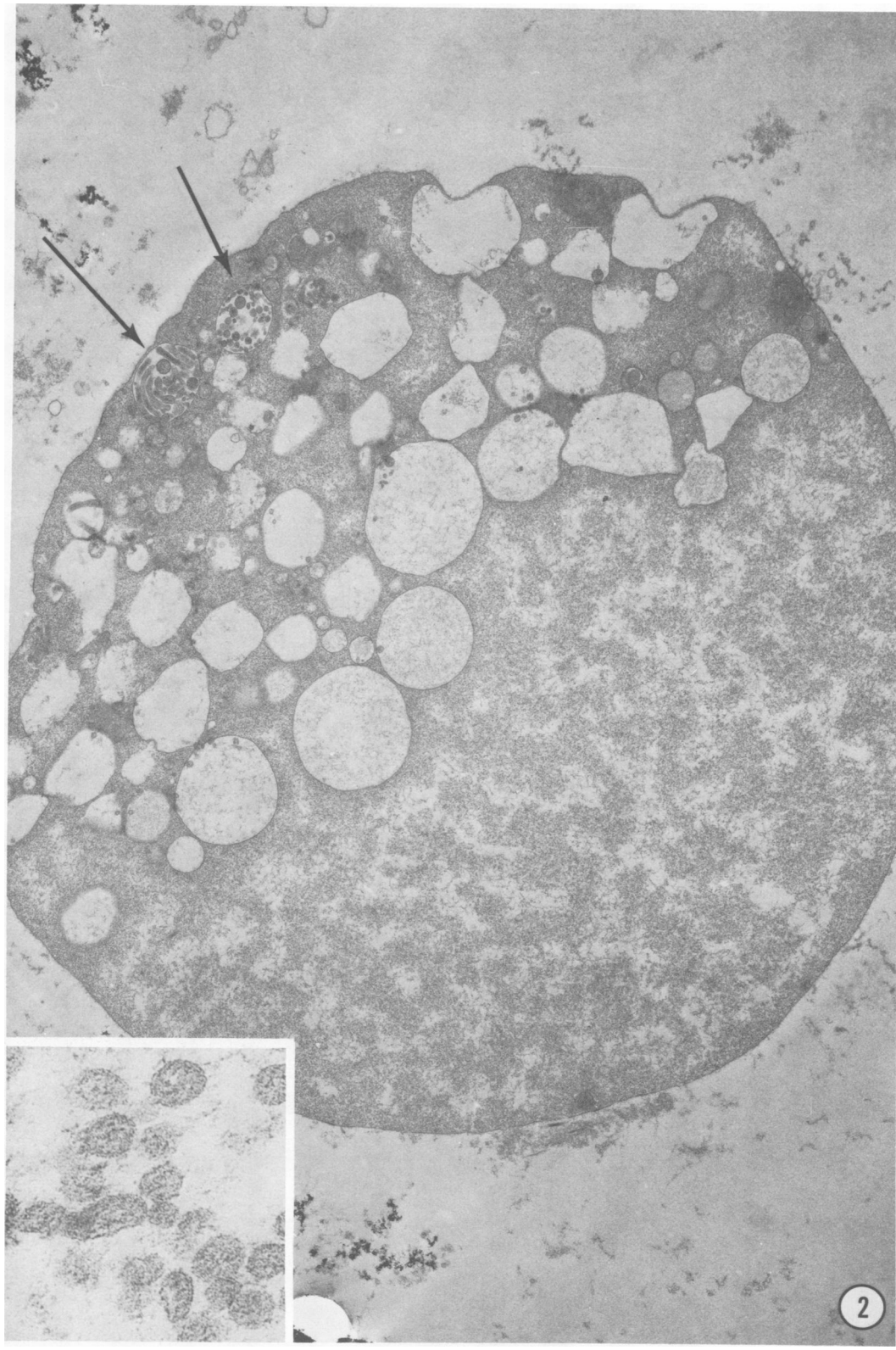


FIG. 2. *Electron micrograph of mature vesiculated M-1 S. faecalis L-form (72 h old, grown in broth). Note aggregation of vesicles at one pole of the L-form with accumulation of dense bodies in certain of the vesicles (arrows). ×11,250. Insert shows detail of dense bodies including trilaminar membrane. ×80,000.*

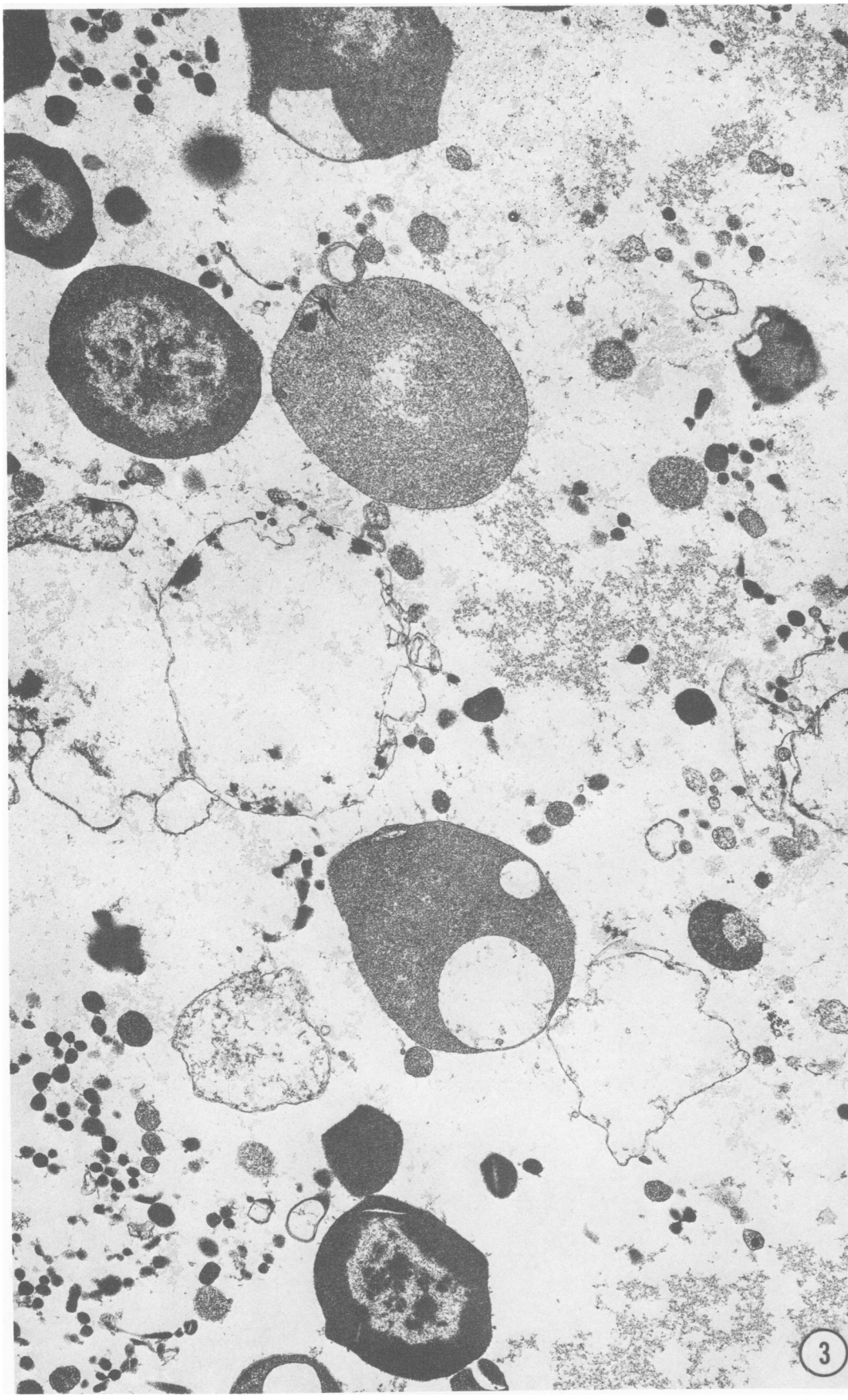


FIG. 3. *Electron micrograph of dense granular B-9 L-forms (grown in broth, 24 h old) along with small free-floating dense bodies.*

TABLE 1. *Cultural results of infection of HEK monolayers with a relatively stable S. faecalis L-form, M-1*

Sampling period (days)	Inoculum (viable L-form units/HEK cells)[a]																		
	Expt 1 ($9 \times 10^4/10^6$)			Expt 2 (5.9×10^4 1.7×10^6)			Expt 3 ($1.1 \times 10^6/10^6$)			Expt 4 ($8 \times 10^5/$ 1.8×10^6)			Expt 5 ($1.5 \times 10^7/$ 1.5×10^6)			Expt 6 ($2.0 \times 10^7/10^6$)		
	Culture	Reversions	Split[b]	Culture	Reversions	Split	Culture	Reversions	Split	Culture	Reversions	Split	Culture	Reversions	Split	Culture	Reversions	Split
0.5	N		0															
1				P	R	0	P	R	0	P		0	P		0	P		0
	N																	
2	N		0	P		0	P		0	P		0						
3				N		0	P		0	P		0	P		0	P		0
4	N	R	1							P		0						
5							P		1	P		0	N	R	1			
6				N		1										N		6
8							P		1									
9										N		1						
13													N	R	1			
14	N		2								R	2						
18				P[c]		3	N		2									
22				N		3												
25	N	R	2			4												
29					R	4	N		3									
30	N	R	3							N						N		2
37										N								
40				N		5							N		4			
49				N		6												
56											R	3						
60							N		5									
63								R	5									
64				N		7												

[a] Abbreviations: N, Negative cultural results; P, positive cultural results; R, reversion.
[b] Number of human kidney cell passages postinfection; 0 indicates no passage.
[c] Cultured day 18; classical growth appeared day 25.

inoculum size to a 1:1 ratio (of viable L-form units to HEK cells), which appeared to be optimal because further increases to ratios of 10:1 and 20:1 resulted in inability to culture L-forms later than 72 h postinfection. L-forms cultured from the cell systems at various time intervals postinfection displayed reversion patterns identical to those of stock L-forms (i.e., portions of certain L-form colonies reverted to classical forms 3 to 10 days after their appearance on solid media). To affirm that both the M-1 and B-9 L-forms could grow in a pour plate, M-1 and B-9 inocula were placed in 4-oz (about 120-ml) tissue culture bottles and overlaid with either $BHIAHS_{10}$ (M-1) or $SFABSA_{7.5}$ (B-9). Heavy growth throughout the agar of both the M-1 and B-9 L-forms was apparent within 24 h.

At certain times during the course of this investigation, often after the cell systems became culturally negative, reversion of the relatively stable L-forms to their parent classical bacterial forms occurred as grossly indicated by turbidity of the tissue culture fluid and destruction of the cell monolayers. The reversions were sporadic, occurring in various experiments at days 4, 5, 14, 25, 29, 30, 56, and 63 postinfection. The remaining test bottles, as well as controls, were culturally negative and grossly normal in appearance. Examination by light microscopy of samples from cell systems at the time of reversion revealed both gram-positive organisms (typical chains of cocci) and gram-variable forms (globase and elongate transitional forms). Electron microscopy confirmed the presence of classical and transitional forms (Fig. 4).

On transfer to blood agar, some of the organisms remained transitional in form, resulting in large mucoid colonies (30%) interspersed with typical *S. faecalis* colonies (70%). Gram stain of

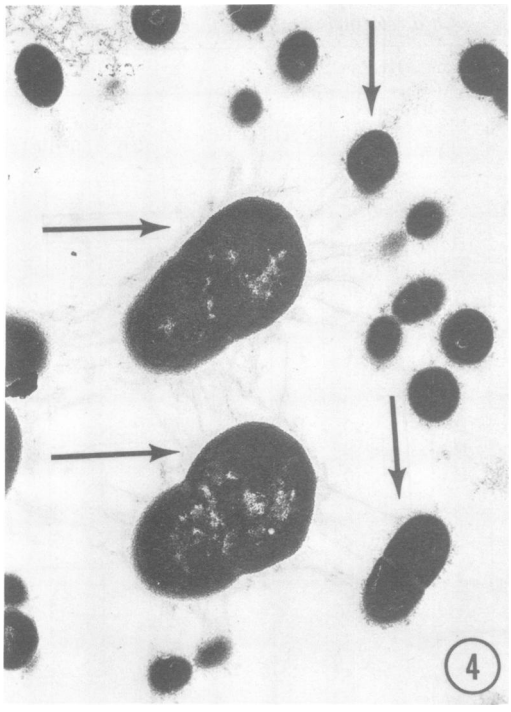


FIG. 4. *Electron micrograph of transitional forms (horizontal arrows) present in tissue culture fluid at reversion 29 days postinfection with the M-1 L-form. Note that transitional forms are approximately three times as large as classical forms (vertical arrows).* ×17,500.

mucoid colonies showed large gram-variable elongate and globose forms, whereas Gram stain of typical colonies showed chains of gram-positive cocci of uniform size. A colony of transitional forms could be reverted to typical bacterial forms by incubation overnight in tryptose broth (Difco) or $BHIBHS_{10}$, but on subculture to blood agar transitional colonies (10%) were once again interspersed with typical colonies (90%). All reverted isolates from the cell systems were Gram + cocci, catalase negative, gamma- to weakly alpha-hemolytic, optochin negative, bacitracin negative, and *S. faecalis* medium positive (definite growth accompanied by color change occurred in *S. faecalis* broth at 48 to 96 h). Additional biochemical reactions of the parent and reverted organisms from cell systems as well as reverted organisms from pure L-form cultures carried on agar were compared (1). In general, the reverted organisms gave slow and somewhat variable reactions, especially with the sugars mannitol and sorbitol. Positive glucose, amygdalin, arabinose, and nitrate reactions were obtained for parent and reverted organisms. Alternatively, all organisms that had at one time been in the L-state gave negative arginine dihydrolase reactions, whereas the parent organisms gave positive reactions. Bacterial tube agglutinations of cell system revertants by immune sera to the parent bacterial form, SF-EM, indicated a further similarity between parent and reverted bacteria.

Bacteriological findings of the remaining six experiments, in which HEK cells were infected with the B-9 L-form, are reported in Table 2. A 1:1 ratio of viable L-form units to HEK cells resulted in positive cultural findings for approximately 5 days postinfection. In contrast to the M-1 inoculum, increased ratios of B-9 inocula to HEK cells (2:1, 3:1, 20:1, 40:1) resulted in routine culture of L-forms from cell systems for 30 days postinfection. In one experiment, L-forms were cultured as late as 73 days postinfection. In another experiment, discontinued after 14 days, one infected bottle inadvertently remained in the incubator. The sample was discovered and cultured 43 days later (57 days postinfection); a heavy growth of L-forms resulted. At no time during the course of this study did the B-9 L-form revert to its parent bacterial form. Of further bacteriological interest is the fact that throughout all of the cell infection experiments, the B-9 L-form, when cultured, grew out more readily and in greater numbers than did the M-1 L-form.

HEK cells. Although there was no evidence of damage to cell monolayers as a result of infection with the M-1 L-form, growth of infected monolayers appeared to be somewhat slower than that of control monolayers during the first 72 h postinfection. In contrast, infection with the B-9 L-form resulted in sloughing of 30 to 50% of the cell monolayer. The remaining cells grew out rapidly, however, after a change of fluid medium (feeding at 48 to 72 h postinfection), and by 5 days postinfection no difference between infected and control monolayers was evident. The particular method of splitting HEK cells utilized in the foregoing experiments resulted in loss of approximately 40% of the cells with each transfer, resulting in constant removal of originally infected cells as the experiment progressed.

Hematoxylin and eosin stains of infected and control monolayers yielded no characteristic and significant histopathological changes unless transitional and classical forms were present. Likewise, no gross abnormality in infected HEK cells as compared with their noninfected controls was noted during electron microscopic examinations of samples from various time periods (Fig. 5). Although several instances of dilated rough endoplasmic reticulum were

TABLE 2. *Cultural reults of infection of HEK monolayers with a stable S. faecalis L-form, B-9*

Sampling period (days)	Inoculum (viable L-form units/HEK cells)[a]											
	Expt 1 ($3 \times 10^7/10^6$)		Expt 2 ($2 \times 10^7/10^6$)		Expt 3 (3×10^6/ 1.8×10^6)		Expt 4 (8.5×10^6/ 1.5×10^6)		Expt 5 ($2 \times 10^6/10^6$)		Expt 6 ($4 \times 10^7/10^6$)	
	Culture	Split[b]	Culture	Split	Culture	Split	Culture	Split	Culture	Split	Culture	Split
1	P	O	P	O	P	O	P	O	P	O	P	O
2	P	O	P	O	P	O	P	O				
3			P	O	P	O			P	O	P	O
4					P	O						
5	P	O	P	1	P	O			P	O	P	O
7	P	1										
8			P	1								
9					P	1	N	1				
12											P	O
13									P	1		
18			P	2			N	2				
30					P	3						
31											P	2
34							N	3				
37					N	3						
39			P[c]	3								
57	P	1			N	4						
62			N	4								
72			P	4								

[a] Abbreviations: N, Negative cultural results; P, positive cultural results.
[b] Number of human kidney cell passages postinfection.
[c] Culture day 39; L-form growth appeared day 48.

noted in both infected and control cells, the phenomenon was consistently more pronounced in infected cells.

Electron microscopy. Electron micrographs suggested that the L-forms (both the M-1 and the B-9) were taken up by the cells at various times over several days after infection (Fig. 6–8). The question arose as to whether L-forms or other debris in the fluid medium could be taken up by the fibroblast. It was resolved by the observation of carbon particles within cytoplasmic vesicles of fibroblasts 24 h after their addition to the system (Fig. 9). These particles, like ingested L-forms, were usually found within membrane-bound cytoplasmic vesicles. Within these vesicles, interpreted as being lysosomal in nature, ingested L-forms underwent degenerative changes early (24 to 48 h) postinfection (Fig. 6–8 and 10–12). The phagocytosis of extracellular L-form fragments has been considered. However, simple phagocytosis of these extracellular fragments is not sufficient to explain the changes in L-form morphology that have been observed, since intracellular L-forms assume a morphology that was never observed in the extracellular medium. Extracellular organisms do not survive in the medium. They were not culturable after 24 to 48 h of residence in the medium, and electron microscopy of such samples revealed only disintegrating organisms. This argues against the random phagocytosis of viable particles from a persisting extracellular pool. A major point is that reversion within cells occurred at 25 days postinfection (see Fig. 20–22). Persisting bodies of varying size were observed in the cells throughout the experimental period. Assessment of viability was based on the reversion of the persisting dense bodies. Reversion did not occur in the tissue culture system (early or late) unless cells were present. This in itself establishes an unquestionable role of cells in the phenomenon of persistence and reversion in this system and justifies, in our view, a working hypothesis of intracellular reversion based on electron microscopic observations of intracellular reversion. Of particular interest was the persistence within fibroblasts of dense bodies, often within vesicles, as early as 2 to 5 days postinfection (Fig. 10–16). Although dense bodies ranging in size from 0.05 to 0.15

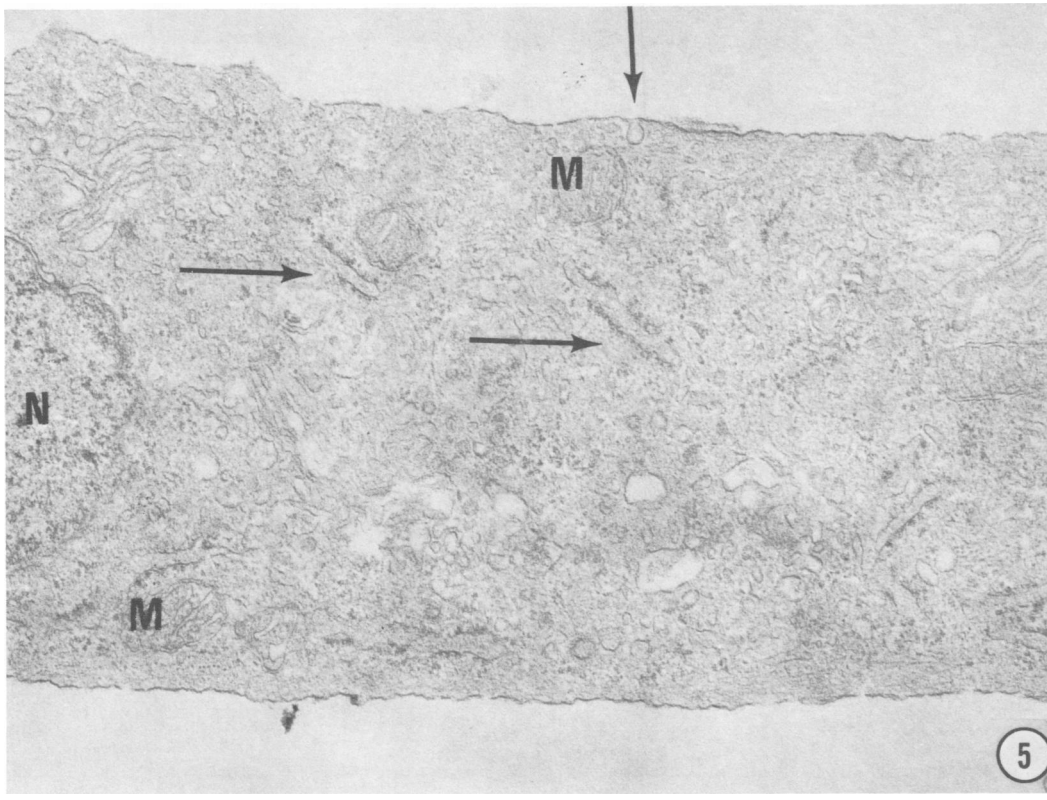


FIG. 5. *Control HEK cell 24 h after initiation of the experiments. Note rough endoplasmic reticulum (horizontal arrows); pinocytosis vesicle (vertical arrow); mitochondria (M); and nucleus (N). The fine structure of infected cells did not differ appreciably from that of control cells.* ×*37,500.*

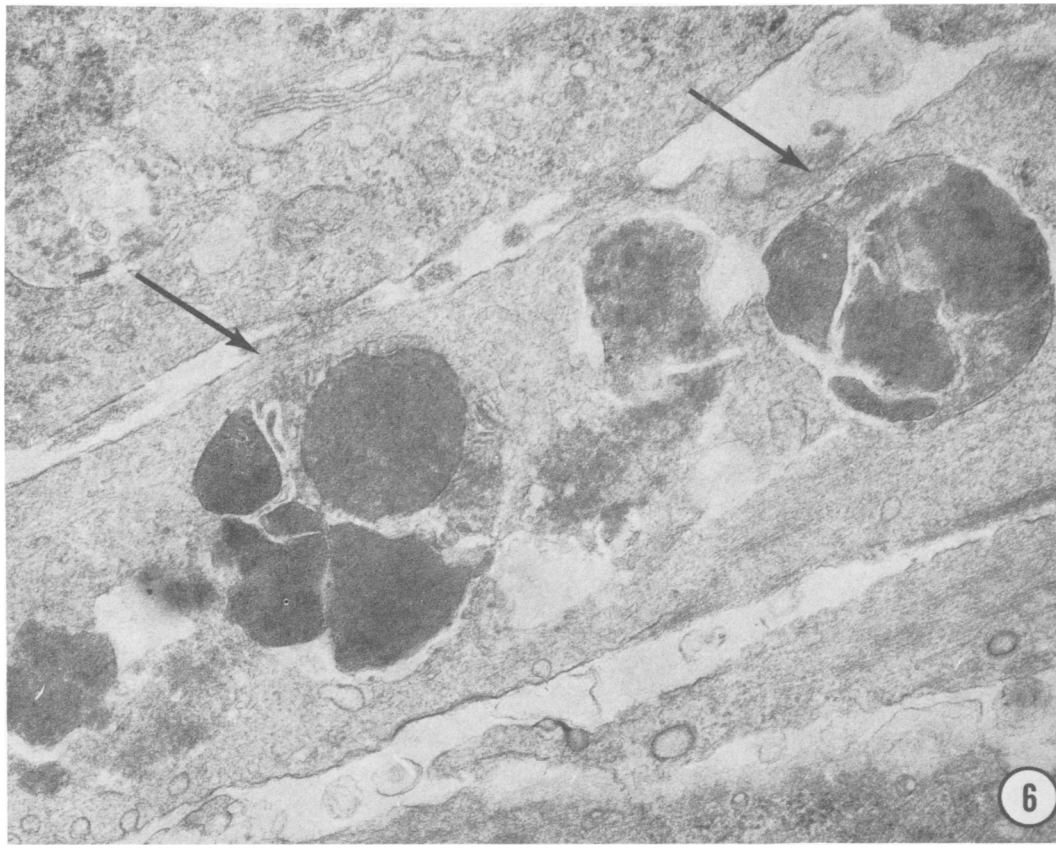


FIG. 6. *Several large M-1 L-forms with dense inclusions (arrows) within HEK fibroblasts 24 h postinfection.* ×*20,625.*

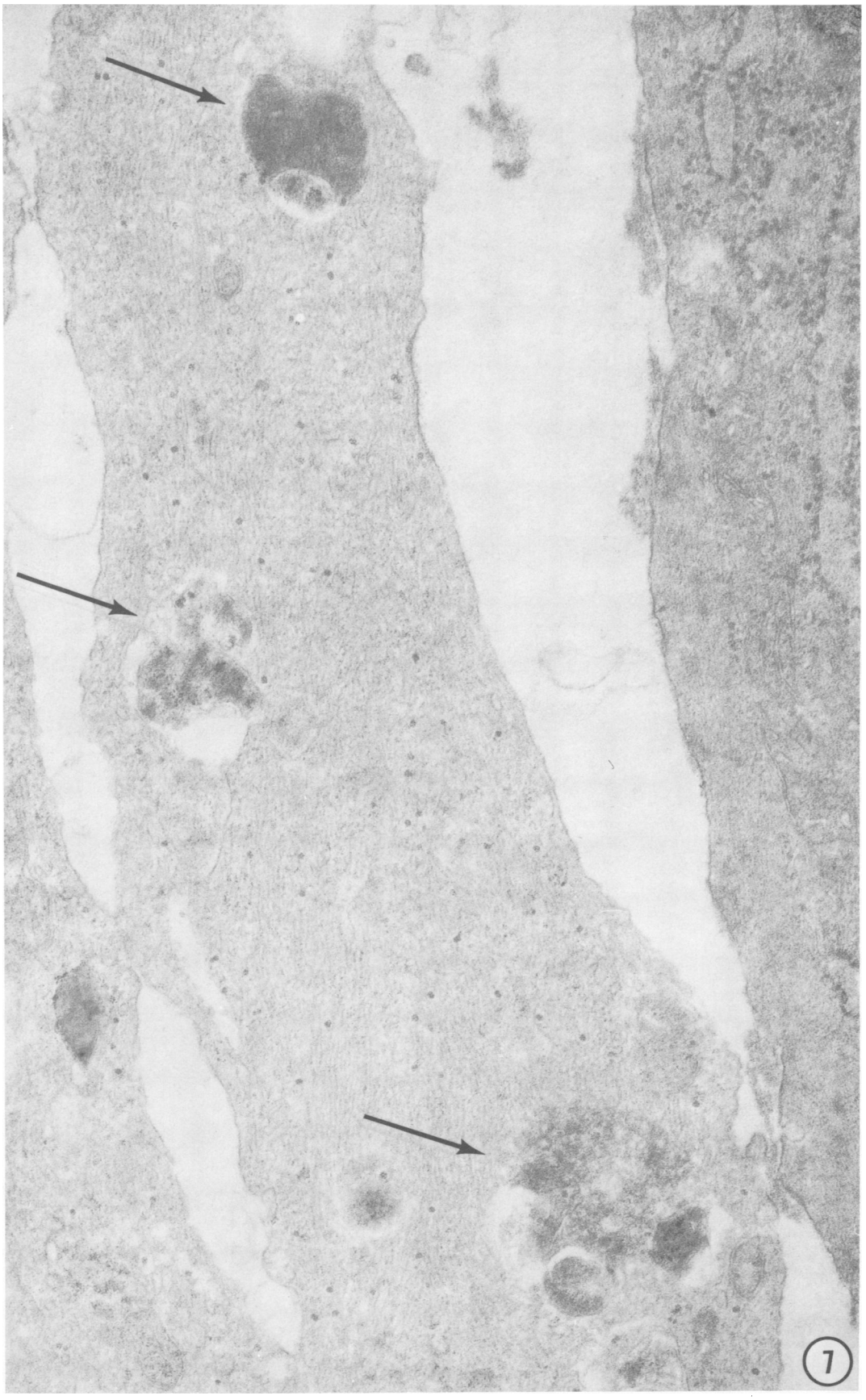


FIG. 7. *Several B-9 L-forms in various stages of degradation within HEK cell 24 h postinfection (arrows).* ×*52,500.*

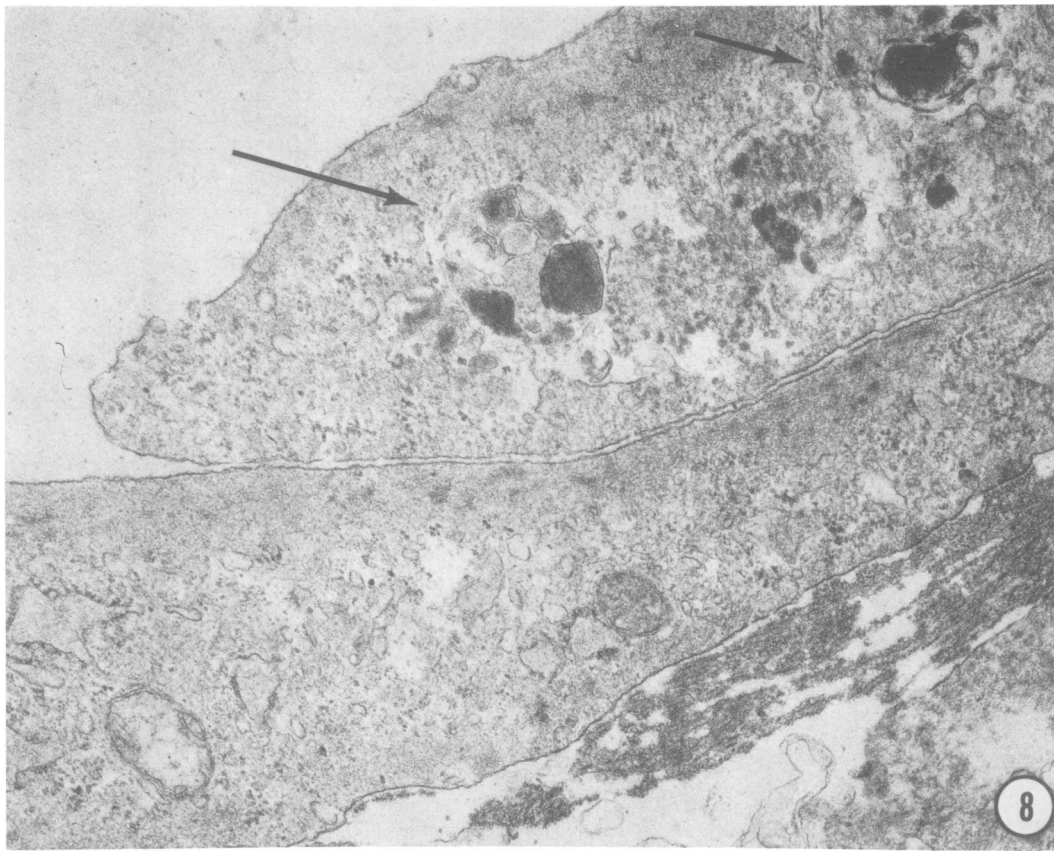


FIG. 8. *Several partially intact M-1 L-forms within HEK cell 48 h postinfection (arrows).* ×*30,000.*

μm were found within cells infected with either the M-1 or the B-9 L-form, dense bodies of 0.3 μm were found only in B-9-infected cells (Fig. 13 and 14). At this time, L-forms could not be cultured from the M-1-infected cells. By 8 and 14 days postinfection, a large dense type of L-form, in addition to small dense bodies, was observed within both M-1- and B-9-infected cells (Fig. 17 and 18). Numerous small dense bodies were found in B-9-infected cells as late as 17 and 42 days postinfection (Fig. 19). In the case of M-1-infected cells, L-forms as well as reverting forms and classical bacteria were found in HEK cells at the time of reversion (25 days postinfection) (Fig. 20–22). The fine structure of these reverting forms bears close resemblance to that of L-forms reverting in pure culture.

Antibody labeling experiments. Labeling of the L-form within infected HEK cells was attempted by using the indirect fluorescent antibody technique. L-form-infected cells labeled intensely with FITC (Fig. 23 and 24). In an experiment (using a 1:20 dilution of rabbit immune sera and a 1:60 dilution of FITC-conjugated goat anti-rabbit gamma globulin), M-1 infected cells were examined 1, 3, and 14 days postinfection and B-9-infected cells were examined 1 and 8 days postinfection. Even though the above cells were diluted by two (1:2) splits with trypsin by day 8 and three (1:2) splits by day 14, test cells were found to fluoresce consistently. Noninfected cells treated with immune sera and FITC-conjugated antibody did not fluoresce. In another experiment (using concentrated rabbit immune globulin and a 1:20 dilution of FITC-conjugated goat anti-rabbit gamma globulin) cells infected with either the M-1 or the B-9 L-form as well as controls were examined 17 and 21 days postinfection; similar results were obtained. In general, globules or granules appeared to fluoresce in M-1-infected cells, whereas smaller pinpoint areas fluoresced in B-9-infected cells.

DISCUSSION

Since 1958, L-forms have been suspected of being vehicles of persistence and subsequent relapse in infectious diseases. In this study the

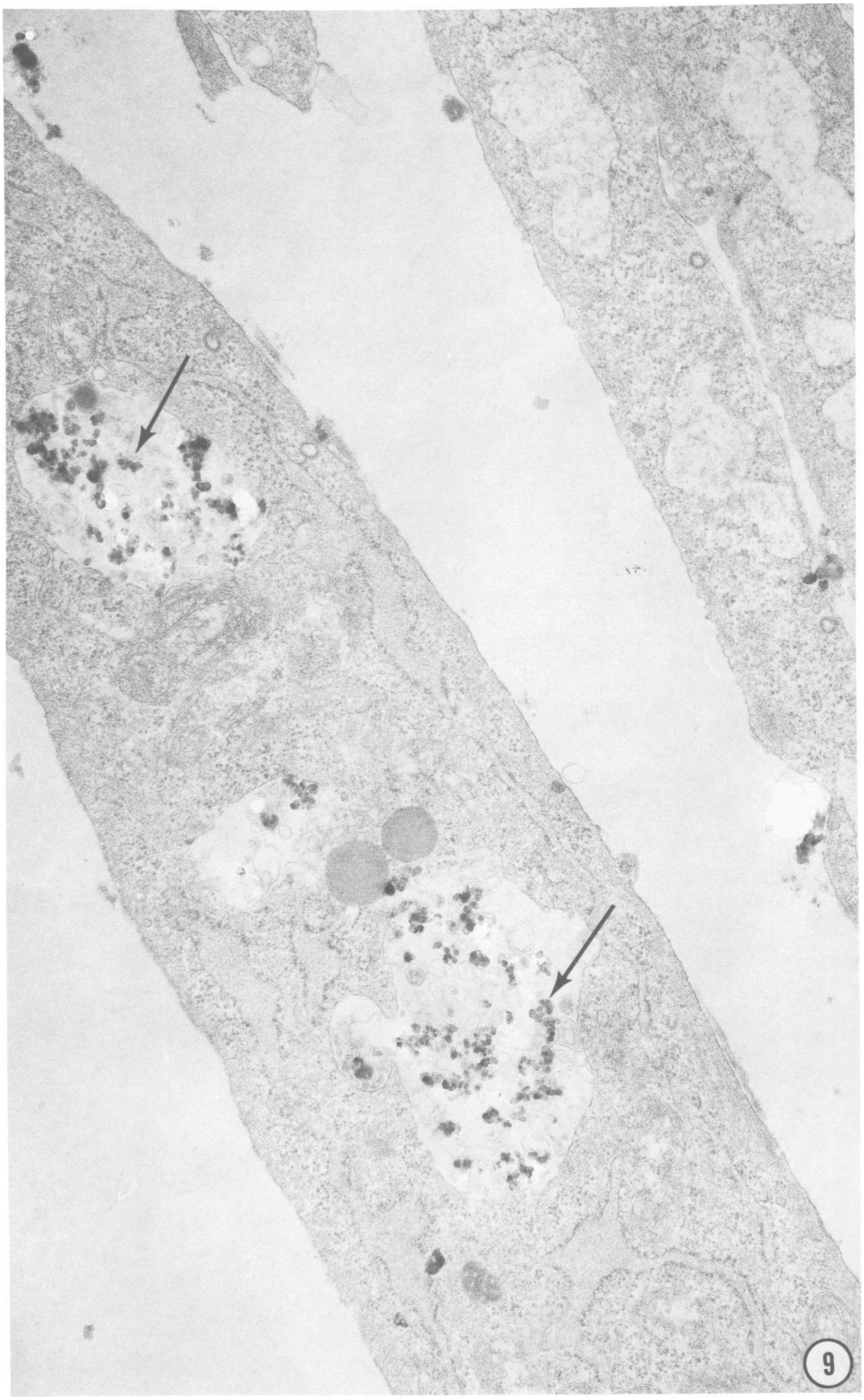


FIG. 9. *Numerous carbon particles sequestered within membrane-bound vesicles of HEK cells 24 h after addition of carbon to medium (arrows).* ×*26,250.*

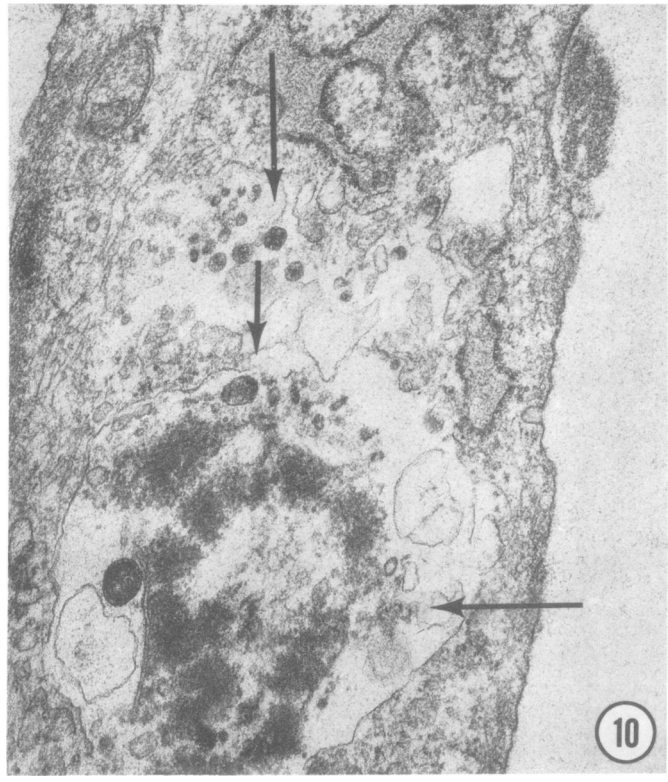


FIG. 10. *Large disintegrating L-form within HEK cell 48 h postinfection with the M-1 L-form. Note dense flocculant material, characteristic degradation product of large membranous L-form bodies (horizontal arrow). Of particular interest are persisting dense round bodies of various sizes (vertical arrows).* ×*28,200.*

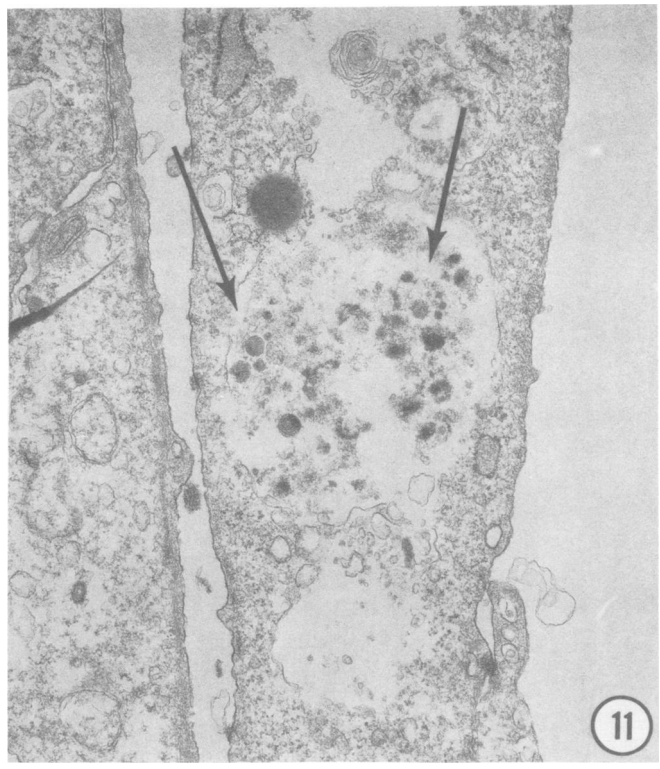


FIG. 11. *Complete breakdown of large L-form within HEK cell 48 h postinfection with M-1 L-form. Note persistence of dense granular bodies within vesicle (arrows).* ×*15,000.*

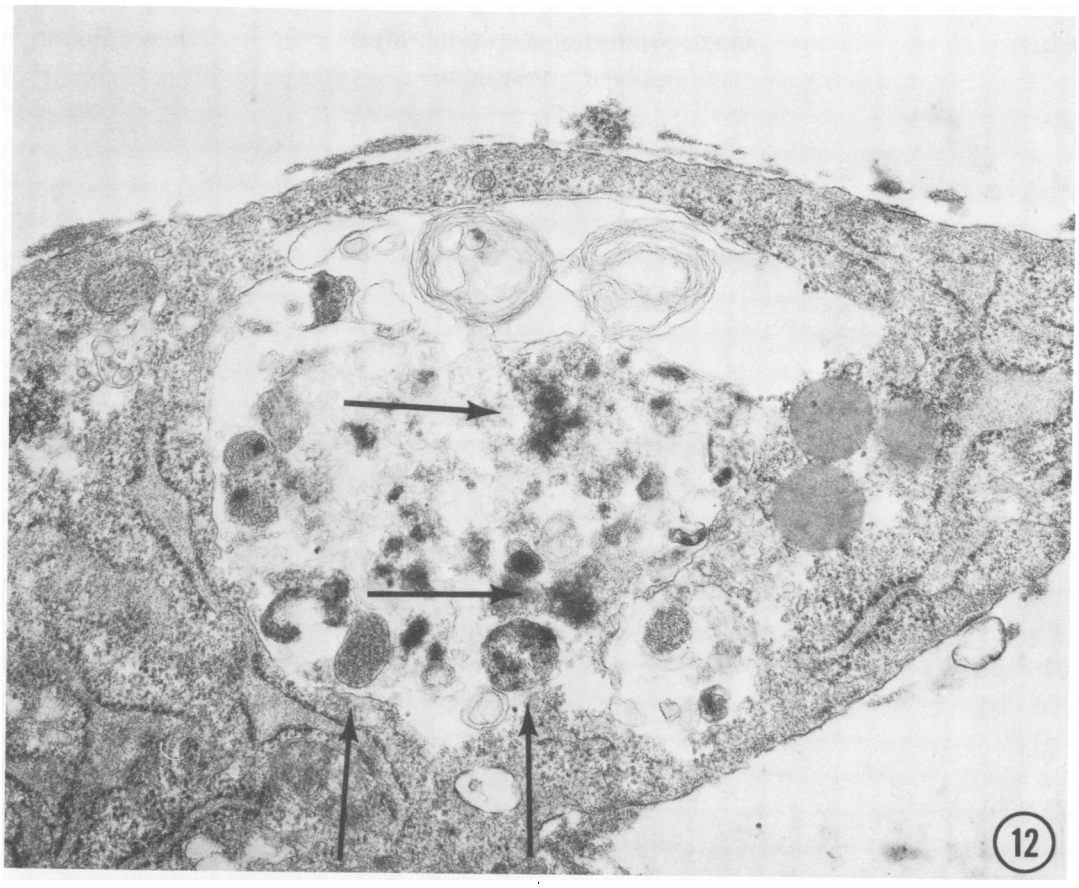


FIG. 12. *Degradation of large L-form with dense amorphous material (horizontal arrows) characteristic of aging and death of membranous L-form body. Note large dense persisting bodies (vertical arrows) within HEK cell 48 h postinfection with the B-9 L-form.* ×24,375.

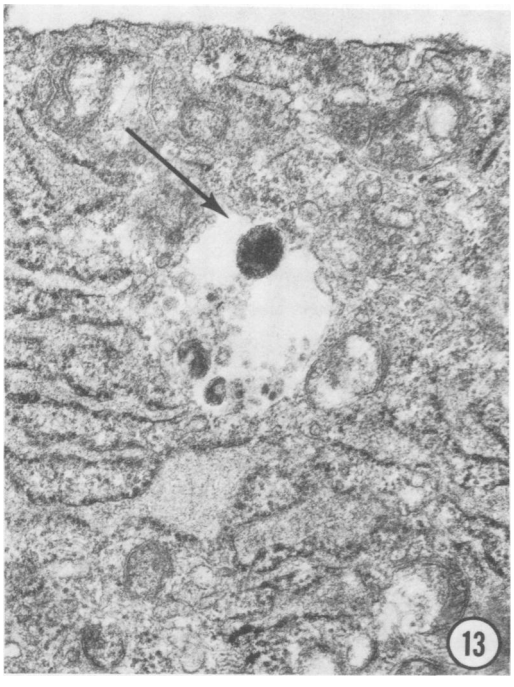


FIG. 13. *Dense persisting bodies in vesicle within HEK cell 3 days postinfection with B-9 L-form. Note larger dense body of 0.38 μm (arrow).* ×32,500.

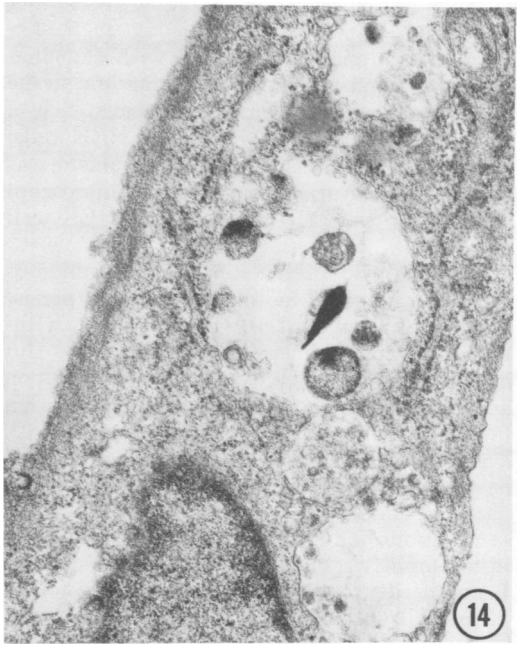


FIG. 14. *Several vesicles containing dense persisting bodies (0.05–0.35 μm) within HEK cell 3 days postinfection with B-9 L-form.* ×24,375.

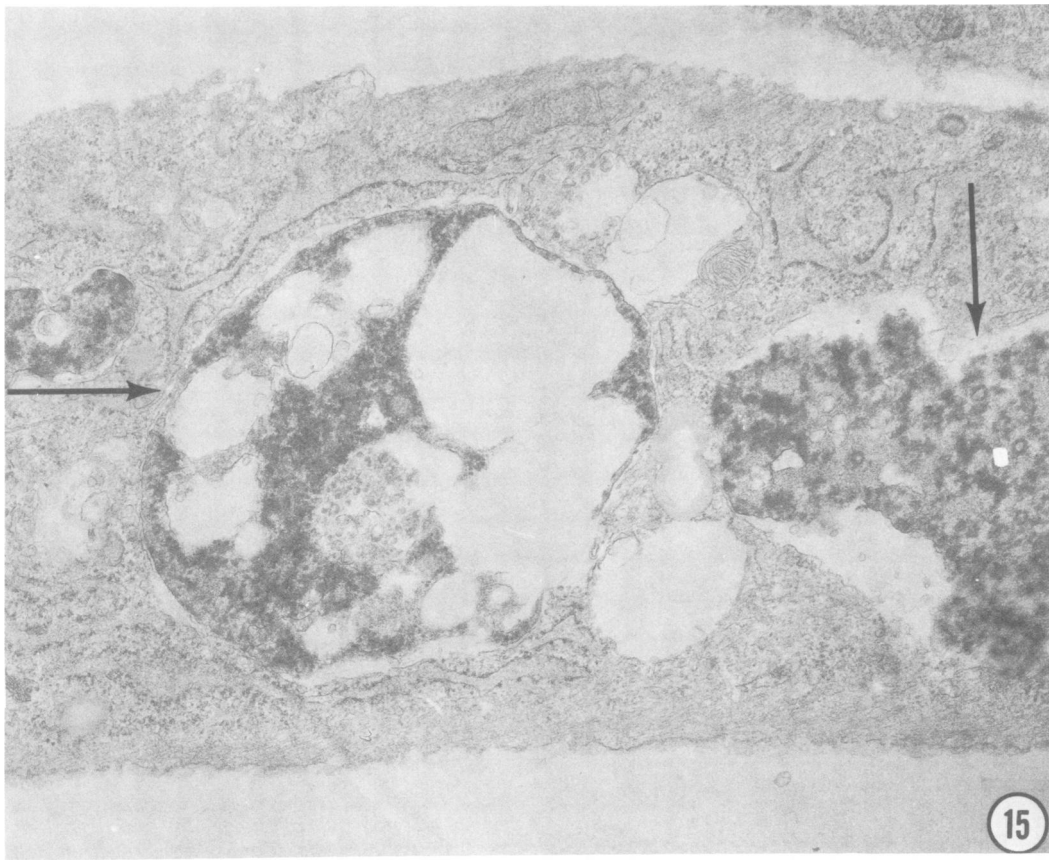


FIG. 15. *Degradation of large vesiculated L-form within HEK cell 5 days postinfection with the M-1 L-form (horizontal arrow). Later stage in degradation of similar L-form (vertical arrow).* ×22,500.

L-form was definitively linked with the phenomena of persistence and reversion in a tissue culture system. After prolonged persistence of the relatively stable L-form in the HEK cells, reversion to the classical parent organism was documented culturally and electron microscopically. This was accompanied by HEK cell death.

It is of considerable interest that numerous large L-forms could be taken up by and persist in some form within the HEK fibroblast without irrepairable damage to the fibroblast. Our finding of the absence of deleterious effects on the cell as a result of long-term infection with a stable L-form per se is in agreement with Schmitt-Slomska et al. (17). Of equal interest is the fact that the L-form could undergo such a dramatic change in morphology (from a large membranous body to small dense particles) and yet apparently retain viability. The persistence of small dense bodies (elementary bodies) within cells after infection with L-forms has been reported previously by Hatten and Sulkin (12) and Schmitt-Slomska et al. (17). Survival of the L-form over long periods of time in this system is particularly fascinating in view of the fact that both the M-1 and the B-9 L-forms survived only 24 to 48 h in cell nutrient media in the absence of HEK cells (based on electron microscopic visualization of dead forms and inability to culture any organisms). One might assume in this case that the HEK cells were providing some mode of protection, such as a privileged site for the L-forms.

Differences in culturability of the stable and relatively stable L-form from infected HEK cells at various time intervals postinfection may reflect the basic morphological differences observed in the M-1 and B-9 L-forms, namely, the prevalence of dense opaque forms and numerous free-floating dense bodies in B-9 cultures in contrast to a preponderance of vesiculated forms containing dense bodies within the vesicles in M-1 cultures.

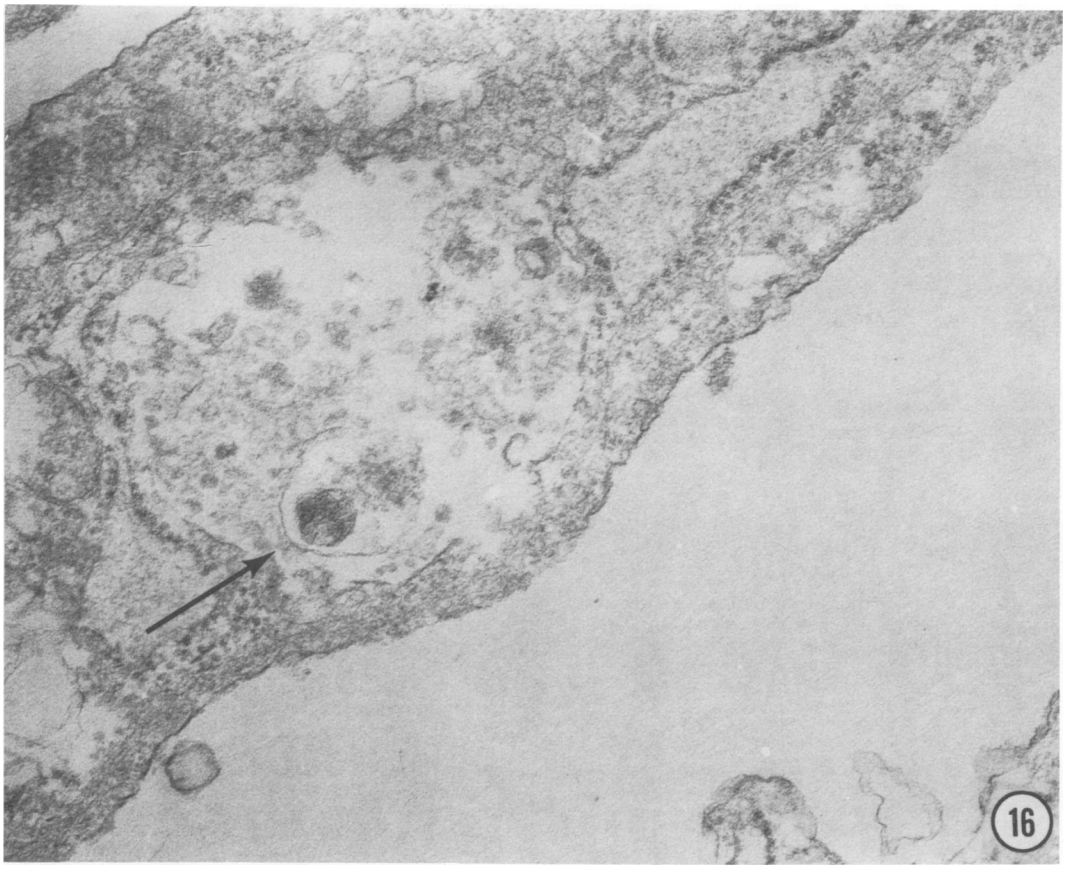


FIG. 16. *Degradation of large vesiculated L-form within HEK cell 5 days postinfection with the M-1 L-form Note persisting dense granular body (arrow).* ×45,000.

Growth of the B-9 L-form after passage through a 0.22-μm filter is not inconsistent with the finding of Dienes and Madoff (5), who determined that the smallest L-form unit capable of reproduction was 0.2 μm, or Coussons and Cole (3), who calculated that a complete bacterial genome and a few ribosomes with some allowance for hydration could be contained within a particle of 0.24 μm. This information allows one to speculate that L-form units of a size corresponding to that of persisting dense bodies (elementary bodies) found within the B-9-infected cells are capable of reproduction and growth.

Two possible explanations for failure of growth of the M-1 L-form after passage through a 0.22-μm filter are that the dense bodies were trapped on the surface of the filter or that the small bodies were too immature to reproduce. The latter explanation seems more plausible in view of the fact that loss of ability to culture L-forms from M-1-infected cells regardless of inoculum size corresponded to disintegration of the membranous L-form body and persistence of dense bodies of 0.05 to 0.2 μm (elementary bodies). Viability of these persisting forms was attested to by the sporadic reversions to classical *S. faecalis* bacteria that occurred in M-1-infected cell systems after various latent periods. In addition, the large size range of particles (0.05 to 0.7 μm) occurring within vesicles of mature L-forms growing in pure culture is consistent with Weibull's speculation that smaller L-bodies inside or connected with larger L-forms may grow and divide (19). It might therefore be speculated that dense bodies can grow into immature, undifferentiated L-forms capable of reproduction only within a protected environment such as that found within the vesicle of the mature L-form, or perhaps within HEK cells. Dense forms were observed by day 14 in M-1-infected HEK cells. It is not illogical

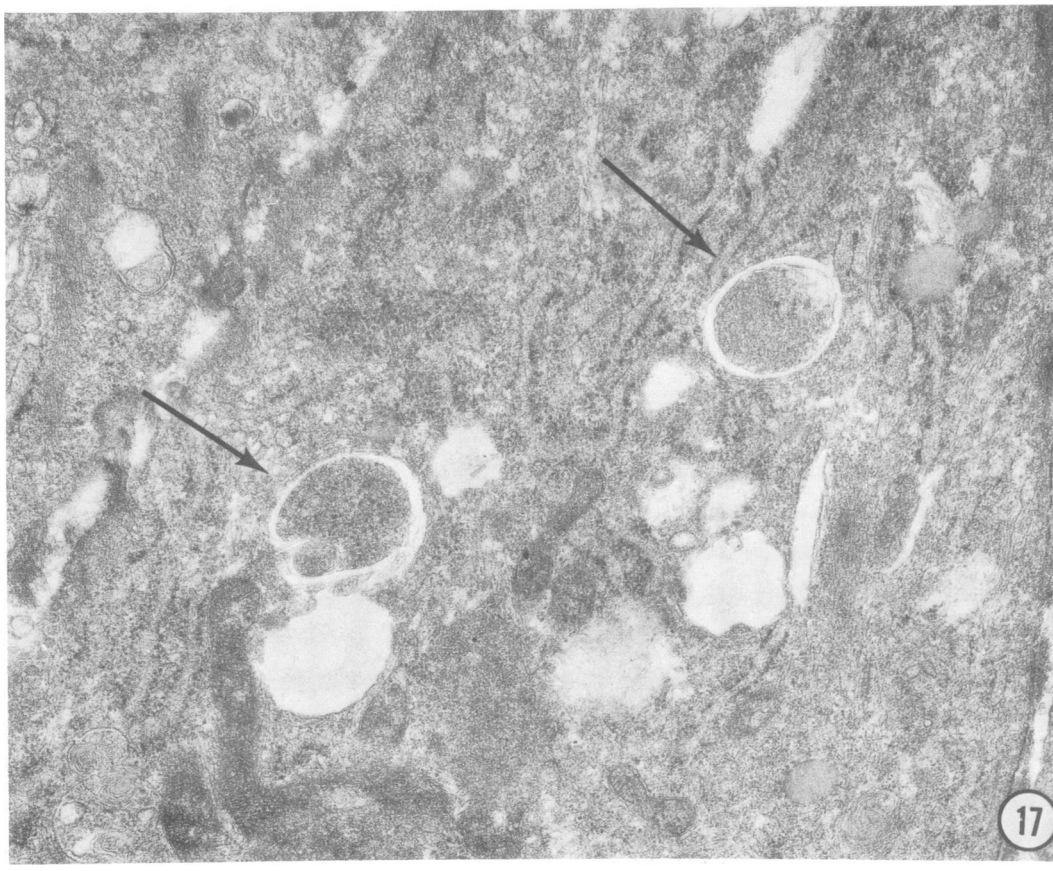


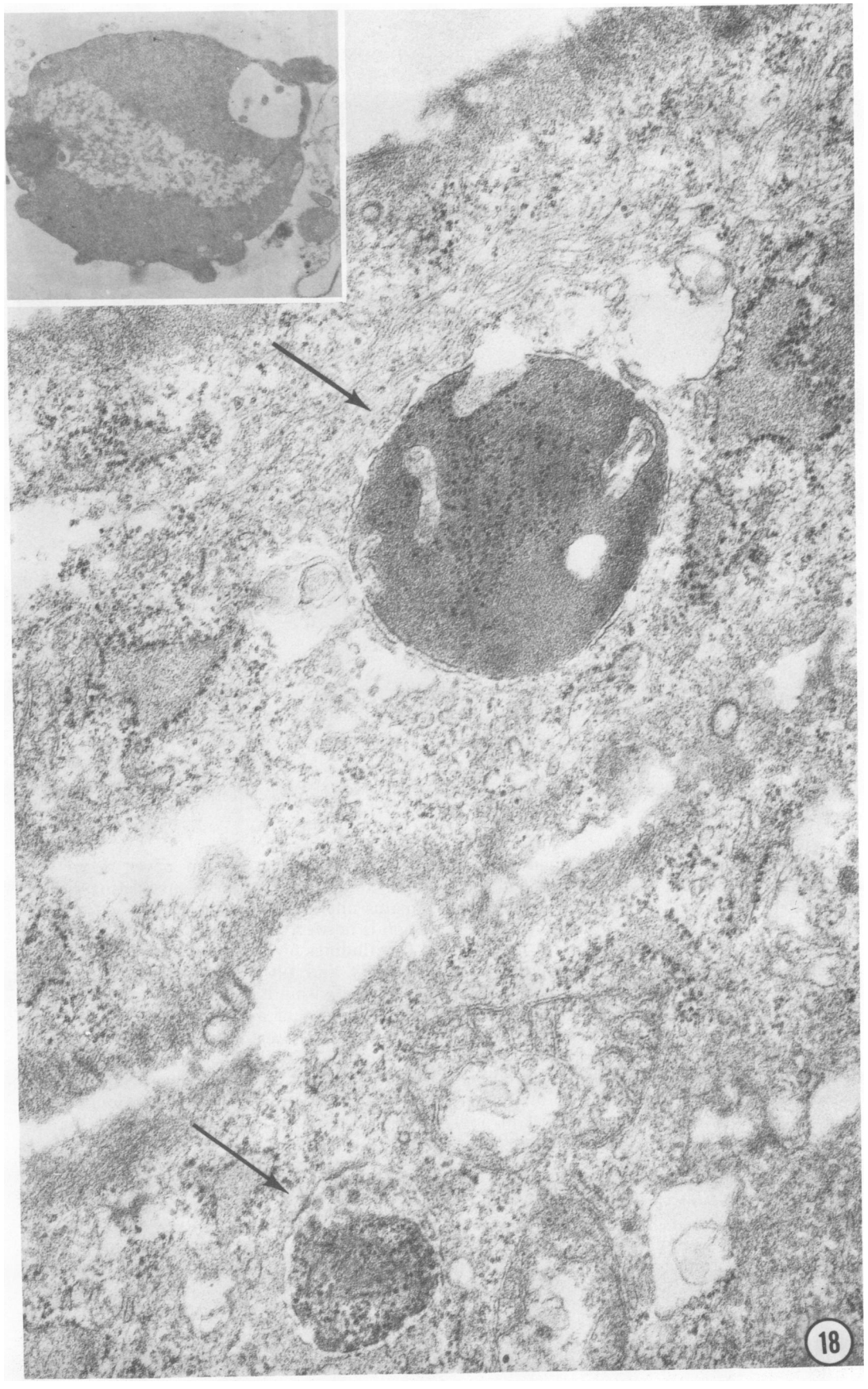
FIG. 17. *Dense granular L-forms within HEK cell 8 days postinfection with the B-9 L-form (arrows).* ×*20,000.*

to assume that these immature, undifferentiated forms, when subjected to an environment unfavorable for growth of the typical membranous body of the mature L-form, could revert to a more advantageous life form, such as an ordinary bacterial form.

Organisms recovered from the tissue culture systems in which they reverted were truly transitional in nature, apparently vacillating between one form and the other (L-form and bacterial form) as manifested by colony and cellular morphology as well as by slow and often variable biochemical reactions. The transitional nature of these organisms may preclude identification by currently established clinical laboratory procedures. A tentative identification of the organism might be gained from biochemical tests, but confirmation may depend on assessment of antigenic properties and genetic parameters.

Worthy of note were the similarities and dissimilarities of results of this study and that of Schmitt-Slomska et al. on group A streptococcus (17). Schmitt-Slomska et al. used a stable L-form and witnessed no reversions. They did, however, lose ability to culture the L-form between 10 and 60 days postinfection, after which the L-form could again be cultured. Between 10 and 30 days, these authors claim to have isolated atypical colonies that could not be subcultured to artificial culture media. No comment is made regarding the 30- to 60-day period. A low initial inoculum size may have been the problem (based on our results). Data on the number of viable units per cell in their study would further elucidate the problem of

FIG. 18. *Dense granular L-forms within HEK cell 14 days postinfection with the M-1 L-form (arrows). Note dense particles associated with L-forms.* ×*35,375. Insert shows for comparison a dense granular M-1 L-form from a 6-day-old pure culture of L-form colony growing on brain heart infusion agar.* ×*13,750.*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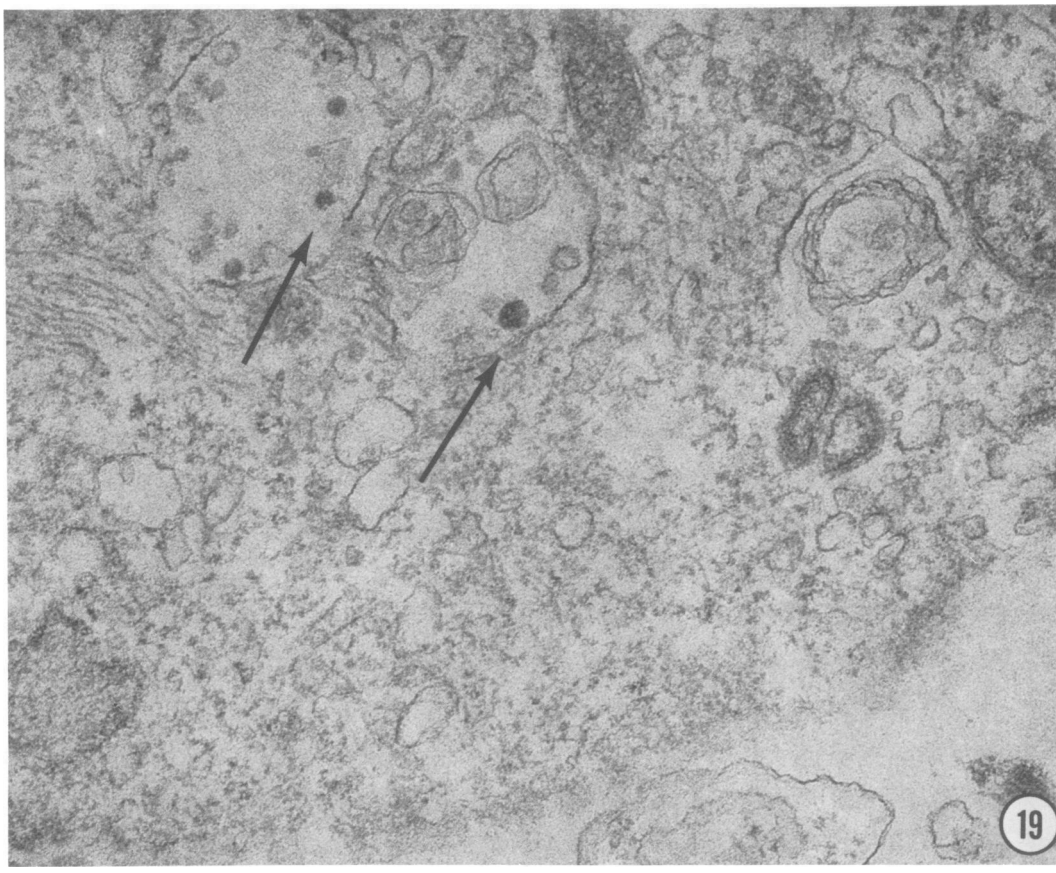


FIG. 19. *Dense persisting bodies in vesicles within HEK cell 17 days postinfection with the B-9 L-form (arrows).* ×*60,000.*

culturability of L-forms from infected cells. In our study, the stable L-forms were cultured throughout the duration of the experiment when high ratios of inoculum to HEK cells were used, whereas the relatively stable L-form became nonculturable after 5 days regardless of inoculum size. We have demonstrated the presence of persisting forms within cells by electron microscopy as well as by fluorescence microscopy during that period when culture was not possible. Schmitt-Slomska et al. suggested persistence during this period based on fluorescence microscopy. Although Trung et al. (18) examined L-form-infected cells by electron microscopy, it is unclear whether the sample was taken during the culturable or nonculturable period. Furthermore, control electron micrographs of uninfected human cells were not shown for purposes of critical comparison. Because the stable L-form appears to coexist with human cells, causing no classical pathology, its role (assuming that it can never regain the ability to revert) in infectious persisting disease remains unclear. Extensive in vivo experimentation is needed to clarify this point.

Our findings for the first time document both culturally and ultrastructurally that L-forms can persist within human cells in a viable state in the absence of antibiotics for long periods of time without apparent harm to the host cell. Furthermore, certain of these persisting agents (relatively stable L-forms) have been observed in the process of reversion after long latent periods, resulting in total destruction of the cell monolayer.

These findings could have broad implications for the entire field of bacterially caused persisting and relapsing infectious diseases. Could the phenomena of persistence and reversion observed with the group D relatively stable streptococcal L-form in a tissue culture system be extrapolated to in vivo situations involving

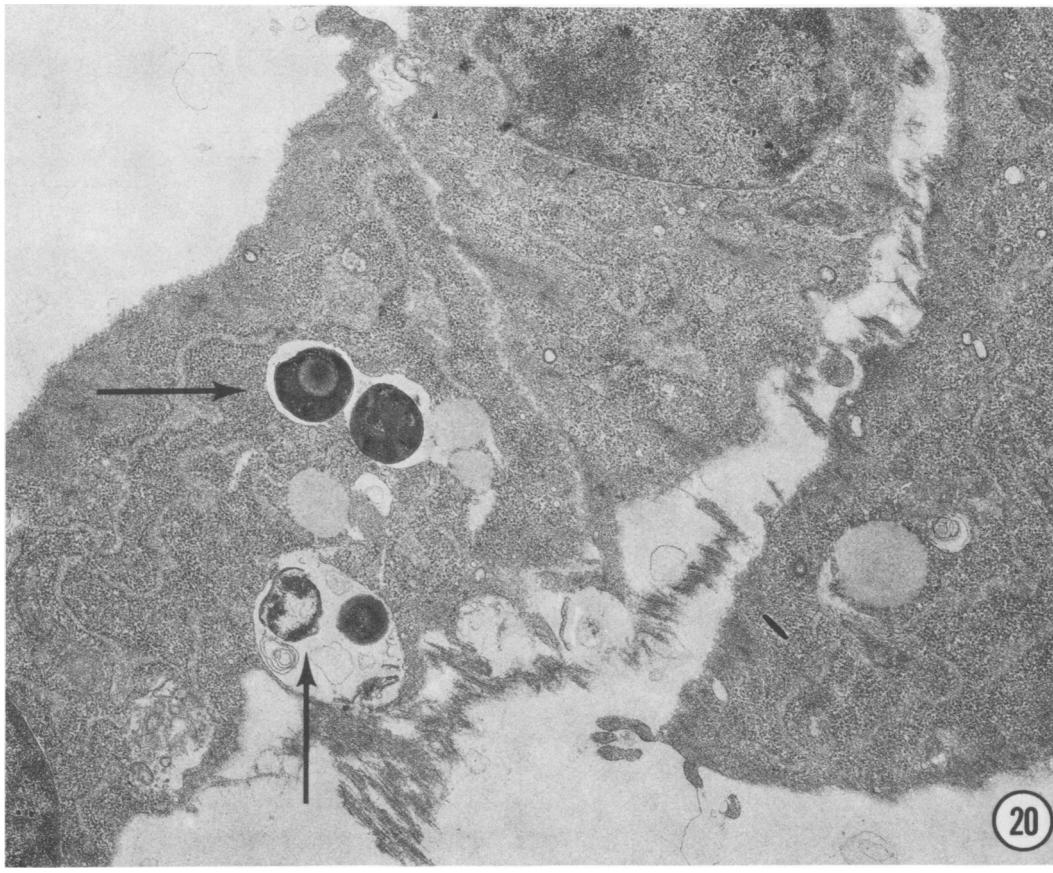


FIG. 20. *Classical S. faecalis bacteria within HEK cell at reversion 25 days postinfection with the M-1 L-form (horizontal arrow). A reverting organism with a partially synthesized cell wall is also seen, which represents the stage intermediate between classical and L-form (vertical arrow). ×15,000.*

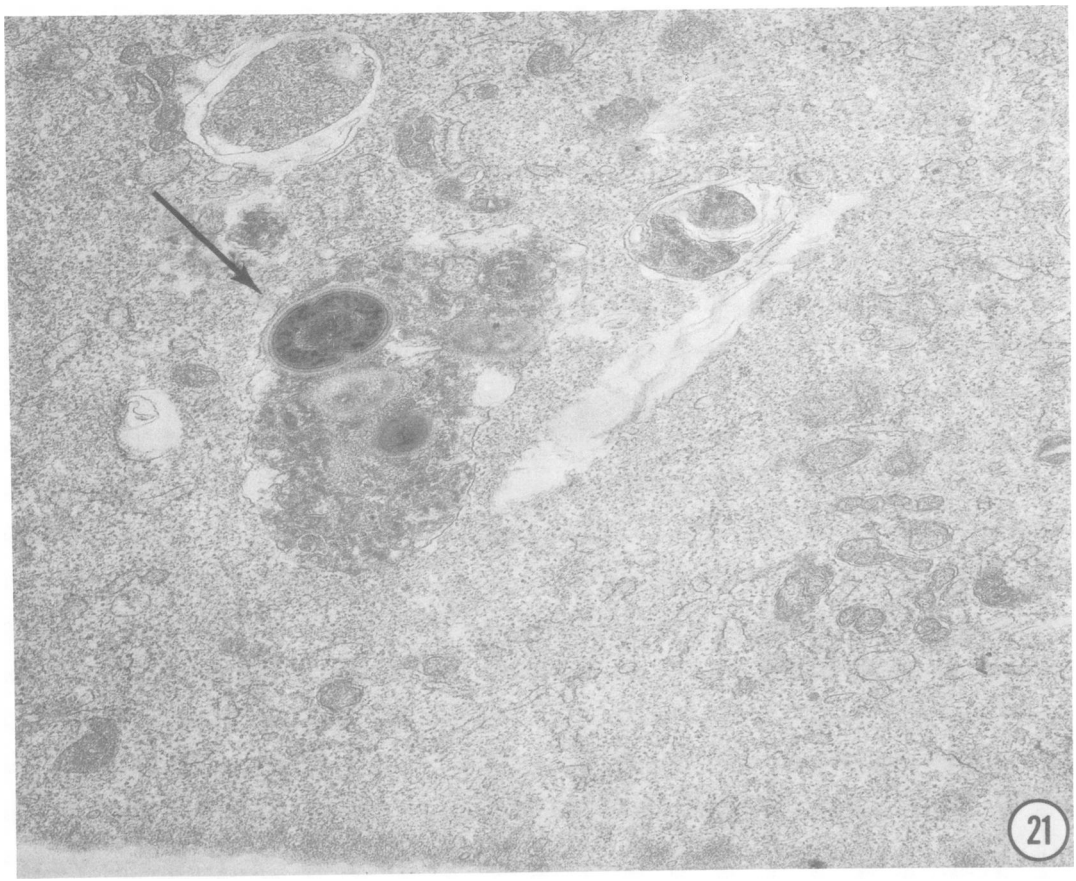
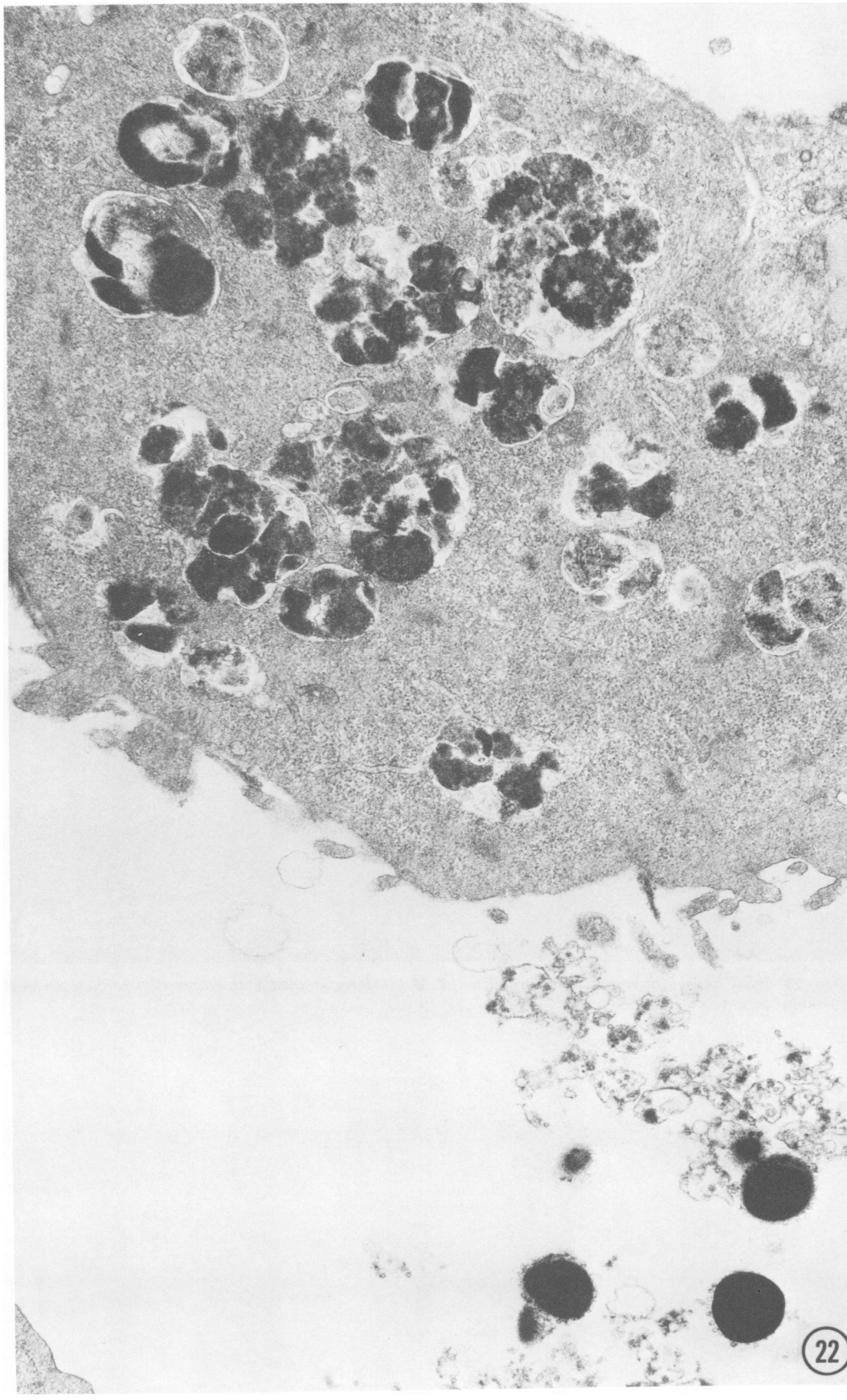


FIG. 21. *Classical S. faecalis bacterium in close proximity to large degraded L-form within HEK cell at reversion 25 days postinfection with the M-1 L-form (horizontal arrow). Note also on adjacent granular L-form (vertical arrow). ×22,500.*

FIG. 22. *Cluster of dense L-forms within HEK cell at reversion 25 days postinfection with the M-1 L-form. ×20,000.*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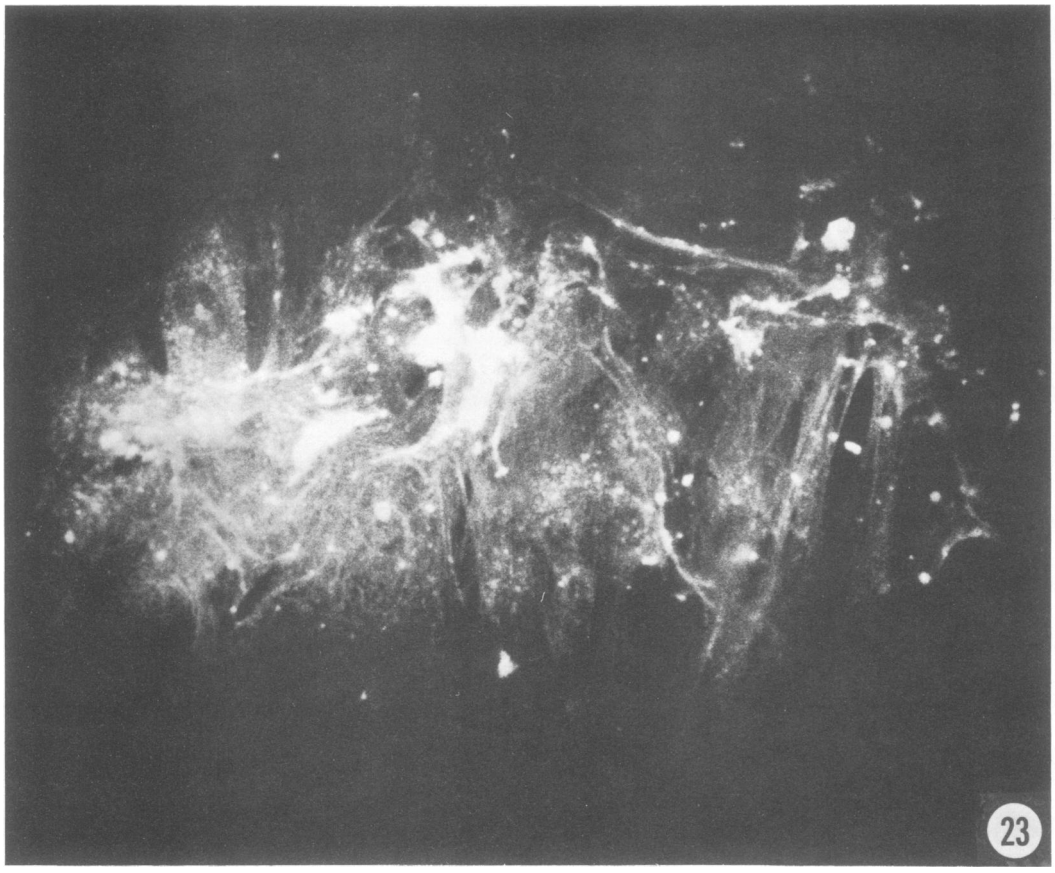

FIG. 23. *HEK cells 8 days postinfection with the M-1 L-form stained with fluorescein-conjugated goat anti-rabbit serum.* ×608.

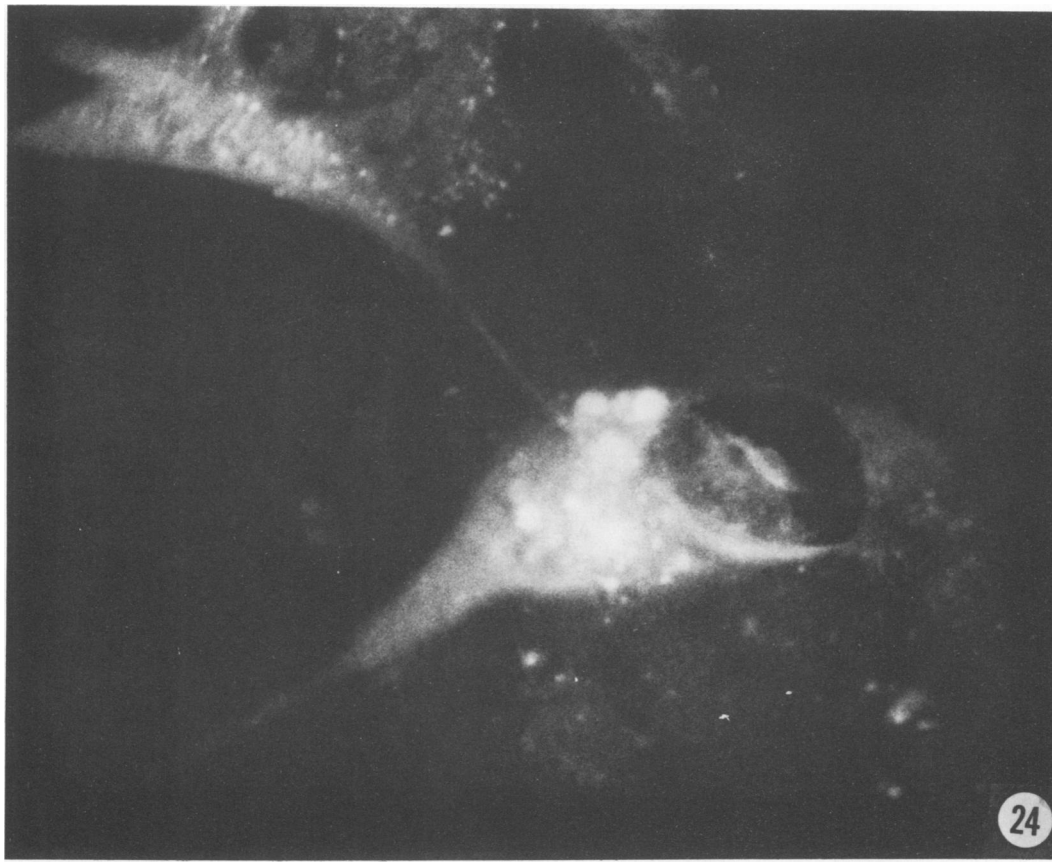

FIG. 24. *HEK cells 21 days postinfection with the B-9 L-form stained with fluorescein-conjugated goat antirabbit serum.* ×2,592.

other kinds of bacteria? Fundamentally, could the occurrence of viable persisting bodies be a universal feature of bacterial L-forms, and if so, what is their chemical and immunological nature? These questions are pertinent because a theme of persistence and relapse runs through a wealth of literature on otherwise diverse diseases with which L-forms have been associated. The above questions might be most expediently approached by isolation and analysis of the dense bodies from pure cultures of L-forms in which they appear, followed by attempted infection of both tissue culture cells and germfree animals with these agents. If dense bodies prove to be the latent persisting forms of infectious bacterial agents, metabolic inhibitors or vaccines directed toward such persisting forms might ultimately break the microbial cycle of persistence and relapse in infectious disease.

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