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Intracellular Production of *Brucella* L Forms

I. Recovery of L Forms from Tissue Culture Cells Infected with *Brucella abortus*[1]

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ABSTRACT

HATTEN, BETTY A. (The University of Texas Southwestern Medical School, Dallas), AND S. EDWARD SULKIN. Intracellular production of *Brucella* L forms. I. Recovery of L forms from tissue culture cells infected with *Brucella abortus*. J. Bacteriol. **91**:285–296. 1966.—Infectivity of virulent *Brucella abortus* strain 3183 was less for hamster macrophages after a 2-hr adsorption period than for an attenuated strain (S19) and its tissue culture variant (30). Both strains S19 and 30 were very toxic for the cells, but 3183 was not toxic. Two types of L forms were recovered from a large percentage of hamster kidney cell cultures when disintegration of infected cells was accelerated by tissue culture medium of high *p*H. One type grew in finely granular microcolonies, was isolated from cells infected for short periods of time, and often reverted to the bacterial form. The other type occurred in small irregularly shaped forms which later developed into round bodies. Both stained specifically with fluorescein-conjugated *B. abortus* antiserum. Semisolid media containing 0.7% agar provided optimal subsurface L-form growth. L forms also grew well in Thioglycollate Medium but grew poorly in other liquid media. Surface L-form growth was supported by several agar media, but CO_2 was required for optimal growth. Monolayers infected with strain 3183 and examined immediately after adsorption contained occasional small, round bodies. Bizarre forms increased in number with time and, after 24 to 72 hr, large pink-staining inclusions were often present which persisted for several days. Also appearing at about the same time were smaller, dark-staining forms which were first seen in clusters but later dispersed and finally occurred in chainlike configurations. Direct fluorescent-antibody stains of infected cells established that the intracellular forms were related to the infecting strain of *B. abortus*.

Production of altered intracellular forms by virulent organisms has been suggested (17, 30). Evidence that virulent and avirulent brucellae differ metabolically when grown in artificial media has been provided (11, 26). In addition, in vitro studies have shown that rapid selection of smooth virulent brucellae occurs in chick embryo cells, and that continuous passage in guinea pig macrophages results in greater multiplication in host cells, more resistance to bovine serum, increased ease of phagocytosis, and less cytopathic effect in cell cultures (12, 32, 33).

Protection of intracellular brucellae from the action of antibiotics was first noted by Shaffer, Kucera, and Spink (28). Although neither penicillin nor streptomycin was bactericidal for intracellular *Brucella abortus*, Richardson and Holt (24) found a synergistic action between these two antibiotics. Even more effective inhibition was obtained with combinations of tetracycline and streptomycin, and tetracycline alone was bacteriostatic at very low levels. A combination of streptomycin and tetracycline is the therapy of choice in treatment of severe cases of brucellosis, but a high incidence of relapses still occurs (8, 31).

This evidence suggests the possible persistence of intracellular brucellae as L forms, since such forms are often induced and subsequently survive in artificial media under conditions which are adverse to bacterial growth (6, 13, 14, 19, 20). L forms of *B. abortus* and *B. melitensis* have

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been recovered in recent isolates from human blood by Nelson and Pickett (21) and from sheep by Carrere and Roux (3). These reports have been neither confirmed nor extended and may only reflect alteration of the organisms due to adverse cultural conditions. No clear-cut evidence has been given to indicate that L forms can be produced or maintained in an intracellular environment. The purpose of the current experiments, therefore, was to determine whether intracellular production of *B. abortus* L forms can occur. The results discussed here demonstrate that organisms resembling L forms can be recovered from tissue culture cells by the experimental methods described.

MATERIALS AND METHODS

Microorganisms. A lyophilized culture of the attenuated vaccine strain of *B. abortus* (S19) was used in comparative experiments on infectivity and toxicity and in preparation, by turbidimetric methods, of a standard curve for measurement of the number of bacteria in broth suspensions.

A recent culture of *B. abortus* 3183 from a case of brucellosis was obtained from Wesley Spink. This microorganism was used in experiments on L-form production in addition to those on infectivity and toxicity.

Stock cultures were maintained on Trypticase Soy Agar (BBL) slants at 4 C and, for infection of tissue culture cells, were grown in Trypticase Soy Broth (BBL) under increased CO_2 tension at 37 C. After 1 week the broth cultures were centrifuged, the supernatant fluid was removed, and the organisms were suspended in Hank's balanced salt solution (BSS), *p*H 7.8. The concentration of organisms in this suspension was adjusted to approximately 3.2×10^9 by comparison with the standard curve. Viability and purity of the cultures were tested by subculture to agar plates at this time.

Macrophage cultures. The technique for obtaining macrophage cultures was modified from that described by Stinebring (32) by substituting hamster for guinea pig exudate cells. The growth medium was also altered slightly by use of 15% calf serum in Hank's lactalbumin hydrolysate (LAH) medium at *p*H 7.4. Cultures (24 to 48 hr) were exposed to 0.5 ml of suspended *B. abortus* for 2 hr, then washed with BSS and returned to the growth medium.

Hamster kidney cell cultures. Hamster kidney cells were trypsinized and grown in tissue culture according to the methods of Dierks and Hammon (7). Leighton tube monolayers were exposed to 0.5 ml of cells or 4-oz prescription bottles to 2.5 ml of suspended *B. abortus* organisms for 5 hr. After adsorption, unattached bacteria were rinsed off the cells with BSS, and the tissue cultures were returned to the growth medium.

Artificial culture media. Bacterial organisms were isolated from infected tissue culture cells on 5% sheep blood-agar plates and in Trypticase Soy Broth under increased CO_2 tension.

L forms were isolated primarily in Difco Thioglycollate Medium, because it did not support growth of the *B. abortus* stock strains. In addition, the following were used to study the morphology and growth characteristics: PPLO agar and broth (Difco), both containing 25% ascitic fluid; Barile, Yaguchi, and Evelyn (BYE) agar (BBL) with 15% human blood and a 1:5,000 concentration of thallium acetate (bacterial growth of *B. abortus* 3183 was inhibited by concentrations of thallium acetate greater than 1:7,500); Trypticase Soy Agar with 10% calf serum and 2% yeast extract; and 5% sheep blood-agar.

Fluorescein-conjugated antiserum. Antiserum was prepared in rabbits by inoculating 72-hr broth cultures of *B. abortus* 3183 in a series of five injections given at 4-day intervals. The first two injections of 0.5 and 1.0 ml, respectively, contained organisms which had been exposed to formalin for 24 hr. The next two inoculations of 2 ml each were made with cultures exposed to formalin for 2 hr. The last injection consisted of 2.0 ml of live organisms. The pooled sera from animals bled 7 days after the last injection (agglutinin titer, 1:256) were conjugated with fluorescein isothiocyanate by a dialysis method recently described by Clark and Shepard (4). After conjugation, the serum was passed through a Sephadex (G-25) column to remove excess fluorescein (10). This conjugate gave 4+ staining of organisms in a 1:8 dilution with essentially no nonspecific staining.

Agglutination tests. Agglutination tests for identification of bacteria recovered from infected monolayers were done by the slide method. Commercially prepared *Brucella* AMS antiserum (Difco) was used for these tests.

May-Grunwald-Giemsa (MGG) stain. The procedure for MGG staining was carried out according to Barile, Malizia, and Riggs (1).

Fluorescent-antibody (FA) stain. The direct FA stains were done on cover slips or culture smears which had been rinsed in distilled water, fixed with acetone for 4 hr at 4 C, and dried at 37 C for 30 min.

Fixation of agar blocks. Agar blocks were cut from plates suspected of having L-form growth, placed face down on microslides, and fixed with Bouin's fixative as described by Klieneberger-Nobel (15).

Enzyme treatment of tissue culture cells. Cover slips containing monolayers of infected cells were exposed to deoxyribonuclease prior to staining (1). The enzyme preparation was a crude extract of pancreatic deoxyribonuclease (Dornavac; Merck Sharp and Dohme, Rahway, N.J.). Infected cells were exposed to a 1:50 dilution of the enzyme for 1 hr at 37 C, or 2 to 4 hr at room temperature.

Microscopic and photographic equipment. Dark-field and fluorescence photographs were made with a Leica M1 camera mounted by a microcamera attachment to a Leitz SM microscope. The light source for fluorescence work was a mercury vapor L2, HBO-200 lamp. The primary ultraviolet filter was a 5840 or 2-mm UG1, and, in addition, a secondary blue absorbing filter was used. Dark-field illumination was provided by a 6-v, 2.5A bulb and Regal transformer set at 6 amp. Time exposures of 3 to 4 min were made for both dark-field and fluorescence photomicrographs

taken with Kodachrome II, Type A, or Kodachrome II daylight and blue flash film. Black and white exposures were made with Kodak Tri X Pan film.

RESULTS

Infectivity and toxicity of B. abortus in tissue culture. Initial experiments were designed to test the infectivity and toxicity of the virulent strain 3183, the avirulent strain S19, and a tissue culture variant of S19, strain 30, in hamster macrophage cultures. Infected cells were observed at several time periods after infection, and the percentage of infected and disintegrated cells was determined by examination of cover slips stained with MGG stain. The per cent of surviving cells infected over a 12-day period after a 2-hr adsorption time is shown in Fig. 1. Strains S19 and 3183 infected approximately the same number of cells initially, whereas a higher percentage were infected with strain 30 at this time. We observed an exponential decrease in the percentage of cells infected with strains S19 and 30 and a precipitous drop in the percentage of cells infected with strain 3183 during the first few hours. After maximal infection of the cells at 2 to 5 days, a decline in the percentage of infected cells was noted with all strains; but, at the end of 12 days, strain 3183 was seen in only a few cells, whereas strains S19 and 30 were still present in a considerable number of the remaining cells.

The marked toxicity of S19 and 30 was evident, however, from the percentage of degenerate cells present after exposure to these organisms (Table 1). Strains S19 and 30 were toxic for hamster macrophage cells within 24 hr after infection; by 4 days, approximately one-half of the macrophages infected with S19 were degenerate, and an even greater number of those infected with 30 were no longer intact. On the other hand, the maximal percentage of disintegrated cells in

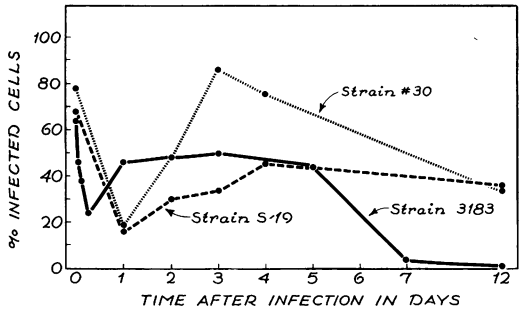

FIG. 1. *Curves comparing the percentage of hamster macrophage cells infected at various times after 2-hr exposure to avirulent Brucella abortus strain S19; a tissue culture variant of S19, strain 30; or virulent B. abortus 3183.*

TABLE 1. *Toxicity of Brucella abortus strains S19, 30, and 3183 for hamster macrophage cells*

Strain	Per cent of cells disintegrated at			
	24 hr	48 hr	72 hr	96 hr
S19	16	32	25	46
30	67	36	33	78
3183	1	2	—	2
Uninfected control cells	1	3	2	3

cultures exposed to strain 3183 was no greater than in control cells. Although mitosis continued in macrophage cells exposed to strains S19 and 30 until 24 hr after adsorption, the cells began to appear abnormal shortly after they were infected. The cells became dense and did not increase in size with age as did the normal control cells. Within 48 to 72 hr most of the infected cells had nuclear and cytoplasmic vacuoles. Macrophage cultures infected with strain 3183 were still able to divide 5 days after infection. Approximately 50% of these cells had spindle shapes and foamy, light-staining cytoplasm, but did not appear to have degenerated.

Several attempts were made to increase the infectivity of the virulent organism, and this was finally accomplished by prolonging the adsorption time to 5 hr, which resulted in a corresponding rise in infected cells to approximately 90%. No appreciable increase in toxicity was noted, and for this reason strain 3183 was used for all subsequent experiments involving the production of L forms.

Recovery of Brucella L forms from hamster kidney cells. Several methods for disrupting infected hamster kidney monolayers were tried, since it was not certain what physiological changes *Brucella* L forms could withstand. Infected tissue culture cells were disrupted with trypsin, 0.05% tryptose, glass beads, or freeze-thawing, and were inoculated into Trypticase Soy Broth and Thioglycollate Medium. The results were disappointing with respect to L-form isolations, and recovery of bacterial organisms was inconsistent, although microscopic examination of MGG-stained cover slips indicated that numerous cells contained intracellular organisms. Recovery of organisms resembling L forms from cells harvested at 2 hr, 3 hr, and 7 days after infection in one experiment after exposure to 2.5 μg/ml each of penicillin and streptomycin suggested that at least some of the intracellular bodies were viable organisms and not "ghost" forms resulting from death of the bacteria.

TABLE 2. *Recovery of bacterial and L forms of Brucella abortus from hamster kidney cells after removal of antibiotics from the growth medium*

Expt*	Exposure time†	L forms		Bacterial forms		
		Thioglycollate medium	Blood-agar (5% CO_2)	Trypticase soy broth (5% CO_2)	Blood-agar (5% CO_2)	Agglutination with *Brucella* antiserum
I	12 days	11/11‡	14/14	1/11	8/14	8/8
II	48 hr	2/2	7/9	0/8	3/9	3/3
Total	—	13/13	21/23	1/19	11/23	11/11
Uninfected control cells	—	0/8	0/8	0/8	0/8	0/8

* *B. abortus* 3183 used in experiment I; infected cells from experiment I used in experiment II.
† Time of intracellular exposure to 2.5 μg/ml each of penicillin and streptomycin.
‡ Number of positive cultures/total number of cultures.

Fragility and inability to adapt to the extracellular environment were considered the most likely reasons for failure to obtain viable L forms with regularity. Two experiments were therefore set up to eliminate these factors. In experiment I, cells were infected with *B. abortus* 3183 suspensions as usual, and were maintained in growth medium containing 2.5 μg/ml each of penicillin and streptomycin. At 5 and 10 days, when the tissue culture medium was changed, the replaced medium was pooled and centrifuged, and the supernatant fluid was discarded. The sediment was rinsed with BSS and resuspended in growth medium. These cells served as the source of infection for hamster kidney cells in experiment II. After a 24-hr adsorption period, the medium was replaced with LAH medium containing 2.5 μg/ml each of penicillin and streptomycin. The antibiotics were removed in experiment I 12 days after the tissue culture cells were infected, and after 48 hr in experiment II. At this time, antibiotic-free growth medium (*p*H 7.8) was added, and the intact cells were incubated for 5 to 7 days without change of medium. During this time the *p*H was maintained at approximately 7.8 in an effort to stimulate L-form growth and to accelerate cellular disintegration. The above procedure thus eliminated physical stress upon the organisms and maintained them under conditions to which they were accustomed. When the infections were heavy, the tissue culture medium became opaque a few days after the cells separated from the glass. In lighter infections, no visible changes were apparent, but a sticky, white mass of material was present in the bottom of the tubes after centrifugation. This sediment was used to inoculate Thioglycollate Medium, Trypticase Soy Broth, and blood-agar plates 10 days to 2 weeks after removal of antibiotics from the tissue culture medium.

Similar results were obtained in both experiments (Table 2), indicating that the previous difficulty in recovering L forms was probably due to their fragility when exposed to physical stress. L forms were recovered from a large number of Thioglycollate Medium (100%) and blood-agar (91%) cultures. Bacteria, on the other hand, were recovered from only one Trypticase Soy Broth culture and from 48% of the blood-agar cultures. It was assumed that the majority of bacterial colonies isolated on blood-agar plates came from L-form reversions because of the early appearance of L forms at 48 to 72 hr, followed by bacterial colonies at 5 to 7 days.

Stability and growth of Brucella L forms under various cultural conditions. The ability of the L forms to produce surface colonies was tested on BYE agar plates (to which thallium acetate had been added in a final concentration of 1:5,000), sheep blood-agar plates, and on Trypticase Soy Agar plates with 10% calf serum and 2% yeast extract. Typical finely granular microcolonies were frequently observed 48 to 72 hr after inoculation. The appearance of these colonies by direct bright-light illumination is shown in Fig. 2a. In addition, smaller colonies were occasionally present which were not readily apparent when the agar surface was examined under a stereomicroscope. Agar-block fixation and staining, however, revealed small granular particles in varied shapes resembling the "T-forms" of *Mycoplasma* described by Shepard (29), and which later became interspersed with round bodies (Fig. 2b). The latter forms usually failed to grow on subculture. Optimal growth occurred only when the plates were incubated under 5 to 10% CO_2 tension, although some multiplication did take place under both anaerobic and aerobic conditions.

The effect of varying concentrations of agar upon L-form growth was also examined. The basic medium consisted of Trypticase Soy Broth

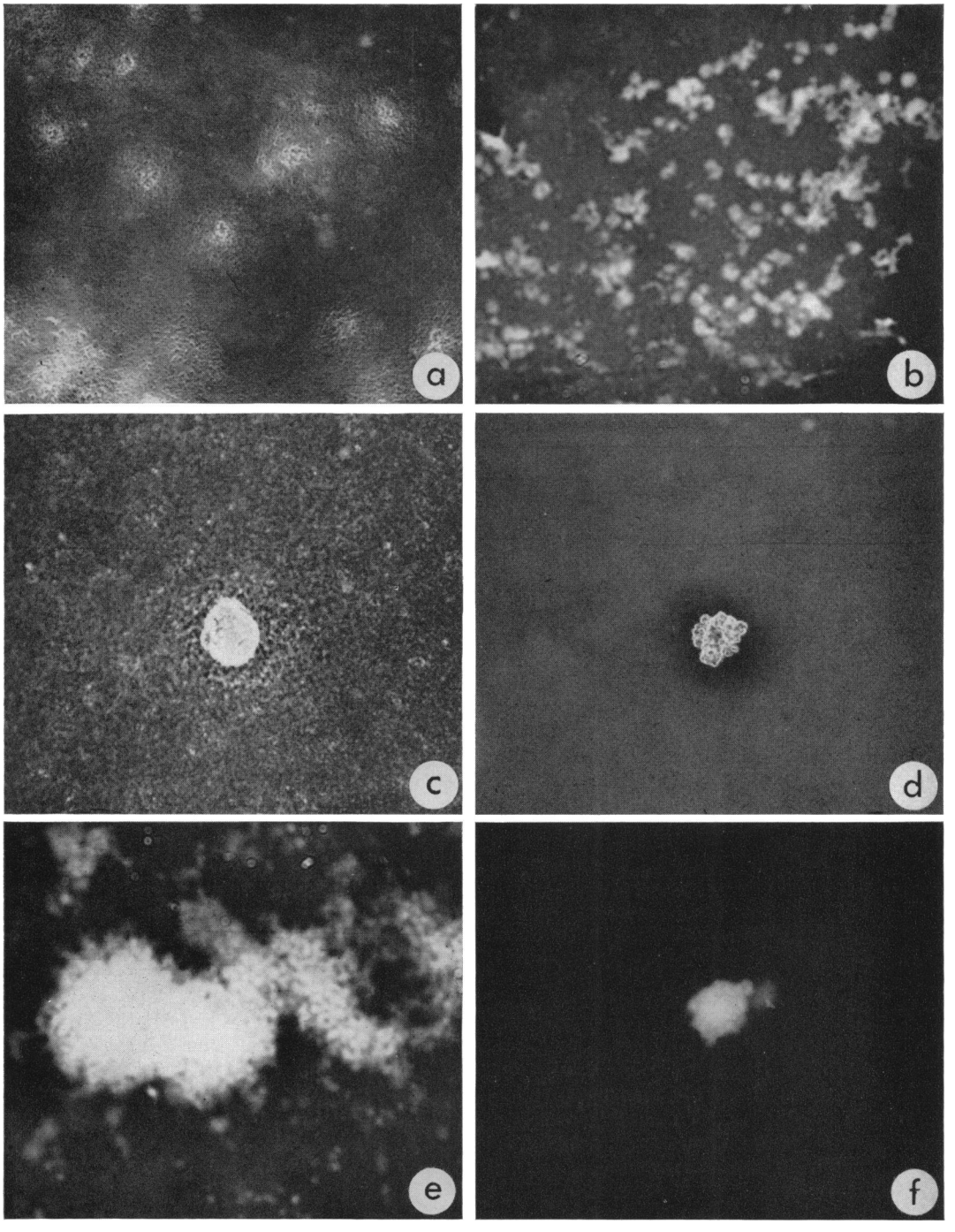

FIG. 2. *(a) Surface colonies of typical Brucella abortus L forms after 72-hr growth on Trypticase Soy Agar containing 10% calf serum. × 120. (b) Small, pleomorphic forms observed with MGG stains of agar-block preparations. × 1,540. (c) Subsurface colony of typical B. abortus L form after 48-hr growth in Trypticase Soy Broth medium containing 0.7% agar and 10% calf serum. × 120. (d) Subsurface colony of B. abortus L form after 5 days of growth, showing multiple, small satellite formations. × 120. (e) B. abortus L-form growth seen in smears from Thioglycollate Medium cultures. MGG stain. × 1,920. (f) Direct FA stain of B. abortus L-form colony showing 4+ fluorescence. × 540.*

TABLE 3. *Dependence of colony characteristics upon duration of intracellular growth*

Period of intracellular growth*	Growth in thioglycollate medium		Type of bacterial growth produced upon reversion of L forms†
	Macroscopic appearance	Microscopic appearance	
0–3 hr	White, flocculent or granular	Typical, finely granular microcolonies	Small to moderate sized smooth colonies
24–72 hr	White, flocculent or granular and/or opaque	Typical, finely granular microcolonies mixed with small pleomorphic forms	Small, flat, slightly spreading colonies
5–7 days	Opaque	Small, pleomorphic "T-type" forms	Moist to mucoid, coalescent colonies with foul odor
Over 7 days	Opaque	Small, pleomorphic "T-type" forms and round bodies	None

* Hamster kidney cells infected with *Brucella abortus* 3183 and maintained in medium with 2.5 μg/ml each of penicillin and streptomycin added.

† All colony types agglutinated with *Brucella* (AMS) antiserum.

with 10% calf serum and a 1:5,000 concentration of thallium acetate, or PPLO broth with 25% ascitic fluid and a 1:100,000 concentration of sodium tellurite. Agar was added to each of the basic media to make final concentrations of 0.35, 0.7, and 1.5%. Each medium was inoculated while warm, mixed, and poured into disposable Cooper tissue culture dishes (60 by 15 mm). Development of the subsurface colonies was then observed by microscopic examination of the plates. Maximal growth was noted in both media when 0.7% agar was present, but Trypticase Soy medium produced heavier growth than did PPLO medium with all concentrations of agar. This observation suggested that L-form growth was not enhanced by the higher *p*H of PPLO medium. Initial growth, present at 48 to 72 hr, consisted of finely granular colonies enclosed by a membrane (Fig. 2c). Continued incubation for 5 to 7 days led to the development of numerous smaller satellite formations, giving the colonies an irregular, three-dimensional appearance (Fig. 2d). Small granular particles seen in some plates were thought to represent the small "T-type" forms seen on the surface growth, but this observation could not be confirmed.

Two types of growth were also found in Thioglycollate Medium. The granular or flocculent growth seen macroscopically was made up predominantly of typical microcolonies when stained with MGG stain (Fig. 2e). The opaque, diffuse type of growth consisted almost entirely of individual pleomorphic forms sometimes interspersed with round bodies similar to those shown in Fig. 2b. Both types of growth occurred in a band about 0.5 inch (1.3 cm) from the surface of the medium, and, in this respect, their growth characteristics were similar to those demonstrated by Nelson and Pickett (21) in the same medium. Specific FA staining of the L forms with *Brucella* antiserum confirmed that they were derived from *B. abortus* 3183 used in the original tissue culture infection. Figure 2f illustrates a microcolony with 4+ fluorescent staining. Considerable variation in the staining ability of the L forms was seen, however, with the variation occuring quite often within a single colony. Growth of the L forms in two other liquid media, Trypticase Soy Broth with 10% calf serum, or PPLO broth with 25% ascitic fluid and a 1:100,000 concentration of sodium tellurite, was limited even with increased CO_2 tension. On the occasions where growth could be detected, the incubation period was prolonged, sometimes extending from 60 to 90 days. Typical microcolonies were rarely seen in the latter broth media and attempts to subculture the organisms generally failed. In both broths, growth consisted of a small amount of stringy white material adherent to the bottom of the tubes.

Relationship of intracellular survival upon L-form morphology and ability to revert to the bacterial form. When the type of L-form growth obtained on the various culture media was compared with the origin of the isolates from which they were subcultured, it appeared that their morphology was correlated with the length of intracellular survival. In addition, the ability of the L forms to revert to the original bacterial type appeared to be altered with extended intracellular growth. A description of the colony types produced by isolates from an experiment in which tissue culture cells were infected with *B. abortus* 3183 and incubated for various periods of time in the presence of 2.5 μg/ml each of penicillin and streptomycin is given in Table 3. It can be seen that, when the brucellae remained in the hamster kidney cells for only a short period of time (0 to 3 hr), the white flocculent or granular growth consisted of finely granular microcolonies. These, in turn,

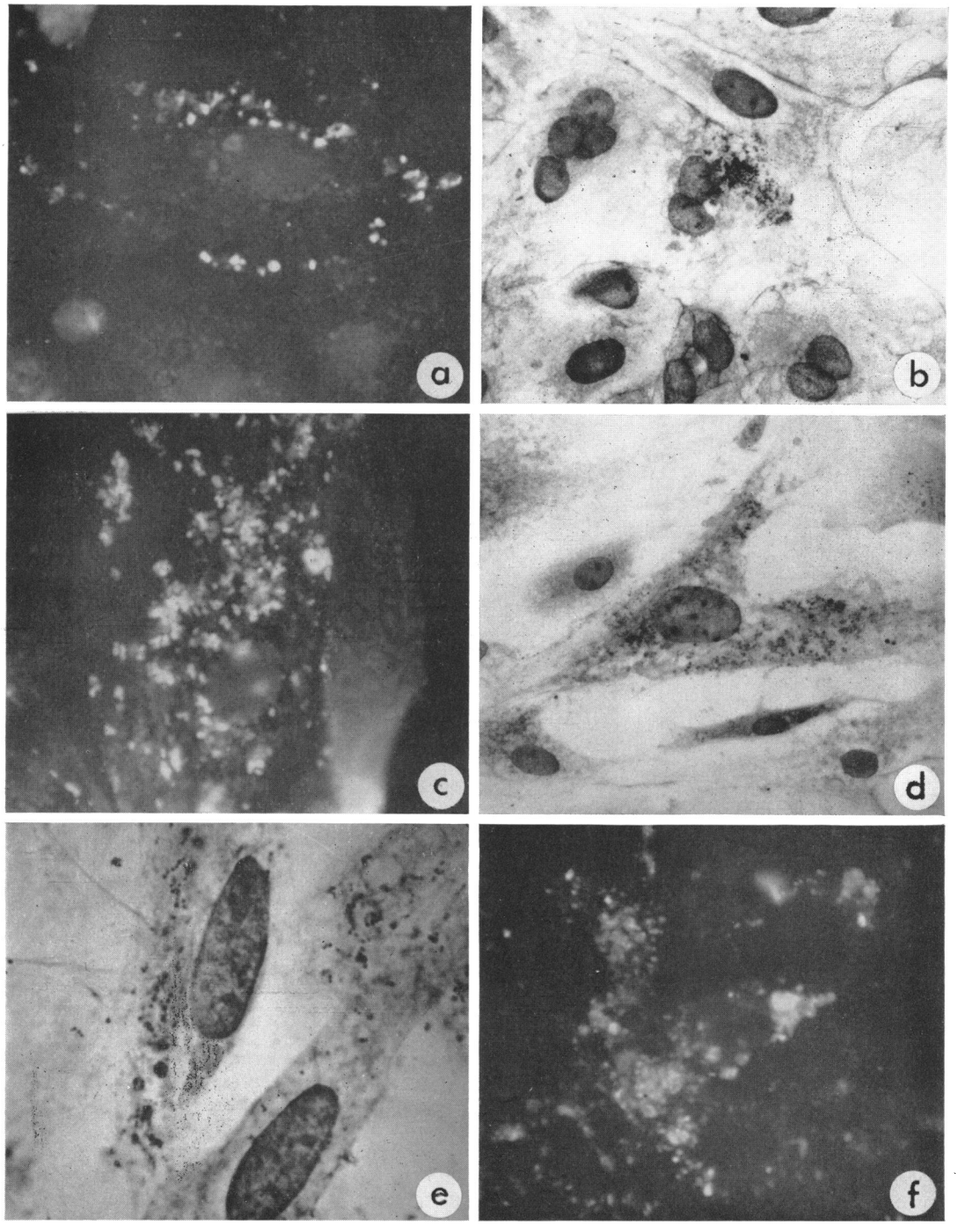

FIG. 3. *(a) Intracellular microorganisms in the peripheral cytoplasm immediately after adsorption. FA stain. × 540. (b) Infected cells containing more centrally located and sometimes aggregated microorganisms at 24 hr. MGG stain. × 385. (c) Pleomorphic forms demonstrated at 24 hr by FA stain. × 540. (d) Small, round bodies present at 72 hr. MGG stain. × 385. (e) Dark-staining, coccoid forms with chainlike configurations seen at later times. MGG stain. × 1,200. (f) Specific FA stain of both large and small granular bodies. × 540.*

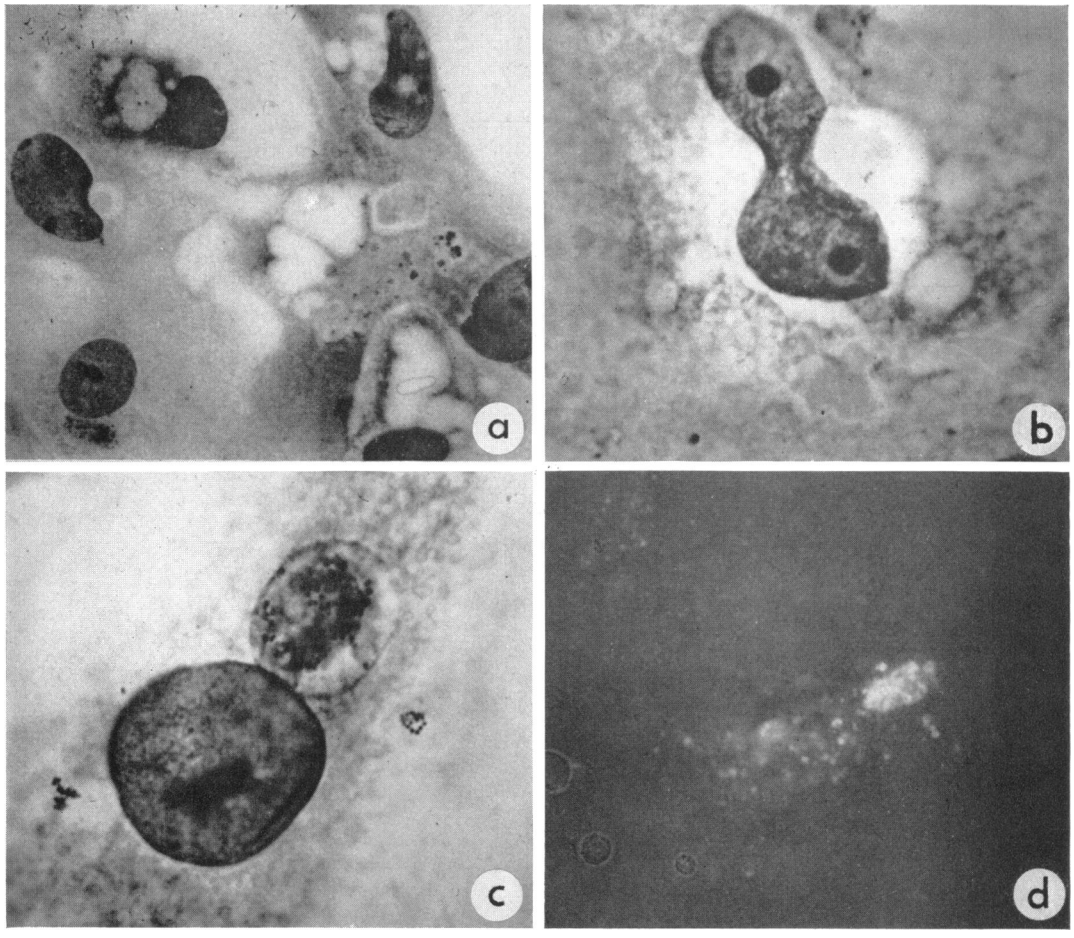

FIG. 4. *(a) Large inclusion separated from the cellular cytoplasm by a narrow, clear zone. Smaller inclusions also present contain small amounts of dark granulation. MGG stain. × 385. (b) Indentation of cellular nucleus caused by growth of a large inclusion. MGG stain. × 1,200. (c) Cytoplasmic inclusion seen 48 hr after removal of antibiotics from the growth medium with an increased amount of granulation. MGG stain. × 1,200. (d) Specific fluorescence of a large area resembling the large inclusions seen in MGG-stained preparations. FA stain. × 540.*

reverted to smooth, small to moderate-sized bacterial colonies resembling the original colony type. As early as 24 to 72 hr after intracellular infection, the organisms produced fewer granular colonies and presented a more diffuse or opaque type of growth. At this time, microscopic examination revealed a mixture of typical microcolonies and small pleomorphic forms again resembling the "T-forms" of Shepard (29). The majority of bacterial colonies produced from these L forms were small, flat, and slightly spreading. Despite their altered appearance, however, they were still agglutinated by *Brucella* antiserum. After the fifth to seventh day of intracellular existence, the isolates grew in an opaque band with no granular colonies and consisted almost entirely of small "T-forms" and round bodies. The resulting bacterial colonies derived from these organisms were white, moist to mucoid colonies which had a foul odor and a tendency to coalesce. These bacteria were also agglutinated by specific *Brucella* antiserum. No bacterial reversions were obtained from L forms isolated after 7 days of growth within tissue culture cells, nor were any typical L-form microcolonies noted.

Morphology of intracellular B. abortus. The morphology of intracellular brucellae was examined by MGG and FA stains at various time intervals after infection of the hamster kidney cells. Cover slips stained immediately after adsorption of the *Brucella* to the hamster kidney cells showed that most of the bacteria were gathered around the periphery of the cells lying just inside the cytoplasmic membrane. At this time

most of the organisms appeared by FA staining to be bacillary forms, although a few rounded and swollen forms were sometimes seen (Fig. 3a). After 24 hr, the organisms had migrated to a more central location within the cytoplasm (Fig. 3b). The majority were of similar size and shape as those seen at earlier times and were singly arranged, but occasionally aggregations were also seen. All of the brucellae were stained light-red with MGG stain and appeared to be morphologically normal bacteria. An FA preparation (Fig. 3c) made from a 24-hr culture of infected cells suggested that many pleomorphic forms were actually present. By 72 hr after infection the alteration of the brucellae was also visible in the MGG-stained cover slips; they now appeared as small, round, light-bluish-purple bodies (Fig. 3d). The final stage of development, which was the predominant form found at later times, is shown in Fig. 3e. The higher magnification shown here allows the chainlike configuration of some of the smaller dark-staining coccoid organisms to be seen. Dark-staining granules within some of the larger bodies, surrounded by lighter-pink-staining material which often had an irregular edge, can also be seen. Figure 3f demonstrates that both the larger and smaller granular bodies reacted with specific *Brucella* antiserum, although the intensity of staining varied within single organisms.

Larger cytoplasmic inclusions were also occasionally seen, beginning as early as 24 hr after infection and persisting until the fifth to seventh day. These inclusions stained light-pink with MGG stain and were generally separated from the cellular cytoplasm by narrow clear zones (Fig. 4a). A cluster of smaller light-staining inclusions can also be seen, one or two of which contain small, dark-staining granules. Inclusions were often situated close to the nucleus and, when present, generally caused distortion of this cellular structure (Fig. 4b). Although this form of inclusion was not noticed in tissue culture cells during prolonged exposure to antibiotics, Fig. 4c illustrates a structure found 48 hr after removal of streptomycin and penicillin from the medium in which the infected hamster kidney cells had been maintained for 12 days. This inclusion resembled those seen earlier in size and proximity to the nucleus of the host cell, but was more dense and contained numerous granules similar in size and shape to those found in the small granular bodies. Some large areas of fluorescence, similar to the one shown in Fig. 4d, suggested that the large pink inclusions originated from the brucellae used to infect the cells, but it is possible that these fluorescent areas simply represent large masses of the smaller granular forms.

Since the pink inclusions had a striking resemblance to intracellular *Mycoplasma pneumoniae*, the staining ability of which was not impaired by pretreatment with deoxyribonuclease (1), cells infected with *Brucella* were exposed to crude deoxyribonuclease by a similar technique. Subsequent stains with MGG after treatment of the infected monolayers for 1 hr at 37 C, or 3 hr at room temperature, did not indicate that any reduction in the staining ability of these inclusions or the smaller granular bodies had occurred, even though nuclear staining was markedly reduced. These results imply that the inclusions were not part of the cellular structure. The irregular outline of some inclusions also supports this contention. Definite proof connecting the large inclusions specifically to *Brucella* has not been shown, but no similar structures were seen in the uninfected control cells examined.

DISCUSSION

Smadel (30) and McDermott (17) suggested that chronic infectious diseases may be the result of in vivo L-form production. Substantial evidence to support this theory has not been given. Toxin-producing bacteria have been induced to give rise to L forms which are also capable of exerting their toxic effect in laboratory animals (16, 25, 27, 34). Because the experiments were generally carried out over a short period of time, none of these studies have demonstrated that L forms could be produced under naturally occurring conditions in vivo, nor have they demonstrated significant survival of L forms once they were introduced into animals or tissue culture cells. Similarly, studies reported by Nelson and Pickett (21) and Carrere and Roux (3) in which *B. abortus* and *B. melitensis* L forms have been related to in vivo survival of these organisms are based on indirect evidence. L-form structures were neither demonstrated in the infected hosts by direct examination of blood and tissues, nor were they isolated in primary blood cultures. It is obvious, therefore, that more direct evidence must be provided before one can assume that L forms of pathogenic organisms can be induced and subsequently survive in vivo. *B. abortus* lends itself well to such studies not only because the infection often persists for long periods of time, but because it is maintained intracellularly. The intracellular nature of the infection further suggests that, if production of an L form constitutes a primary mechanism of survival during a course of therapy, conversion to the L phase should occur within the cells. A tissue culture system which simulates conditions occurring in natural infections, such as the one used in the

present experiments, should be a reliable means of determining whether intracellular induction of *Brucella* L forms is possible and, if so, whether they play a vital role in perpetuating the infection.

Selection of the virulent *B. abortus* 3183 for use in these studies was based on preliminary experiments which indicated that this strain had little or no toxic effect upon the host cells. At the same time, the avirulent strain S19 and a variant (strain 30) derived by two passages of S19 in tissue culture cells were shown to be very toxic for hamster macrophage cells. This observation confirmed several reports (2, 9, 12) that virulent strains of *B. abortus* were less toxic for tissue culture cells. The enhanced infectivity of the variant strain 30 over that of the parent S19 strain also verified a report by Stinebring and Kessel (33) that tissue culture passage increased infectivity and survival of the intracellular brucellae. The lower percentage of cells infected with the virulent strain 3183 was not anticipated, although conflicting reports have been made on the uptake and subsequent survival of smooth strains by tissue culture cells. Holland and Pickett (12) and Braun et al. (2) agreed that smooth *Brucella* strains were taken up more rapidly and survived better than nonsmooth strains, whereas Freeman et al. (9) observed no significant differences between the two types. Comparison of such results is difficult, however, since the techniques and strains of organisms examined were not the same. The preference of the *B. abortus* L forms for media of lower *p*H is another characteristic similar to *Mycoplasma* "T-form" growth (29). The inability to obtain growth upon subculture or transformation to other forms was not surprising, since it has been reported by Shepard (29) that *Mycoplasma* "T-forms" could not be induced to produce typical microcolonies. Large bodies of *Diplococcus pneumoniae* and *Neisseria gonorrhoeae* also did not develop into typical L-form colonies (5, 18).

Difficulty in recovering L forms after disruption of the hamster kidney cells was not expected, at least to the degree encountered, since both mycoplasmas and L forms generally withstand trypsinization, freeze-thaw techniques, and ultrasonic treatment (22). Survival in hypotonic solutions, such as 0.05% tryptose, would be dependent upon the osmotic stability of the L form. Substitution of antibiotic-free tissue culture medium in which the *p*H had been raised to accelerate death and disintegration of the infected cells led to the recovery of organisms which not only differed morphologically from the bacterial forms but grew well in Thioglycollate Medium and grew poorly, or not at all, in Trypticase Soy Broth. Their appearance in Thioglycollate Medium varied from floccular or granular to a diffuse band of growth, but in either case occurred in a zone just below the band of diffused oxygen. This type of growth, as well as the appearance of finely granular microcolonies on solid or semisolid media, was similar to that reported by Nelson and Pickett (21). The small forms observed were morphologically similar to the "T-forms" described by Shepard (29) and eventually evolved into small round bodies more closely resembling the type of growth described by Carrere and Roux (3). *B. abortus* round bodies observed in the current studies did not develop into larger colonies and did not fragment in artificial media, although some indications that fragmentation of these forms did occur intracellularly were seen after removal of antibiotics from the tissue culture medium. A discrepancy between the *B. abortus* round bodies and those described by the French workers pertained to their ability to grow in liquid medium; although *B. melitensis* L forms grew well in peptone broth, *B. abortus* L forms did not.

Morphological alterations of the *B. abortus* L forms noted both intracellularly and upon subculture of infected cells into artificial media, which corresponded with the duration of intracellular survival, are of considerable interest. The L forms isolated after short periods of intracellular infection produced, for the most part, typical L-type colonies. Subculture of these generally gave rise to bacterial colonies resembling those produced by the parental organism. After longer periods of intracellular infection, more of the "T-forms" and round bodies were isolated, but these could be induced only occasionally to revert to the bacterial form. When reversions did occur at the later time periods, the bacterial colonies produced were atypical, resembling the "porteuese" or coalescent colonies described by Nelson and Pickett (21) and Renoux and Suire (23). Finally, after approximately 7 days of intracellular survival virtually no bacterial reversions could be obtained. The increased difficulty in obtaining bacterial reversions after prolonged intracellular survival suggests stabilization of the L forms. Intracellular changes in the morphology of the organisms were also revealed by direct observation of infected cells. Stains made of infected cells soon after infection showed a predominance of bacteria as well as small granular forms which were often in clusters resembling small microcolonies and occasionally small round bodies. At later times more round bodies were noted, although fewer bacteria could be detected, and finally only the small granular and round bodies

were detected. There was a striking similarity between the larger round bodies and mycoplasmas, particularly intracellular *M. pneumoniae* described by Barile et al. (1), not only in the morphology but also with respect to localization adjacent to the cell nucleus. The intracellular *B. abortus* organisms underwent a transition from the typical coccobacillary form of the true bacteria through at least two morphologically distinct forms. A variety of intermediate forms were also apparent, suggesting that each step in the complete transformation from one morphological type to another is a complex process. Some types are probably more capable of intracellular survival, multiplication, or eventual reversion to the bacterial parent under the proper circumstances than others; likewise, some intracellular forms may retain their ability to grow readily on artificial media, whereas others do not. Whether these altered organisms represent true L forms cannot be ascertained from the evidence supplied in the present study. This question could be answered by determining whether they lack cell walls and whether they are filterable. Electron microscopic studies should also provide information regarding their structure, development, and relationship to the host cell.

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