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Intracellular Production of *Brucella* L Forms

II. Induction and Survival of *Brucella abortus* L Forms in Tissue Culture[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. II. Induction and survival of *Brucella abortus* L forms in tissue culture. J. Bacteriol. **91**:14–20. 1966.—Intracellular survival of altered brucellae, possibly L forms, was not greatly affected by penicillin or streptomycin in concentrations ranging from 5.0 to 40 μg/ml, but a combination of these two antibiotics (2.5 to 20 μg/ml each) reduced the number of positive L-form cultures. Tetracycline (2.0 μg/ml) decreased the number of positive L-form cultures at about the same rate as combinations of the higher concentrations of penicillin and streptomycin. Various concentrations of tetracycline (0.1 to 2.0 μg/ml) with 5.0 μg/ml of penicillin or streptomycin significantly reduced the number of positive L-form cultures. L forms were recovered for several days after elimination of bacteria from the cultures by all of the antibiotics tested. L-form production was not dependent upon the presence of antibiotics in the culture medium, but they were recovered in greater numbers when bacteria were still present in the hamster kidney cells. Addition of thallium acetate to infected cells (at varying intervals of time after infection) to control bacterial growth and conversion to the L phase during cellular disintegration decreased the number of positive L-form cultures obtained over a 10-day period. Comparison of the antibiotic sensitivity of bacteria recovered from infected tissue culture cells with the stock strain of *Brucella abortus* indicated that some resistance to penicillin and tetracycline had developed. A marked resistance to streptomycin was observed in those bacteria recovered from cells maintained in the presence of this antibiotic.

Evidence that most antibiotics are capable of permeating cellular membranes and remaining active intracellularly has been accumulated (2, 4, 7, 8, 13, 20, 23). Intracellular survival of bacteria throughout a course of antibiotic treatment must therefore be dependent upon development of resistant bacterial organisms or conversion to more resistant forms. No increased resistance of *Brucella abortus* after intracellular exposure to antibiotics has been shown (20), but greater antibiotic resistance of L forms over their related bacterial parents is well known (5, 9, 10, 12, 24). Recovery of growth resembling L forms from hamster kidney cells infected with *B. abortus* is highly suggestive that such forms play an important role in the persistence of *Brucella* infections (6). The experiments described in this paper were designed to establish the effect of several antibiotics upon induction and survival of these forms in hamster kidney cells.

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MATERIALS AND METHODS

Methods for cultivating and infecting hamster kidney cells with *B. abortus* strain 3183 were the same as those described in the preceding paper (6). After infection, growth medium containing the following was added: penicillin or streptomycin (5 to 40 μg/ml), combined penicillin and streptomycin (2.5 to 20 μg/ml each), tetracycline alone (0.1 to 2.0 μg/ml), and tetracycline (0.1 to 2.0 μg/ml) combined with streptomycin or penicillin (5.0 μg/ml). At various time intervals, this medium was replaced with antibiotic-free medium, *p*H 7.8. Ten days later the tubes were centrifuged and 0.1 ml of the sediment from each tube was inoculated into Thioglycollate Medium (Difco) and Trypticase Soy Broth (BBL). The latter cultures were incubated under 5% CO_2 tension. At the end of 7 days, the cultures were examined for L-form and bacterial growth. L forms were detected by May-Grunwald-Giemsa (MGG) stain, and their identity

was confirmed by the direct fluorescent-antibody (FA) staining technique, both of which have also been previously described (6). In those cultures with few L forms, making detection and identification difficult, subcultures were made to Barile, Yaguchi, and Evelyn (BYE) agar (BBL) plates containing 15% human blood and a 1:5,000 concentration of thallium acetate. The presence or absence of growth was determined by examination of stained agar-block preparations. Bacterial cultures were examined grossly for the presence of growth, and positive cultures were subcultured to blood-agar plates from which agglutination tests were made.

Antibiotic sensitivity testing of recovered bacterial organisms was carried out by an agar-plate technique. The antibiotics were added to Trypticase Soy Agar (BBL) in the same range of concentrations as those employed in the tissue culture medium. *B. abortus* organisms reisolated directly from the hamster kidney cell cultures were grown on blood-agar plates for 72 hr under CO_2 tension. Suspensions containing approximately 3.2×10^9 organisms were made in Trypticase Soy Broth. A loopful of the suspended organisms was inoculated in a straight line across the antibiotic-containing plate and across plates with no antibiotic added. The plates were then incubated for 7 days at 37 C under increased CO_2 tension before final readings were made.

RESULTS

Recovery of B. abortus L forms after intracellular exposure to penicillin and streptomycin. Table 1 shows the frequency of occurrence of bacteria and L forms of *B. abortus* (strain 3183) after infected hamster kidney cells were maintained from 1 to 14 days in the presence of varying concentrations of penicillin, streptomycin, or penicillin combined with streptomycin. The number of positive cultures obtained immediately after adsorption of the organisms to the cells is given at zero-time. The figures shown are the combined results of several experiments. The number of positive bacterial cultures is given for comparison with the number of positive L-form cultures.

Incorporation of penicillin or streptomycin into the tissue culture medium produced similar inhibitory patterns. The number of positive bacterial and L-form cultures was less after 24 hr of exposure of the infected cells to all concentrations

TABLE 1. *Recovery of Brucella abortus L forms and bacteria from hamster kidney cells after intracellular exposure to penicillin and streptomycin*

Antibiotic	Amt added	Type of growth	Period of intracellular exposure to antibiotics in days					
			0	1	3	7	10	14
	μg/ml							
Penicillin	5	Bacteria	7/11*	6/9	8/9	8/9	8/9	6/9
		L form	8/11	6/9	8/9	8/9	8/9	6/9
	10	Bacteria	7/11	3/9	4/9	3/9	8/9	9/9
		L form	8/11	5/9	7/9	7/9	8/9	8/9
	20	Bacteria	6/9	4/7	4/10	2/10	1/10	2/10
		L form	7/9	4/7	8/10	9/10	9/10	10/10
	40	Bacteria	6/9	5/7	3/10	1/10	0/13	2/13
		L form	7/9	2/7	9/10	8/10	8/13	9/13
Streptomycin	5	Bacteria	7/11	3/9	5/9	6/9	6/9	5/9
		L form	8/11	3/9	9/9	5/9	5/9	6/9
	10	Bacteria	7/11	1/9	3/9	9/9	9/9	6/9
		L form	8/11	5/9	8/9	9/9	9/9	6/9
	20	Bacteria	6/9	3/7	3/10	1/10	3/10	2/12
		L form	7/9	2/7	6/10	9/10	9/10	9/12
	40	Bacteria	7/10	3/7	0/11	1/11	4/11	3/13
		L form	8/10	5/7	7/11	6/11	9/11	10/13
Penicillin and streptomycin	2.5	Bacteria	4/6	2/8	2/8	3/8	1/2	0/7
		L form	2/6	8/8	7/8	6/10	2/2	3/7
	5	Bacteria	4/6	3/6	4/10	3/12	0/6	0/6
		L form	2/6	6/8	8/10	8/12	6/7	5/9
	10	Bacteria	4/6	0/6	0/6	0/8	0/6	0/6
		L form	2/6	6/6	5/7	6/6	5/6	4/6
	20	Bacteria	4/6	0/7	0/5	0/9	0/6	0/6
		L form	2/6	7/7	3/5	6/9	4/6	1/6

* Number of positive cultures/total number of cultures.

of penicillin or streptomycin, but an increase in the number of positive cultures occurred after 3 days of growth in cells maintained in medium containing 5.0 or 10 μg/ml of penicillin or streptomycin. Higher concentrations of penicillin or streptomycin controlled bacterial growth. No reduction in the number of positive L-form cultures was observed.

The combined inhibitory effect of penicillin and streptomycin upon the bacterial phase can readily be seen. A significant decrease in the number of positive bacterial cultures was observed after the first day of exposure to 2.5 and 5.0 μg/ml each of penicillin and streptomycin, and, when exposed to higher concentrations, no positive bacterial cultures were obtained after this time. The number of L-form cultures was not appreciably lowered during the first 10 days of exposure to any of the concentrations of penicillin and streptomycin used, and L forms were not eliminated from all of the cultures even after 14 days of exposure to 10 and 20 μg/ml each of these two antibiotics.

Recovery of B. abortus L forms after intracellular exposure to tetracycline. The results of several experiments in which infected hamster kidney cells were maintained for various periods of time in the presence of medium containing tetracycline alone or in combination with penicillin or streptomycin are shown in Table 2. When tetracycline alone was added to the tissue culture medium, there was a rapid rise in the number of positive bacterial and L-form cultures 1 to 3 days after exposure to the antibiotic. The increase was not as great with the higher concentrations of tetracycline. At later time periods, fewer cultures were positive for bacteria or L-forms; however, bacteria were inhibited more rapidly than the L forms. No bacteria were recovered from cells exposed to 2.0 μg/ml of tetracycline for 10 days, but L forms were isolated in numerous Thioglycollate Medium cultures made from cells exposed to 2.0 μg/ml of tetracycline for 14 days.

Tetracycline had an increased inhibitory capacity against the bacterial phase when combined with penicillin or streptomycin, as expected. Al-

TABLE 2. *Recovery of Brucella abortus L forms and bacteria from hamster kidney cells after intracellular exposure to tetracycline*

Antibiotic	Amt added	Type of growth	Period of intracellular exposure to antibiotics in days					
			0	1	3	7	10	14
	μg/ml							
Tetracycline	0.1	Bacteria	5/13*	11/11	11/11	5/9	5/10	2/10
		L form	5/13	11/11	11/11	9/9	7/10	6/10
	0.5	Bacteria	5/13	11/11	11/11	5/9	4/10	2/12
		L form	5/13	11/11	11/11	7/9	9/10	7/12
	1	Bacteria	5/13	8/11	8/11	5/11	0/14	1/9
		L form	5/13	10/11	9/11	8/11	9/14	3/9
	2	Bacteria	5/13	5/11	6/10	5/9	0/10	0/10
		L form	5/13	6/11	7/11	6/9	3/10	1/10
Tetracycline with 5 μg/ml of penicillin	0.1	Bacteria	5/6	6/7	0/5	3/6	0/7	—
		L form	5/6	7/7	3/5	1/6	1/7	—
	0.5	Bacteria	5/6	2/7	0/5	0/6	0/7	—
		L form	5/6	1/7	4/5	1/6	4/7	—
	1.0	Bacteria	5/6	0/6	0/5	0/6	0/7	—
		L form	5/6	5/6	2/5	4/6	2/7	—
	2.0	Bacteria	5/6	0/6	0/5	0/6	0/8	—
		L form	5/6	6/6	3/5	1/6	2/8	—
Tetracycline with 5 μg/ml of streptomycin	0.1	Bacteria	5/6	5/6	1/5	1/6	0/6	—
		L form	5/6	5/6	4/5	1/6	0/6	—
	0.5	Bacteria	5/6	0/7	0/5	0/6	0/8	—
		L form	5/6	5/7	0/5	3/6	6/8	—
	1.0	Bacteria	5/6	1/6	0/4	0/6	0/6	—
		L form	5/6	1/6	2/4	3/6	0/6	—
	2.0	Bacteria	5/6	0/7	0/6	0/6	0/7	—
		L form	5/6	4/7	4/6	1/6	2/7	—

* Number of positive cultures/total number of cultures.

though all bacterial cultures were not negative until after 10 days of exposure to 0.1 μg/ml of tetracycline with 5.0 μg/ml of penicillin or streptomycin, higher concentrations of tetracycline inhibited bacterial growth within 1 day. L-form growth was present throughout the 10-day period, but most Thioglycollate Medium cultures were negative by the end of 7 to 10 days. Inhibition of the latter forms was not enhanced by increasing the concentration of tetracycline combined with penicillin or streptomycin.

Intracellular production of L forms in the absence of antibiotics and in the presence of thallium acetate. The experiments described in the preceding sections demonstrated that the number of L forms increased rapidly in those cells grown in the presence of tetracycline and also, after a brief lag period, in the presence of penicillin or streptomycin. It was possible, therefore, that L forms might not be produced in the absence of antibiotics. It was also possible that the L forms were being produced during disintegration of the cells and were not actually present in the intact monolayers. Two experiments were set up to test these possibilities. The first was designed to determine whether L forms could be recovered from hamster kidney cells maintained in antibiotic-free medium. This was accomplished by growing the infected cells in antibiotic-free medium at *p*H 7.4. At various time intervals, this medium was replaced by medium with a *p*H of 7.8, which did not allow further cellular metabolism and caused disintegration within a few days. The second experiment was similar, except that, when the *p*H was adjusted to 7.8, thallium acetate in a final concentration of 1:5,000 was added to inhibit bacterial growth and prevent conversion to the L phase.

The results of the two experiments are given in Table 3. In the first experiment in which no bacterial inhibitor was added, the number of positive bacterial and L-form cultures was high. The presence of thallium acetate, added in the second experiment at zero-time and at 1, 3, 7, and 10 days, inhibited all bacterial growth. The number of positive L-form cultures was also less in the latter experiment, but a relatively constant number of Thioglycollate Medium cultures contained L-form growth throughout the 10-day period.

Antibiotic sensitivity of B. abortus before and after intracellular exposure to antibiotics. Antibiotic sensitivity determinations were carried out on a number of reisolated organisms, as well as on the stock strain of *B. abortus* 3183, by an agar dilution technique. The concentration of antibiotics utilized corresponded to those levels used in the intracellular studies. The results (Table 4) indicate the maximal concentration of the antibiotic or combinations of antibiotics to which the recovered *Brucella* organisms and stock strain were resistant. The original stock strain and a subculture which had been maintained on artificial media had the same antibiotic sensitivity patterns; both were sensitive to the lowest levels of antibiotics used in all cases. *B. abortus* organisms recovered from infected tissue culture cells after exposure to penicillin or tetracycline for various periods of time had a slightly increased resistance to tetracycline, from 0.1 μg/ml or less observed with the stock strains to 0.5 μg/ml with the recovered cells, and a slightly increased resistance to penicillin of from 5.0 to 10 μg/ml in the stock strains and recovered cells, respectively.

Although the development of slight penicillin and tetracycline resistance in these organisms did not explain their survival during treatment of tissue culture cells with high concentrations of these antibiotics, bacteria exposed to streptomycin intracellularly had developed a high level of resistance. After 10 and 14 days of intracellular exposure to streptomycin, the recovered bacteria were resistant to 40 μg/ml or more of streptomycin, 10 μg/ml of penicillin, and 5.0 μg/ml each of penicillin and streptomycin. Because the recovered bacteria were generally sensitive to levels of the antibiotic concentration being used in the tissue culture medium, reversion from the altered state presumably occurred at a time immediately prior to, or after, removal of antibiotics from the tissue culture medium.

TABLE 3. *Recovery of Brucella abortus L forms and bacteria from tissue culture cells in the absence of antibiotics and in the presence of thallium acetate*

Condition	Period of intracellular growth in days				
	0	1	3	7	10
No bacterial inhibitor*					
Bacteria	14/17†	7/7	6/6	6/7	6/7
L form	13/17	7/7	6/6	6/7	6/7
Thallium acetate (1:5,000) added at the end of growth period					
Bacteria	0/17	0/7	0/7	0/7	0/7
L form	9/17	4/7	4/7	6/7	3/7

* At the end of the growth period, the growth medium (*p*H 7.4) was replaced and the *p*H was adjusted to 7.8.

† Number of positive cultures/total number of cultures.

TABLE 4. *Antibiotic sensitivity of Brucella abortus before and after intracellular exposure to antibiotics*

Antibiotic	Amt added	Time after exposure	Level of antibiotic resistance*					
			P	S	PS	T	TP	TS
	μg/ml							
Tetracycline	0.1	24 hr	10	<5	<2.5	0.5	<0.1	0.1
		72 hr	10	<5	<2.5	0.5	<0.1	0.1
		10 days	10	<5	<2.5	0.5	<0.1	0.1
		14 days	10	<5	<2.5	0.5	<0.1	0.1
	1.0	72 hr	10	<5	<2.5	0.5	<0.1	0.1
		5 days	10	<5	<2.5	0.5	<0.1	0.1
		10 days	10	<5	<2.5	0.5	0.1	0.1
Penicillin	40.0	14 days	10	<5	<2.5	0.5	0.1	0.1
Streptomycin	10.0	10 days	10	>40	5.0	<0.1	0.1	0.1
	40.0	10 days	10	>40	5.0	<0.1	0.1	0.1
		14 days	10	>40	5.0	<0.1	0.1	0.1
Original culture†			5	<5	<2.5	<0.1	<0.1	0.1
Subculture			5	<5	<2.5	<0.1	<0.1	0.1

* Amounts given are maximal concentrations of antibiotic or combinations of antibiotics allowing bacterial growth. The ranges of concentrations used were: 5.0 to 40 μg/ml of penicillin (P) or streptomycin (S), 2.5 to 20 μg/ml each of penicillin and streptomycin (PS), 0.1 to 2.0 μg/ml of tetracycline (T) or tetracycline with 5 μg/ml of penicillin (TP), or tetracycline with 5 μg/ml of streptomycin (TS).
† *B. abortus* 3183.

DISCUSSION

Penicillin was expected to promote intracellular production of L forms to a greater extent than streptomycin, but, upon examination of the results obtained over a 14-day period, only slight differences could be seen between the number of positive L-form cultures obtained after exposure of the infected cells to penicillin and the number obtained after exposure to the same concentrations of streptomycin. The results confirmed the findings of Richardson and Holt (20) that neither penicillin nor streptomycin was capable of controlling intracellular *B. abortus* multiplication effectively when used separately. Although in the present studies L forms were recovered from a greater number of cultures than were bacterial forms, it could not be determined whether the L forms were capable of maintaining themselves for any length of time in the tissue cultures containing penicillin or streptomycin alone, owing to the continued sporadic recovery of bacterial forms. However, the results obtained when infected cells were exposed to both penicillin and streptomycin not only verified the report of Richardson and Holt (20) regarding a synergistic bactericidal activity of these two antibiotics upon intracellular *B. abortus*, but also demonstrated the continued presence of L forms throughout the 14-day culture period. The number of positive cultures decreased as the concentrations of the combined antibiotics increased, but isolation of L forms for periods from 2 to 13 days after viable bacterial forms were no longer detected indicated that some L forms either survived intracellularly for this entire period or were multiplying at a high enough rate to maintain a low level of infection throughout this time.

Tetracycline proved to be the most effective antibiotic in controlling both bacterial and L-form growth, especially when combined with 5 μg/ml of streptomycin. Although exposure of infected cells to 0.1 and 0.5 μg/ml of tetracycline produced results similar to those obtained with 5 and 10 μg/ml of streptomycin or penicillin, an increase in the concentration of tetracycline to 2.0 μg/ml resulted in an inhibitory pattern comparable to that obtained with 20 μg/ml each of penicillin and streptomycin. Elimination of all bacterial forms was attained within a short period of time when 1.0 or 2.0 μg/ml of tetracycline alone was used, and with all concentrations of this antibiotic when combined with penicillin or streptomycin; however, L forms were still recovered in small numbers from some of the infected tissue culture cells for a period of 8 to 10 days longer. The greater effectiveness of low concentrations of tetracycline in reducing the number of positive L-form cultures may explain its ability to decrease the percentage of recurrent infections in vivo (3, 22), and also verifies the results of Richardson and Holt (20) concerning the striking in vitro inhibition of *B. abortus* by tetracycline, particularly in combination with streptomycin.

Because L forms were present in Thioglycol-

late Medium cultures obtained from tissue culture cells after 5 hr of exposure to *B. abortus* 3183, the influence of antibiotics upon their production was questionable. In addition, a possibility existed that L forms were being produced in the antibiotic-free medium after disintegration of the tissue culture cells. The Thioglycollate Medium cultures inoculated with infected cells which had been maintained in antibiotic-free medium also contained L-form growth. This confirmed the suspicion that production of L forms by the intracellular bacteria was not dependent upon the presence of antibiotics. In identical experiments in which thallium acetate was added to the medium at various time periods after infection of the tissue culture cells to prevent further growth or reversion of the bacterial forms, the number of positive L-form cultures was reduced by 40 to 60%. Addition of thallium acetate to the replacement medium demonstrated, however, that a small number of intracellular L forms were persistent throughout the 10-day period after the cells were infected.

It is difficult to determine whether reversions from L forms to bacteria have occurred intracellularly. The ability of the intracellular L forms to undergo this reverse step, of course, is theoretically essential to initiation of recurrent active infection. Indirectly the antibiotic sensitivity tests carried out by an agar-plate technique suggested that some reversions had occurred. Whether this transition took place intracellularly or at the time of cellular disruption could not be ascertained. Development of bacterial resistance was apparent to a significant degree only in those organisms which were recovered from hamster kidney cells maintained in medium with streptomycin. It does not seem likely, therefore, that organisms recovered from occasional tissue cell cultures after 10 to 14 days of exposure to 40 μg/ml of penicillin or 1.0 μg/ml of tetracycline had survived in the bacterial state throughout this period. Although the bacteria may have survived in an inactive state or were protected by the host cell, spontaneous reversions from the antibiotic-resistant forms may have occurred. No reports of increased bacterial resistance to antibiotics after their recovery from the L phase have been noted (1, 24).

Several potential interrelationships between *B. abortus* and the host cell which may promote spontaneous intracellular induction of L forms can be discussed. L forms were demonstrated immediately after adsorption of *B. abortus* 3183 to the hamster kidney cells, both by direct observation and by isolation in Thioglycollate Medium. Since the organisms were situated just inside the cell membrane at this time, it is possible that a lysozyme-like substance or amino acids were present at the cellular surface, which enhanced L-form production. Lysozyme and glycine have been used to induce L-form production in artificial media (21, 14), and it has been reported that glycine and a lysozyme-like substance from rabbit monocytes inhibited intracellular *Brucella* organisms (17, 18). Another possible mechanism of L-form induction is implied by studies in which a bacteriophage has been found in recently isolated *B. abortus* strains (15, 16, 19). The bacteriophage was described as weakly lytic, and it was noted that the lysogenic bacteria produced atypical colonies. Pickett and Nelson (15, 16) also demonstrated the presence of brucellaphage in plasma from patients with brucellosis and concluded that it was responsible for inducing L forms in vivo. Intracellular activity of brucellaphage has since been shown in vitro (11), but no attempt was made to show L-form induction. Recovery of L form-like stages of *B. abortus* which often reverted to atypical bacterial types (6) suggests that the L forms may be produced as the result of intracellular phage activity. Further studies on recovery of L forms after treatment of intracellular brucella with phage, lysozyme-like substances, or amino acids may provide some evidence pertaining to the role of these agents in the spontaneous induction of intracellular L forms.

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