IMMUNIZATION AGAINST BRUCELLA INFECTION

I. Isolation and Characterization of a Streptomycin-Dependent Mutant[1]

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An examination of the present status of active immunization against brucella infection as practised upon domestic animals leads one to the conclusion that the only relatively effective procedure utilizes a living organism unique in its combination of avirulence with immunogenic capacity. Although several promising investigations are in progress in which dead cells or antigens extracted therefrom are being used, such studies are still in a stage precluding profitable discussion. The working hypothesis in immunization studies against brucellosis has in common with that for tuberculosis the idea that successful immunization against infection requires that the host undergo an active infection of some duration, that bacterial multiplication, or at least persistence, be induced, following which the host develops a relative resistance to reinfection. Whether this immunization is followed by sterilization of the host's tissues or whether the vaccine strain persists indefinitely in the host's tissues appears not to have been decided conclusively.

In view of the pressing need for an effective control of *Brucella melitensis* infection the investigations to be detailed in this first report were begun. These studies had for their objective the isolation of a strain of *B. melitensis*, the peculiar growth requirements of which could be used to advantage in controlling the extent and severity of the immunizing infection. By the ability to control the growth of the organism, the immunizing infection could theoretically be terminated whenever desired and then the organisms would be cleared eventually from the host as a result of the newly acquired immune state.

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EXPERIMENTAL METHODS

Experimental animals. Male mice of the BRVS Webster strain weighing 18 to 20 grams were obtained from a single source. Guinea pigs of both sexes weighing 300 to 500 g were obtained from various sources. The mice when used experimentally were allotted to the various groups by random selection; no attempt was made to randomize guinea pigs.

Typing of cultures. The dye resistance and H_2S production tests used for classification were those described by Huddleson (1943). The urease test was that described by Hoyer (1950). Strain 6056 was kindly sent by Dr. M. R. Castañeda and was inhibited by pyronin (1:100,000) and methyl violet (1:100,000) but grew in the presence of basic fuchsin (1:50,000) and thionin (1:50,000). No H_2S was produced during 4 days' incubation, and no urease activity was demonstrable after 24 hours.

Agglutination reactions were incubated at 37 C for 24 hours followed by 24 hours at 5 C. The titer of the sera was expressed in terms of the highest dilution of serum giving complete agglutination.

Population studies. To study population changes of the smooth and rough type, heavily seeded plates of "2-1 agar" were observed after 4 days of incubation under 3× magnification, using indirect transmitted oblique light according to the method of Henry (1933). All cultures were studied frequently for their inagglutinability in acriflavine according to the method of Braun and Bonestell (1947).

Experimental infection. Animals were infected routinely via the subcutaneous route. The procedure followed that of previous studies (Elberg *et al.*, 1951).

Infection was evaluated in each animal by spleen culture. This procedure was found to be most effective as this organ invariably contained viable organisms whenever generalized brucella

infection occurred in mice and guinea pigs (Striedter, 1939). The spleen was homogenized in a sterile mortar and pestle with a little sand, and saline (1.0 ml per mouse spleen and 5.0 ml per guinea pig spleen) was added. The homogenates were cultured in triplicate on Albimi or tryptose agar. An animal was considered to be infected if a single *Brucella* colony were isolated from the homogenate. The number of colonies per plate allowed an approximation of the spleen count.

Tests for significance. When groups were compared, the significance of the differences between groups was evaluated by the "chi square" method (Fisher, 1948) using the Yates *Correction for Continuity* when small numbers were involved (Yates, 1934). Values of P less than 0.05 were interpreted as indicating nonhomogeneity of the data.

Virulence screening test in mice. Upon comparing the splenic infections produced in mice by the virulent *B. melitensis*, strain MM472, and by the relatively avirulent *B. abortus*, strain A19, it was found that the greatest difference between the two strains was manifest two weeks after an infective dose of 5×10^4 to 1×10^5 organisms had been administered subcutaneously (see figure 1). At this period the avirulent organism could not be isolated from the spleen whereas the virulent organism was cultivable from 4/5 to 5/5 of the animals.

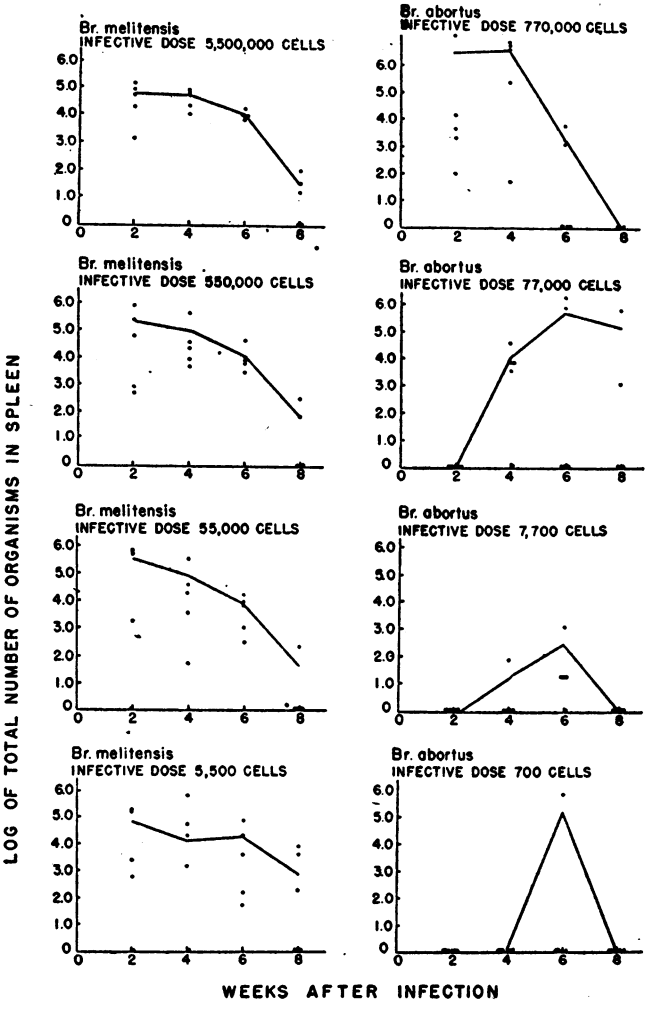


Figure 1. Splenic infection in mice inoculated with varying numbers of virulent *Brucella melitensis* (strain MM472) and avirulent *Brucella abortus* (strain A19) after varying periods of time.

RESULTS

A single colony grew from an inoculum of 10^8 cells of *B. melitensis*, strain 6056, on a plate of Albimi agar containing 5,000 μg streptomycin per ml. This was subcultured onto agar containing 1,000 μg drug per ml, the growth from which grew well on further subculture in agar containing 1,000 μg drug per ml but only slightly in the absence of the antibiotic.

A suspension of dependent cells was inoculated onto streptomycin agar. Of the 66 colonies on the plate of streptomycin agar (none having grown on streptomycinless agar), 30 were tested for dependence upon streptomycin. Growth occurred in each of the 30 cases only in streptomycin agar. Portions of these cultures were transferred to slants containing 500 μg streptomycin per ml and carried henceforth as the dependent strain.

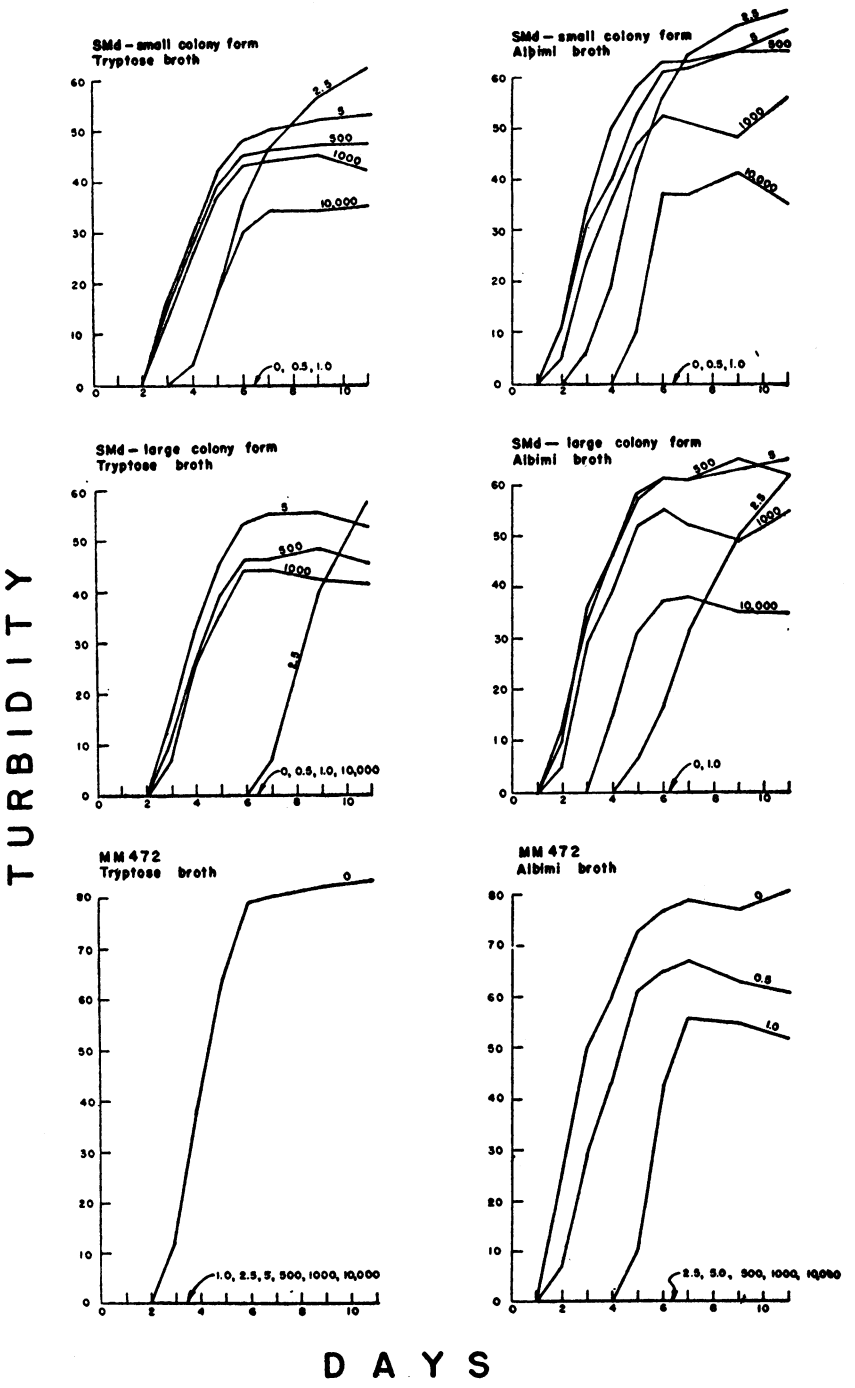
Equally good growth could be obtained on agar containing amounts of streptomycin ranging from 5 to 500 μg per ml; concentrations above 50 μg per ml increased the lag phase when as few as 1,000 cells constituted the inoculum. When 10^6 cells were inoculated, the delayed growth effect was not apparent.

To study the effects of the concentration of streptomycin in liquid medium on growth of small numbers of organisms, tubes containing three different media and varying concentrations of streptomycin were inoculated with 10^4 cells per ml. (These cells had been cultivated on 500 μg streptomycin per ml and had not been washed.) Changes in transmission compared with an appropriate blank were measured on the Universal Coleman spectrophotometer at 6500 A daily for eleven days. A similar experiment was carried out with a streptomycin-sensitive strain of *B. melitensis*.

The results appear in figure 2. Maximal total growth and rate of growth were obtained with concentrations ranging from 5 to 500 μg streptomycin per ml. A concentration of 1,000 μg per ml consistently yielded less total growth while 10,000 μg per ml inhibited growth and in one case prevented visible turbidity entirely. The lowest concentration (2.5 μg per ml) just inhibiting the susceptible strain was the concentration which just supported the growth of the dependent strain. The growth of the dependent organism at this concentration was delayed, but eventually total growth reached the same level as in higher concentrations of streptomycin. Concentrations below 2.5 μg per ml failed to support the growth of the organism. The Gerhardt-Wilson synthetic medium failed to support the growth of the streptomycin-dependent variant regardless of streptomycin content just as it also failed to support the growth of the parent strain (MM6056). No differences were observed between Albimi and tryptose broths in terms of streptomycin requirement although the lag phase in Albimi broth was consistently shorter. No essential differences in streptomycin requirement or rate of growth were demonstrable between the large and small colony forms of the streptomycin-dependent variant.

Since a factor other than streptomycin, namely oxygen tension, was involved in limiting the growth in static culture, the experiment was repeated with the following modifications, starting with approximately 5×10^7 to 1×10^8 cells per ml which yielded a reading of 80 to 90 per cent transmission on the spectrophotometer enabling the lag phase to be measured. The organisms (streptomycin-dependent/small) which had been grown on agar containing 500 μg streptomycin per ml were washed three times in saline and resuspended in saline. This was done to remove all extracellular streptomycin. The inoculum was placed in the tubes containing varying concentrations of the drug in Albimi broth. The tubes were shaken at a rate of 200 oscillations per minute with a 1.5 inch stroke in a water bath held at 37 C. A duplicate series was incubated statically. The turbidities were measured then as per cent transmission against a clear broth blank in the Coleman Universal spectrophotometer at 4250 A at varying intervals.

The results are presented in figure 3. After a lag period of about 20 hours, turbidity increased rapidly in streptomycin concentrations from 25 to 1,000 μg per ml. The maximum growth was attained at 72 hours. Concentrations of 10 and 5 μg per ml yielded somewhat delayed growth, but the same total growth was attained at 65 and 82 hours, respectively. In 2.5 μg per ml the growth was greatly delayed, requiring 204 hours to attain maximum turbidity. This delayed growth appeared to be due to a slower rate of growth rather than a longer lag period. Concentrations of 2,500 and 5,000 μg per ml inhibited growth to a certain extent, but the final yield was the same as had occurred in the more optimal concentrations.



NUMBERS ON EACH CURVE DESIGNATE THE CONCENTRATION OF SM IN THE MEDIUM (MICROGM./ML.)

Figure 2. Effect of streptomycin on growth of strain of *Brucella melitensis*.

Some growth was evident in the absence of streptomycin. This probably was due to the intracellular carry-over of the drug, enabling the cells to multiply in the absence of the antibiotic in the medium until the intracellular supply was consumed. This phenomenon of "residual growth" in aerated cultures due to the limiting effect of oxygen tension.

It was noted that growth obtained with a large inoculum yielded a predominance of small colonies interspersed with a few large colonies. The large colony forms bred true on streptomycin

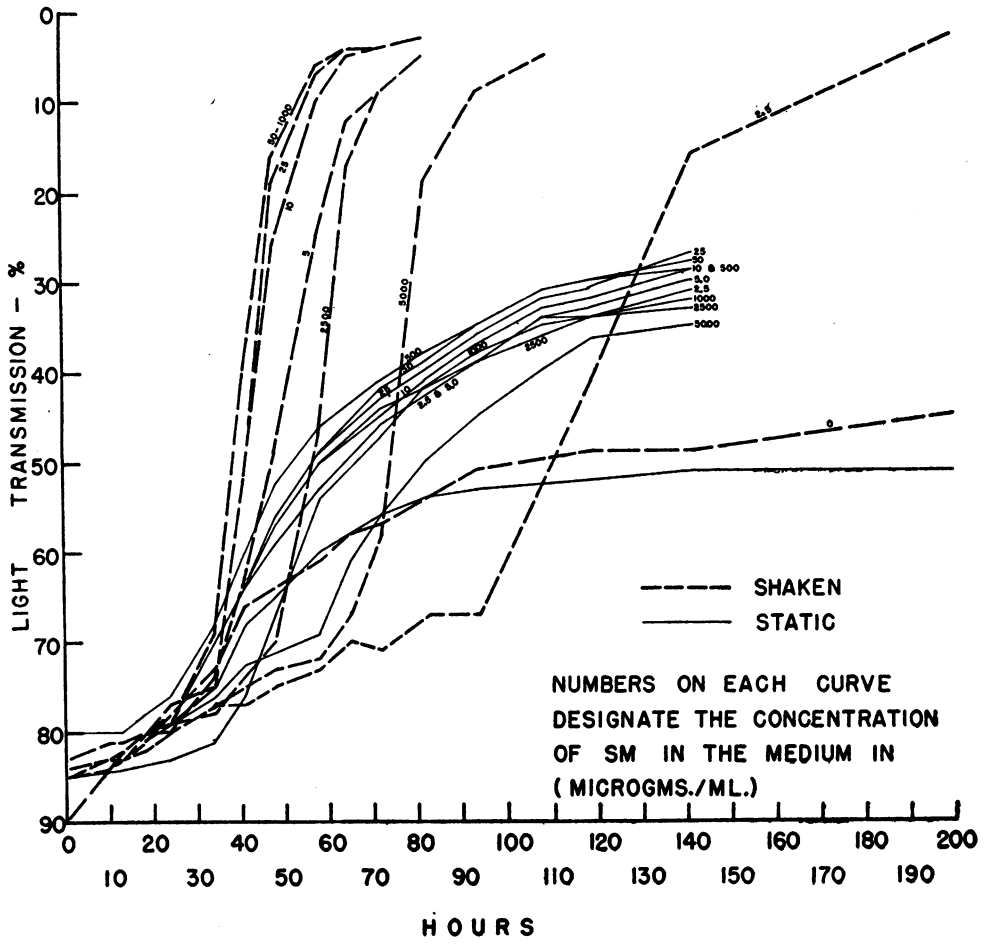


Figure 3. Effect of shaking on the growth of the dependent mutant in streptomycin broth.

(Bertani, 1951) will be described in a later section of this study.

In static culture the organism exhibited a greatly retarded rate of growth and total cell crop. It was evident that this strain was highly aerobic and required large amounts of oxygen in order to obtain optimal growth conditions in the medium. Differences in rate of growth and total growth were not as striking in static culture as containing media while the small forms continued to throw off large colonies. These two variants were designated *streptomycin-dependent/small* and *streptomycin-dependent/large.*

Gram stain preparations of the streptomycin-dependent variants revealed the typical morphology of *B. melitensis*, being small, gram negative coccobacilli. The colonial appearance of the variants was that of the classically smooth, ir-

ridescent, even-edged, raised brucella colony. The individual colonies of the streptomycin-dependent/small strain were minute after 96 hours of incubation but became normal in size after 144 hours.

The streptomycin-dependent variant possessed the same characteristic susceptibility to the presence of various dyes incorporated into the medium as did the parent strain.

The small colony variant, killed with 0.5 per cent phenol, was tested for agglutinability in the serum of a goat infected with *B. melitensis* (MM6015). This serum, which agglutinated a standardized abortus agglutinating antigen to a titer of 1:1,280, agglutinated the streptomycin-dependent suspension completely at a dilution of 1:2,560. The antigenicity of the streptomycin-dependent variant, both in the living and heat killed (100 C) state, was tested in the guinea pig. Single or repeated inoculations of 10^{10} viable cells stimulated the appearance of agglutinins (range 1:20 to 1:320, medians 1:40 and 1:160). The titers so induced were much lower than those obtained in guinea pigs infected for a similar period with a virulent strain of *B. melitensis* (range 1:20 to 1:640+, median 1:640). Heat killed cells, on the other hand, yielded slightly higher titers than those resulting from the inoculation of viable streptomycin-dependent cells (range 1:40 to 1:320, median 1:160).

Normal goats inoculated subcutaneously with large numbers of viable streptomycin-dependent cells yielded high agglutination titers (range 1:160 to 1:2,560) when their sera were tested against a standardized abortus antigen. These titers were much higher for the time periods involved than were the titers produced by the virulent strain (MM6015) administered to normal goats (range 1:80 to 1:320). These results are to be contrasted with those obtained in the guinea pig, and it appears that the two host species may differ either in their capacity to allow growth of the streptomycin-dependent variant or in their capacity to produce agglutinins.

A *Macaccus rhesus* monkey inoculated with 10^6 viable streptomycin-dependent cells intravenously produced an agglutinin titer of 1:80 when tested against streptomycin-dependent phenolized antigen and 1:40 against a standardized abortus agglutinating antigen when the serum was tested 20 days after inoculation. Another monkey inoculated with 10^{10} viable streptomycin-dependent cells subcutaneously produced titers of 1:80 and 1:20 against the respective agglutinating suspensions after the same period.

Thus, the streptomycin-dependent variant possesses essentially the same antigens as the wild type *B. melitensis* and *B. abortus*.

Since the guinea pig is so highly susceptible to small numbers of *Brucella*, it was selected as the best indicator of virulence of the streptomycin-dependent/small variant. As many as 65,000 viable cells, inoculated subcutaneously, failed to persist in the spleens of guinea pigs for 4 weeks. The spleens were perfectly normal in size and appearance, and the spleen weights, expressed as per cent of total body weight, in all groups varied within normal limits (DeRopp, 1945). The regional lymph nodes draining the site of inoculation (inguinal) were small and uninjected. Thus, 65,000 organisms was less than the minimal infecting dose for this strain in the guinea pig. In another experiment (see table 4) 10^8 to 10^{11} viable cells administered subcutaneously or intravenously failed to yield viable organisms from the spleens 4 weeks after inoculation. These animals failed to show any evidence of splenomegaly, adenopathy of the regional lymph nodes, or any gross evidence of lesion formation or tissue damage. Organisms were recovered from 4/11 of the animals which had been inoculated intravenously, but they occurred in extremely small numbers and were still dependent on streptomycin for growth.

In order to approximate the effects of administration of the dependent strain in an animal closely related to man, two *Macaccus rhesus* monkeys were inoculated, one with 10^6 cells intravaneously and the other with 10^{10} cells subcutaneously. The animal inoculated intravaneously maintained normal body temperature during the 33 day observation period. Subcutaneous inoculation produced a small wheal at the site which disappeared within 6 days, a slight temperature rise which returned to normal after 6 days, and no enlargement of the axillary lymph nodes was observed. Streptomycin-dependent organisms were recovered from the blood of both animals on the 10th day, but the blood cultures were negative on the 14th and 20th days.

The effect of administration of streptomycin to the host on the growth of the organism *in vivo* was studied in groups of 15 mice. Then the groups were treated according to different sched-

ules with streptomycin at levels just below the predetermined toxic dose of the antibiotic for the mouse. The antibiotic was administered to some groups in a water-in-oil emulsion[2] so as to give a prolonged streptomycin effect because of its slow absorption. Other groups received multiple doses of the antibiotic in saline and two control groups received no antibiotic. After 1, 2, and 4 weeks, 5 animals from each group were sacrificed and spleen homogenates were cultured quantitatively on both streptomycin containing and streptomycin-free media.

The results appear in table 1. Viable organisms were cultured in one week from 5/5 of the control animals which did not receive streptomycin (groups G and H). However, these colonies appeared only on the streptomycin containing agar, hence were still dependent on the antibiotic for their growth. Since the spleens were not heavily infected and large numbers of organisms had been administered, it could not be determined whether the organisms recovered were simply survivors of the original inoculum or whether they represented progeny due to *in vivo* growth of the organism. It is difficult to conceive of such lengthy survival (some even to the 2nd and 4th week) in the absence of what appears to be a necessary growth substance for this mutant, but such a possibility exists. In either case the organisms may have been able to obtain some substitute or analogue of the drug either for survival or multiplication, or their metabolism *in vivo* was shunted around the streptomycin requirement. These results are contrasted strongly in the guinea pig where much larger inocula failed to yield organisms 4 weeks after inoculation.

The administration of the drug to the host had little effect upon recovery of the organisms from the spleens. Infections were light in all groups as compared with the infections produced by virulent strains using smaller numbers of cells. In all groups the animals had practically cleared themselves of infection by the 4th week.

All the organisms which could be recovered from these animals were still dependent on streptomycin. No growth was observed when replicate samples of homogenates were inoculated onto streptomycin-free media. Their growth rate after isolation from the host was still very slow, requiring 6 to 7 days for microscopic colonies to appear. The absence of microscopic growth was checked carefully under the dissecting microscope.

The experiment was repeated with some modifications using larger numbers of animals. Smaller doses of streptomycin were employed due to the greater toxicity of this particular batch of drug. The drug was administered intravenously to some groups in order to determine if by this route it was more readily available to the organisms. Two groups received the drug one week after infection in order to provide a stimulus at a period when the animal was actively eliminating the organisms.

The results of the second experiment (table 2) were essentially similar to the first. However, the effect of survival of the original inoculum (10^8 cells) was even more marked. In all cases 91 to 100 per cent of the animals yielded organisms from their spleens two weeks after inoculation and 50 to 69 per cent contained organisms even at a period 5 weeks after inoculation. Again the administration of the drug had no significant effect on the recovery of the organisms, either in the numbers of animals infected or in the numbers of organisms isolated. The following analysis bears this out. A comparison of all groups at 4 weeks' infection yielded a chi square value of 1.149, which for 5 degrees of freedom yielded a probability of $0.95 - 0.90$. This would indicate that the data were homogenous and that any differences observed could easily have occurred by chance. The analysis of the groups after 5 weeks' infection revealed a chi square value of 4.064, which for 5 degrees of freedom yielded a P value of $0.70 - 0.50$, again demonstrating the homogeneity of the data. Although 50 to 69 per cent of the animals were infected at 5 weeks, the numbers of organisms isolated were extremely small and the animals would undoubtedly have cleared their spleens within a short subsequent period.

It therefore was concluded that the drug was unavailable to the organisms, probably due to the intracellular position of the bacilli in the host tissues. Magoffin and Spink (1951) have demonstrated that the streptomycin is unable to penetrate polymorphonuclear leucocytes and

[2] One part streptomycin:1 part "bayol F":0.1 part "arlacel A", prepared by adding the solution of streptomycin slowly to the oily mixture in a mortar with continuous grinding with a pestle (Freund and Bonanto, 1944).

TABLE 1

Effect of antibiotic treatment on recovery of streptomycin-dependent variant from mice

GROUP	NUMBER CELLS USED TO INFECT	AMOUNT OF STREPTOMYCIN (SUBCUTANEOUSLY)	TOTAL ORGANISMS IN SPLEEN (WEEKS)			INFECTED/TOTAL (WEEKS)		
			1	2	4	1	2	4
A	10^8	5 daily doses of 10,000 μg each in saline	16 550 2,150 0 0	150 50 2,300 16 400	0 16 65 — —	3/5	5/5	2/3
B	10^6	5 daily doses of 10,000 μg each in saline	300 50 0 0 0	5,500 400 300 0 0	0 — — — —	2/5	3/5	0/1
C	10^8	1 dose of 10,000 μg*	50 16 16 0 0	33 0 0 0 0	0 0 0 0 0	3/5	1/5	0/5
D	10^8	1 dose of 50,000 μg*	16 2,100 200 50 0	16 2,150 200 0 0	80 0 0 0 0	4/5	3/5	1/5
E	10^6	1 dose of 10,000 μg*	6,000 1,700 1,100 33 0	550 150 200 0 0	0 0 0 — —	4/5	3/5	0/3
F	10^6	1 dose of 50,000 μg*	20,000 16 0 0 0	4,700 1,200 400 0 0	33 0 0 0 0	2/5	3/5	1/5
G	10^8	None	250,000 18,000 900 200 33	400 250 33 0 0	0 0 0 0 0	5/5	3/5	0/5
H	10^6	None	15,000 4,300 50 33 16	2,500 700 0 0 0	33 0 0 0 0	5/5	2/5	1/5

* Streptomycin mixed with "arlacel A" and "bayol F" (see text).

fails to act upon brucellae which are within these cells. Mackaness (1952) has demonstrated the same lack of effect of streptomycin on *Mycobacterium tuberculosis* when located within the macrophage in tissue culture. That brucellae occupy an intracellular position in the host has been amply demonstrated (Castañeda, 1947), and this undoubtedly held true for the streptomycin-dependent variant.

On original isolation the streptomycin-dependent variant was an antigenically smooth clone. It was also inagglutinable by acriflavine. All subsequent transfers of stock cultures, of both large and small colony forms, and vaccine preparations failed to exhibit nonsmooth mutants in the population when studied on "2-1 agar" or tested by the acriflavine reaction.

Cultivation for 11 days in broth containing varying concentrations of streptomycin yielded some dissociation although in no case was it excessive. Prolonged cultivation of streptomycin-dependent variants for 11 days in the presence of varying concentrations of streptomycin failed to yield organisms which were nutritionally independent of the drug, for in no case was growth observed when transplants were made on streptomycin-free media. Although the turbidities of these cultures indicated the presence of 10^9 to 10^{10} cells per ml, reversions to independence did not appear. This is in marked contrast to the behavior of the susceptible strain growing in the presence of small concentrations of streptomycin wherein large numbers of drug resistant variants were isolated.

In order to determine if the delayed growth observed in figure 2 was due to the presence of a mutant which either required less streptomycin or was independent of the drug, the growth from those cultures which contained 2.5 μg streptomycin per ml and which had grown slowly was diluted and inoculated into fresh media containing 0, 2.5, and 500 μg streptomycin per ml. The dilutions were such that the fresh medium received approximately 10^4 cells per ml as determined turbidimetrically. Again the pattern of delayed growth in 2.5 μg per ml, the more rapid growth in 500 μg per ml, and the absence of growth in the absence of streptomycin was dem-

TABLE 2

Effect of antibiotic treatment by various routes on recovery of streptomycin-dependent variant from mice

GROUP	SCHEDULE OF STREPTOMYCIN ADMINISTRATION	INFECTED/TOTAL (WEEKS AFTER INFECTION)				AVERAGE SPLEEN COUNT (WEEKS AFTER INFECTION)			
		1	2	4	5	1	2	4	5
A	5,000 μg s.c. simultaneous with infection	11/11 100%	12/12 100%	7/12 59%	2/4 50%	10,000	5,000	900	60
B	As above, then 5,000 μg s.c. daily for 5 days	12/12 100%	12/12 100%	8/12 67%	2/4 50%	20,000	9,000	500	20
C	1,500 μg i.v. simultaneous and daily 5 days after infection	12/12 100%	12/12 100%	7/12 59%	0/3 0%	5,000	5,000	100	0
D	5,000 μg s.c. 1 week after infection	— —	11/12 91%	10/12 83%	9/16 56%	—	2,000	200	200
E	5,000 μg s.c. daily during 2nd week after infection	— —	11/12 91%	10/12 83%	9/16 56%	—	30,000	500	50
F	None	12/12 100%	12/12 100%	9/12 75%	11/16 59%	40,000	20,000	300	30

All animals received an initial inoculation of 1.95×10^8 viable streptomycin-dependent organisms, subcutaneously.

onstrated. This would indicate that cultivation of the streptomycin-dependent variant in broth culture, even in suboptimal amounts of the drug, had failed to yield mutants of lesser dependence on streptomycin.

Strains of streptomycin-dependent organisms described in the literature are known to revert to lack of dependence by a mutational step. Bertani (1951) describes such a reversion rate in *Escherichia coli* as 1.4 per 10^8 per generation. Since large numbers of streptomycin-dependent organisms were to be used for immunization, the possibility of reversions was important. If such

TABLE 3

Reversion of streptomycin-dependent variant from dependence to independence

NO. OF ORGANISMS PLATED	NO. OF REVERTED COLONIES*	NO. OF REVERSIONS PER 10^8 ORGS. PLATED
Albimi agar		
2.06×10^9	71	3.4
2.06×10^8	11	5.3
2.06×10^7	1.4	6.8
2.06×10^6	0	—
2.06×10^5	0	—
"Tissue-extract-Albimi agar"		
2.06×10^9	64	3.1
2.06×10^8	6	2.9
2.06×10^7	1.4	6.8
2.06×10^6	0	—
2.06×10^5	0	—

* Each number represents arithmetic mean of 5 plates on drug-free medium.

mutants were characterized by streptomycin resistance or sensitivity, they might also regain their virulence, assuming that dependence of the organism on a substance foreign to the host was the governing factor originally in its lack of virulence for such a host.

In order to estimate the approximate number of nondependent organisms in the stock cultures, organisms were grown on Albimi agar containing minimal amounts of streptomycin (5 to 10 μg per ml). This cultivation on small amounts of streptomycin was necessary in order to minimize "residual growth" on the streptomycin-free medium due to carry-over of the drug either intra- or extracellularly. Such background growth might have created the impression of larger numbers of reversions per unit of the parent population. A heavy suspension was prepared by washing off the plate cultures with normal saline, and a viable count was performed on streptomycin containing medium. The original suspension and 4 tenfold dilutions were plated then in 0.1 ml amounts onto each of 5 plates per dilution on Albimi agar free of streptomycin. The dilutions were plated also to obviate the possibility of inhibition of the growth of the reversions by crowding effects of the large nongrowing dependent population as described by Bertani (1951). This experiment also was repeated on Albimi agar enriched with 1.0 per cent filtrate

TABLE 4

In vivo reversion of streptomycin-dependent variant from dependence to independence

GROUP	NUMBER OF CELLS USED	ROUTE	CULTURED ON STREPTOMYCIN-FREE MEDIUM: INFECTED/ TOTAL	CULTURED ON STREPTOMYCIN CONTAINING MEDIUM: INFECTED/ TOTAL
A	1.06×10^{11}	s.c.	0/8	0/8
B	1.06×10^{10}	s.c.	0/10	0/10
C	1.06×10^9	s.c.	0/10	0/10
D	1.06×10^8	s.c.	0/10	0/10
E	1.06×10^{11}	i.v.	0/4	2/4*
F	1.06×10^{10}	i.v.	0/7	2/7*

* These animals were infected very lightly as only 1, 2, or 3 colonies were cultured from each.

of homogenized spleen to simulate *in vivo* conditions. The results are presented in table 3. Approximately 3 to 6 reversions were present for every 10^8 dependent cells when plated on either medium when the initial cultivation had been conducted on low concentrations of streptomycin.

One hundred reverted colonies were tested then by double-plating onto streptomycin containing (500 μg per ml) and streptomycin-free media of which 98 clones grew only on streptomycin-free media and hence had reverted to a streptomycin-sensitive state. The other two clones were streptomycin-resistant. Since most of the reversions were sensitive, it is not surprising that none were detected in liquid culture even in the presence of a concentration of streptomycin suboptimal for the growth of the parent dependent strain.

The effect of such reversions *in vivo* was

studied in the guinea pig, which is susceptible to infection by small numbers of virulent brucella. It would act, therefore, as a sensitive indicator for the presence of virulent cells contained in a large population of avirulent dependent cells. The protocol of this experiment is presented in table 4. The intravenous route was studied as well as the subcutaneous route on the assumption that large numbers of avirulent cells surrounding a small number of virulent cells in the inoculum might influence the activity of the virulent cells when compressed in a small area as occurs in subcutaneous inoculation. Intravenous administration would conceivably dilute out the inoculum so that the virulent cells could better express themselves and not be affected by the host response to a large amount of foreign substance injected in a localized area.

In order to determine the number of reversions inoculated, viable cell counts on 0.1 ml of each suspension were performed on streptomycin-free medium and on streptomycin agar to determine the total dependent population. The results (table 5) indicated that fewer reversions were detected per 10^8 dependent cells than in the original reversion experiment (table 3). However, the organisms used in this experiment had been cultivated originally on 500 μg streptomycin per ml and had not been washed (as opposed to 5 to 10 μg per ml in the previous experiment), thus allowing for considerable residual growth of the streptomycin-dependent cells in the absence of the antibiotic. Thus, the effects of crowding of the reversions by the background growth of the dependent cells are real. The dependent organisms had been cultivated on high concentrations of streptomycin as this had been adopted as the standard medium for maintaining the strain for immunization purposes. Using the calculated number per 10^8 cells of group C in table 5 as the most reliable estimate due to lack of the crowding effect, the number of reversions inoculated in 1.0 ml of the suspensions used for infection were:

Group	*Reversions inoculated*
A & E	600
B & F	60
C	6
D	0.6

Such an estimate was closer to that obtained in the original reversion experiment (table 3).

The animals were inoculated with 1.0 ml of the various dilutions either subcutaneously in the inguinal region or intravenously by intracardial inoculation. They were sacrificed 4 weeks after infection, and cultures of spleen-liver homogenates were made in triplicate on both streptomycin containing and streptomycin-free media. The results appear in table 4. Of all the animals inoculated only 4 animals in groups E and F were infected, and these were infected only with dependent organisms. Each of these animals yielded only 1, 2, or 3 colonies totally on culture. None of the animals yielded cultures of a non-dependent organism. Considering the possible number of reversions inoculated originally, it would appear that these were avirulent as 600 such cells failed to yield infected spleens or livers

TABLE 5

Determination of number of reversions to drug independence

GROUP	NO. OF ORGS. PLATED ON DRUG-FREE MEDIUM	NO. OF COLONIES*	REVERSIONS PER 10^8 cells	NO. OF REVERSIONS IN INFECTING INOCULUM
A & E	1.06×10^{10}	6.8	0.064	68
B & F	1.06×10^{9}	2.0	0.19	20
C	1.06×10^{8}	0.6	0.64	6
D	1.06×10^{7}	0	—	—

* Each number represents the mean 5 plate determination of colonies growing in absence of antibiotic.

4 weeks after inoculation. The organs were perfectly normal in size and appearance. This also held true for the lymph nodes draining the site of subcutaneous inoculation.

Four weeks after infection 3 animals from each group were tested for cutaneous hypersensitivity by use of a highly sensitive reagent developed by Benedict and Elberg (1953). None of the animals exhibited cutaneous reactions to a dose (0.005 mg N) which detects a state of hypersensitivity in guinea pigs infected for one week with 1,000 organisms of the parent type virulent strain. Fleischner *et al.* (1919) concluded that cutaneous hypersensitivity was present only after an active infection. The fact that this state could not be elicited by killed suspensions was confirmed by others (Stubbs and Live, 1942; Meyer and Eddie, 1941). While such a state can

be elicited by dead cells or fraction thereof in the presence of adjuvants (Elberg, 1952), such means were not employed in the experiment described, and the lack of cutaneous hypersensitivity in guinea pigs inoculated with large numbers of living streptomycin-dependent variant speaks against any growth of the organism *in vivo*. The other possibility which may clarify this finding is that these organisms may not possess skin-sensitizing antigens since this appears to be correlated with the virulence of strains (Benedict and Elberg, 1953).

TABLE 6

Virulence of clones characterized by streptomycin independence

CULTURE	NUMBER OF ORGANISMS INOCULATED ($\times 10^4$)	INFECTED/TOTAL (TWO WEEKS AFTER INJECTION)
1	6.7	2/5*
2	6.0	4/5
3	4.1	1/5
4	8.3	1/5*
5	4.5	0/4
6	6.1	0/5
7	8.7	2/5*
8	4.8	1/5
9	0.9	1/5*
10	7.6	3/4
11	5.1	0/5
12	11.0	1/5
13	5.1	5/5
14	5.3	3/4
15	3.0	4/5
Brucella abortus, strain A19	11.0	0/5
Brucella melitensis (MM6015)	8.6	5/5
Brucella melitensis (MM6056)	15.0	4/5
Streptomycin-dependent	12.0	0/5

Note: For procedure see Experimental Methods.

* Infections were very light. Only 1 to 2 colonies cultured.

Of the reverted clones obtained in the previous experiment 98 per cent were unable to to grow in the presence of 500 μg streptomycin per ml. These organisms had mutated to streptomycin sensitivity. Streptomycin resistance therefore was not a necessary step involved in returning to the parent type. The reverted clones grew slowly on Albimi agar, requiring 5 to 6 days to yield macroscopic colonial growth. They were all smooth by virtue of their inagglutinability by acriflavine.

Fifteen of the reverted clones were tested for virulence in the mouse utilizing the screening test described in the Experimental Methods section. The results appear in table 6. It is apparent that these clones were reduced in virulence as compared with the virulent parent strain, MM6056, and a second virulent strain, MM6015.

TABLE 7

Infection of guinea pigs induced by two clones which had reverted to streptomycin independence

REVERSION NO.	NO. CELLS USED	ANIMAL NO.	TOTAL SPLEEN COUNT	AGGLUTINATION TITER	INFECTED/TOTAL
1	2.6×10^3	A	25,000	1:40	3/3
		B	500	1:20	
		C	500	1:20	
	2.6×10^3	A	25,000	1:320	2/3
		B	0	1:20	
		C	50	1:20	
	2.6×10^4	A	50	1:20	3/3
		B	50	1:20	
		C	2,500	1:80	
	2.6×10^5	A	500	1:20	3/3
		B	1,250	1:20	
		C	50	—	
2	1.7×10^3	A	0	1:20	2/3
		B	500	1:160	
		C	500	1:20	
	1.7×10^3	A	25,000	1:80	3/3
		B	100	1:20	
		C	150	1:20	
	1.7×10^4	A	0	1:20	2/3
		B	50	1:80	
		C	5,000	1:20	
	1.7×10^5	A	500	1:40	3/3
		B	500	1:20	
		C	1,000	1:160	

Infection induced via subcutaneous route.
Necropsy performed 4 weeks after infection.
Agglutinin titers determined at necropsy.

In order to estimate the degree of virulence, two of the reverted clones were titrated for their minimal infective dose in the guinea pig. The results in table 7 indicated that the smallest doses used were sufficient to infect the animals on the basis of the 4 week spleen test. However, in

the great majority of animals studied it was apparent that the degree of infection and agglutinating titer of the sera were low, regardless of the numbers of organisms injected, when these animals were compared with animals injected with a virulent strain of *B. melitensis* for the same period. These streptomycin-dependent cells appear to be of diminished virulence for the guinea pig and may themselves find use as relatively avirulent immunizing agents.

When 10^9 to 10^{10} streptomycin-dependent cells were plated onto streptomycinless medium, visible confluent growth occurred in 3 to 4 days. When washed cells were inoculated heavily into streptomycinless broth, turbidity was observed to increase (see figure 3). This phenomenon, described as "residual growth" by Bertani (1951), was of interest as the amount of growth of the streptomycin-dependent variant could be conditioned by the concentration of streptomycin on which it had been cultivated. It is precisely this *in vivo* growth which could determine the degree of immunity induced in vaccinated animals. The residual growth of the small and large strains of streptomycin-dependent variant was determined by culturing the cells on solid medium containing 10, 100, and 500 μg streptomycin per ml. The cell crops were harvested by washing the surfaces of the plates with saline. The cells were washed by centrifugation in 3 changes of saline to eliminate all extracellular streptomycin. Tubes of Albimi broth were inoculated in triplicate with the washed cell suspension to a turbidity just visible to the naked eye (5×10^7 to 1×10^8 cells per ml). Control tubes containing 500 μg streptomycin per ml also were inoculated. The results demonstrated that minor increases in total population occurred up to a period of approximately 114 hours with major increases commencing at this point. This secondary rise in population was due to the outgrowth of mutants not requiring streptomycin. Whenever a major change had occurred, the resulting population was primarily streptomycin independent. Thus, the minor changes up to 88 hours were due exclusively to the residual growth of the streptomycin-dependent strain, and no contribution to the turbidities could have been made by the reversions at this point.

An analysis of the amount of residual growth occurring at 48 and 88 hours of incubation was made by determining the changes in optical density of the suspensions and utilizing these figures as a rough measure of increases in cell populations. The streptomycin-dependent/small strain originally cultured on 10 μg per ml increased approximately twofold in the absence of streptomycin at 48 hours with no demonstrable increase occurring after 88 hours of incubation. The same strain when originally cultured on 100 and 500 μg streptomycin per ml increased twofold at 48 and fourfold at 88 hours of incubation in streptomycin-free broth, thus demonstrating the effect of the concentration of drug on which the organisms had been cultivated originally. The streptomycin-dependent/large strain was influenced less by the concentration of the drug upon which it had been cultivated initially. The amount of increase by this strain was maximal in all cases at 48 hours and was not nearly as great as the increase shown by the small strain. This was the only difference between these two colonial variants which was demonstrable during the course of these investigations.

The streptomycin-dependent/small variant therefore can store enough streptomycin intracellularly to enable it to undergo one or two divisions in the absence of the drug in the medium, and such growth occurring *in vivo* could enhance conceivably the immunogenic capabilities of the vaccine. The possibility also arises that streptomycin-dependent organisms cultivated on higher concentrations of the drug would induce a greater degree of immunity due to a greater degree of residual growth in the tissues of the host.

DISCUSSION

The hypothesis which was tested in these investigations, that a streptomycin-dependent mutant of *B. melitensis* would be avirulent, has been borne out. The mutant which has been isolated follows the pattern of streptomycin-dependent strains isolated from such organisms as the avirulent dependent strains of meningococci (Miller and Bonhoff, 1947*a*,*b*) and *M. tuberculosis* (Doane and Bogen, 1951).

The brucella mutant required the drug for *in vitro* growth but otherwise possessed the parent strain's biochemical, morphological, and antigenic characteristics, as far as was determined. While this requirement was not absolute due to the phenomenon of "residual growth", it sufficed to render the organisms avirulent because such growth was limited to the amount of stored, intracellular drug. While this requirement for streptomycin was easily demonstrable *in vitro*,

the question arises whether or not it was as obligatory *in vivo*.

The best evidence for avirulence of the mutant was its inability to persist or to cause gross tissue damage in the guinea pig. This animal is extremely susceptible to infection by virulent brucellae. Yet, the mutant was not recovered from the spleens of guinea pigs receiving as many as 10^{11} cells when the animals were necropsied four weeks after inoculation. The spleen is an excellent indicator of generalized infection, and while the organisms may have persisted in the other tissues, the absence of the organisms in the spleen certainly attests to their lack of invasive powers. The lack of multiplication in the guinea pig was borne out further by the low agglutinin titers obtained in comparison with the levels achieved in animals infected with virulent brucellae.

The situation in the mouse was not as clear-cut. The organism could be isolated from the spleen 1 to 5 weeks after inoculation. Whether these organisms constituted the residue of the large population inoculated, simply persisting in the tissues in a viable state, or whether these isolates were the resultant population due to slow multiplication and clearance from the host, could not be definitely ascertained. Simple persistence, as an explanation, would require the assumption that the organism, which is not resistant in the sense of bacterial spores, remained viable but dormant in host cells and was cleared from the animal in the manner of an inert particle. Such a situation is difficult to conceive as no analogy exists in other bacterial infections. Slow growth of the organism *in vivo* would require the assumption that the dependent organism obtained an analogue of the drug in the host tissues or that it utilized the enzyme systems of the host cells to circumvent the streptomycin requirement. Neither of these hypotheses was tested, but they remain as possible explanations of the experimental findings. The relative avirulence of the organism for the mouse was based on (1) the large dose necessary to yield infected spleens 2 to 4 weeks after inoculation; (2) the fact that few organisms were recovered from the spleen; (3) the steady clearance of organisms as indicated by numbers of organisms isolated and numbers of animals infected, occurring over a period of 5 weeks, with complete clearance probably achieved by 6 weeks.

Further evidences of avirulence were obtained in the monkey. Large numbers of the mutant failed to induce the typical fever observed when such animals are infected with virulent cells. Although the organism was isolated from the blood 10 days after inoculation, its presence was not accompanied by a rise in body temperature and did not induce high agglutinin titers in the serum.

The administration of the drug failed to enhance the number of organisms which could be recovered from the spleens of mice, indicating that the infection is not one susceptible to easy alteration.

The phenomenon of reversion from streptomycin dependence to independence was demonstrated. When the streptomycin-dependent strain was grown on low concentrations of the drug, 3 to 6 reversions per 10^8 cells could be demonstrated. When the streptomycin-dependent strain was cultivated on higher concentrations, fewer reversions were demonstrable. Such a phenomenon can be explained either by (a) overgrowth of the independent organisms by the extremely large numbers of dependents due to the residual growth of the latter in the absence of the drug, or (b) destruction of the independent (sensitive) mutants by drug being released from lysing dependents. The importance of the presence of the reversions in the population of streptomycin-dependent organisms cannot be underestimated if the streptomycin-dependent strain is to be used as a living vaccine. A concomitant return to virulence associated with return to drug independence was demonstrated in *M. tuberculosis* by Doane and Bogen (1951). However, the facts which speak for the safety of the strain for use as a vaccine in spite of the presence of streptomycin-independent organisms are as follows: (1) In no case were streptomycin-independent organisms isolated from any animal inoculated with large numbers of living, dependent cells. This held true for mice in spite of the long persistence of viable dependent organisms in the spleen. The guinea pig which is extremely susceptible to virulent organisms was not infected by as many as 600 reverts administered in the presence of the avirulent dependent organisms. (2) The reverts appear to be susceptible to the concentration of streptomycin employed for the cultivation of the dependent strain. Hence their viability is greatly impaired under the conditions of growth used in preparation of the dependents as a vaccine.

(3) The reverts appear to be avirulent by the mouse screening test. This was not completely borne out in the titration in the guinea pig due to its high susceptibility. However, the low grade splenic infection produced in these animals and the low agglutinin titers manifested are important as evidence for a reduced virulence.

SUMMARY

A test, utilizing mice, was developed for screening virulent strains of brucellae.

A streptomycin-dependent variant was isolated from a virulent strain of *Brucella melitensis*. The characteristics of the streptomycin-dependent strain studied were similar to the parent strain except for complete dependence on streptomycin for its growth *in vitro*. Residual growth of the variant occurred from large inocula in media which did not contain streptomycin due to storage of the drug intracellularly. Large doses of the streptomycin-dependent variant persisted in the mouse and were subsequently eliminated, failed to infect the guinea pig, and did not induce fever in the monkey. Reversions of the streptomycin-dependent variant to streptomycin independence appeared to be characterized by a return to drug sensitivity and some loss in virulence. Administration of streptomycin to the streptomycin-dependent variant *in vivo* failed to influence its growth or persistence.

It is proposed that the streptomycin-dependent variant may be useful in the living form as an avirulent vaccine against virulent *B. melitensis* infections in the goat, and such investigations are now in progress.

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