

IMMUNIZATION AGAINST BRUCELLA INFECTION¹

II. EFFECTIVENESS OF A STREPTOMYCIN-DEPENDENT STRAIN OF BRUCELLA MELITENSIS

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In previous reports (Elberg and Herzberg, 1953; Herzberg and Elberg, 1953) the isolation and characteristics of a streptomycin-dependent mutant of *Brucella melitensis* were described.² The streptomycin-dependent strain retained the biochemical and antigenic characteristics of the virulent parent strain but differed in a requirement for streptomycin for its growth. Growth *in vivo* was not influenced detectably by *in vivo* administration of streptomycin.

The following studies describe the capabilities of this strain for inducing protection of mice and guinea pigs against experimental infection with virulent *B. melitensis*.

MATERIALS AND METHODS

These were described in considerable detail in the first paper of the series (Herzberg and Elberg, 1953) and need not be restated here. Suffice it to say at this point that protection was assessed in terms of the ability of the animal to sterilize spleen tissue of the organisms constituting the "challenge".

EXPERIMENTAL RESULTS

The first sets of observations deal with mice which had received one injection of streptomycin-dependent strain of *B. melitensis*.

Mice were inoculated subcutaneously with doses ranging from 10⁶ to 10⁸ organisms, both large and small colony forms being employed. After a rest period of 5 weeks, the immunities were tested against graded challenge doses. Three

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² While this paper was in preparation, Olitzki and Szenberg (1953) reported data on *Brucella abortus*, strain 19, which confirm that reported earlier (Elberg and Herzberg, 1953) and in this manuscript relative to virulence and antigenicity.

weeks after the challenge infection the animals were sacrificed and spleen homogenates were cultured in triplicate.³

The results which are presented in table 1 indicate that significant protection, with small probabilities of the differences between control and test animals occurring by chance, was achieved in only two groups. These animals had received the lightest challenge dose and had been given the largest immunizing dose of vaccine. The challenge dose was approximately equal to one ID₅₀⁴ as shown by the rate of infection in the control series. No significant protection was demonstrable in any other group when the differences were tested by the chi square method. No difference in protective ability was shown between the small and large colony variants of the streptomycin-dependent strain.

Since little protection had been conferred by one injection of vaccine in the previous experiment, two doses of vaccine prepared from the small colony variant were administered to mice subcutaneously. Three weeks elapsed between the two immunizing inoculations. Four weeks later the protection was tested against two challenge doses and the animals were examined three weeks after the challenge infection had been given. As shown in table 2, significant protection was obtained in all cases except one (group G) as tested by the chi square method, indicating the extremely low probability that the difference between test and control could have

³ In all cases, in this and in subsequent experiments with streptomycin-dependent vaccine, cultures of spleen homogenates also were plated in duplicate on streptomycin containing agar (500 µg streptomycin per ml). In no case were vaccine organisms isolated at necropsy.

⁴ An ID₅₀ is defined in this work as the minimal number of cells required on subcutaneous injection to produce after 3 to 4 weeks an infection in 50 per cent of the animals.

occurred by chance. Protective rates as high as 93 per cent and 78 per cent were achieved (groups A and B) against challenges which infected 94 to 100 per cent of the control animals. These challenge doses were approximately 20 and 200 ID₅₀. This high degree of immunity was induced by the largest dose of vaccine (10¹⁰ cells), and correspondingly smaller degrees of immunity were induced in animals receiving smaller doses of vaccine.

An analysis of the results of varying the dose of vaccine revealed that in those groups receiving

vised to test the effect of drug administration upon the resulting immune state.

A single dose of 10¹⁰ streptomycin-dependent cells of the small colony mutant was administered subcutaneously, and various schedules of streptomycin administration were followed for each group. Two control groups were included, one of which received only the vaccine whereas the other received neither vaccine nor drug. Four weeks after vaccine administration all groups were challenged, and four weeks later the animals were sacrificed. In table 3 the data make it

TABLE 1

Response of mice immunized with living streptomycin-dependent mutant to subsequent challenge with a virulent strain of *Brucella melitensis*

GROUP	IMMUNIZING INFECTION DOSE	CHALLENGE INFECTION DOSE	TEST ANIMALS		CONTROL ANIMALS		CHI ²	P
			Infected/total	Per cent infected	Infected/total	Per cent infected		
A	1.7 × 10 ⁸	4.1 × 10 ⁷	13/15	86.6	9/10	90	0.14	0.7-0.5
B	small colony	4.1 × 10 ⁶	6/14	42.9	7/10	70	0.81	0.5-0.3
C	form	4.1 × 10 ³	1/15	6.6	5/10	50	4.03	0.05-0.02
D	9.7 × 10 ⁶	3.5 × 10 ⁷	14/15	93.3	7/7	100	0.12	0.8-0.7
E	small colony	3.5 × 10 ⁵	8/16	50.0	5/7	71	0.24	0.7-0.5
F	form	3.5 × 10 ³	6/14	42.8	3/6	50	0.53	0.5-0.3
G	1.9 × 10 ⁸	4.1 × 10 ⁷	13/15	86.6	9/10	90	0.68	0.5-0.3
H	large colony	4.1 × 10 ⁶	10/15	66.6	7/10	70	0.02	0.9-0.8
I	form	4.1 × 10 ³	0/15	0	5/10	50	4.50	0.05-0.02
J	6.0 × 10 ⁶	3.5 × 10 ⁷	12/14	85.6	7/7	100	0.06	0.9-0.8
K	large colony	3.5 × 10 ⁵	10/15	66.6	5/7	71	0.07	0.8-0.7
L	form	3.5 × 10 ³	7/14	50.0	3/6	50	0	1.0

A 5 week period elapsed between immunization and challenge by strain MM472 of *Brucella melitensis* and a 3 week period elapsed between challenge and sacrifice.

the smaller challenge dose (groups A, D, G, J, M) the chi square value was 19.197, where *n* is 4, and *P* is equal to less than 0.01. For groups receiving the higher challenge (groups B, E, H, K), chi square was 7.79, where *n* is 3, and *P* is equal to less than 0.01. These values indicate that variation in the dose of vaccine did have an effect on protection since the results could not have been due to chance.

In the experiments reported previously (Herzberg and Elberg, 1953) it was shown conclusively that administration of streptomycin to the host had no perceptible influence on the growth or persistence of the streptomycin-dependent mutant *in vivo*. The following experiment was de-

evident that all groups had been protected significantly as compared with their controls (groups 153 and 154). However, the analysis of all immunized groups, including those which had received no postvaccinal streptomycin therapy, established the fact that any difference between groups could have arisen by chance. That is, the chi square value for groups receiving the smaller challenge (odd numbered groups 141 to 151) was 5.472, where *n* equals 5 and *P* equals 0.5 to 0.3. The chi square value for those groups receiving the larger challenge (even numbered groups 142 to 152) was 4.935, where *n* equals 5 and *P* equals 0.5 to 0.3. Thus, the administration, or lack of administration, of the drug had no

measurable effect on the immunity produced against two doses of virulent cells although in all cases the immunity so produced was significant. One inoculation of 10^{10} streptomycin-dependent cells was sufficient to protect 77.8 and 72.3 per cent of animals receiving challenges which infected 70 and 90 per cent of the control animals. Such protection was not as high as that

mentioned groups. No protection was found in any other group when tested against larger doses of virulent cells. Thus, the guinea pig was not immunized as easily as the mouse.

DISCUSSION

It has generally been accepted that active immunization against brucellosis requires stimula-

TABLE 2
Response of mice immunized with two inoculations of the streptomycin-dependent mutant

GROUP	IMMUNIZING INFECTION DOSE	CHALLENGE INFECTION DOSE	INFECTED/TOTAL	PER CENT INFECTED	CHI ²	P
A	1st— 1.7×10^{10} 2nd— 4.1×10^{10}	8.0×10^4	1/14	7	20.8	0.01
B	1st— 1.7×10^{10} 2nd— 4.1×10^{10}	8.0×10^5	3/14	21	17.2	0.01
D	1st— 1.7×10^9 2nd— 4.1×10^9	8.0×10^4	4/15	27	13.4	0.01
E	1st— 1.7×10^9 2nd— 4.1×10^9	8.0×10^5	10/14	71	10.7	0.01
G	1st— 1.7×10^8 2nd— 4.1×10^8	8.0×10^4	8/11	71	1.19	0.3-0.2
H	1st— 1.7×10^8 2nd— 4.1×10^8	8.0×10^5	9/14	64	4.84	0.05-0.02
J	1st— 1.7×10^7 2nd— 4.1×10^7	8.0×10^4	6/15	40	9.05	0.01
K	1st— 1.7×10^7 2nd— 4.1×10^7	8.0×10^5	8/13	61	5.32	0.05-0.02
N	None	8.0×10^4	17/18	94	—	—
O	None	8.0×10^5	17/17	100	—	—

Three weeks between immunizing inoculations.

Challenged 4 weeks after last immunizing dose with strain MM472.

Sacrificed 3 weeks after challenge.

resulting from two inoculations but was significant, however.

When attention was directed to the guinea pig, it was observed that an entirely different situation existed, for barely significant protection was achieved after 3 inoculations (table 4). One inoculation of vaccine when tested against a challenge dose infective for 80 per cent of the control animals appeared more effective than two inoculations against a challenge which was approximately twice that administered to the above

tion of the immune mechanisms of the host over a relatively protracted time. Such a situation has been achieved by (1) twice-daily inoculation of a soluble antigen for 14 days (Elberg, 1952), (2) less frequent administration of a soluble antigen in adjuvants (Live, 1949), and (3) by the growth and persistence of a living avirulent organism in the cells and fluids of the host, as practised currently in control of the natural disease in cattle.

Since no data in brucella investigations existed

TABLE 3
Effect of streptomycin administration to mice upon their active immunization against *Brucella melitensis* infection

GROUP	POSTVACCINAL ADMINISTRATION OF DRUG	CHALLENGE DOSE	INFECTED/TOTAL	PER CENT INFECTED	CHI ²	P
141	5,000 µg subcutaneously simultaneous with vaccine inoc.	7.5×10^3	4/18	22.2	6.9	0.01
142		7.5×10^5	7/20	35.0	10.6	0.01
143	As above then 5,000 µg subcutaneously daily for 5 days	7.5×10^3	2/20	10.0	12.6	0.01
144		7.5×10^5	3/20	15.0	19.3	0.01
145	1,500 µg intravenously simultaneous and daily for 5 days after vaccine inoc.	7.5×10^3	3/19	15.7	9.3	0.01
146		7.5×10^5	5/20	25.0	15.2	0.01
147	5,000 µg subcutaneously 1 week after vaccine inoc.	7.5×10^3	6/17	35.3	3.13	0.05-0.10
148		7.5×10^5	8/9	42.1	7.8	0.01
149	5,000 µg subcutaneously daily during 2nd week after vaccine inoc.	7.5×10^3	6/17	35.3	3.13	0.05-0.10
150		7.5×10^5	3/16	18.7	15.8	0.01
151	None	7.5×10^3	4/18	22.2	6.9	0.01
152		7.5×10^5	7/19	36.8	9.7	0.01
153	None*	7.5×10^3	14/20	70.0	—	—
154		7.5×10^5	18/20	90.0	—	—

* Groups 153 and 154 received no vaccine. All others received 10^{10} viable streptomycin-dependent cells before streptomycin administration.

Four weeks elapsed between vaccine administration and challenge, and 4 weeks elapsed between challenge and sacrifice.

TABLE 4
Response of guinea pigs immunized with living streptomycin-dependent mutant

GROUP	VACCINE ADMINISTRATION	CHALLENGE	TEST		CONTROL		CHI ²	P
			Infected/total	Per cent	Infected/total	Per cent		
A	3 inoculations: 1.7×10^{10} , 4.7×10^9 , 3.3×10^8 , two weeks apart	59	6/15	40	8/9	89	3.7	0.05-0.10
B		590	15/15	100	10/10	100	—	—
C		5,900	8/8	100	10/10	100	—	—
D	2 inoculations: 1.7×10^{10} , 4.7×10^9 , two weeks apart	190	6/13	46	6/10	60	0.066	0.70-0.80
E		1,900	9/14	64	10/10	100	2.6	0.10-0.20
F		19,000	14/14	100	6/6	100	—	—
G	1 inoculation: 1.7×10^{10}	96	2/12	17	8/10	80	6.4	0.02-0.01
H		960	11/14	78	9/9	100	0.73	0.30-0.50

Vaccine and challenge doses were administered subcutaneously in 1 ml volumes.

All challenges were administered 3 weeks after the last vaccine inoculation, and all animals were sacrificed 4 weeks after challenge.

which would make one optimistic over the chances of any but the third approach at the moment for goat, sheep, or even man, an organism was sought, the *in vivo* phases of which would not induce excessive pathologic changes and which after a period of viability *in vivo* would be eliminated; thus, the organism would be classed as relatively avirulent. The second aspect of the approach, tactical as it were, was the hypothesis that a streptomycin-dependent strain of *B. melitensis* might possess such properties with the further advantage that the *in vivo* growth of the organism be controlled by administration and subsequent withdrawal of the drug.

Experimentally the mutant has proven to be sound if judged by the findings in mice, whereas it has shown little success in the immunization of guinea pigs. Furthermore, administration of an antibiotic to the host did not affect the results in the mouse.

The effectiveness of the vaccine strain in mice has not been attributed conclusively to multiplication of the strain *in vivo* although the alternate possibility that the organisms merely persisted for the number of weeks indicated is more difficult to accept biologically. If the antibiotic is not available to the mutant *in vivo*, and the results of Magoffin and Spink (1951) amply indicate that it is not available to the intracellularly located *Brucella*, the organisms are by-passing *in vivo* the drug if multiplication is occurring. On the other hand, the very large numbers of cells required for immunization speak against effective multiplication and in favor of persistence in which case the data on *in vivo* administration of the drug are further clarified.

The ease with which mice were immunized when compared with guinea pigs emphasizes again the problem of using only the latter species of laboratory animal in assessing the immunogenic capabilities of brucella vaccines for larger animals and man. Mice are generally much more susceptible to immunization against *Brucella* species than guinea pigs. Data on the native antigens (Elberg *et al.*, 1951), acetone killed *Brucella* cells (Smart, 1949), and now on the mutant strain attest to this. The guinea pig is extremely susceptible to *Brucella* infection and can be infected by very small numbers of organisms administered via a variety of routes. And it has been accepted by most investigators that if an animal as susceptible as the guinea pig can be protected by a vaccine, more resistant animals

will also experience the protection. Since species do differ in their ability to be immunized by different preparations as illustrated by findings of Baker *et al.* (1951) in studies on *Pasteurella pestis* and by Jawetz and Meyer (1943) on the same organism, the use of only one species of laboratory animal to screen possible immunogenic agents in *Brucella* infections may have hindered the uncovering of effective vaccines.

The guinea pig is extremely variable in its response to immunization, and often experiments cannot be replicated. Elberg *et al.* (1951) could find little correlation between the variables studied in their immunizing procedures with the native antigen and the extent of protection resulting in the guinea pig although the results as a whole indicated a protective effect by the antigen. Other workers have noted this same phenomenon. Lisbonne *et al.* (1938) speak of their method of immunization "misfiring" on occasion, and McEwen and Roberts (1936) state that "although in the majority of experiments clear-cut results were obtained, this was not always the case. Why in a few instances vaccinated groups of animals showed little or no more resistance than the control animals is still obscure." These facts point to a great genetic variability in the guinea pig in regard to its response to immunization.

Administration of the streptomycin-dependent mutant is a method of presenting the animal with the living organism containing its complement of antigens unaltered by any special method of extraction, killing, etc. Irrespective of the occurrence of *in vivo* multiplication it is possible to administer safely massive doses of the organism to mice and guinea pigs. Thus, it is possible to influence the results by varying the initial dose of vaccine.

Results of active immunization studies on the goat and monkey will be presented in a subsequent paper of this series.

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SUMMARY

A living streptomycin-dependent mutant of *Brucella melitensis* was able to confer significant protection on the mouse against experimental infection. Administration of the antibiotic to the

immunized mice failed to enhance the immunity so induced. The streptomycin-dependent mutant protected guinea pigs only against small doses of virulent organisms.

REFERENCES

- BAKER, E. E., SOMMER, H., FOSTER, L. E., MEYER, E., AND MEYER, K. F. 1951 Studies on immunization against plague. I. The isolation and characterization of the soluble antigen of *Pasteurella pestis*. *J. Immunol.*, **68**, 131-145.
- ELBERG, S. 1952 *Unpublished observations*.
- ELBERG, S., AND HERZBERG, M. 1953 Immunity in brucellosis. *Federation Proc.*, **12**, 441.
- ELBERG, S., HERZBERG, M., SCHNEIDER, P., SILVERMAN, S., AND MEYER, K. F. 1951 Studies on the immunization of guinea pigs and mice to brucella infection by means of "native antigen". *J. Immunol.*, **67**, 1-13.
- HERZBERG, M., AND ELBERG, S. 1953 Immunization against *Brucella* infection. I. Isolation and characterization of a streptomycin-dependent mutant. *J. Bact.*, **66**, 585-599.
- JAWETZ, E., AND MEYER, K. F. 1943 Avirulent strains of *Pasteurella pestis*. *J. Infectious Diseases*, **73**, 124-143.
- LISBONNE, M., ROMAN, G., AND RENOUX, G. 1938 Vaccination "vraie" du cobaye contre *B. melitensis*. *Comm. du lab. de Microbiol. de la faculté de médecine de Montpellier et du centre de recherches sur la fièvre ondulante de l'Institute Bouisson-Bertrand, Montpellier*.
- LIVE, I. 1949 Effect of adjuvants upon the immunizing quality of ether killed *Brucella abortus*. *Am. J. Vet. Research*, **10**, 347-350.
- MC EWEN, A. D., AND ROBERTS, R. S. 1936 Bovine contagious abortion. The use of guinea pigs in immunization studies. *J. Comp. Pathol. Therap.*, **49**, 97-117.
- MAGOFFIN, R. L., AND SPINK, W. W. 1951 The protection of intracellular brucella against streptomycin alone and in combination with other antibiotics. *J. Lab. Clin. Med.*, **37**, 924-930.
- OLITZKI, A. L., AND SZENBERG, E. 1953 Toxicity, infectivity and antigenicity of a streptomycin dependent mutant of *Brucella abortus* (strain 19). *Proc. Soc. Exptl. Biol. Med.*, **82**, 539-541.
- SMART, K. L. 1949 Immunity studies in *Brucella* infection of the mouse. Thesis. University of California, Berkeley.