IMMUNIZATION AGAINST BRUCELLA INFECTION

III. Response of Mice and Guinea Pigs to Injection of Viable and Nonviable Suspensions of a Streptomycin-Dependent Mutant of *Brucella melitensis*[1]

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In previous papers of this series (Herzberg and Elberg, 1953; Herzberg, Elberg, and Meyer, 1953) the response of mice and guinea pigs to living cells of a streptomycin-dependent mutant of *Brucella melitensis* was studied in terms of their ability to resist reinfection. Unpublished data in the case of the monkey have shown that the immunizing ability of the mutant was retained after exposing the cells to flowing steam for 20 minutes. It was therefore important to determine in some detail whether in fact any differences in protection conferred on mice or guinea pigs by injection of living or nonliving cell suspensions of the mutant could be demonstrated. Since a negative answer to this question was obtained in the results reported here, attention was directed again to the isolation of several nonstreptomycin requiring clones from a population of streptomycin-dependent cells (Herzberg and Elberg, 1953). These provided an opportunity to study immunization against brucella infection, a process which appears to require multiplication *in vivo* of the vaccine, because these clones combined relative avirulence with the ability, in the case of at least one, to multiply *in vivo*.

MATERIALS AND METHODS

Preparation of cell suspensions for immunization. The dependent mutant cells described earlier (Herzberg and Elberg, 1953) referred to as SMd were grown on Albimi agar to which 0.5 mg streptomycin per ml had been added. After six days at 37 C the growth was harvested in sterile saline, and a viable cell count was performed at this time. The suspension was stored at 5 C. A portion of the suspension was heated in flowing steam for 20 minutes, and after a sample was removed to test for viability, the rest of the suspension was stored at 5 C.

The streptomycin-nondependent reversion strain was originally isolated from the dependent population by plating heavy suspensions of SMd on drug-free Albimi agar. Three to six cells per 10^8 SMd cells were capable of growing in the absence of streptomycin.

Of about 100 "reversion" clones, 98 were inhibited by 500 μg streptomycin per ml. The virulence of 15 of these sensitive clones was determined in mice and guinea pigs (Herzberg and Elberg, 1953). One such clone was designated as strain Rev. 1 and was used in all subsequent experiments. It has the characteristic morphological, colonial properties and sensitivity to bacteriostatic dyes typical of *Brucella melitensis*. It, however, grows very slowly in broth and on agar and is agglutinated to titer by antimelitensis serum.

The cells were grown on Albimi agar, and suspensions were prepared exactly as described for the dependent strain.

Experimental animals. Male mice of the BRVS Webster strain, weighing 18–20 g were allotted to the experimental group by random selection from the colony. Guinea pigs of both sexes and weighing 300–500 g were obtained from a single source. All animals were injected subcutaneously in the inguinal region.

Except where otherwise noted, the presence of infection in mice and guinea pigs was determined by recovery of the organisms from spleen homogenates, according to procedures described earlier (Elberg *et al.*, 1951).

Tests for significance. Comparisons of the significance of the difference between groups were made by the "chi square" method utilizing the tables of Finney (1948) and Latscha (1953) for groups of 20 animals or less. When larger groups

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were compared, the fourfold tables of Fisher (1948) with the Yates (1934) correction for continuity were used. The "one-tailed" distribution was used in calculating differences between test groups and their respective controls since the results could vary in only one direction, i.e., it was assumed that the vaccine would not increase susceptibility to infection. The "two-tailed" distribution was used when comparing two or more immunized groups. Values of P of 0.05 or less were taken as indicating a rejection of the null hypothesis.

RESULTS

Comparative activity of viable and nonviable SMd cell suspensions. When groups of 16–20 mice were inoculated with one or two doses of the living or heat-killed SMd suspensions, no difference, significant at the one per cent level, in response to subsequent injection of virulent cells was observed although the protection conferred by the SMd cell suspensions was highly significant when compared with the respective untreated control groups. The activity of the two SMd cell suspensions was not significantly altered under the following conditions: (a) when the dose of SMd cells of either suspension varied in tenfold steps from 10^6–10^{10} cells; (b) when the number of virulent cells injected was raised in tenfold steps from 10^3 to 10^6; (c) when *B. suis* strain PS3 or *B. melitensis* strain M6015 was used to test the immunity; (d) when the rest period before challenge was increased from 4 to 12 weeks; and (e) when the dead cells were emulsified in the adjuvant system of Freund and Bonanto (1944).

Influence of method used to kill SMd cells. When it became apparent that viable and nonviable cells of the mutant strain were equally active, it was of interest to learn whether the mutant cells possessed any unique qualities useful in immunization. Nonviable cells of the SMd strain were compared with similarly prepared cells of *B. suis* strain PS3, a virulent strain.

Suspensions of both strains were killed either by exposure to flowing steam for 20 minutes or by exposure to a final concentration of 0.5 per cent (w/v) phenol or formaldehyde for 48 hours at 37 C. The cells were then washed three times in sterile saline and resuspended in the adjuvant system of Freund and Bonanto (1944). For each preparation studied, the effect of omitting killed *M. tuberculosis* from the adjuvant mixture was determined. All vaccines were administered to groups of 18–20 mice and guinea pigs in two doses, contaning $1 \cdot \times 10^{10}$ cells per dose, two weeks apart. The immunity was measured against graded doses of *B. suis* strain PS3, four weeks after the second injection of dead vaccine. Four weeks later the tissues of the animals were examined for the presence of brucellae and compared with those from infected animals not previously treated with dead cells.

When the data for this experiment were analyzed by the "chi square" method, the following facts emerged: (a) Differences between the immunized groups and their controls were highly significant ($P = 0.01$). (b) No such differences could be detected in the potencies of the various vaccines, irrespective of the method of killing or of the presence of killed *M. tuberculosis* in the adjuvant mixture. (c) The responses to the killed cell suspensions of both the highly virulent suis and the dependent melitensis cells were not significantly different at the one per cent level. Hence the mutant did not appear to have any protective type antigens uniquely resistant to heat, phenol, or formaldehyde, and which were absent from the suis strain.

The similarity in immunizing potency between the living and nonliving cells of the SMd mutant was assumed to be a function of the inability of the living SMd cells to grow or persist for a sufficient period *in vivo*. Attention was then directed to the nondependent clones, isolated from a dependent population and described in tables 3 and 4 of the earlier study (Herzberg and Elberg, 1953). Screening tests of the virulence of 15 such clones led to the selection of one, referred to as "Rev. 1", for a detailed comparison with the virulent *B. melitensis* strain 6015 as to its ability to grow and multiply in the mouse and guinea pig.

Enough animals of each type were injected with 10^3, 10^4, or 10^5 cells of each strain to allow the sacrifice of 5–10 animals from each dosage group at weekly intervals. Clearance studies over a period of several weeks showed certain differences between the virulent strain 6015 and the reversion strain in several important qualities. In the mouse and guinea pig the virulent strain maintained itself through the eleventh week, actively multiplied in the spleen, induced agglutinin formation to a uniformly high titer, and caused

TABLE 1

Immunization of mice and guinea pigs by a streptomycin-nondependent clone isolated from a streptomycin-dependent population

Experiment	Immunization (Dose)	Size of Challenge	Immunized		Controls			
			Infected / Total	Per cent protected	Infected / Total	Per cent infected	Chi²	P
Mice								
1	1.1×10^5 cells	220,000	13/22	41	16/16	100	6.4	0.02–0.01
		22,000	10/20	50	14/14	100	7.1	0.01
		2,200	7/23	70	14/16	87.5	9.1	0.01
Guinea pigs								
2	1.15×10^3 cells	2,150	7/27	74	16/16	100	19.2	0.01
		215	7/23	70	18/18	100	18.2	0.01
		21	4/25	84	15/18	83	16.5	0.01
3	1.1×10^4 cells	4,400	8/13	39	9/11	82	0.408	0.7–0.5
		440	3/18	83	15/15	100	22.9	0.01
		44	2/16	87.5	7/8	87.5	9.7	0.01

a significant increase in spleen weight. In contrast, Rev. 1 multiplied during the first five weeks but was cleared by the eleventh week. It induced only minor agglutinin formation and caused little or no increase in spleen weight. In mice, using the Webster BRVS strain, Rev. 1 organisms were cleared by the eighth week which is about four weeks longer than the SMd cells persist in the mouse. No evidence of active multiplication of Rev. 1 cells in the mouse could be obtained after exhaustive study. However, in the NAMRU strain of mice, the Rev. 1 cells persisted for at least ten weeks, but no evidence of multiplication in the spleen could be obtained.

Immunizing ability of Rev. 1. Mice (BRVS) and guinea pigs, injected with 10^5 and 10^3 Rev. 1 cells, respectively, were exposed after 12 weeks to graded doses of *B. melitensis* strain 6015. The data in table 1, drawn from one of several experiments similar in design and result, show that Rev. 1 was effective in conferring protection on both animal species.

DISCUSSION

The intracellular location of brucella in infected animals protects them from the action of streptomycin, among other antibacterial agents, according to the studies of Magoffin and Spink (1951). Presumably this explains the failure of the drug to support *in vivo* growth of the SMd mutant (Herzberg *et al.*, 1953), and consequently the equal effectiveness of the viable and nonviable forms of SMd is, on this basis, not difficult to understand.

The problems encountered in immunity to brucella infection appear to have much in common with those encountered in immunity to *M. tuberculosis*. It is possible to confer an immune state against *M. tuberculosis* in mice by the administration of phenol-killed cells (Dubos *et al.*, 1953). The immunity induced by such a cell suspension is of the same order as that conferred by the immunizing dose of the living avirulent strain, BCG. Presumably the avirulent strain induces immunity by virtue of its growth *in vivo*, and when growth has reached a point where sufficient bacterial mass is present at the required sites to insure a sufficient response on the part of the tissues, immunity will result. The studies reported in this paper indicate that an immunity against *B. melitensis* infection can be achieved in mice and guinea pigs by the administration of small numbers of a relatively avirulent mutant which does multiply in the guinea pig and persists for several weeks in the mouse.

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SUMMARY

The protection conferred on mice and guinea pigs by the injection of one or two doses of 10^{10} living cells of a streptomycin-dependent mutant of *Brucella melitensis* was compared with the response to the same number of cells killed by phenol, formaldehyde, or heat. No significant differences were detected.

The addition of an adjuvant mixture containing killed tubercle bacilli did not affect the protective activity of the nonviable cell suspensions.

The clearance of a virulent strain of *B. melitensis* was compared with that of a nondependent streptomycin clone (Rev. 1) isolated from the dependent population. The nondependent living cells were cleared from the tissues of mice and guinea pigs by eight and eleven weeks, respectively, whereas the virulent cells were still present in the spleens 12 weeks after injection.

Mice and guinea pigs which had previously cleared themselves of Rev. 1 cells were protected against the subsequent injection of virulent cells to the extent that 41, 50, and 70 per cent of the mice resisted 222,000, 22,000 and 2,200 cells, respectively. In the guinea pig similar percentage protection was observed against smaller challenge doses.

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