IMMUNIZATION AGAINST BRUCELLA INFECTION

VI. Immunity Conferred on Goats by a Nondependent Mutant from a Streptomycin-Dependent Mutant Strain of *Brucella melitensis*[1]

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The isolation of streptomycin-dependent mutants from different Brucella was suggested by the results of Hall and Spink (1947), and described in detail by Olitzki and Szenberg (1953), Olitzki and Sulitzeanu (1953) and Herzberg and Elberg (1953). From populations of the dependent strain of *Brucella melitensis* nondependent mutants were isolated and their immunogenic capacity for mice and guinea pigs was described (Herzberg, Elberg and Meyer 1953; Herzberg and Elberg, 1955). In contrast to the behavior of *B. melitensis*, Olitzki and Szenberg (1953) could not isolate nondependent mutants in a culture of *Brucella abortus* (strain 19), although Olitzki and Bienstock (1955) did record the observation that on a drug-free medium a "temporary growth of a streptomycin-dependent *B. abortus* was observed on 1–2 subcultures. . . . In the third subculture the growth was finally arrested."

The attenuation of virulence of the dependent and "derived" nondependent strains is best compared on the basis of their rate of clearance from mice and the population levels they reach in the spleens of mice. The dependent strain is cleared from the NAMRU strain of Webster BRVS mice in 5 weeks whereas the "derived" nondependent strain is cleared between the 9th and 11th weeks, when the same numbers of each strain are injected. No multiplication of the dependent strain can be detected whereas the derived strain does increase approximately 100 fold in the spleen over the period of study before it decreases in numbers and finally disappears. Neither strain induces any sign of gross tissue response characteristic of brucella infection.

The very significant ability of the "derived" nondependent mutant of *B. melitensis* to immunize mice and guinea pigs suggested that *in vivo* proliferative tendencies of the strain were more highly developed than had been the case with the dependent strain (Herzberg and Elberg, 1955); Elberg, Henderson, Herzberg and Peacock, 1955; Olitzki and Olitzki, 1956). In the light of greater qualities of persistence *in vivo*, the "derived" nondependent strain was tested in the goat and evidence was obtained of its effectiveness as an immunizing agent for that host species.

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EXPERIMENTAL METHODS

Bacterial strains. The "vaccine strain" was originally isolated as one of a number of colonies which appeared when the streptomycin-dependent strain of *B. melitensis* was placed in the absence of the drug on Albimi agar (Herzberg and Elberg, 1955). The nondependent clone had been compared with the standard virulent strain 6015 of *B. melitensis* and the relative attenuation of the former established for mice and guinea pigs (Herzberg and Elberg, 1955). Further studies of the strain led to the isolation of a stable small colony form on Albimi agar which was used in all subsequent work and hereinafter called the "derived" nondependent strain. The strain grows well on Albimi agar and requires 3–4 days for good colony development. The virulent strain (6015) used to establish infection in the experimental animals was described earlier (Herzberg and Elberg, 1953). It was maintained on Albimi agar slants at 5 C.

Preparation of cell suspension for estimation of numbers of viable cells and "smoothness" of cells. The methods have been described in previous papers (Herzberg and Elberg, 1953) and were followed without change. The crystal violet method of White and Wilson (1951) was used in addition to the tests of Henry (1933) and Braun and Bonestell (1947) for checking the presence of smooth and nonsmooth colonial forms in the cultures of vaccine and challenge infection strains. When heat-killed cells were to be used a portion of the suspension of the living vaccine

strain was exposed to flowing steam for 20 min after thermal equilibration.

Bacteriological studies. Tests for dye resistance and H_2S production were those described by Huddleson (1943). The urease test was that of Hoyer (1950). No H_2S was produced by the vaccine strain over a four day period at 37 C and no urease activity was demonstrable after 24 hr. The vaccine strain was completely inhibited on agars containing 1:200,000 crystal violet, 1:50,000 thionin, 1:400,000 pyronin and 1:50,000 basic fuchsin.

Agglutination tests. The antigen for tube agglutination was kindly supplied by the Agriculture Research Service, U. S. Department of Agriculture. The reactions were incubated at 37 C for 24 hr followed by 48 hr at room temperature. The titer of a given serum has been expressed in terms of the final dilution of serum giving 50 per cent agglutination.

Blood culture. Five ml of blood were transferred to 50 ml of Albimi broth and incubated at 37 C. Samples of these broth cultures were removed to plates of Albimi agar twice during a four to six week incubation period and the culture was recorded as negative at that time if no brucellae were detected on the plates.

Experimental infection was evaluated at autopsy in the goats and their kids or fetuses by culture of various organs and body fluids (table 3). All organs were macerated separately with pestle and sand in a mortar, and suspended in a minimal amount of saline; $\frac{1}{10}$ to $\frac{2}{10}$ ml of the brei was cultured on each of 3 to 6 plates of Albimi agar and, where necessary, on the inhibitory agar of Kuzdas and Morse (1953). Occasionally, in order to check the sensitivity of the culture method, a large portion of the spleen and the entire prescapular lymph node brei were cultured in their entirety employing for this purpose between 60 to 70 plates per organ. Goats and kids were sacrificed within 48 to 72 hr following delivery.

Experimental animals. Four- to five-year-old female Angora goats[2] were obtained from Sonora, Texas. The animals were maintained on a diet of alfalfa, supplemented weekly with a ration of Purina goat chow, and water. Fecal examinations[3] of all animals were made for the presence of intestinal worms. Therapeutic doses of an antihelminthic were instituted before any experimentation was undertaken and a maintenance regimen adhered to when repeated fecal specimens showed that the infection had been eliminated.

RESULTS

The persistence of the mutant strain of *B. melitensis* in the goat was determined first in order to plan the date of the challenge infection. In the light of the reports of Pollack, Kelly, Gorelick, Braun and Victor (1952) and Kelly, Gorelick, Silverman and Braun (1953*a*, *b*) it was desired to avoid the problem of superinfection with its complications in the interpretation of an immune response and to study the immunity in the time period of the so-called sterile immunity. Accordingly nine goats received 1.5×10^9 cells of the vaccine strain subcutaneously in the left prescapular region. At 2, 4, 5, 7, 10, 11 and 14 weeks, animals were sacrificed and tested for the presence of brucellae.

At the third week organisms were recovered from the spleen, bone marrow, left and right prescapular nodes, left and right supramammary nodes, right precrural and the mediastinal nodes from one animal and only from the left prescapular node in the second animal autopsied. In two goats sacrificed at the fifth week, the spleen, left prescapular, and mediastinal nodes yielded brucellae from both animals whilst only the left precrural and right prescapular were infected in one animal of the two. Autopsies carried out during the seventh and eighth weeks yielded brucellae from the left and right prefemoral nodes of one animal and from the left prescapular of both animals sacrificed. By the fourteenth week the infection was confined in the one remaining animal of the series to the left prescapular node from which only one colony was isolated. It was assumed that at this time the animals had reached a stage of freedom from infection in which the phenomenon of superinfection, while not entirely eliminated, would not be of overriding importance.

Daily blood cultures on the animals for the first ten days after injection of the mutant

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[3] Kindly performed by the Laboratory Diagnostic Service of the School of Veterinary Medicine, University of California, Davis.

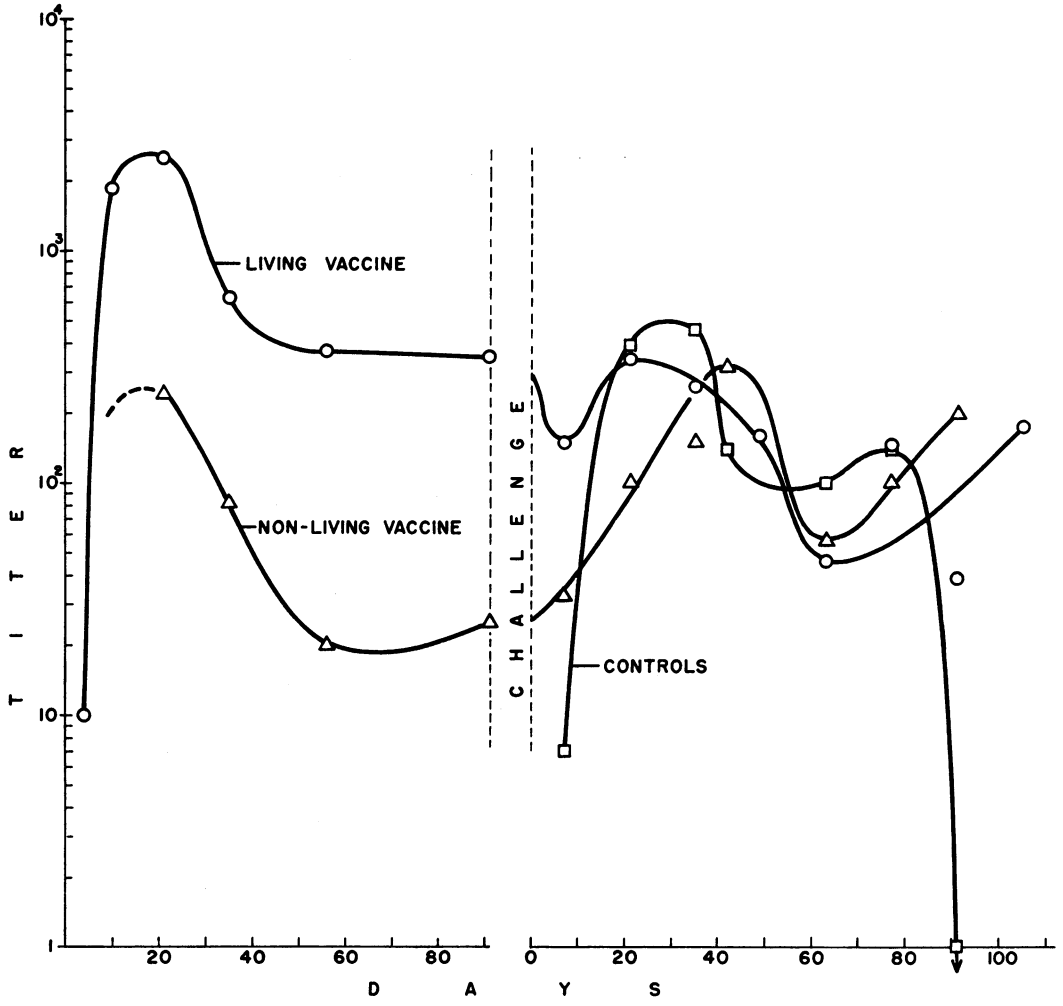

Figure 1. Agglutinin titer development in response to immunization and infection

strain yielded brucellae only in two of the nine animals. From one of these two goats organisms were recovered only on the first and sixth day after injection whereas a recovery was made from the second goat only on the seventh day.

Agglutination tests indicated that the mutant strain stimulated the antibody-producing mechanism within the first ten days. Figure 1 presents these and other agglutination titers observed during the entire period of the experiment. Each point represents the average titer of the 12 or 13 animals in the respective groups. Animals which received living cells of the vaccine strain responded serologically more intensely than those which received heat-killed cells of this strain before the challenge infection was given.

Figure 1 tends to give a somewhat peculiar impression of a dramatic loss of serological response in the control group towards the end of the observation period. This, however, is clarified by the fact that by the 91st observation day the survivors in the control group comprised those animals which had throughout the experiment responded serologically only slightly. It must be pointed out that in the experience of this and earlier experiments the agglutination titer of a goat contributed little if any information as to its infection status. However, in many cases the phenomenon of abortion was preceded by a dramatic rise in the titer but not regularly enough to allow fruitful prediction.

Rectal temperatures of goats receiving viable mutant cells rose during the ten day post-injection period from a preimmunization range of

TABLE 1

*Isolation of the challenge strain of Brucella melitensis from the blood**

Group	Day after Receiving Challenge Infection										
	14	21	28	35	42	49	56	63	70	77	112
Live vaccine† (13 in group)	0‡	0	0	0	0	0	0	0	0	0	0
Heat-killed vaccine (12 in group)	2	2	7	4	3	4	1	0	1	0	0
Nonvaccinated control (13 in group)	3	6	6	3	3	0	1	2	1	1	1

* Distinguished by size of colony on Albimi agar at 72 hr.

† On the first, sixth and seventh day after injection of the vaccine strain, one animal out of nine studied yielded a positive blood culture when tested on 10 successive days.

‡ Numbers in table refer to numbers of goats from which brucella were isolated by blood culture.

102.5–103.5 F to 104 F for 1 to 3 days in 4 out of 8 animals studied; in 3 animals the temperature rose to 104.2–106.1 F for a period of 3 to 6 days. One animal showed no rise in temperature over the 10-day observation period.

Of 12 goats receiving heat-killed cells, only three showed temperatures above 103.5 F and then only on the day following injection.

The ability of the mutant to immunize against experimental infection was studied in 25 goats. Thirteen animals were inoculated subcutaneously with 1.5×10^9 viable cells; twelve animals received 1.5×10^9 heat-killed cells. Twelve weeks following the first inoculation all animals received a dose of 2×10^9 heat-killed cells, and two weeks later all animals were infected with 830,000 cells of the virulent *B. melitensis* strain #6015 which, based upon earlier unpublished data, was equivalent to 33 ID_{50} doses. A third group of 12 nonimmunized goats was added to the experiment at this time to serve as a control group; animals in this group were infected with the same number of virulent cells.

Breeding of the animals was carried out with the aid of two male goats which were allowed to run with the females two months after the first injection of the vaccine strain. Breeding was completed about two weeks before the challenge infection was given.

Agglutination tests and blood culture data from the three groups of animals are recorded in figure 1 and table 1, respectively. It is of interest that the animals which received living cells of the mutant strain did not differ significantly from the controls as far as agglutination titers following infection are concerned. On the other hand the absence of organisms from the blood in the animals immunized with the living vaccine stands in marked contrast to the response of the other two groups of animals.

The data on resistance to the challenge infection are presented in table 2. No evidence of infection was obtained in the goats or their kids in the group which received the vaccine strain in the living state. The pronounced ability of the living vaccine to immunize thus is strongly contrasted to the inability of the nonliving cells of the same strain to protect against the dose of virulent cells employed.

The incidence of abortion (table 2) in the three groups of animals is of considerable interest. Again, the ability of the living form of the vaccine strain to protect against this manifestation of the infection is clearly evident.

The distribution of brucella in the tissues of animals which aborted was determined at au-

TABLE 2

Resistance to infection induced by a mutant strain of Brucella melitensis

Vaccine Employed	No. Adult Animals at Risk	Goats Infected*
Living†	13	0
Nonliving‡	12	10
None, controls§	12	10

* Judged on the basis of recovery of brucella from tissues.

† Twelve live, uninfected kids delivered. One uninfected kid born dead at full term.

‡ Nine abortions occurred in this group; all fetuses were infected. Three live kids delivered, one of which was infected.

§ Nine abortions occurred in this group; all fetuses were infected. Three live kids delivered, one of which was infected. One nanny in this group was not bred successfully but when it was sacrificed 160 days after challenge it was found to be infected. Of the two uninfected adult goats one delivered an infected kid.

TABLE 3

Isolation of brucellae from tissues

Tissue at Autopsy	Infected Animals*
Blood	3/19
Spleen	15/19
Liver	3/19
Lung	10/19
Marrow	2/15
Kidney	9/19
Uterus	18/19
Left prescapular L.N.	15/19
Right prescapular L.N.	15/19
Left precrural L.N.	16/19
Right precrural L.N.	15/19
Left supramammary L.N.	16/20
Right supramammary L.N.	17/19
Mesenteric	6/17
Hepatic	12/17
Left prefemoral L.N.	16/19
Right prefemoral L.N.	15/19
Placental cotyledons	17/18

* All animals in this series had aborted within 48 hr prior to autopsy.

topsy within 48 hr after abortion. The results are summarized in table 3. Marked differences are to be noted between the spleen, lymph nodes, uterus and lung tissue as sites of localization of brucellae on the one hand and the liver, kidney, blood and marrow on the other hand.

DISCUSSION

One of the most interesting aspects of the active immunity conferred on goats by the mutant strain, isolated originally when a streptomycin-dependent population was placed on streptomycin-free Albimi agar, is the great difference in effectiveness between the living and nonliving forms of the mutant. In earlier studies on mice, guinea pigs and monkeys employing the drug-dependent strain, equal effectiveness of the living and dead forms of the strain was observed (Herzberg and Elberg, 1955; Elberg, Henderson, Herzberg and Peacock, 1955). Unpublished data on immunization of goats with the dependent strain followed the same pattern. However, in the earlier experiments on monkeys and goats smaller doses of the challenge strain had been used. The data reported in the present paper, therefore, differ in the important matter of the size of the challenge infection. Hence it is proposed that the immunity conferred by the viable and nonviable forms of the mutant strain has been tested more rigorously in the present experiment than heretofore.

In earlier experiments the thesis that effective immunity to brucella infection requires a quality of persistence and an ability to multiply on the part of the vaccine strain was proposed (Elberg, Henderson, Herzberg and Peacock, 1955). There is no question of the greater persistence of the mutant strain used in these studies when compared with that of the streptomycin-dependent strain of *B. melitensis* used in earlier studies. It is unfortunate that it was not possible to introduce a fourth group of animals which received the nonviable cells of the vaccine strain in a suitable adjuvant in order to examine further whether persistence itself, rather than the act of multiplication of the vaccine strain, is prerequisite to establishing an immunity against an infection essentially intracellular. Subsequent experiments on the drug-dependent strain in adjuvant did not lend support to the idea that persistence with slow adsorption of antigen is a major determinant of immunity (Herzberg and Elberg, 1955).

In the light of the observations recorded here it is of importance to refer to the experiments of Zdrodowski (1953), Vershilova (1954), Kral (1955), Dranken and Simagina (1955) and Dranken and Malyutin (1955) who have used a clone originally isolated from *Brucella abortus* strain 19 for highly successful immunization of humans, guinea pigs and other animal species against *B. melitensis* infection. The strain gives dye tests and H_2S production typical of *B. abortus*. The phenomenon of cross-immunization which these workers have apparently established raises the question of the effectiveness of the derived nondependent strain against infections by *B. abortus* and *Brucella suis*. No data are yet available on which to base a precise statement about this possibility.

Comparison of the results obtained in guinea pigs by Vershilova and Kokorin (1954*a*, *b*) who employed the living vaccine derived from *B. abortus* strain 19, with the results obtained in mice by Elberg, Steiner and Doll (1955) using the streptomycin-dependent strain of *B. melitensis*, reveals in both experiments a response to vaccination characterized by early generalization and hyperplasia of the reticulo-endothelial elements of the liver, spleen and regional lymph

nodes. The former workers observed the same events in the lung and kidney. The hyperplasia increased during the first period and then gradually decreased as the organisms were eliminated, the immune process causing no permanent histopathologic changes. Both groups of workers observed that when heat-killed cells were injected, the histopathologic events were shortened still further and, in the case of the former group, the immunity was not as pronounced as conferred by the living vaccine. In contrast, the nonviable cells of the streptomycin-dependent strain were as effective as the viable cells against the dose of virulent brucellae used to test the immunity. The greater persistence of the nondependent mutant in the goat (observed also in unpublished results on the mouse and guinea pig) and the highly effective immunity resulting therein confirms the view of Vershilova and Kokorin (1954*a*) that the production of a high level of immunity requires an intensive and prolonged generalized vaccination process, that is, a prolonged, nonsterile phase, yet relatively shorter than that induced by typical brucella infection with fully virulent strains.

The precise role of antibody activity in brucella immunity remains enigmatic at this time. In the case of another intracellular parasite, *Mycobacterium tuberculosis*, it has been demonstrated (Fong, Schneider and Elberg, 1956) that antibody delays or prevents, respectively, the destruction of the mononuclear cell (depending on whether "normal" or "immune" mononuclears are used) which normally occurs as a sequel to ingestion of tubercle bacilli. Thus, the antibody, serving to protect the integrity of the phagocytic cell from damage following phagocytosis of virulent bacilli, may augment or complete the resistance acquired by the phagocyte by active immunization. The possible implications of such phenomena for applied immunization practices against brucella infection could be most critical when the results in figure 1 are considered. It is not uncommon in brucellosis control work to regard the development of antibody following immunization as undesirable because it interferes with the detection of infected animals by the agglutination test. The admissibility of such a view is open to more serious question if the observations referred to above on antibody function in tuberculosis are extended to brucellosis. It is possible that a reagent which fails to induce a significant serologic response may merely be reflecting a minor, temporary protection, one in which the mononuclear cells have been insufficiently impressed. Such a situation appears to occur as a response to certain colonial variants or antigenic fractions (Elberg, Herzberg, Schneider, Silverman and Meyer, 1951).

SUMMARY

A nondependent mutant isolated from a streptomycin-dependent population of *Brucella melitensis*, when injected in the viable state, conferred an immunity in goats which enabled the animals to resist a challenge infection of 33 ID_{50} doses of *B. melitensis*.

Nonviable cells of the mutant strain failed to establish an immunity to the challenge dose of virulent *B. melitensis*.

An explanation for the mechanism underlying the effectiveness of the viable vaccine is presented based upon the act of multiplication *in vivo* and consequent dissemination of the vaccine strain.

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