Resistance to Reinfection in Experimental Brucellosis. (20703)

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Elberg and Silverman's report(1) contains an elaborate discussion and bibliography on immunology of brucellosis up to 1950. Few publications have appeared during the last 3 years, while this work was being done(2,3). Information in the literature, about the effect of the presence or absence in tissues of the brucella of primary inoculation on the duration of resistance to reinfection in experimental animals, is meager. The purpose of this work was to study this aspect of the problem.

Materials and methods. Following McEwen's technic(4), 2 smooth strains of *Br. abortus* were used: Strain No. 1489, a Co_2 dependent organism, and strain "Sablin" which grew aerobically and under partial CO_2 tension. The anaerobic culture was used for primary inoculations; reinoculations were done with the aerobic organisms. Animals were inoculated subcutaneously or/and intracardially with .1 ml of a saline suspension matching $BaSO_4$ standard No. 1, prepared with a 48-hour-old growth on tryptose agar. Preliminary experiments showed that this amount produced infection consistently in all animals, when given subcutaneously or intracardially, as evidenced by abundant growths from spleen and/or lymph nodes one month after inoculation.* Castañeda's method(5) was used routinely for blood cultures and organs were cultured by streaking the cut surface of tissues or macerated material on tryptose agar. The number of organisms in tissues was estimated by depositing measured amounts of diluted macerated tissue on the surface of agar plates. The history of the strains and description of methods are given in detail in previous publications(6,7).

In preliminary experiments cultures were made of the bile, urine and all tissues of guinea pigs infected subcutaneously with the 2 strains of *Br. abortus* utilized in this study. Organisms were recovered most frequently from the spleen, pooled lymph nodes, bone marrow and lungs. Infection lasted longest in spleen and lymph nodes. Brucellae were recovered from these tissues in all animals 3 months after inoculation but were isolated only occasionally after 6 months. Brucellae have been recovered only once from the urine and twice from bile in approximately 100 animals examined from one week to 6 months after infection. The acriflavine test, applied routinely to a large number of colonies recovered from the tissues, was consistently negative.

Results. The data presented in the following experiments are typical of the results ob-

TABLE I. Resistance to Reinfection among Guinea Pigs Experimentally Infected with *Br. abortus*.

Days after infection‡ when animals were reinoculated	No. of animals reinoculated	Cultivation of Brucellae from spleen and/or lymph nodes	
		Organism of original infection	Organism of re-inoculation
11	6	All,* 4+†	5 —
			1 2+
30	6	" 4+	All, —
58	6	" 3+	" —
94	1	3+	" —
	1	1+	
	1	—	
128	2	—	" —
	1	1+	
170	2*	4+	—
	1	—	1+
	2	—	—
225	2*	—	—
	1	—	—
	1	2+	—
	1	—	1+
420	1*	—	—
	2*	—	4+

Controls not included; all positive.

* Animals killed 2 wk after reinfection was attempted; other animals killed 4 wk after reinoculation.

† 1+ = 1 to 5 colonies, 2+ = 6 to 15 colonies, 3+ = 16 to 30 colonies, 4+ = 30 to 75 colonies, 5+ = more than 75 colonies.

‡ All animals gave a positive blood culture at one time or another during exp.

* The disadvantages of using a 100% endpoint were eliminated by determining in each case, active primary infection by blood cultures.

TABLE II. Resistance of Guinea Pigs, Experimentally Infected with *Br. abortus*, to Intracardial and Subcutaneous Reinoculation.*

Days after reinoculation when animals sacrificed	Route of reinoculation	No. of animals		Cultures from spleen and/or lymph nodes	
		Normal control	Reinoculated†	Brucella of original infection	Brucella of reinoculation
3	S	2			3+
			2	—	—
			1	C	1+
			1	1+	1+
	I	2			5+
			1	1+	—
			1	—	—
			2	1+	1+
10	S	2			5+
			2	—	—
			1	1+	—
			1	—	2+
	I	2			5+
			1	1+	—
			1	—	1+
			1	—	—
40	S	2			5+
			2	—	—
			1	1+	—
	I	2			5+
			1	1+	—
			2	—	—
8	I	3			5+
			2‡	—	—
			2§	4+	—

* 5 mo. after primary infection. † 3 animals died and discarded. ‡ Reinoculated 7 mo. after infection. § Reinoculated 2 mo. after infection.
S = Subcutaneous, I = Intracardial, C = Contaminated.

tained repeatedly with relatively small groups of animals.

Exp. 1 was done to study any correlation between resistance to reinfection and the presence in the tissues of the organisms of primary inoculation. Forty-eight guinea pigs were injected subcutaneously and subsequently challenged, at different times, by the same route. Animals were sacrificed and tissues were cultured 2 or 4 weeks after reinoculation. Eleven guinea pigs died of intercurrent infection and were discarded. Results are shown in Table I. From 3 to 6 normal controls, not included in the table, were always injected at the time of reinoculation and all gave abundant growths from spleen and lymph nodes. Cultivable brucellae disappeared from the tissues in some animals earlier than in others. The bacteria of primary inoculation were recovered in one instance 225 days after infection; this animal was resistant to reinoculation. Two animals challenged 170 days and 225 days respectively after primary inoculation, from which organisms of the original infection were not cultured, were susceptible to reinfection. Three animals, reinoculated 420 days after infection gave negative cultures for brucellae of the original infection. One of these animals was resistant and 2 were susceptible to reinoculation. The organisms of reinoculation were never recovered from the tissues in the presence of the brucellae of primary infection, except in one instance. The exception was a guinea pig reinoculated 11 days after infection.

Exp. 2. Failure to recover the organisms of subcutaneous reinoculation from the spleen and lymph nodes of resistant animals, at a time when abundant growth was obtained from the tissues of controls, may be due to 1) inability of organisms to penetrate into the deep

tissues 2) rapid disposal of the bacteria after reaching the internal organs. The purpose of this experiment was to investigate these possibilities. Twenty-four animals were divided into 2 groups of 12 each, 5 months after primary subcutaneous inoculation. One group was reinoculated subcutaneously and the other intracardially. Animals from each group were sacrificed 3, 10 and 40 days after reinoculation. Results are shown in Table II. The results obtained in a separate experiment with 4 animals challenged intracardially 2 or 7 months after infection, and sacrificed 8 days after reinoculation, are also included in this table. Brucellae penetrated the internal organs when injected subcutaneously into animals 5 months after primary infection, but they disappeared rapidly. Similarly, organisms injected intracardially also cleared promptly from the tissues in resistant animals. Infected guinea pigs reinoculated subcutaneously exhibited the local reaction described by Burnet(8) characterized by small abscesses which in many instances ruptured to the outside and eventually healed. In normal controls this reaction was less frequent and milder. The pus always gave abundant growth of brucellae. This local reaction was not a major contributing factor in resistance to reinfection among previously infected animals challenged subcutaneously. Cultures from spleen and/or pooled lymph nodes have been reported here as the sole criterion of infection because when cultures from these tissues were negative those of the blood, bone marrow, liver and other organs were also negative. In many cases streaked cultures of the cut surface or organs and macerated tissue, as well as a quantitative estimation of the number of organisms, were done in the same animal. Under these circumstances the number of animals sacrificed on any single day was necessarily small. These experiments, however, have been repeated using small groups of animals and the same results reported above have been obtained consistently.

Exp. 3 was attempted in order to meet criticism based on the assumption that intracellular brucellae cannot be cultured by the methods employed. Exp. 2 and 3 were repeated using, in addition to the culture technics utilized previously, cultivation of tissues after submitting them to rapid vibration in a Mickle tissue disintegrator. Preliminary experiments showed that disintegration of 10 ml of tissue suspensions, at a given amplitude, for 5 minutes gave best results. Estimation of the number of organisms in tissue suspensions (lymph nodes, spleen, and liver) before and after disintegration showed no significant differences. The results obtained with disintegrated materials were essentially the same as with the other methods and the findings reported above were fully corroborated. When disintegrated citrated blood was deposited directly on the surface of agar media growth appeared earlier than in Castañeda's double medium(5). On solid media, inoculated with same amount of undisintegrated and disintegrated blood, growth was more abundant and colonies were usually larger after disintegration. This observation, made in resistant animals and in those in which agglutinins had not yet developed, suggests that some bacteria may have been dislodged from the phagocytes, making the nutrients in the medium more readily available.

Summary and conclusions. 1. The purpose of the present work was to determine if a relationship exists between resistance to superimposed infection and the presence of the organisms of primary inoculation in the body of guinea pigs infected with *Br. abortus*. The organisms of reinoculation never persisted in the tissues in the presence of the brucellae of primary infection, except in one instance. The exception was a guinea pig reinoculated 11 days after infection. Some animals, from which the organisms of the original infection were not recovered from any of the tissues, were resistant to reinfection. Resistance waned gradually after the organisms of primary inoculation could no longer be recovered from the tissues. 2. A local reaction with abscess formation was produced at the site of subcutaneous inoculation in infected guinea pigs; brucellae were recovered in large numbers from these lesions. The organisms of reinfection penetrated into the deep tissues but disappeared promptly. This suggests that the local reaction was not a major contributing factor in resistance to reinfection. When

previously infected animals were injected intracardially the brucellae of reinfection also disappeared rapidly from the tissues. 3. In order to meet the criticism that intracellular brucellae could not be cultivated by routine methods, all tissues were cultivated after disintegration by rapid vibration, under optimal conditions. The results obtained with this technic corroborated the findings obtained with other methods. 4. It is puzzling to see that the *Brucella* of original infection can persist for months in the tissues from which the organisms of reinoculation disappear rapidly. An explanation for this phenomenon is not apparent and these studies are being extended in an attempt to clarify this point. 5. Cultivation of disintegrated blood on the surface of solid media constituted an improvement over other methods used routinely for the isolation of brucellae from the blood.

1. Elberg, S. A., and Silverman, S. J., *Immunology of brucellosis,* in *Brucellosis,* a symposium, AAAS, 1950.

2. Pollack, A. D., Kelley, E. H., Gorelick, A. N., Braun, W., and Victor, J., *J. Inf. Dis.,* 1952, v90, 267.

3. Braun, W., Gorelick, A. N., Kraft, M., and Mead, D. D., *ibid.,* 1952, v89, 286.

4. McEwen, A. D., *Vet. Rev.,* 1940, v52, 441.

5. Ruiz-Castaneda, M., PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 114.

6. Pomales-Lebrón, A., and Fernández, C., *J. Bact.,* 1952, v64, 837.

7. Pomales-Lebrón, A., Hall, E. H., and Fernández, C., *Chemotherapy of Br. abortus infection,* 3rd Interam. Congress on Brucellosis Pan American Sanitary Bureau, 1950.

8. Burnet, E., *Arch. Inst. Pasteur Tunis,* 1925, v19, 62.

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Relative Biological Effectiveness of Fast Neutrons, Gamma Rays, X-Rays on Grasshopper Nymph Ovarioles. (*Melanoplus differentialis*) (20704)

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In previous communications(1-3) it was demonstrated that anabolic processes are more susceptible to ionizing radiations than catabolic processes. In order to examine this phenomenon further a tissue was required in which growth, in the sense of the synthesis of cellular material, takes place, in the absence of cell division. These requirements are fulfilled by the nymphal grasshopper ovariole and its developing eggs. During early oogenesis in the ovarioles, the newly differentiated unfertilized egg is approximately 35 μ in diameter, but within 45 days this cell attains a size of 4 cu mm, increasing in volume by a factor of 1.6×10^5. Throughout this period (still before fertilization) the egg contains the diploid number of chromosomes. Meiotic divisions begin just prior to laying and are completed after the eggs are laid, as previously shown by Slifer and King(4).

In accordance with expectation the developing eggs were found to be highly radiosensitive: Whereas a total-body dose of 1500 r of 200 kv x-rays was necessary to kill 50% of the grasshopper nymphs in a 10-day period, only 350 r or its equivalent was sufficient to destroy completely most of the eggs in the ovarioles. In this paper the relative biological effectiveness of Co^{60} gamma rays and fast neutrons is compared with 200 kv x-rays for the production of this irradiation effect.

Material and methods. Fifth or sixth instar grasshopper nymphs were exposed to whole body x-radiation in doses ranging from 52 to 801 r, from a 200 kv, 15 ma, oil-cooled x-ray machine; HVL: 0.25 mm Cu; no additional filtration. The grasshoppers were exposed in a covered Petri dish made of cellulose acetate. In each dosage series 10 or 12 grasshoppers were kept as controls and an equal number were irradiated. Gamma and fast neutron exposures were all made in the gamma-neutron radiation chamber recently described by Vogel *et al.*(5). Slow neutrons from the thermal