Toxicity, Infectivity and Antigenicity of a Streptomycin Dependent Mutant of *Brucella abortus* (Strain 19). (20172)

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Streptomycin-resistant *Neisseriae* and *Escherichiae* were found to be non-pathogenic for mice unless the animals were treated with the drug(1,2). It was therefore to be expected that strictly dependent mutants should be applicable for immunization purposes in untreated animals. However, *Brucellae* strictly dependent on streptomycin have not yet been described, although some strains grow more abundantly in its presence(3). The following experiments were carried out with a strictly dependent mutant of *Br. abortus* which required for its optimal growth a concentration of 0.01% of streptomycin.

Origin of the strain. In the course of studies on the number of resistant mutants in cultures of *Br. abortus*(4) several broth cultures derived from single colonies were exposed to a 0.01% concentration of streptomycin. In one culture some mutants which survived after an exposure to 0.01% of the antibiotic grew better at this concentration than at 0.001% and 0.0001%. They were continuously subcultured on broth containing 0.01% of streptomycin, lost gradually their ability to grow at lower concentrations of the antibiotic and became finally dependent upon it.

Toxicity. The strains to be examined were grown on trypticase-soy-agar and washed off with small amounts of saline. Then cooled acetone was added, the dead cells were removed by centrifugation, resuspended in acetone, centrifuged again, dried *in vacuo* and prepared as 1% suspensions in saline. One half of each suspension was left unheated, while the other half was heated at 100°C for 2 hours. Corresponding groups of 10 mice received 12, 10, 8, 6 and 4 mg of unheated and heated bacteria intraabdominally and were held under observation for 14 days. The LD50 was 9.2 mg for the streptomycin-dependent, 9.6 mg for the streptomycin-resistant and 7.3 mg for the non-resistant *Br. abortus* 19 parent strain. No differences were observed between toxicity of unheated and heated bacteria.

Infectivity. Fresh agar cultures were washed off with broth and suspensions were prepared which contained 3.2 x 10^9 *Brucellae* per cc. Suitable dilutions of this suspension were poured into plates and the dose to be injected checked by plate counts. Groups of 20-25 mice received intraabdominally varying quantities of *Brucellae* suspended in 0.5 cc of broth. After suitable time intervals groups of five mice were killed by ether, the organs removed aseptically, ground with glass powder in porcelain mortars and the homogenous suspensions of fine particles were taken up in 50 cc of streptomycin broth. From these suspensions series of tenfold dilutions were made and 0.2 cc of suitable dilutions plated. The average number of *Brucellae* per organ was determined by plate counting. Table I shows the bacterial counts in the organs of mice infected with the non-resistant parent strain (NR) and the streptomycin-dependent mutant (D). When 3 x 10^6 NR-bacteria were injected, concentrations of more than 10^6 bacteria per spleen were observed and the bacteria persisted after 60 days. When the same quantity or 10 and 100 fold quantities of D-bacteria were injected, no such concentrations were ever observed, and the bacteria disappeared within 45 days from the spleen. When a 1000-fold infective dose of D-bacteria was given, more than 10^6 bacteria per spleen were counted, but they disappeared within 60 days. The administration of streptomycin did not prevent the final disappearance of the D-bacteria from spleen and liver. The spleen enlargements produced by D-bacteria were relatively small, and 5 x 10^7 of them were needed in order to produce enlargements for which about 5 x 10^3 NR-bacteria were sufficient as shown in Table II.

Agglutinogenic power. Twelve rabbits were divided into 4 groups and received at 3-day

TABLE I. Number of *Brucellae* in the Organs of White Mice Infected with Strains NR (Non-Resistant against Streptomycin) and D (Streptomycin-Dependent).

Organ	Strain	No. of bacteria inj.	No. of *Brucellae* per organ at intervals (days) after onset of infection 8	14	30	45	60
Spleen	NR	3×10^6	3×10^6	2×10^6	1×10^5	2×10^5	6×10^4
	D	2×10^6		5×10^6	5×10^2	1×10^2	0
		5×10^5		2×10^5	8×10^2	0	0
		5×10^7		7×10^4	3×10^2	0	0
		5×10^6	8×10^4	8×10^3	2×10^1	0	0
		$5 \times 10^{6*}$	9×10^3	6×10^3	1×10^2	0	0
		$5 \times 10^{6\dagger}$		2×10^4	1×10^3	0	0
Liver	NR	3×10^6	4×10^5	2×10^4	1×10^4	5×10^3	8×10^2
	D	2×10^6		4×10^3	1×10^2	0	0
		5×10^5		2×10^4	2×10^2	0	0
		5×10^7		2×10^3	2×10^2	0	0
		5×10^6	2×10^3	8×10^2	0	0	0
		$5 \times 10^{6*}$	3×10^3	0	0	0	0
		$5 \times 10^{6\dagger}$		2×10^3	2×10^1	0	0

* Received daily 1 mg streptomycin 7 days following onset of infection.
† " " 1 mg " 14 " " " " " " .

TABLE II. Average of Relative Spleen Weight, Expressed as % of Body Weight, in Group of Infected Mice. Normal value 0.6.

Strain	Time after inj. (days)	Relative spleen wt after inj. of No. of *Brucellae* 5×10^5	5×10^7	5×10^6	5×10^4	5×10^3	5×10^2
NR	8			.8	1.0	.7	.5
	15			1.4	1.1	1.0	.6
	30			1.1	1.2	1.0	.7
D	8	.8	.9	.6	.5		
	15	.9	1.1	.6	.6		
	30	.9	.6	.7	.5		

intervals intravenously 1.0, 2.0 and 3.0 cc of suspensions containing 10^9 D- or NR-bacteria per cc, both living and killed by acetone. At different time intervals after the last injection blood was taken and the agglutinin titers determined. Table III shows that the average agglutinin titer in the groups immunized with dead bacteria decreased quickly and reached the value of 1:200 after 45 days. No differences of agglutinogenic power were observed between dead NR- and D-bacteria. On the other hand, the agglutinins persisted for a longer period after the administration of living bacteria. Although the initial titers were nearly equal for both living NR- and D-bacteria, they decreased more quickly when living D-bacteria were employed.

Guinea pigs received 3 injections of 3 x 10^9 bacteria at 10-day intervals. Thirty days after the last injection, titers of 1:1000 were observed in sera of the animals treated with living NR-bacteria; 1:500 in those which were treated with living D-bacteria, while in the groups treated with acetone-killed bacteria the titers did not rise over 1:200.

Immunization experiments. Groups of 20 mice received intraabdominally varying quantities of living D- and killed D- and NR-bacteria. Three injections were given at 7-day intervals. 30 days after the last injection, all immunized mice and a group of 20 untreated mice received 10^6 *Brucellae* (*Br. abortus* 2308) intraabdominally. Five mice were killed at different periods after the challenge infection and the average number of *Brucellae* per spleen determined. Bacterial counts on plain agar and streptomycin-agar showed how many organisms of the immunizing D- and the non-resistant challenge strain were present. Table IV shows that in the group immunized with 1.5 x 10^{10} living D-bacteria the NR-bacteria did not appear in the spleen so long as D-bacteria were still present. In the groups treated with killed bacteria the number of the NR-challenge bacteria was markedly reduced as compared with their number in

TABLE III. Average Agglutinin Titers in Serums of Rabbits Immunized with Living and Acetone-Killed D- and NR-Bacteria.

Bacteria inj.	Titers observed in sera at different intervals (days) after last inj., ×1000					
	7	14	30	45	60	90
NR, living	1: 90	1:110	1:70	1:5	1:2	1:1
D, living	1:100	1:120	1:50	1:2	1:1	1:0.5
D & NR, killed by acetone	1: 40	1: 22	1: 5	1:0.2	1:0.2	1:0.1

TABLE IV. Immunizing Effect of 3 Injections of Living and Acetone-Killed D- and Acetone-Killed NR-Bacteria.

Antigen inj.	No./inj.	No. of *Brucellae*/spleen (D and NR) at different intervals (days) after inj. of challenge dose				
		7		15		30
		D	NR	D	NR	NR
D living	1.5×10^{10}	2×10^{2}	0	0	3×10^{2}	0
	$\times 10^{8}$	3×10^{2}	3×10^{2}	0	3×10^{2}	0
	$\times 10^{6}$	0	2×10^{3}	0	4×10^{3}	6×10^{2}
D killed	$\times 10^{10}$	0	3×10^{2}	0	2×10^{3}	4×10^{2}
	$\times 10^{8}$	0	2×10^{3}	0	2×10^{4}	7×10^{3}
	$\times 10^{6}$	0	2×10^{3}	0	2×10^{4}	3×10^{4}
NR killed	$\times 10^{10}$	0	3×10^{2}	0	3×10^{2}	9×10^{2}
	$\times 10^{8}$	0	8×10^{2}	0	2×10^{3}	7×10^{3}
	$\times 10^{6}$	0	1×10^{4}	0	7×10^{3}	4×10^{4}
Controls untreated		0	3×10^{6}	0	4×10^{4}	1×10^{5}

the untreated animals. The number of the NR-challenge bacteria was lower in the spleen of mice immunized with living D-bacteria than in those immunized with dead D- or NR-bacteria. Living NR-bacteria were not employed for the immunization since by the method employed it was impossible to determine whether the bacteria isolated after the challenge injection belonged to the immunizing or to the challenge strain.

Stability of the D-mutant. The D-strain was subcultured 6 times at monthly intervals on agar slants containing 0.01% of streptomycin and the appearance of non-dependent mutants tested for by inoculation of 10^{10} bacteria on plain agar and broth. No growth appeared without the presence of streptomycin.

Summary. A streptomycin-dependent mutant (D) of *B. abortus* (strain 19) showed the following properties: 1. Its toxicity for mice was almost equal to that of the streptomycin-resistant (R) and non-resistant (NR) parent strains. It produced infections of limited duration with relatively low concentrations of microorganisms in the spleens of white mice, and disappeared from the spleen within 45 to 60 days. Spleen enlargements were observed when 5 x 10^{7} or more microorganisms were injected. Subcutaneous injection in man did not result in serious reactions. 2. The agglutinogenic power of the acetone killed D-bacteria in rabbits and guinea pigs was equal to that of NR-bacteria. As living antigens D- and NR-bacteria produced high initial titers, but those produced by the D-bacteria decreased more quickly. Three injections of living D-bacteria prevented completely the appearance of virulent NR-bacteria given as challenge dose in the spleen or lowered their number markedly as compared with the non-immunized controls. Killed vaccines prepared from NR- and D-strains did not exert such marked effects.

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