THE DURATION OF BACTERAEMIA IN RELATION TO THE VIRULENCE OF *BRUCELLA* STRAINS

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Guinea-pigs inoculated subcutaneously with infecting dosages of Brucellae show a bacteraemia which persists for a more or less prolonged period. The duration of the bacteraemia may be a measure of the pathogenicity of the strain and, if so, might be made the basis of a test for virulence. A reliable test of this kind would be of value in the selection of suitable strains for vaccines and in assessing the possible significance of certain strains in epidemiology. It would be simpler and much more economical than the usual tests in which infectivity is estimated by injecting graded dosages of organisms into groups of guinea-pigs, necessarily large in number.

Jacotot & Valleé (1956) found that, in guinea-pigs inoculated subcutaneously with large dosages of various strains of Brucellae (20–60 thousand million organisms), bacteria were present in the blood 1 hr. after inoculation and remained in diminishing numbers for 2 or 3 days. A difference was then observed between 'pathogenic' strains (*Brucella abortus* and *Br. melitensis* from aborted foetuses) with which the bacteraemia persisted for 2–4 weeks and 'attenuated' strains (such as are used for bovine vaccines) which tended to disappear from the blood from about the 7th day. These workers suggested a standard test in which groups of four guinea-pigs were bled each week for 4 weeks after infection. The pathogenic strains yielded 2–4 positive blood cultures each week over the period of observation, as against 0–2 with the attenuated strains. In the authors' view a strain giving five or more positives out of the sixteen possible is probably too virulent for use in a live vaccine.

In the present work this test has been examined and modified, and various strains of *Brucella* have been investigated, especially a number of strains of *Br. melitensis* isolated from cow's milk in Great Britain, whose pathogenicity has been regarded as doubtful since no human infections with them have been recorded.

EXPERIMENTAL METHODS

The following strains of *Brucella* were used:

Br. abortus S19, the strain of low virulence used in veterinary vaccines.

Br. abortus 544 (Weybridge), designated as type strain by the Expert Committee on Brucellosis of the World Health Organization.

Br. melitensis 16M (Beltsville), similarly designated as type strain.

Br. melitensis B61, a thermo-agglutinable and probably avirulent stock culture (London School of Hygiene and Tropical Medicine).

The other strains were isolated in England from cow's milk and were submitted by the Directors of various laboratories of the Public Health Laboratory Service.

Br. melitensis 29208, isolated by Dr N. S. Mair in 1950 from milk from one cow on a farm in Leicestershire.

Br. melitensis B 156, isolated by Dr Lynette M. Dowsett (Norwich) in 1950 from a routine bulk sample from a farm in Norfolk.

Br. melitensis 169934, isolated by Dr J. E. Jameson (Brighton) in 1956 from bulk milk from a herd in Sussex.

Br. melitensis 175862, also isolated in 1956 by Dr J. E. Jameson from bulked tuberculin-tested milk from another herd in Sussex.

Br. abortus 2245, isolated in 1956 by Dr J. H. C. Walker (Maidstone).

The identity of the *Br. melitensis* strains isolated from milk in England was confirmed by Dr A. W. Stableforth, Director of the Ministry of Agriculture and Fisheries Veterinary Laboratory, Weybridge.

In preliminary experiments it was shown that these strains were able to grow from small inocula on ordinary media and on the selective media to be used. Suspensions in saline were standardized by opacity and aliquots from decimal dilutions were plated on liver agar and on the same medium containing 1 in 700,000 crystal violet. Colony counts from the higher dilutions showed that there was no inhibition of growth on the selective medium. An inoculum of a few viable organisms also produced growth consistently in liver broth containing the same concentration of crystal violet.

In each of the two main experiments five groups, each consisting of six guinea-pigs, were inoculated subcutaneously with different *Brucella* strains. *Br. abortus* S 19 was included in both experiments. At intervals of a week 1·0 ml. of blood was taken from each guinea-pig by cardiac puncture, and 0·3 ml. was spread on the surface of a liver agar plate and a crystal-violet liver agar plate with a glass spreader. The remainder of the blood was inoculated into crystal-violet liver broth. The cultures were incubated at 37° C. in CO_2 and were inspected at intervals for 7 days. The liver broth cultures were inoculated on to liver agar slopes and on to sections of crystal-violet liver agar plates after 3 and 6 days' incubation. The identity of colonies was confirmed by slide agglutination with specific antisera.

The following method was adopted for recording positive results. Plates showing 1–10 colonies were recorded as one plus (+), those with 11–50 colonies two plus (++) and those with more than 50 colonies three plus (+++). Positives obtained from the broth cultures were assigned one plus (+). The final score for any one guinea-pig was obtained by adding the plus sign for a positive broth culture to those of the plate with the greater count (since one of the two plates was occasionally partly overgrown with a mould). The maximum score obtainable was thus four plus (++++). It was not unusual, specially in the later stages, for plate cultures to be positive when the corresponding broth culture was negative. The presence of antibodies in the blood might possibly account for this.

RESULTS

First series

The results obtained in the first series are shown in Table 1. The strains used were *Br. abortus* S 19, a strain of *Br. abortus* and two strains of *Br. melitensis* isolated from milk in England, and the W.H.O. type strain of *Br. melitensis*. Following the

method of the French workers mentioned above, very large dosages were used—a total of about 50,000 million organisms, half the inoculum being injected subcutaneously in each thigh. Later work showed that these dosages were unnecessarily large, and some of the guinea-pigs inoculated with strains other than S19 developed ulcers at the site of injection.

It will be noted that all the blood cultures made on the 7th day after infection were positive. None of the later cultures from the guinea-pigs infected with strain S19 were positive, but, with the other four strains, positive cultures were obtained from nearly all the animals up to the 28th day. The ratio of positive blood cultures to the total possible for each of the strains over the four weeks is: *Br. abortus* S19, 6/24: *Br. abortus* 2245, 24/24: *Br. melitensis* 16M, 20/20: *Br. melitensis* 29208, 22/24: *Br. melitensis* B156, 18/20.

During the 5th week all the guinea-pigs were killed, except for two animals in each of the groups other than that receiving strain S19. The weight of the spleen in the dead animals is shown in Table 1. It was thought that the spleens of the guinea-pigs with a prolonged bacteraemia might be much larger than of those in which the bacteraemia was of short duration. There was, however, no very marked or consistent difference, but the animals with much enlarged spleens were, in fact, in the former groups.

In order to determine how long bacteraemia could last, blood cultures were taken from the surviving guinea-pigs each week for a further period, as shown in Table 1. The last positive culture was obtained from both guinea-pigs infected with *Br. abortus* 2245 in the 10th week after infection: with *Br. melitensis* 16M in the 5th and 6th weeks; with *Br. melitensis* B156 in the 5th week. The two surviving guinea-pigs inoculated with *Br. melitensis* 29208 yielded positive cultures in the 17th week. By the 22nd week, when the experiment was terminated, one guinea-pig had died and the other still gave a strongly positive blood culture. In some of the guinea-pigs one or more negative cultures were succeeded by a positive culture in the following week.

Second series

The strains used in this series were *Br. abortus* S19, *Br. abortus* 544, *Br. melitensis* 169934 and 175862 (both from cow's milk in England) and the rough stock strain of *Br. melitensis* B61. A smaller infecting dosage was given—about 18,000 million viable organisms. The same procedure was used for taking blood cultures and recording results as in the first series. The results, which are shown in Table 2, were even more clear-cut. During the first 4 weeks positive cultures were obtained on each occasion from all the guinea-pigs inoculated with the W.H.O. strain of *Br. abortus* and both the recently isolated strains of *Br. melitensis*. Only three of the six guinea-pigs receiving *Br. abortus* S19 gave positive cultures, one for 2 weeks, the others for only 1 week. No positive cultures were obtained from any of the animals inoculated with the old stock culture *Br. melitensis* B61. The ratios of positive cultures to 'possibles' were: *Br. abortus* S19, 4/24: *Br. abortus* 544, 24/24: *Br. melitensis* 169934, 24/24: *Br. melitensis* 175862, 24/24: *Br. melitensis* B61, 0/24. A final blood culture was taken at 8 weeks from

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Table 1. ***Result of blood cultures in successive weeks after infection with different*** **Brucella** ***strains***

Groups of six guinea-pigs infected with

Weeks after infection	*Br. abortus* S19						*Br. abortus* 2245						*Br. melitensis* 16M					
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	−	−	−	−	−	−	+	+	+	+	+	+	+	d	−	+	+	+
3	−	−	−	−	−	−	+	+	+	+	+	+	+	*	+	+	+	+
4	−	−	−	−	−	−	+	+	+	+	+	+	d	*	+	+	+	+
5†	510	660	670	620	700	510	580	640	+	−	1690	750	*	*	+	+	2150	890
6	.	.	.	.	.	.	.	.	+	+	.	.	.	.	+	−	.	.
7	.	.	.	.	.	.	.	.	+	−	.	.	.	.	−	−	.	.
8	.	.	.	.	.	.	.	.	+	+	.	.	.	.	−	−	.	.
9	.	.	.	.	.	.	.	.	+	+	.	.	.	.	−	−	.	.
10	.	.	.	.	.	.	.	.	+	+	.	.	.	.	−	−	.	.
11		.	.	.	.	.	.	.	−	*	.	.	.	.	−	−	.	.
12		.	.	.	.	.	.	.	−	−	.	.	.	.	*	*	.	.
13	.	.	.	.	.	.	.	.	−	−	.	.	.	.	*	*	.	.
14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
16	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
17	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
22	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Groups of six guinea-pigs infected with

Weeks after infection	*Br. melitensis* 29208						*Br. melitensis* B156					
	1	2	3	4	5	6	1	2	3	4	5	6
1	+	+	+	+	+	+	+	+	+	+	d	+
2	+	+	−	+	+	+	+	+	+	+	*	+
3	+	+	−	+	+	+	+	+	+	+	*	+
4	+	+	+	+	+	+	+	−	−	+	*	+
5†	+	780	620	700	+	630	650	−	720	380	*	+
6	+	.	.	.	+	.	.	−	.	.	.	−
7	+	.	.	.	+	.	.	−	.	.	.	−
8	+	.	.	.	+	.	.	−	.	.	.	−
9	+	.	.	.	+	.	.	−	.	.	.	−
10	+	.	.	.	+	.	.	−	.	.	.	−
11	+	.	.	.	+	.	.	−	.	.	.	−
12	+	.	.	.	+	.	.	*	.	.	.	*
13	+	.	.	.	+	.	.	*	.	.	.	*
14	+	.	.	.	+	.	.	.	.	.	.	.
15	+	.	.	.	+	.	.	.	.	.	.	.
16	+	.	.	.	+	.	.	.	.	.	.	.
17	+	.	.	.	+	.	.	.	.	.	.	.
22	+	.	.	.	d	.	.	.	.	.	.	.

+, + +, + + +, + + + + = positive blood cultures of differing intensities (see text).
− = negative.
d = dead.
* = not done.
† = the figures give the weights in mg. of the spleens of guinea-pigs killed in the 5th week.

Table 2. *Result of blood cultures in successive weeks after infection with different* Brucella *strains*

Weeks after infection	Groups of six guinea-pigs infected with: *Br. abortus* S19						*Br. abortus* 544						*Br. melitensis* 169934					
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
1	+	+	−	+	−	−	+ + + +	+ +	+ +	+	+	+	+ + + +	+ +	+ + + +	+ +	+ + +	+ + +
2	−	−	−	+	−	−	+ + + +	+ + + +	+ + +	+ + +	+ + +	+ +	+ + + +	+ + +	+ + + +	+	+ +	+ + +
3	−	−	−	−	−	−	+ + +	+ + +	+ + +	+ + +	+ +	+	+ + +	+ +	+ + + +	+ + +	+ + + +	+ + +
4	−	−	−	−	−	−	+ + +	+ + + +	+ + +	+ + +	+	+	+ + +	+ + + +	+ + + +	+ +	+ + +	+ + +
8	*	*	*	*	*	*	+ + +	+ +	+	+	+	−	+ +	−	+ +	+	+ +	+ + +

Weeks after infection	Groups of six guinea-pigs infected with: *Br. melitensis* 175862						*Br. melitensis* B61					
	1	2	3	4	5	6	1	2	3	4	5	6
1	+ + +	+	+ +	+	+	+ +	−	−	−	−	−	−
2	+ +	+	+	+	+ +	+	−	−	−	−	−	−
3	+	+	+	+	+ +	+ +	−	−	−	−	−	−
4	+	+	+ +	+	+	+	−	−	−	−	−	−
8	+	−	+	−	−	+	*	*	*	*	*	*

all the guinea-pigs in the three groups showing sustained bacteraemia. Positive cultures were obtained from five of the six animals infected with *Br. abortus* 544, from five of the six infected with *Br. melitensis* 169934 and from three of the six infected with *Br. melitensis* 175862.

DISCUSSION

Virulence and pathogenicity are relative terms, and when they are used the conditions under which the observations were made, including the host species, should be specified. In natural *Brucella* infection the virulence of a strain implies its capacity to produce undulant fever in man or clinical manifestations such as contagious abortion in cows, goats or other farm animals. Experimentally it is usual to estimate virulence in guinea-pigs, though larger animals have also been used. In general it would seem that observations in the field tend to be confirmed by laboratory tests for virulence. For example, long experience of living vaccines of *Br. abortus* S19 has established its innocuity for cattle, and its virulence for guinea-pigs is low. Strains of *Br. abortus* responsible for undulant fever in man have been traced to their origin in herds containing cows suffering from contagious abortion, and the same strains have been isolated in the laboratory by their capacity to produce lesions in guinea-pigs inoculated with the suspected milk. Complete correlation of observations regarding virulence to the guinea-pig and to man is of course not to be expected. Occasional accidental infections in man with strain S19 have been reported. Also, when the herds in this country were heavily infected with brucellosis and much raw milk was drunk, the estimated number of human infections did not exceed the relatively small figure of about 1000 annually. Clearly then, the occurrence of infection in man depends on such factors as dosage, portal of entry of the organism and the various specific and non-specific mechanisms that may contribute to resistance. Thus, while it is difficult to state definitely that a strain shown to be virulent to the guinea-pig is potentially virulent to man, the evidence suggests that this is probably so.

In the experiments now reported, *Br. abortus* S19, accepted as a strain of low virulence, was found in the blood stream of most of the guinea-pigs infected with it after 1 week, but was with one exception not found in subsequent weeks. The rough laboratory strain of *Br. melitensis* B61, as was to be expected, was not isolated from the blood on any occasion. The typical strains of the World Health Organization, *Br. abortus* 544 and *Br. melitensis* 16M, and the strain of *Br. abortus* 2245, taken at random from many isolated from cow's milk in England, produced in all the guinea-pigs a bacteraemia lasting for at least 4 weeks. Some of the animals were examined for longer periods and continued to show a bacteraemia for periods up to 10 weeks.

The other four organisms examined were strains of *Br. melitensis* isolated from cow's milk in different parts of England. Menton (see Duke, 1940) first reported the isolation in this country of strains with all the biochemical and serological characters of *Br. melitensis*. This led to the introduction of the Brucellosis-melitensis Order 1940, which empowered the authorities to slaughter the infected animals. Since

that time the organism has been isolated from milk in various parts of the country, as often as 6 times in some years (Cruickshank, 1954). No human infections with *Br. melitensis* (apart from those due to laboratory accidents) have been reported as arising in Great Britain, and some authorities have expressed the view that these strains are devoid of virulence (Dalrymple-Champneys, 1953). Stabbleforth (1954) has commented on the absence of human cases, although infected cows have aborted and been handled in the usual way, and their milk has been drunk raw in many instances. He states that the organism appears to be completely lacking in the usual pathogenicity of *Brucella* with the characters of *Br. melitensis*.

If the method used in the present work does in fact give a measure of virulence, the results do not support the suggestion that these strains are of low virulence. The four strains tested gave rise to bacteraemia which persisted for 4 weeks in all the guinea-pigs, except with one of the strains, in which the duration of bacteraemia was 3 weeks for two of the animals. With two of the strains bacteraemia continued for at least 8 weeks in five out of six and three out of six guinea-pigs. With another strain (29208) blood cultures in the two animals that were retained were still strongly positive respectively up to the 17th and the 22nd week, when the observations were terminated.

Even if the strains of *Br. melitensis* found in this country are in fact virulent for man, the absence of reported cases is perhaps not so puzzling as it might seem. The number of herd milks containing *Br. melitensis* at any one time must be an extremely small proportion of the total of infected milks and the expected number of human cases due to this organism must be correspondingly minute. Further, such cases would only be detected if diagnosis was made by isolation of the organism (as opposed to the agglutination test) and its precise identification by full biochemical and serological tests, including the use of monospecific absorbed antisera. This can rarely be done as a routine. It seems possible therefore that the occasional human case due to *Br. melitensis* might occur in this country and not be precisely recognized bacteriologically.

Two other points not hitherto fully documented have emerged from this investigation. One is the surprisingly long duration of bacteraemia that can be easily demonstrated after subcutaneous inoculation of brucellae. Most of the guinea-pigs gave positive blood cultures for at least 8–10 weeks, and two animals were still strongly positive after 17 and 22 weeks. The other points refer to the number of circulating organisms. This can be inferred very approximately from the symbols in the tables, since +, + + and + + + signs for the plates indicate respectively about 1–33, 34–166 and over 166 viable organisms per ml. of blood.

In general, the results now reported are in accord with those of Jacotot & Vallée (1956). It appears, however, from their paper that these workers used sixteen guinea-pigs for each strain, and that a different set of four animals was bled each week. In the present work only six were used, and the results were so consistent that four would have sufficed, but for possible losses during cardiac puncture. It seems probable that an economical method for the biological screening of antibiotics for brucellosis might be developed from this technique.

SUMMARY

The duration of bacteraemia in guinea-pigs following the subcutaneous inoculation of brucellae can be used as a measure of the virulence of the strains. Strains of low virulence (e.g. *Brucella abortus* S19) are almost always cleared from the blood stream within a week, whereas virulent strains produce a bacteraemia that regularly persists for 4 weeks and may last very much longer.

All of four strains of *Br. melitensis* isolated from cow's milk in Great Britain behaved in the same way as the typical and virulent strains of *Br. abortus* and *Br. melitensis* tested, regularly producing prolonged bacteraemia. These results do not support the suggestion that the strains of *Br. melitensis* isolated in this country are of low virulence.

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