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# KINETICS OF PLACENTAL COLONIZATION OF MICE INOCULATED INTRAVENOUSLY WITH *BRUCELLA ABORTUS* AT DAY 15 OF PREGNANCY

N. BOSSERAY

*From the Station de Pathologie de la Reproduction, Institut National de la Recherche Agronomique, 37380 Nouzilly, France*

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**Summary.—When 15-day pregnant mice were challenged i.v. with 1 to $2 \times 10^4$ virulent *Brucella abortus* Strain 544, the bacteria were recovered from all the placental discs and spleens of the dams, as few as 5 min post-challenge. Kinetics in both organs differed greatly. In spleens, the fraction of the inoculum recovered increased from 3–20%, from 5 min–6 h post-challenge. Brucella multiplication was low from 24–72 h. In each placenta, there was first an immediate dissemination of less than 0·1% of the inoculum. Then, some brucella disappeared leaving about 20% of the placentas free of infection, the others being infected with as few as 3 Brucella CFU on average, 4–6 h post-challenge. Brucella then multiplied in colonized placentas, leading to an average of $10^4$ brucella CFU per placenta at 72 h post-challenge.**

BRUCELLA ORGANISMS infect the reticulo-endothelial system, spleen, liver and the genital area of many mammalian species including man. Invasion of most target organs by this facultative intracellular bacteria is normally limited by the immune response of the host, developed during infection. Experiments recently made on different mouse models showed that liver and spleen brucella infections decrease until spontaneous cure (Young *et al.*, 1979; Ho and Cheers, 1982; Bosseray, Plommet and De Rycke, 1982). In contrast, placental infection can increase up to high levels. Smith *et al.* (1961) and Alexander, Schnurrenberger and Brown (1981) enumerated up to $10^{13}$ brucella per infected bovine delivery. The infected host seems to be unable to restrict development of placental infection. Environmental conditions, for example the presence of foetal erythritol (Smith *et al.*, 1962) and/or the special immunological status of placenta might explain this enormous proliferation of brucella. Placenta might also be among the target organs, which concentrate the majority of bacteria, at the early stage of infection.

The pregnant mouse was a satisfactory experimental model for studies of placental infections with *E. coli* (Coid *et al.*, 1978), *B. streptococcus* (Coid and Nicholson, 1981) and *Brucella* (Bosseray, 1980). This model was used in the present work to compare early events of *Brucella abortus* infection in individual placentas and spleens of the dams during the first 72 h post i.v. challenge.

A very small part of the inoculum reached and colonized placentas where brucella multiplied until 72 h post-challenge. In contrast. spleens concentrated the inoculum until 6 h post-challenge and brucella multiplication was low.

## MATERIALS AND METHODS

*Mice.*—CD-1 (Charles River, Elbeuf, France) and OF1 mice (Iffa-Credo, Saint-Germain sur l'Arbresle, France) were born in the station's environment-controlled animal building (temperature 21°, relative humidity 60%, sterile filtered air with 12 removals/h, water and

sterilized food *ad libitum*) and used according to availibility.

They were mated when 7–9 weeks old with males of respective strains. Day 1 of pregnancy was the day when the vaginal plug was observed.

*Challenge.*—A standard lyophilized inoculum of *B. abortus* Strain 544 prepared as previously described (Plommet and Bosseray, 1977) was diluted in buffered saline solution (BSS) before use. The brucella colony forming units (CFU) inoculated were enumerated by plating on Trypticase Soy Agar (TSA, Bio-Mérieux, Marcy l'Etoile, France) after 5 days at 37° in 10% $CO_2$.

The i.v. challenge (in 0·1 ml) was injected into the tail vein after vasodilatation under incandescent light for 3 min, at Day 15 of pregnancy.

*Autopsy—brucella enumeration.*—Mice were killed by cervical dislocation. Using an asceptic technique, the uterus and spleen were removed. The uterus was opened on a sterile board. Placental discs (placentas) were dissected from foetuses. Spleens and placentas were individually stored at −20° until bacterial count.

Number of brucella per spleen was determined by plating of appropriate dilutions of spleen homogenates in BSS. This technique, previously described in detail (Plommet and Bosseray, 1977), had a minimum detection level of 5 CFU per spleen.

Placentas were individually homogenized in 0·2 ml of BSS and each seeded on one TSA plate. Brucella were enumerated after 5 days at 37° in 10% $CO_2$. This technique detects as few as 1 CFU per placenta. It gives the real number of brucella CFU up to $10^3$ per placenta and a rough estimation of it by comparing colony density to a scale containing $10^3$, $10^4$, $10^5$ and $10^6$ colonies.

*Expression of data.*—A placenta was considered infected when at least 1 brucella CFU was isolated.

Frequency of placental infection was the ratio of infected placentas to total examined in a group of mice.

Degree of infection was the average number of brucella per infected placenta or per spleen. From 5 min to 6 h post-challenge, when the number of brucella was low, degrees were given by the arithmetic average of brucella per organ, and expressed as a fraction (%) of the inoculum. From 24–72 h post-challenge, data were first transformed into $\log_{10}$ values before calculation of average degree and standard error.

*Experiments.*—Experiments 1 and 2, using two strains of mice in lots of 5, analysed the responses to different challenge doses, 72 h after challenge.

Experiments 3–5, using two strains of mice in lots of 5 to 6 analysed the kinetics of placental and splenic infection to one challenge dose (Table I). This dose was chosen so that the scale of measured responses would be as large as possible. As indicated by preliminary assays it was about $1–2 \times 10^4$ Brucella.

## RESULTS

### *Dose-response curves of placentas and spleens were similar for CD-1 and OF1 mice, killed 72 h post-challenge*

Both strains of mice gave similar dose–response curves to either placental or

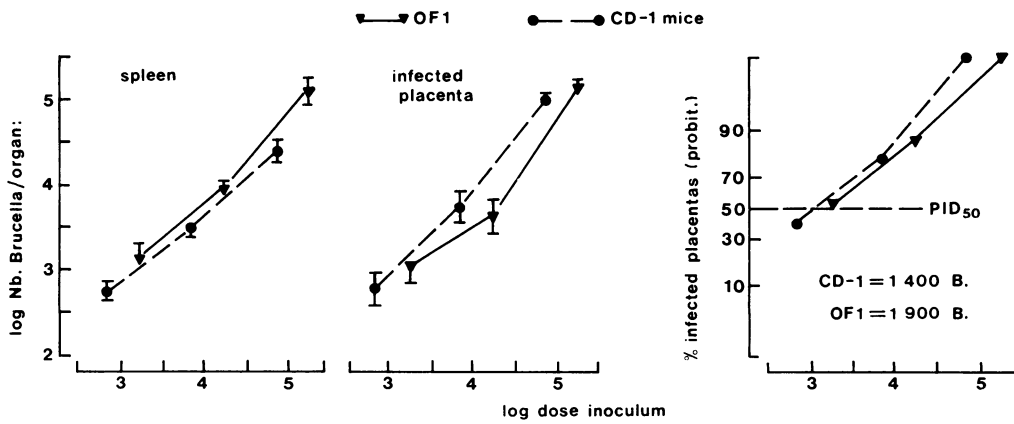


FIG. 1.—Frequency and degree of splenic and placental infections in response to challenge dose, 72 h after i,v. inoculation of *B. abortus* Strain 544 in OF1 and CD-1 mice, at Day 15 of pregnancy. All the spleens were infected but only a fraction of placentas. Thus, a placental infective dose 50% ($PID_{50}$) can be computed. Both mouse strains behave similarly. Five mice (46–60 placentas) per dose. CD-1 mice challenged with $0·7 \times 10^{3-5}$ and OF1 mice with $1·8 \times 10^{3-5}$ CFU. Vertical bar: standard error.

TABLE I.—*Frequency of isolation of Brucella from placenta after challenge. It declined from 100% to 60–80% and remained stable until 72 h.*

| Time post-challenge | Placenta infected/total (%) Experiment No. 3 | 4 | 5 |
|---|---|---|---|
| 5 min | 71/71 (100) | | |
| 10 min | 55/55 (100) | | |
| 20 min | 54/59 (91·5) | 79/80 (98·7) | |
| 40 min | 61/68 (89·7) | | |
| 1 h | 56/64 (87·5) | 68/74 (91·9) | 47/48 (97·9) |
| 1 h 30 | 55/62 (88·7) | | |
| 2 h | | 76/79 (96·2) | 48/55 (87·3) |
| 4 h | | 64/79 (81·0) | 35/55 (63·6) |
| 6 h | | 78/87 (89·6) | 38/56 (67·9) |
| 24 h | | | (*)98/117 (83·7) |
| 48 h | | | 42/50 (84·0) |
| 72 h | | | 45/56 (80·3) |
| Mouse strain | OF1 | OF1 | CD-1 |
| Challenge dose (CFU) | $1·8 \times 10^4$ | $1·0 \times 10^4$ | $0·7 \times 10^4$ |
| No. of mice per group | 5 | 6 | 5 |

(*) 10 mice.

TABLE II.—*Distribution of infected placentas in classes of infection (CFU per placenta) in relation to time post-challenge*

| Time post-challenge (h) | No. of infected placenta (%) <10 CFU | 10–$10^2$ | $10^2$–$10^3$ | $10^3$–$10^4$ | >$10^4$ | Total* |
|---|---|---|---|---|---|---|
| 6† | 111 (95·7) | 5 (4·3) | 0 | 0 | 0 | 116 |
| 24 | 44 (44·9) | 48 (49·0) | 6 (6·1) | 0 | 0 | 98 |
| 48 | 6 (14·3) | 7 (16·7) | 7 (16·7) | 12 (28·6) | 10 (23·8) | 42 |
| 72 | 1 (2·2) | 3 (6·7) | 1 (2·2) | 11 (24·4) | 29 (64·4) | 45 |

* From Table I.
† Pooled results from experiments 4 and 5.

splenic infections, expressed in frequency or in degree of infection. The dose required to infect 50% of the placentas (placental infective dose 50%, $PID_{50}$) according to the Reed and Muench method was 1400 CFU with CD-1 strain and 1900 CFU with OF1 strain (Fig. 1).

Because both strains behave similarly, whichever was available was used in the following experiments.

*Colonization of placenta followed a two-step process*

Five–10 min post-i.v. challenge, brucella were disseminated to all placentas (frequency of infection, 100%) (Table I). On average, there were 13 and 15 CFU per placenta (Fig. 2), that is 0·1% of the inoculum, according to challenge dose (Fig. 3).

Then, number of brucella decreased. Some placentas became free of detectable infection, the others (64–90%) remained infected with 3 CFU on average, 4–6 h post-challenge (Table I, Fig. 2).

Frequency of infection remained about stable from 4–6 h, until 72 h post-challenge One may consider that colonization, that is irreversible fixation of brucella in placentas took place 4–6 h post-challenge.

*Brucella quickly multiplied in the colonized placentas*

Degree of placental infection increased from 6–72 h post-challenge (Fig. 2). Most of the colonized placentas (93·9%) were infected with less than 100 CFU, 24 h post-challenge. None of them had more than $10^3$ CFU (Table II). Seventy-two h

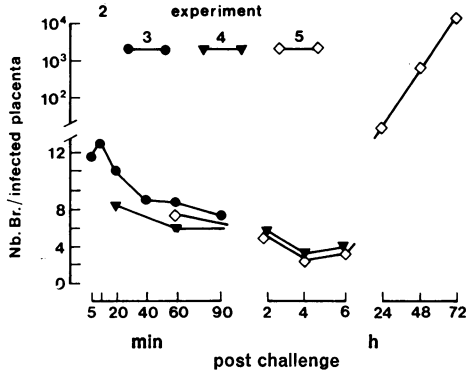


FIG. 2.—Kinetics of placental infection. The average number of brucella per infected placenta decreased from 10 min post-challenge to about 3 CFU at 4 h post-challenge, when it stabilized. It then increased to $10^4$ CFU within 72 h. Three experiments (Table I). At least 5 mice (35–98 infected placentas) per point.

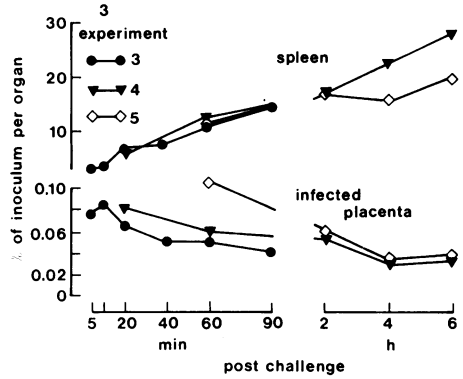


FIG. 3.—Evidence that each infected placenta retained only a small and decreasing fraction of the inoculum, whereas the spleen concentrated a progressively higher amount. Three experiments (Table I). At least 5 mice (38–79 infected placentas) per point.

post-challenge, 64·4% of the colonized placentas had more than $10^4$ CFU.

*Colonization of spleens was a continuous process with limited brucella multiplication*

All spleens were infected.

On average, there were 524 and 596 CFU per spleen, 5 and 10 min post-challenge. These averages represent 2·9 and 3·3% respectively of the inoculum (Fig. 3). Initial splenic infection was 38 times greater than initial placental infection.

Degree of splenic infection increased steadily during the first 6 h (Fig. 2). Slower increase was observed later with an average of $3{\cdot}3 \pm 0{\cdot}09$, $3{\cdot}29 \pm 0{\cdot}11$ then $3{\cdot}52 \pm 0{\cdot}11$ degrees, 24, 48 and 72 h respectively post-challenge.

Brucella multiplication is much slower in spleens than in colonized placentas. Seventy-two h post-challenge, 64·4% of the colonized placentas (29/45) were more intensively infected than the spleens of respective dams.

## DISCUSSION

*Brucella abortus* Strain 544 inoculated i.v. to 15-day pregnant mice was recovered in small amounts from all placentas within the first 10 min following injection. Then both number of brucella per infected placenta and number of infected placentas decreased. Stabilized state was reached 4–6 h after challenge. At this time, about 80% of placentas examined were still infected and average number of brucella per infected placenta was 3 CFU. Frequency of infection remained approximately stable until 72 h post-challenge, while placentas supported strong brucella multiplication.

Splenic colonization kinetics differed strongly from that of placentas. Spleens received the largest amount of brucella in the first minutes after challenge, and followed a regular concentrating process during the first 6 h of infection. Brucella proliferation during the following 3 days was much slower than in placentas.

The course of splenic infection illustrates the blood clearance function of the organ where, once concentrated, brucella can either be phagocytosed and destroyed, or survive both in intracellular and extracellular state (reviewed in Spink, 1956). The slowly increasing number of brucella per spleen between 24 and 72 h after challenge resulted from concentration from the blood, killing by phagocytic cells and multiplication of surviving bacteria.

The kinetics of placental infection suggests two successive mechanisms

during colonization. Dissemination of brucella to the dam's blood stream within the first minutes of infection. The number of brucella per placenta probably depended on maternal blood supply of placental discs. Brucella could have been either in the maternal blood compartment of the placenta or just trapped at the surface of placental cells.

Blood clearance by organs of the reticuloendothelial systems including the spleen, could contribute to the discharge of brucella from placenta, hence the decrease of frequency of placental infection.

Colonization was effected when frequencies and numbers of brucella per infected placenta were stabilized, 4 to 6 h post-challenge. Frequency of placental colonization remained approximately stable until 72 h post-challenge. This suggests that new colonization after 6 h were scarce or impossible.

Brucella quickly multiplied in placentas. Generation time was estimated on enumerations made at 6 and 24 h post-challenge. There were 3 CFU on average per colonized placenta, 6 h post-challenge. Eighteen hours later, there were 27 CFU (arithmetic average). This progression corresponded to 3 or 4 generations, that is, the number of brucella per placenta might double every 4 to 6 h.

This estimated generation time *in vivo* is close to that previously estimated *in vitro* by Richardson and Holt (1964) on *B. abortus* cultivated in calf spleen cells (8 h), and uterine mucosal and foetal calf skin cells (4 h).

This research shows that the sensitivity of the mouse placenta to infection by *B. abortus* strain 544 is not due to an early local concentration of bacteria. Only a few bacteria (less than 0·1% of the inoculum) become attached to this organ and colonize it. The placenta becomes, within a few days, the main centre of infection in the pregnant mouse because it is a site of intense proliferation of brucella.

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