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Intraperitoneal infection with *Salmonella abortusovis* is partially controlled by a gene closely linked with the *Ity* gene

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SUMMARY

The aim of the present study was to determine whether the *Ity* gene, which controls the resistance to *S. typhimurium* infection in mice, also governs the resistance to *S. abortusovis*, a serotype specific for goat and sheep. During either i.v. or i.p. infection, BALB/c mice (Ity^s) were not able to control the growth of *S. abortusovis* and eventually died from infection. In contrast CBA (Ity^r) or (C.CB)F1 ($Ity^{r/s}$) mice were able to control the growth of these bacteria. Using congenic C.D2 Ity^r mice, we found that the gene controlling resistance to *S. abortusovis* was tightly linked to the *Ity* gene on chromosome 1. Furthermore, in the spleen and the liver of backcross BALB/c × (CBA × BALB/c) mice, the *S. abortusovis* resistance phenotype cosegregated with the two alleles of the *Len-1* gene, a gene tightly linked to the *Ity* gene. By contrast, in these backcross mice, the level of infection of the peritoneal cavity, the site of inoculation, did not correlate with the *Len-1* phenotype of the animal. These results provide evidence that after i.p. inoculation the control of *S. abortusovis* growth in the spleen and the liver is controlled by the *Ity* gene, but also suggest that additional gene(s) regulate the number of bacteria at the site of inoculation.

Keywords *Salmonella abortusovis* genetic control peritoneal infection *Ity* gene

INTRODUCTION

The genetic control of *S. typhimurium* has been extensively studied in mice (review by [1]). The early phase of the infection is controlled by the *Ity* gene. This gene exists on two allelic forms, Ity^r (resistant) and Ity^s (susceptible), and regulates the early *in vivo* net growth rate in the reticuloendothelial cell system of *S. typhimurium* [2] *S. enteritidis* [3] as well as of other unrelated pathogens *Leishmania donovani* [4] and different species of mycobacteria [5–9]. The mechanism of action of the *Ity* gene remains unknown, but considerable evidence points to the macrophages as the effector cells [10–12]. The *Ity* gene is mapped on the mouse chromosome 1 very close to the *Len-1* gene which encodes a lens γ-crystallin protein [13–15].

Inbred strains of mice infected subcutaneously or intravenously with another serotype of salmonella, *S. abortusovis*, can also be characterized as either resistant (CBA and DBA) and or susceptible (C57BL/6 and BALB/c) on the basis of their ability to control the proliferation of the bacteria in the spleen and the liver ([16], Lantier *et al.*, manuscript submitted). Classical Mendelian analysis has shown that resistance/susceptibility to *S. abortusovis* infection is controlled by an autosomal dominant gene. In all four of the inbred mouse strains tested, the pattern of resistance/susceptibility to infection with *S. abortusovis* was identical to that observed with *S. typhimurium* [16]. It was therefore interesting to test whether the *Ity* gene was also controlling the resistance to *S. abortusovis* infection.

In this study we report that the *S. abortusovis* resistance gene is expressed during both i.v. and i.p. infection. By the use of congenic strains and formal linkage on backcross animals, this gene was mapped on the mouse chromosome 1 to the same location as the *Ity* gene. However, in the backcross mice we observed a low correlation between the number of bacteria recovered in the peritoneal cavity and the *Len-1* phenotype which suggest that other gene(s) may be involved in the control of salmonella growth in the peritoneal cavity.

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MATERIALS AND METHODS

Mice

BALB/c and CBA mice were from breeding pairs originally obtained from Iffa Credo (Saint Germain-sur-l'Arbresle, France). The C.D2 mice are congenic with *S. typhimurium*-susceptible BALB/c mice except for a 30-cM segment of chromosome 1 derived from *S. typhimurium*-resistant mice

DBA/2. This portion of chromosome 1 contains the DBA/2 *Idh-1*^{b}, *Ity*^{r}/*Lsh*^{r}/*Bcg*^{r}, *Pep-3*^{b} markers [17].

The (BALB/c × CBA)F1 and (CBA × BALB/c)F1 are herein designated (C.CB)F1, and (CB.C)F1, respectively. All experimental mice, including (C.CB)F1, (CB.C)F1 and backcross BALB/c × (BALB/c × CBA) mice were bred in our own facilities, in a filtered air-conditioned building. The mice were given free access to filtered water and sterilized food, and were used at 10–15 weeks of age.

Bacterial strain

S. abortusovis strain 15/5 was described previously [18]. To maintain constant virulence, it was passaged in mice and stored at −70 C in infected spleen. Overnight cultures from homogenates of thawed spleens were subcultured (24 h, 37 C) onto Trypticase Soy Agar slopes (TSA, Biomerieux, Lyon, France) and used to prepare suspensions in phosphate-buffered saline (PBS), pH 6·9. These were turbidimetrically standardized and tested for purity and number of viable organisms by plating serial dilutions. After s.c. inoculation *S. abortusovis* strain 15/5 has an LD50 of $7{\cdot}7 \times 10^{1}$ in BALB/c mice and $2{\cdot}0 \times 10^{6}$ in CBA mice (Lantier *et al.*, manuscript submitted).

Experimental design and bacterial enumeration

Groups of mice were injected intraperitoneally or intravenously with 0·2 ml of *S. abortusovis* suspensions containing the desired concentration of bacteria. At various times after infection, mice were killed by cervical dislocation. The peritoneal cavities were washed with 5 ml Minimum Essential Medium (MEM 1031, Eurobio, Paris, France) and the total number of *S. abortusovis* in the cell suspension was determined by adding 1 ml of the peritoneal fluid from each mouse to 1 ml distilled water containing 0·1% Triton-X 100. We have previously verified that Triton-X 100 at a concentration of up to 1% has no effect on *S. abortusovis* growth. Serial 10-fold dilutions of this fluid were plated onto TSA medium, and bacterial colonies were counted after incubating plates for 2 days at 37 C.

In addition, spleens and livers were aseptically removed and weighed. The number of bacteria per organ was determined by tissue homogenization and plate counting of serially diluted homogenates.

Len-1 *phenotype*

Encapsulated lenses were removed from the eyes of freshly killed mice and frozen at −70 C until analysis. Lens proteins were homogenized by sonication in 200 μl of 50 mM NH_4HCO_3, 3 mM β-mercaptoethanol buffer [13] and centrifuged for 5 min at 8000 ***g***. *Len-1* alleles were distinguished by electrophoresis of lens homogenate supernatants on Titan III cellulose acetate plates (Helena Laboratories, TX). Electrophoresis was conducted at 4 C for 20 min at 270 V in 25 mM Tris, 190 mM Glycine, pH 8·5 [19]. Proteins were stained with a 0·5% amido-black solution containing 4 vol of water, 5 vol of methanol and 1 vol of acetic acid.

This method allowed us to easily distinguish the *Len-1*^{a} phenotype (1 cathodal band) from BALB/c mice and the *Len-1*^{ab} phenotype (2 cathodal bands) from (C.CB)F1 or backcross mice [13].

Statistical analysis

Viable counts of *S. abortusovis* per organ, or per peritoneal cavity, were expressed as $\log_{10}$ units; means and s.e.m. were calculated from the logarithmic values.

Classical F and *t*-test were used for statistical analysis. Maximum likelihood estimates of recombination probabilities (c), their variances (V_c), and standard errors (S_c) among backcross progeny were calculated according to Green [20]: $c = r/n$, $V_c = (c\ (1-c))/n$, $S_c = \sqrt{V_c}$, where r is the number of recombinations in a sample of size n.

RESULTS

Both intravenous and intraperitoneal route of inoculation differentiate mice genetically susceptible and genetically resistant to S. abortusovis *infection*

We first compared the *in vivo* growth of *S. abortusovis*, following i.v. or i.p. challenge, in mice carrying the resistant (*Ity*^{r}, CBA) or the susceptible (*Ity*^{s}, BALB/c) allele of the *Ity* gene (Fig. 1). Whatever the route of inoculation used, 24 h post-inoculation more bacteria were recovered from the spleen and the liver of BALB/c mice than from the organs of CBA mice. Thereafter, the bacteria showed an exponential growth curve and the doubling time of the bacteria was 1·4–2·8 times longer in CBA mice than in BALB/c mice. Interestingly, 2 days after the i.p. inoculation, the number of bacteria in the peritoneal cavity differed between the two strains of mice. Of note, there was no multiplication of *S. abortusovis* in the peritoneal cavity of CBA mice (Fig. 1). After i.v. injection, no bacteria could be found in the peritoneal cavity of the infected animals (data not shown).

We then investigated whether the inoculum size could have any effect on the phenotypic expression of resistance to *S. abortusovis*. A range of doses of bacteria was inoculated in the peritoneal cavity of BALB/c mice and (C.CB)F1 mice (Fig. 2). As the resistance allele of the *Ity* gene is dominant, the (C.CB)F1 mice, hybrid between BALB/c and CBA mice, express also the resistant phenotype but this hybrid is genetically more closely related to the BALB/c mice than its CBA parent. With decreasing inoculum, the difference in the number of bacteria recovered from the peritoneal cavity of both strains of mice could be detected earlier. When mice were given $1{\cdot}08 \times 10^{5}$ *S. abortusovis*, three-fold more bacteria were detected in the cavities of BALB/c mice than in (C.CB)F1 mice at day 1 after inoculation ($P < 0{\cdot}05$), and by day 3, BALB/c mice had 350-fold more bacteria than (C.CB)F1 mice ($P < 0{\cdot}001$). Even with a higher infectious dose ($1{\cdot}3 \times 10^{6}$ bacteria), the bacterial colonization of the peritoneal cavity differed between the two strains only from day 3 post-inoculation and the difference remained lower than eight-fold. The highest dose ($0{\cdot}9 \times 10^{7}$ bacteria) led to the death of all BALB/c by day 2 post-inoculation. In the spleen and the liver, bacterial proliferation was more intense than in the peritoneal cavity in both strains of mice. As expected, the mean numbers of viable bacteria in the spleen and the liver were higher in BALB/c mice than in (C.CB)F1. The differences were maximal on day 3 after inoculation and increased with decreasing inoculum (Fig. 2).

In conclusion, the i.v. route, which was the route originally used to study the *in vivo* growth of *S. typhimurium* in *Ity*^{r} and *Ity*^{s} mice, also allowed us to distinguish between mice susceptible and resistant to *S. abortusovis* infection. After inoculation of the

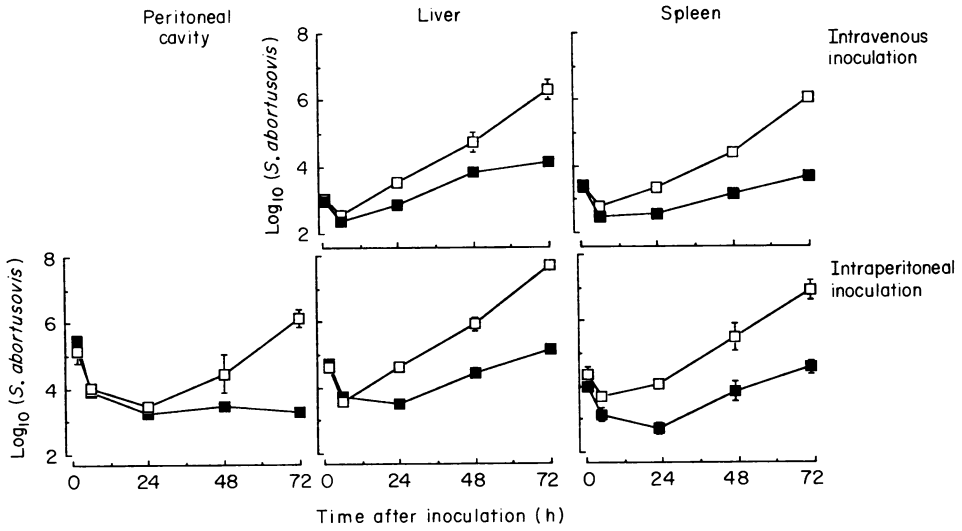

Fig. 1. Time course of infection in BALB/c (*Ity*^{s}, □) and CBA (*Ity*^{r}, ■) mice inoculated intravenously (top panel) or intraperitoneally (bottom panel) with respectively $7{\cdot}3 \times 10^3$ and $1{\cdot}5 \times 10^5$ *S. abortusovis* 15/5. Each point represent the geometric mean count ± s.e.m. from a group of 4–5 male mice.

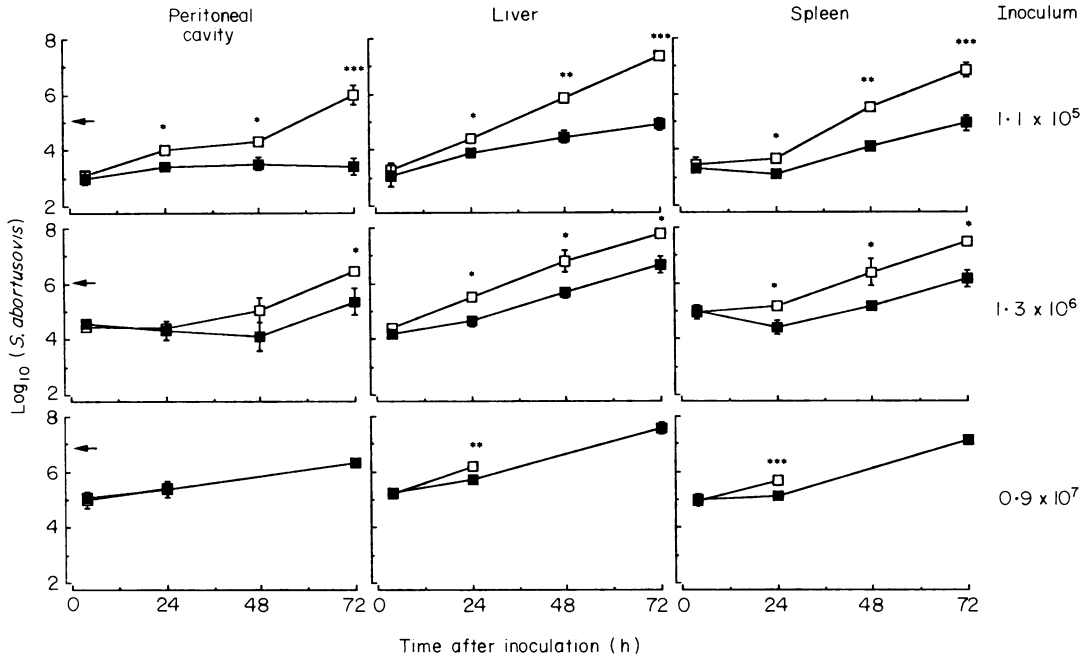

Fig. 2. Time course of infection in BALB/c (susceptible, □) and (C.CB)F1 (resistant, ■) mice inoculated intraperitoneally with a range of doses of *S. abortusovis* strain 15/5 ($1{\cdot}1 \times 10^5$; $1{\cdot}3 \times 10^6$ or $0{\cdot}9 \times 10^6$ bacteria/mouse). Each point represents the geometric mean count ± s.e.m. from a group of 3–4 male mice. Arrows indicate doses of inoculation. Comparison between BALB/c and (C.CB)F1 mice by a t-test: $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$.

bacteria by the i.p. route, the bacterial growth followed the same pattern as that observed after i.v. inoculation. Moreover, the difference between resistant and susceptible mice could be seen whatever the size of the inoculum.

The gene which controls resistance to S. abortusovis *infection and the* Ity *gene are on the same fragment of mouse chromosome 1*

Mice of the C.D2 strain differ from BALB/c mice by a chromosomal segment surrounding the *Ity* gene which controls resistance to *S. typhimurium*. The kinetics of *S. abortusovis* infection were compared in BALB/c and C.D2 congenic mice inoculated by the i.p. route to find out whether this chromosomal segment was involved in resistance to *S. abortusovis* (Fig. 3). Resistant (C.CB)F1 mice were included as controls. As expected, *S. abortusovis* multiplied extensively in the peritoneal cavity, spleen and liver of susceptible BALB/c mice. Bacteria in C.D2 and (C.CB)F1 mice multiplied slower than those in BALB/c mice, and after 3 days bacteria in each location began a gradual decline. Thus, in contrast to BALB/c mice, C.D2 mice possess the gene conferring resistance to *S. abortusovis*.

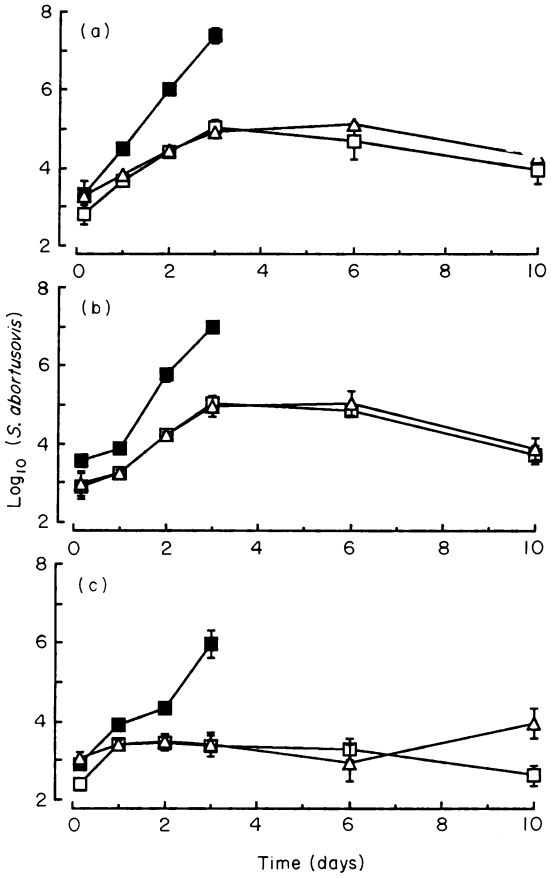

Fig. 3. Time course of infection in BALB/c (susceptible, ■), (C.CB)F1 (resistant, △) and congenic C.D2 (□) mice inoculated intraperitoneally with $1{\cdot}08 \times 10^5$ *S. abortusovis* strain 15/5. Each point represents the geometric mean count ± s.e.m. from a group of 5 mice (a) Liver; (b) spleen; (c) peritoneal cavity.

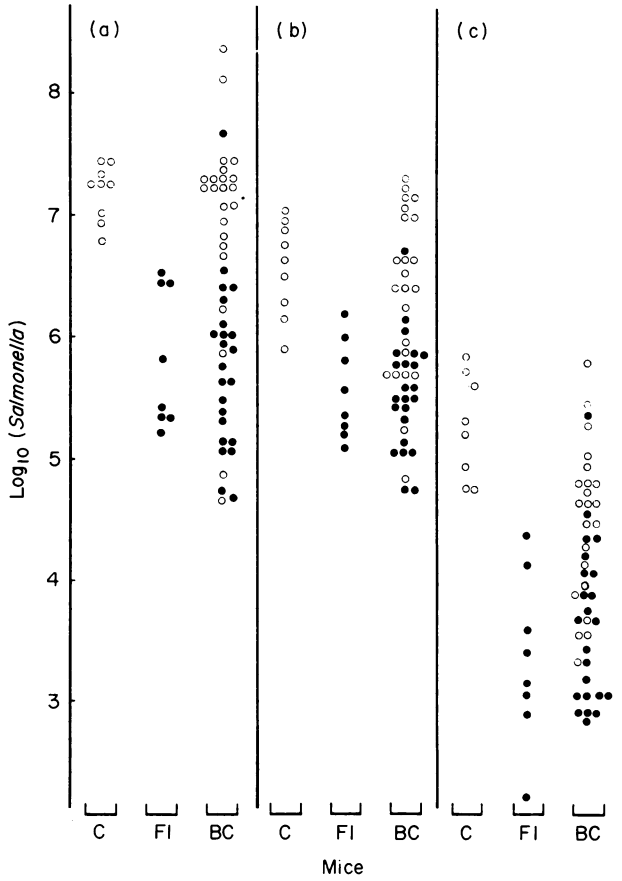

Fig. 4. *Len-1* phenotype and infection level in BALB/c, (C.CB)F1 and BALB/c × (CBA × BALB/c) mice inoculated by the i.p. route with *S. abortusovis* strain 15/5. *Len-1* phenotype was determined by electrophoresis of lens protein: (○) homozygous *Len-1*^{a} phenotype, (●) heterozygous *Len-1*^{ab} phenotype. The number of bacteria recovered from (a) liver, (b) spleen and (c) the peritoneal cavity of each animal was determined on day 3 after inoculation of $1{\cdot}25 \times 10^6$ bacteria/mouse.

Cosegregation of the trait of resistance/susceptibility to S. abortusovis *with the* Len-1 *phenotype in backcross BALB/c × (CBA × BALB/c) mice*

To localize more precisely the gene controlling the resistance to *S. abortusovis* infection inside the 30 cM, *Idh-1-pep-3*, fragment of chromosome 1, we estimated its distance to the *Len-1* gene, a gene mapped between *Idh-1* and *Pep 3* and which is very close to the *Ity* gene. As expected, 23 out of the 46 tested backcross mice presented the BALB/c-associated *Len-1*^{a} phenotype and 23 presented the (C.CB)F1-associated *Len-1*^{ab} phenotype (Fig. 4).

A cut-off point of $10^{6{\cdot}6}$ bacteria in the liver completely discriminated between parental BALB/c and (C.CB)F1 mice and allowed us to determine the resistant or susceptible phenotype of backcross mice. By this criterion 20/46 (43%) mice were susceptible and 26/46 (57%) resistant (Fig. 4). This is consistent with the hypothesis that the trait of resistance to *S. abortusovis* is controlled by a single dominant gene ($\chi_2 = 0{\cdot}70$, $P > 0{\cdot}3$). In 41/46 animals, we observed cosegregation of either resistance to *S. abortusovis* and *Len-1*^{ab} phenotype (22 mice) or susceptibility to this bacteria and *Len-1*^{a} phenotype (19 mice). These data suggest that the two loci are closely linked; the recombination frequency obtained between the gene which controls resistance to *S. abortusovis* and the *Len-1* gene was estimated according to Green [20] to be $0{\cdot}109 \pm 0{\cdot}046$.

The bacterial spleen counts from the same backcross animals gave similar results to those obtained from the livers (Fig. 4); the correlation between the colonization of these two organs was 0·894. However, the distribution of the individual values from the two parental strains of mice (BALB/c and (C.CB)F1) was overlapping between $10^{5{\cdot}75}$ and $10^{6{\cdot}25}$ bacteria per spleen.

The peritoneal cavities of BALB/c mice contained 1·6-fold more bacteria than those of (C.CB)F1 mice. The backcross mice classified as 'resistant' by their liver counts were similar to (C.CB)F1 mice in terms of peritoneal cavity-associated Salmonella. However, animals classified as 'susceptible' by their liver counts contained variable numbers of peritoneal cavity-associated bacteria. This result suggests that the peritoneal cavity colonization by *S. abortusovis* is governed by more than one gene in these mice.

DISCUSSION

The i.p. course of infection proceeded in at least three distinct phases: (i) early disappearance of peritoneal cavity-associated bacteria, greater than 95%, and simultaneous colonization of

the liver and the spleen; (ii) exponential growth of surviving bacteria in the peritoneal cavity, the spleen and the liver occurring between days 1 and 3 post-inoculation; and (iii) either host clearing of the bacteria (resistant mice) or host death (susceptible mice). Thus i.p. injection of *S. abortusovis* led to a rapidly disseminated infection, and the bacterial growth in the liver and spleen of mice mimicked that in the peritoneal cavity. The peritoneal cavity was not colonized during i.v. infection. Thus, the increase of the number of *S. abortusovis* in the peritoneal cavity was caused by multiplication of the orginal inculum rather than by a secondary contamination. Nevertheless, the possibility that bacteria may colonize an infected cavity more easily than a non-infected one cannot be excluded.

The mechanisms of natural resistance to *S. abortusovis* seemed to prevent fulminant bacterial growth in the initial phase of infection as has been described for mouse resistance to *S. typhimurium* infection [21,22]. Whatever the infectious inoculum, bacteria grow exponentially in the peritoneal cavity of susceptible mice until host death. However, in resistant mice the replication rate of the bacteria was reduced and the level of colonization of the different organs was limited during the early phase of infection (Fig. 1). This impeding of bacterial growth could allow the infected host to survive until specific cellular and humoral immune responses develop [23,24].

We focused our study on the exponential growth phase which permitted us to distinguish between susceptible and resistant animals. We demonstrated that after an i.p. inoculation the net growth rate of *S. abortusovis* in the spleen and the liver is controlled by a dominant gene, as already shown using other routes of inoculation (Lantier *et al.*, manuscript submitted). Using congenic strains and backcross mice, we have shown that this gene is identical to or closely linked with the *Ity* gene. The *Ity* gene, also called *Lsh* or *Bcg*, controls the multiplication of *S. typhimurium* [2,22], *S. enteritidis* [3], *L. donovani* [4] and different species of mycobacteria [5–9] in the reticuloendothelial system of the mice. The C.D2 mice differ from BALB/c mice by a 30-cM fragment of chromosome 1 which contains the *Idh-1*, *Ity/Lsh/Bcg* and *Pep-3* genes [17]. Comparison of the i.p. course of infection in BALB/c and C.D2 mice (Fig. 3) showed that this chromosomal segment also conferred resistance to *S. abortusovis* infection, suggesting that the *S. abortusovis* resistance gene is located between the *Idh-1* and *Pep-3* genes that mark the boundaries of the 30-cM fragment. This localization was confirmed by mapping the *S. abortusovis* resistance gene of 46 backcross mice, to very near the *Len-1* gene, a gene located in the *Idh-1-Pep-3* fragment of chromosome 1.

Genetic maps of the proximal chromosome 1 region surrounding the *Ity/Lsh/Bcg* gene have been recently established [14,15]. The closest marker is the villin gene, which is 1–4 cM [15] or less than 0·3 cM [14] from the *Ity/Lsh/Bcg* gene. These maps located the *Len-1* gene at 7·2 cM [14] or at 7·9 cM [15] from the *Ity/Lsh/Bcg* gene. In this study we mapped the gene which controls the resistance to *S. abortusovis* infection at $10{\cdot}9 \pm 4{\cdot}6$ cM. Additionally on BALB/c × (CBA × BALB/c) backcross mice inoculated subcutaneously there was no recombination between the *S. abortusovis* resistance gene and the villin gene (Oswald *et al.*, manuscript in preparation). Taken together these results indicate that the *S. abortusovis* resistance gene is identical to or closely linked with the three other resistance genes, *Ity*, *Lsh* and *Bcg*. The formal proof of the identity of these four genes requires molecular cloning of the DNA segment which confers this multi-resistance.

The genetic control of the number of bacteria at the site of inoculation, i.e. in the peritoneal cavity, seemed to be more complex. The low correlation obtained in backcross mice between the bacterial counts in the liver and in the peritoneal cavity suggests that other gene(s) may be involved in the control of the bacteria at the site of inoculation. The inflammatory level, which is known to differ between CBA and BALB/c mice, may interfere with the *S. abortusovis* resistance gene to determine the bacterial level in the peritoneal cavity. Thus inflammatory macrophages and polymorphonuclear cells which are recruited very early during the infection could be responsible for a rapid clearance of the bacteria. These cells could constitute, in addition to the resident macrophages whose activity is controlled by the *Ity* gene, another step of regulation of the number of bacteria in the peritoneal cavity. Thus the i.p. route may be a useful tool to study the genetic control of the early phase of an infection and to determine the role of the inflammatory cells.

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