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The *in vivo* division and death rates of *Salmonella typhimurium* in the spleens of naturally resistant and susceptible mice measured by the superinfecting phage technique of Meynell

C. E. HORMAECHE *Microbiology Division, Department of Pathology, University of Cambridge, Cambridge*

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Summary. *Salmonella typhimurium* appears to divide faster in the spleen of naturally susceptible BALB/c than in resistant (B10 × A/J)F_1 mice. *S. typhimurium* M526 is an LT2 derivative lysogenic for a non-excluding P22 mutant which allows superinfection with a second, non-replicating, P22 phage so that the proportion of superinfected organisms halves at each division. The true *in vivo* division and death rates can be calculated from successive determinations of the proportion of superinfected organisms and the viable count. It was found that the division time was 2·86 h in BALB/c and 5·02 h in (B10 × A/J)F_1; the death rate was low and actually greater in the susceptible BALB/c strain. These results suggest that the gene controlling *in vivo* salmonella net growth rate, which is very important in natural resistance to salmonella infection, acts very early by regulating the division rate, perhaps inside macrophages. The actual mechanism remains unknown.

INTRODUCTION

Inbred mouse strains can differ markedly in their natural resistance to many organisms, including salmonellae. In some susceptible mice, the number of viable organisms in the reticulo-endothelial system (RES) can increase far more rapidly than in resistant mice during the early stages of the infection. This difference in early net growth rate is controlled by a single autosomal dominant gene (*Ity*) whose function is at present unknown. (Plant & Glynn, 1974, 1976, 1979; Hormaeche, 1979a, b, c). More specifically, it is not known whether the slower net growth rate seen in resistant mice is due to a bactericidal or bacteriostatic effect as compared with susceptible mice. Such information could be useful in elucidating the mechanism.

This question was investigated using the superinfecting phage P22 technique described by Maw & Meynell (1968). The method employs salmonellae carrying a non-replicating genetic marker, which dilutes out in a predictable manner during bacterial division. Wild-type P22 is normally excluding, not allowing lysogenized cells to become superinfected. Walsh & Meynell (1967) succeeded in isolating non-excluding mutants of P22. These allow a lysogenized strain to become superinfected with a differentially marked mutant P22 which does not become integrated in the bulk of the organisms and is passed on to only one cell, so that the proportion of superinfected organisms halves at each division. Therefore, successive determinations of the proportion of superinfected organisms allow an estimation of the division rate. Using this method, Maw & Meynell determined the true *in vivo* division rate (*l*) in mice, the death rate (*m*)

Correspondence: Dr Carlos E. Hormaeche, Microbiology Division, Department of Pathology, University of Cambridge, Cambridge.

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being calculated by difference from the observed overall net growth rate ($l-m$) determined by colony counts on organ homogenates. This paper describes the results obtained applying this system to resistant and susceptible mice.

MATERIALS AND METHODS

Animals

BALB/c (susceptible) and (B10 × A/J)F_1 (resistant) mice (Hormaeche, 1979a) were bred in the Department from stock purchased from the Laboratory Animals Centre, Carshalton, England. Animals of either sex weighing 20–25 g were used.

Organisms and phages

These were generously provided by Prof. G. G. Meynell. *S. typhimurium* M526 is an LT2 devivative with an intravenous LD50 of $10^{4.5}$ for BALB/c and $10^{5.5}$ for (B10 × A/J)F_1 mice. It is lysogenic for a P22 mutant which is non-excluding (x), gives a cloudy plaque (c^+), cannot plate on semi-rough mutants (h^+) and is sensitive to 0·1 M $MgCl_2$ (mg^s). The superinfecting P22 mutant gives a clear plaque (c_2), can plate on semi-rough mutants (h), and is magnesium resistant (mg^r). The streptomycin-resistant *S. typhimurium* indicator strains were the smooth SL1207 on which both phages will form plaques, and the semi-rough SL1208 on which only the superinfecting phage will plaque in 0·1 M $MgCl_2$ agar.

Media

Cultures were grown overnight on Oxoid Nutrient Broth No. 2 (OB2), also used for *in vitro* growth experiments. Plating media contained 1% Bacto-Tryptone, 1·5% agar and 0·01% glucose. Overlay medium was similar but with 0·7% agar.

Preparation of superinfected cultures

A stationary overnight culture of M526 in OB2 was shaken for 80–100 min at 37°. An aliquot was mixed with superinfecting phage at 37° for 10 min; antiphage serum was added and allowed to act for a further 10 min, a procedure which reduced the free phage concentration by 10^{-4}. Cultures to be used *in vivo* were prepared in a similar manner but the incubation times were reduced to 5 min and immediately snap-frozen and stored in liquid nitrogen.

In vitro *growth experiments*

A superinfected culture was diluted 10^{-4} into OB2 at 37°; the viable count was determined by pour plates and the proportion of superinfected bacteria determined at successive intervals as below.

Infection of animals

Mice were injected with a dilution of organisms in PBS in 0·1 ml in a tail vein. For viable counting and phage assays, spleens were homogenized in 10 ml distilled water in a Colworth Stomacher 80. Viable counts were performed in duplicate pour plates in Tryptic Soy agar (Difco) and phage assayed as below.

Estimation of the number of superinfected organisms

Samples from *in vitro* experiments were diluted 1/10 in PBS pH 7·2. A 5 ml sample was rocked in a petri dish 60 cm under a 15 W UV light for 45 s. Spleen homogenates were irradiated for 2 min. (This inactivated most of any free phage.) Plaques were counted by the overlay technique. The number of UV-induced organisms in a given sample was determined by plating (before lysis occurred) on SL1207, and the number of superinfected organisms by plating on SL1208 on $MgCl_2$ agar. The ratio of plaques on SL1208/plaques on SL1207 gives the proportion of superinfected organisms.

Calculation of the true in vivo *division and death rates*

There is little death when bacteria are in the phase of exponential growth *in vitro*, and the division rate can be calculated from the observed increase in viable counts. *In vivo*, where bacteria are being killed by the host, the growth equation becomes

$$N_t = N_i\, 2^{(l-m)t}$$ (Maw & Meynell, 1969)

where N_t is the number of viable organisms at time t (days), N_i the initial number of viable bacteria and l and m the true division and death rates. The overall net growth rate *in vivo* ($l-m$) is approximately linear during the early stages of the infection and can be estimated by viable counts on organ homogenates. The division rate l can be estimated from the rate of decrease in the proportion of superinfected organisms, p, during this phase of growth, and m is obtained by difference.

RESULTS

Experiments *in vitro*

Initial experiments were hampered by an inability to obtain a high initial proportion (p_0) of superinfected

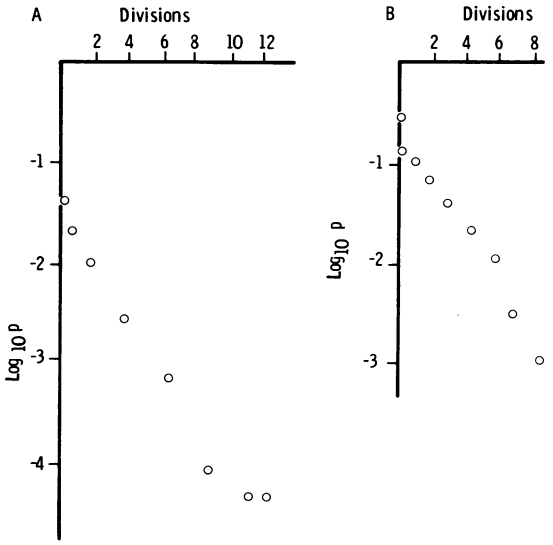

Figure 1. Problems with superinfected cultures *in vitro*. (A) Low p_0. A moi of 7·2 gives a small initial proportion of superinfected organisms. (B) Unstable superinfection. A moi of 11·5 gives a higher p_0 value, but this rapidly decreases during the lag phase.

bacteria, and by a tendency to an unpredictable, pronounced drop in p during the lag phase, an effect described by Meynell as 'unstable superinfection' (Meynell, 1959). Experiment A in Fig. 1 shows the progress of a culture in which a multiplicity of infection (moi) of 7·2 gave a p_0 of only 0·04. Thereafter, however, the culture behaved as expected, p decreasing at a rate of −0·28, close to the theoretical −0·301 ($\log_2$). As described by Meynell, p tends to become constant after about ten generations due to interference from a small fraction (1/1000) of organisms in which the superinfecting phage appears to have become integrated. This artefact sets a limit to the number of generations that can be accurately followed by this technique.

A higher moi was sometimes effective in giving a higher p_0, although this tended to kill many organisms ('lysis from without'). Experiment B in Fig. 1 shows a culture started with a moi of 11·5. This gave a p_0 of 0·47 but this rapidly decreased during the lag phase (unstable superinfection).

The combined effects of low p_0 and unpredictable unstable superinfection made the system unusable for *in vivo* studies. As bacteria are being killed, if p_0 is low the total number of superinfected organisms will drop below detectable levels after only a few divisions. Stable cultures with high p_0 are required, such as obtained by Maw and Meynell.

The reason for unstable superinfection is unknown. A marked improvement was obtained by shaking the overnight M526 culture before superinfection as described by Meynell (1959) with *E. coli* K12 and phage lambda. A further improvement was obtained by using superinfecting phage stocks derived from plaques obtained in experiments such as in Fig. 1 after the unstable culture had reached the linear phase (after the first hour). Superinfecting phage was taken from such plaques and suspensions prepared from overlays grown on SL1208 on magnesium plates. After such passages, the resulting phage gave results more in keeping with those described by Maw and Meynell. Figure 2 shows one such experiment in which the moi was 11·4; p_0 was 0·41 and did not show the marked instability observed in earlier cultures.

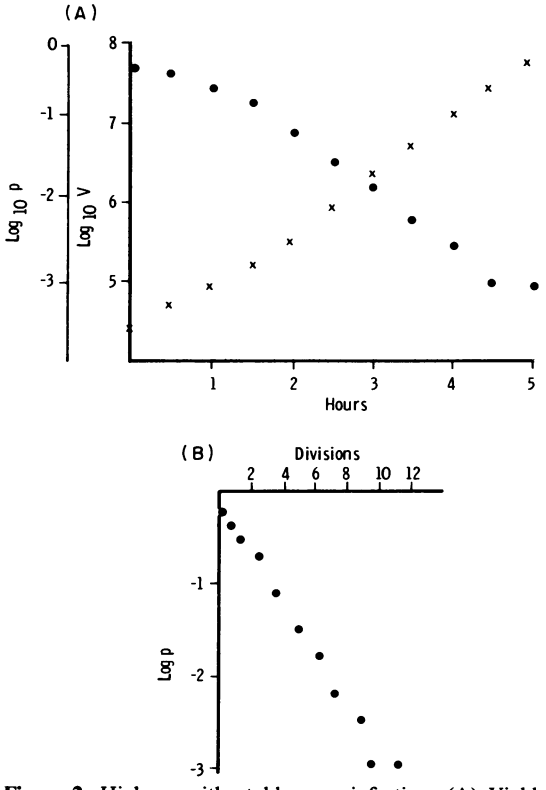

Figure 2. High p_0 with stable superinfection. (A) Viable counts (crosses) and p values (circles) in a stable superinfected culture growing in broth. (B) $\log_{10} p$ and observed divisions in the same experiment.

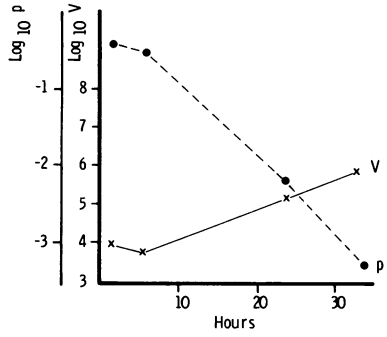

Figure 3. Growth of a superinfected culture in BALB/c mice (pilot experiment). Animals were injected with $10^{5.93}$ organisms. Log_{10} viable count (crosses) and $log_{10} p$ (circles) in the spleens of groups of two mice.

Preliminary trial *in vivo*

A pilot trial was made in BALB/c mice. A superinfected culture was prepared, the animals were injected immediately, and groups of two mice were killed at intervals. The viable counts and p values are shown in Fig. 3. The inoculum was $10^{5.93}$; at 1·5 h 10^4 organisms were recovered from the spleens and this dropped to $10^{3.8}$ by 5·75 h, after which it increased steadily up to 33 h when the experiment was terminated. There was a slight drop in p in the first 1·5 h, after which it appears to drop linearly.

This limited first experiment gave the following results: $l-m=6{\cdot}16$; $l=8{\cdot}11$; $m=1{\cdot}95$. The division time was 2·96 h.

Freezing a superinfected culture

The effect of freezing on a superinfected culture was studied, as there are obvious advantages in working with aliquots of a culture which can be checked before a larger scale *in vivo* experiment. Figure 4 shows that a snap-frozen sample stored in liquid nitrogen behaved in a manner similar to that seen in previous experiments.

Comparison of resistant and susceptible mice

BALB/c mice are very susceptible to salmonellae, $(B10 \times A/J)F_1$ are resistant. Animals were injected with $10^{6.38}$ organisms from a frozen culture and groups of three killed at 7·6, 23, 32 and 49·5 h. Viable spleen counts were performed on all, and p determinations on the first three (the superinfecting phage appeared to be markedly increased in the last sample making it unsuitable for estimating the division rate). Figure 5 shows the results.

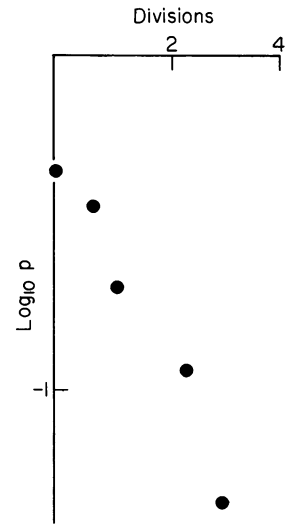

Figure 4. Growth of a stably superinfected culture *in vitro* after storage in liquid nitrogen.

At 7·6 h (0·32 days) the viable counts were $10^{4.19}$ in the F_1 and $10^{4.9}$ in BALB/c mice, and successive counts showed that the exponential growth phase had commenced (Fig. 5A). Net growth rate was faster in BALB/c than in the F_1 mice. There was little scatter, the counts being tightly grouped.

It is immediately apparent from Fig. 5B that p was dropping at a far greater rate in BALB/c mice. This means that the division rate was faster in the susceptible than in the resistant mice. Table 1 shows the division and death rates calculated from this data. The

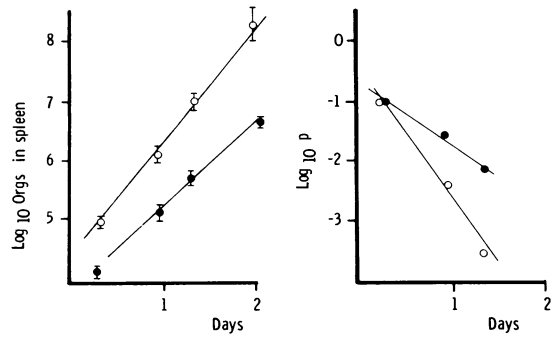

Figure 5. Growth of a stably superinfected culture in spleens of BALB/c (open symbols) and $(B10 \times A/J)F_1$ (solid symbols) mice. Viable counts and p values in groups of three mice.

Table 1. Net growth rate ($l-m$), division rate (l), death rate (m) and doubling times (d.t.) of *S. typhimurium* M526 in the spleens of BALB/c and (B10 × A/J)F_1 mice

	BALB/c	(B10 × A/J)F_1
$l-m$	6·79	4·87
l	8·38	4·78
m	1·59	0·09
d.t.	2·86 h	5·02 h

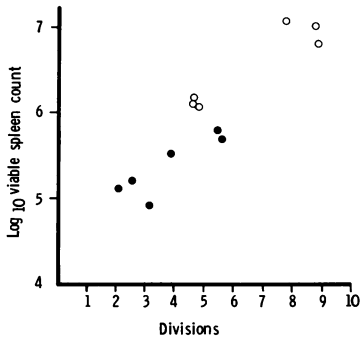

Figure 6. viable counts and p values for individual mice from the experiment shown in Fig. 5; the same symbols apply.

values for BALB/c agree well with those observed in the pilot experiment described above. The Table shows the surprising fact that, under these experimental conditions, the whole process–division and death—appears to be occurring at a faster rate in the susceptible mice. There was no indication of resistant mice killing bacteria better than susceptible mice; the reverse was the case, with a very small death rate in the resistant mice.

The results on individual mice were plotted separately, taking the 7·6 h values as the starting point, to see if higher viable counts correlated with a higher number of divisions (Fig. 6). There is good agreement for both resistant and susceptible mice, the viable counts attained seem to depend mainly on the number of divisions and the results are closely grouped.

DISCUSSION

Meynell employed non-replicating genetic markers to study the *in vivo* multiplication of organisms, using abortive transductants (Meynell & Subbaiah, 1963) or the more sensitive superinfecting phage technique (Meynell, 1959; Maw & Meynell, 1968). This was originally used with *E. coli* and phage lambda; this organism is not pathogenic for mice, and it was found that the viable count did not increase and the proportion of superinfected organisms did not drop, suggesting that the low virulence was due to an inability to divide *in vivo*.

Using the superinfecting phage P22 technique, Maw and Meynell found that the process of a salmonella infection in the RES was relatively sluggish. There was no indication that the slow overall net growth rate could be accounted for by rapid division and death; the maximum *in vivo* division rate was only 5–10% of that seen *in vitro*.

The present results are in general agreement with those of Maw and Meynell. The actual values for division and death rates are different, but this is to be expected as different mice were used. The most striking find was that the difference between resistant and susceptible mice seemed to be in the division rate. The division rate (l) values of 8·38 and 4·78 for susceptible and resistant mice represent doubling times of 2·86 and 5·02 h respectively (contrasting with approximately 30 min in broth). The other noteworthy observation was that m, the death rate, was actually higher in the susceptible mice, suggesting that, at least under these experimental conditions, once bacterial division has commenced the death rate may depend on the division rate (Meynell, 1959).

Several workers have suggested that differences in macrophage activity could determine variations in natural resistance. Maier & Oels (1972) found that peritoneal macrophages from resistant BRVR mice killed salmonellae better *in vitro* than those from susceptible BSVS mice. We have been unable to obtain comparable results with BALB/c and (B10 × A/J)F_1 mice (unpublished observations). BRVR mice were selected from an outbred stock for resistance to salmonellae, and it may be that other genes, different to the one controlling *in vivo* net growth rate, determine bactericidal efficiency in short-term *in vitro* assays such as described by Maier and Oels. *In vitro* studies show that many salmonellae are killed following phagocytosis; this may be the mechanisms responsible for the marked drop in the viable count of an inoculum immediately after intravenous challenge. It is the growth of the survivors of this initial kill that determines the course of the infection, and it appears to be during this

phase of exponential growth that the gene controlling net growth rate comes into effect.

While the present results suggest that this net growth rate controlling gene operates by regulating bacterial division, there are drawbacks to this system. One is the number of divisions that can be followed, due to the emergence of the small proportion of organisms in which the superinfecting phage has become integrated, setting a limit of about 10–12 divisions to the experiment. Another, perhaps more serious, is the high dose of organisms required. The challenge of 10^6 organisms is necessary because the initial kill which is always seen when mice are challenged i.v. leaves a relatively small proportion of organisms in the spleen. As the organisms are being killed, the actual number of superinfected organisms decreases, so that if more conventional doses (10^3–10^4) are used the marker is undetectable after only a few divisions. The penalty for these high doses is that the viable count increases very rapidly to values close to those at which grossly visible lesions appear, and if these are allowed to progress too far one can no longer assume that macrophage handling alone regulates division. It is by no means certain that the actual values obtained here would hold for lower doses. Unfortunately, there is at present no system which will allow these experiments to be done with lower doses.

These considerations suggest that more weight should be given to the comparative result between susceptible and resistant mice than to the actual values obtained. The data leave little doubt that division was proceeding faster in the susceptible BALB/c mice, and this was apparent from the early stages of the experiment. The difference was clear by 24 h, by which time the challenge had increased to 10^6 in BALB/c mice. This is well below the 'lethal numbers' of 10^8–10^9. This is very early for a primary immune response, and suggests that an innate mechanism, perhaps operating in macrophages, may be regulating bacterial division.

The nature of the mechanisms by which this gene operates is unknown. Using outbred mice, Lowrie, Aber & Carrol (1979) studied the intracellular division rate of salmonellae in peritoneal macrophages *in vitro* by determining the effect of penicillin on overall growth. The low penicillin-induced death rate suggested that the slow intracellular growth was due to a bacteriostatic rather than a bactericidal effect. They estimated the division rate to be 1·5–2·5 h; the death rate was very low. Under these conditions salmonellae appeared to be quite resistant to intracellular killing; they did not prevent phagolysosome formation, but appeared to be relatively resistant to the effect of intracellular bactericidal enzymes—unlike glutaraldehyde-treated organisms which were readily digested (Carrol, Jackett, Aber & Lowrie, 1979).

The present results therefore suggest that specific differences in *in vivo* net growth rate may be due to regulation of bacterial division rate. How this may occur is at present a matter for speculation, as little is known of intracellular growth regulation by macrophages. It may be that non-specific factors such as intracellular iron-binding proteins may be important (Weinberg, 1978), but this must await further investigation.

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