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Systemic *Mycobacterium lepraemurium* Infection in Mice: Differences in Doubling Time in Liver, Spleen, and Bone Marrow, and a Method for Measuring the Proportion of Viable Organisms in an Inoculum

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Counts of acid-fast bacilli were made on homogenates of whole liver, whole spleen, and two femurs of CBA mice killed at various time intervals after intravenous infection with *Mycobacterium lepraemurium*. The growth curves so obtained showed that the bacillus multiplied faster in bone marrow than in liver or spleen. No evidence of redistribution during the early part of infection was obtained. The time of appearance of significant numbers of bacilli (10^7) in the bone marrow was used to make estimates of viability of *M. lepraemurium* suspensions. Several applications of the techniques described are discussed.

This work was initiated when we were studying the effect of various mycobacterial vaccines on the course of *Mycobacterium lepraemurium* infection in mice. Sensitive methods were needed to assess the growth of bacilli after intravenous inoculation and the viability of suspensions of the organism.

The method most commonly used to assess growth was to count the number of bacilli present in samples of splenic or hepatic tissue at various time intervals after inoculation (2). We have adapted this method in view of possible sampling errors to whole-spleen and whole-liver homogenates, and to give a full assessment of growth throughout the reticuloendothelial system, we have introduced counts of bacilli in the femoral bone marrow.

The methods then available to assess viability were determination of in vitro elongation of the bacillus (4), determination of mouse mortality after intravenous injection or of the time of appearance of gross lesions after subcutaneous injection (5), or the method described above, which depends on sampling of spleen and liver (2). However, the study of in vitro elongation of bacilli was only an indirect assessment of viability, and the other methods were either inaccurate or took too long. The method to be described is simple, reproducible, and gives accurate estimates of viability in a matter of weeks. It involves an assessment of the growth of *M. lepraemurium* after intravenous injection into mice by counting the total number of acid-fast bacilli (AFB) present in the liver, spleen, and femoral bone marrow at intervals over a period of 6 to 8 weeks. Reference is made to several applications of the technique.

MATERIALS AND METHODS

Mice. All mice were obtained from minimal-disease colonies at the National Institute for Medical Research. Inbred CBA mice were 1- to 3-month-old males or females. Thymectomized, irradiated, bone-marrow-reconstituted (T900R) female CBA mice were thymectomized at 4 weeks of age and given 900 R of lethal whole-body irradiation from a ^{60}Co source 2 weeks later. Just after irradiation, each mouse was reconstituted by injecting 0.3 ml of femoral bone marrow suspension prepared from normal female CBA mice. Three recipients were injected with the marrow prepared from one donor. T900R mice were 2 to 3 months old when used for infection. Parkes strain mice were random-bred albino mice of either sex, initially infected at about 6 weeks of age.

Bacteria. Colonies of Parkes or CBA strain mice were infected intravenously with approximately 10^{10} *M. lepraemurium* (Douglas strain) to provide bacteria for inoculation. Using a clean but not sterile technique, inocula were prepared from the heavily infected livers and spleens of mice infected 4 to 6 months previously. Pieces of infected tissue were homogenized in saline, and the suspension obtained was centrifuged at 440 × *g* for 5 min to remove cell debris. The supernatant was then re-

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centrifuged at 2,200 × *g* for 10 to 30 min, depending on turbidity, and the upper white layer of bacilli in the resulting deposit was diluted to a suitable volume with saline. The total number of AFB per milliliter in this suspension was counted with the spot-slide method of Hart and Rees (3). A 3-μl drop of suitably diluted suspension was spread over a circle 8 mm in diameter marked on a glass slide. After fixation, the smears were stained by the Ziehl-Neelsen method. Using an oil immersion objective, the number of AFB was counted in duplicate spots either in one diameter or in eight fields spaced 1 mm apart across the diameter of each spot. The number of bacilli in the original sample was calculated using calibration factors for the optical system, the area of the smear, and the dilution factor.

In some experiments, bacilli prepared as above were exposed to 2.5 Mrads of ^{60}Co irradiation before injection.

Counting of bacilli in the tissues of infected experimental mice. Three tissues only were studied: liver, spleen, and bone marrow (as represented by the washouts from the two femoral bones). Each mouse was killed by exposure to ether vapor to avoid internal bleeding, and the body weight was recorded. The liver and spleen were removed, weighed, and then homogenized in glass homogenizers in, respectively, 6 ml and 2 ml of 0.1% bovine albumin (fraction V, Armour) in saline (albumin-saline). The two femoral bone marrow plugs of each mouse were syringed out with 2 ml of albumin-saline, and the cells were dissociated by 12 passages through a 25-gauge needle and then disrupted by subjecting the suspension to two 20-s bursts at the maximum setting of a Luziesa 800KHZ ultrasonic generator (Luziesa, Paris, France). The resulting bacillary suspensions were thoroughly mixed with a Whirlimixer (Fisons Scientific Apparatus), diluted in albumin-water, and counted as described above. Counts were recorded as the number of AFB present in the whole liver or spleen of each mouse or in the bone marrow as represented by the femoral washouts.

Because there was little variation among mice in a group, in some experiments pool counts for each tissue were made by mixing equal volumes of the homogenates prepared from the tissues of three to four mice and then counting in the usual way.

Taking account of the various dilution factors involved in the use of this method, minimum detectable levels of about 10^4/ml (diameter count) and 10^5/ml (eight high-power-objective fields count) were obtained (3). Details of the number of bacilli injected into mice are given later, but, routinely, between 10^8 and 10^9 bacilli were given intravenously in a volume of 0.2 ml of saline. The thresholds mentioned above were a controlling factor in the early detection of AFB in any particular tissue.

Plan of experiments. Details of individual experiments are given below, but typically a group of some 20 male CBA mice was injected with each suspension or dilution of suspension to be tested. On day 1 and at intervals of 2 weeks thereafter, groups of three to four mice were killed for counts of bacilli.

RESULTS

Distribution of killed bacilli. Counts made 24 h after inoculation on the liver, spleen, and bone marrow of mice injected with bacilli that had been exposed to 2.5 Mrads of ^{60}Co irradiation (designated "killed") showed that most of the bacilli were taken up by the liver (Table 1). However, the count of AFB per unit weight of tissue was virtually the same for liver and spleen.

Irradiated bacilli injected into mice remained acid fast for at least 3 months. After this time, harvested bacilli did not stain well and difficulty was experienced in counting. The counts of AFB remained essentially at the same level in the liver, spleen, and bone marrow of those mice killed during the 3-month period (Fig. 1). Only during the last month was there a slight drop in count, and a possible reason for this has already been mentioned. *M. lepraemurium* are removed from the bloodstream within 24 h of intravenous injection; this observation would suggest that no significant redistribution of the irradiated bacilli occurred within the period of study.

Distribution and multiplication of viable bacilli. An intravenous inoculum of between 10^8 and 10^9 bacilli was required to recover countable numbers of bacilli from the liver, spleen, and bone marrow of mice 24 h after injection (see day 1 distributions and recorded bone marrow counts in Tables 1 and 2). At this dose level, animals injected with freshly harvested bacilli died of infection at about 7

TABLE 1. *Initial distribution of irradiated M. lepraemurium in CBA mice after intravenous infection*[a]

Expt	Inoculum	Liver			Spleen			Femur: log total AFB
		Wt (mg)	Log total AFB	Log AFB/mg	Wt (mg)	Log total AFB	Log AFB/mg	
1	10^9	1,208	8.69	5.61	87	7.31	5.37	5.66
2	10^9	1,301	8.50	5.58	95	7.35	5.37	5.55

[a] Counts were made after 24 h. The figures given represent arithmetic means of observations on four mice in each experiment.

months. Signs of infection were apparent by 4 to 5 months, and mice died bearing massive systemic loads of bacilli. Examination of mice dissected for various reasons during infection revealed the increasing hepatomegaly and splenomegaly characteristic of this infection (Table 2).

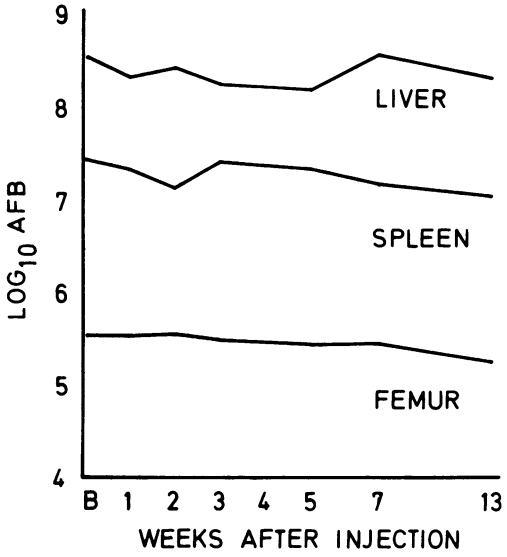

FIG. 1. *Counts of acid-fast bacilli over a 13-week period in the liver, spleen, and bone marrow of CBA mice injected intravenously with 10^9 bacilli from a M. lepraemurium suspension previously exposed to 2.5 Mrads of ^{60}Co irradiation. B, Base line distribution count made at 24 h. Each point represents the arithmetic mean of counts made on four mice at the time intervals indicated. The bone marrow counts were made on the two femurs (pooled) of each mouse and represent approximately 1/10 of the total bone marrow.*

Although the initial distribution of viable bacilli was similar to that described for irradiated bacilli, the ability of the former to grow and divide resulted in an increase, with time, in the number of bacilli harvested from the tissues of infected mice over their remaining life-span. Results of a typical experiment are summarized in Table 2. Four mice were killed on each occasion for individual tissue counts of AFB. The figures given in the table represent the arithmetic means for each group.

Mixtures of viable and killed bacilli. To investigate whether or not dead bacilli affected the growth curve of viable bacilli, we created various suspensions containing different proportions of dead bacilli. A suspension of *M. lepraemurium* was prepared in the usual way and divided into two volumes. One volume was designated "live" bacilli and the other was subjected to 2.5 Mrads of ^{60}Co irradiation and subsequently designated "dead" bacilli. The live and dead suspensions were mixed in different proportions to give suspensions ranging from 100% live to 100% dead. Groups of mice were injected with 10^8 bacilli from each of these several preparations, and sequential counts of AFB were performed on mice of all groups as described above. We observed no significant effect of dead bacilli on the growth of live bacilli as reflected in liver and spleen counts over the next 9 weeks. The latter are shown in Fig. 2.

Doubling time of M. lepraemurium. Using the counts shown in Table 2, doubling times of bacilli in the liver, spleen, and bone marrow

TABLE 2. *Sequential counts of AFB in the liver, spleen, and bone marrow of CBA mice injected intravenously with 10^8 M. lepraemurium*

Time after infection	Body wt[a] (g)	Liver wt[b] (mg)	Spleen wt[b] (mg)	Log AFB		
				Whole liver	Whole spleen	Bone marrow (2 femurs/mouse)
Day 1	19.2	1,184 (6.18)	83 (0.43)	7.78	6.68	4.44
Week 1	20.7	1,772 (6.15)	103 (0.50)	7.87	7.24	5.24
2	17.1	1,050 (6.14)	82 (0.48)	7.94	7.21	5.62
3	19.0	1,144 (7.61)	98 (0.52)	8.13	8.01	6.54
4	20.4	1,545 (7.58)	167 (0.82)	8.42	8.12	6.73
6	20.6	1,750 (8.52)	327 (1.59)	8.82	8.42	7.58
8	23.3	1,922 (8.27)	253 (1.09)	8.90	8.90	8.29
12	22.6	1,758 (7.77)	330 (1.46)	9.79	9.37	9.59
16	22.0	1,885 (8.57)	370 (1.68)	10.95	10.36	10.62
21	22.5	2,462 (10.94)	429 (1.90)	ND[c]	ND	11.56

[a] Mean of four mice.
[b] Parenthetic expressions show percent ratio of organ to body weight.
[c] ND, Not determined.

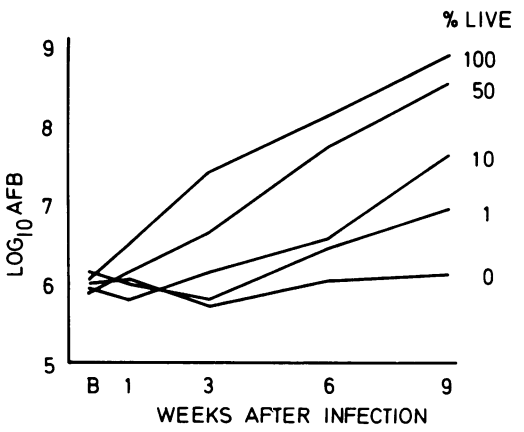

FIG. 2. *Sequential spleen counts of AFB in groups of CBA mice injected with 10^8 M. lepraemurium from suspensions containing freshly harvested bacilli and irradiated (dead) bacilli. The proportion of live bacilli is indicated to the right of the growth curves. B, Base line count of AFB at 24 h. Compare the slope of the spleen growth curves in this figure with the slope of the bone marrow growth curves shown in Fig. 3.*

were calculated over the whole period (16 weeks), over the first 8 weeks, and during the period 8 to 16 weeks after injection (see Table 3).

Over the whole period, the doubling time of bacilli in the bone marrow was shorter than that of bacilli in either the liver or spleen. This was true also if the first 8 weeks or the last 8 weeks was considered. The doubling time of bacilli in the spleen was similar to that of bacilli in the liver over 16 weeks. However, during the first 8 weeks, bacilli lodged in the spleen showed the shorter doubling time, whereas, during the second 8-week period, bacilli in the liver showed the shorter doubling time. The most extreme differences were detectable early in infection.

Viability test. The distribution and growth curves we have described were reproducible and were made the basis of a viability test. The assumption was made that no gross redistribution of bacilli occurred within the first few weeks of infection. Dilutions of live bacillary suspension (4×10^8, 4×10^7, or 4×10^6 bacilli per mouse) after intravenous injection yielded bone marrow growth curves (see Fig. 3) that showed striking parallelism. During the early part of infection at least the rate of growth was the same regardless of the number of bacilli inoculated. A standard curve was plotted (log inoculum versus time taken to reach a bone marrow count of log 7) from the growth curves shown in Fig. 3. The rationale of this approach is discussed by Youmans and Youmans (7) in connection with the rate of growth of tubercle bacilli in culture. Dilutions of the same suspension of bacilli were used to inoculate cell-free cultures, and the viability of cultured bacilli was assessed by mouse inoculation. Growth curves obtained by injection of mice with known numbers of bacilli "cultured" for 1, 4, 7, and 12 weeks were used to calculate retrospectively from the standard curve the viability of bacilli in the culture tubes. The result (Table 4) indicates a rapid loss of viability on this occasion. Nevertheless, the technique permitted the detection of extremely small numbers of viable bacilli injected by the intravenous route. From sequential harvests made over 6 months to a year, we were able to estimate that fewer than 100 viable bacilli were injected as part of an inoculum of 4×10^6 bacilli.

TABLE 3. *Doubling times of M. lepraemurium in the liver, spleen, and bone marrow of CBA mice given 10^8 bacilli intravenously*

Organ	Doubling time (days)[a]		
	Day 1-week 16	Day 1-week 8	Week 8-week 16
Liver	10.6	15.0	8.2
Spleen	9.1	7.6	11.5
Bone marrow	5.9[b]	4.8[c]	7.2

[a] Doubling time *(dt)* in days calculated according to the formula: $dt = (\log_2 \times [t^2 - t^1 \text{ in days}])/(\log \text{AFB } t^2 - \log \text{AFB } t^1)$.

[b] Calculated over the period week 1 to week 16.

[c] Calculated over the period week 1 to week 8.

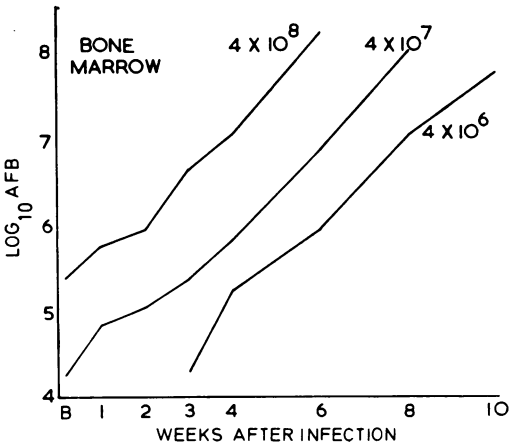

FIG. 3. *Bone marrow counts of AFB in groups of CBA mice over a 10-week period after injection with 4×10^8, 4×10^7, or 4×10^6 freshly harvested M. lepraemurium. B, Base line count made at 24 h.*

M. lepraemurium infection in T900R mice. The effect of T cell depletion and irradiation on the growth of *M. lepraemurium* was examined in T900R mice. Two groups of female CBA mice (normal and T900R) were infected intravenously with approximately 10^8 freshly harvested bacilli. The course of infection in the mice was followed by counting the number of AFB in liver and spleen at 5, 9, and 13 weeks. The spleens of T900R mice did not enlarge as did the spleens of normal mice within 6 weeks of infection. Only in those mice harvested at 13 weeks did splenomegaly occur. Despite the smallness of their spleens, these mice eventually harbored higher numbers of bacilli, as judged by liver and spleen counts (Table 5).

DISCUSSION

The method we have described is reproducible and enables estimates of the viability of *M. lepraemurium* suspensions to be made within a matter of weeks. It has already been applied to a study of the viability of bacilli cultured in a cell-free system and also to the determination of the cooling rate giving optimal survival of *M. lepraemurium* suspended in 10% dimethyl sulfoxide in liquid nitrogen (1; I. N. Brown, H.-N. Krenzien, and J. Farrant, manuscript in preparation). Several aspects of the technique warrant further discussion here. These include the suitability of the growth curve in each organ for an assessment of viability, the particular sensitivity of bone marrow tissue as an indicator of recent systemic *M. lepraemurium* infection, and the possible reasons for the differences in doubling time among the tissues studied.

The slope of the growth curve varied according to the tissue studied. Extreme differences were seen during the first 2 months of infection at the dose levels used. After this period, the apparent growth rate of bacilli in the liver increased considerably, whereas that of bacilli in the spleen and, to a lesser degree, in bone marrow, decreased. This change coincided with the appearance of new foci of infection in the liver and spleen (I. N. Brown and H.-N. Krenzien, unpublished data), and a possible explanation is that the infection became more widely disseminated at about this time. Dissemination could result either from bacteremia due to the bursting of cells or perhaps from a redistribution of infected cells themselves. We have not looked for *M. lepraemurium* bacilli in the blood of infected mice.

Because of these changes in observed doubling time, calculations of viability were routinely based on the early curves of growth in bone marrow, which showed an increase over several logarithms of count within a relatively short period. The doubling time of *M. lepraemurium* in the bone marrow during the first 8

TABLE 4. *Estimates of the viability of M. lepraemurium cultured in vitro made by injection of the bacilli into mice and determination of the growth rate in their bone marrow*

Time of harvest from culture	Mouse inoculum	Time in weeks to $\log_{10}$ 7	Estimated viable inoculum	Estimated viability (%)
0	4×10^8	3.9	4×10^8	[a]100
0	4×10^7	6.2	4×10^7	100
0	4×10^6	7.9	4×10^6	100
1 week	4×10^6	10.6	2.8×10^5	7
1 week	4×10^5	12.5	3.6×10^4	9
4 weeks	4×10^6	18.5	55	0.0014
7 weeks	4×10^6	23	1-10	0.00025
12 weeks	4×10^6	>52	0	0

[a] For the purpose of this experiment and calculations, the initial inoculum was assumed to be 100% viable.

TABLE 5. *Growth of M. lepraemurium in the liver and spleen of normal and T900R CBA mice after the intravenous injection of 10^8 bacilli*[a]

Week	Normal mice					T900R mice				
	Wt			Log AFB		Wt			Log AFB	
	Body (g)	Liver[b] (mg)	Spleen[b] (mg)	Whole liver[c]	Whole spleen[c]	Body (g)	Liver (mg)	Spleen (mg)	Whole liver	Whole spleen
5	18.9	1,387 (7.36)	232 (1.23)	8.34 (5.20)	8.09 (5.72)	18.6	1,101 (5.93)	74 (0.40)	8.48 (5.44)	7.83 (5.96)
9	17.1	1,431 (8.36)	230 (1.35)	9.10 (5.94)	8.74 (6.38)	16.1	1,215 (7.57)	90 (0.56)	9.36 (6.29)	9.23 (7.27)
13	16.6	1,441 (8.68)	265 (1.60)	10.35 (7.19)	9.82 (7.41)	15.2	1,229 (8.09)	141 (0.93)	10.78 (7.69)	10.66 (8.50)

[a] Doubling times (in days) were calculated for 5 to 13 weeks and were as follows. For normal mice, doubling time in whole liver was 8.35 days, and in whole spleen, 9.71 days. For T900R mice, doubling time in whole liver was 7.30 days, and in whole spleen, 5.93 days.

[b] Figures in parentheses are the organ to body weight percent ratios.

[c] Figures in parentheses are the logs of AFB per milligram of liver or spleen.

weeks of infection was 4.8 days (see Tables 2 and 3). Similar estimates of doubling time can be made using the set of three inocula on day 0 in Table 4. Serial tenfold dilutions gave differences of 2.3 and 1.7 weeks in time to 10^7 AFB/bone marrow sample, indicating doubling times of 4.9 and 3.6 days. A disadvantage of bone marrow was the requirement of high inoculum (between 10^8 and 10^9) so that a base line count of bacilli could be obtained. However, in several experiments in which inocula of a lower order were used, spleen growth curves were successfully used as an alternative.

Where base line counts were not required, bone marrow counts formed a sensitive indicator of low-order systemic infection or of systemic spread of small numbers of bacilli from, e.g., subcutaneous injection sites or inoculated footpads. As we have illustrated in our description of the viability assay of cultured bacilli, sequential bone marrow counts enable the estimation of between 1 and 10 viable bacilli in an inoculum, and this sort of approach has obvious application to tissue culture experiments where extremely small numbers of bacilli are used as inocula. A simple calculation, using the formula given in Table 3 and assuming doubling times of 5, 10, and 15 days for bone marrow, spleen, and liver, respectively, shows that if three viable bacilli were injected and one went to each of the tissues concerned, aside from other considerations, a detectable level of 10^5 bacilli would be reached in the bone marrow in 12 weeks, in the spleen in 24 weeks, and in the liver in 36 weeks. For accurate calculations we have found that several points in time should be plotted on the growth curve and that the time taken to reach a significant number of bacilli (e.g., $\log_{10} 7$; see Results) in the tissue concerned be determined by extrapolation.

Why the doubling time varied among the three tissues studied is not known. No evidence was obtained to suggest that massive redistribution of bacilli occurred in the early part of infection with which we were mainly concerned. On the contrary, our results with irradiated bacilli and the pathology of early infection would suggest otherwise. Also, the uptake of particulate matter by the bone marrow is depressed at an early stage of *M. lepraemurium* infection (I. N. Brown and V. S. Šljivić, manuscript in preparation), which would suggest that infiltration from other tissues is not a major factor contributing to the short doubling time in this tissue. The suspensions used were always prepared in a similar way and came from the tissues of mice infected 4 to 6 months previously. If it is assumed that the suspensions of bacilli were uniform, then presumably every bacillus had the potential to multiply at the fastest observed rate, i.e., every few days. We suggest that the generation time is determined in part by the macrophage population found in a particular tissue. Thus, in the liver, bacilli would be taken up predominantly by Kupffer cells, which are long-lived, differentiated macrophages, showing a low rate of mitosis. Within such cells, we found that *M. lepraemurium* initially showed pedestrian multiplication. In contrast, both the spleen and, in particular, the bone marrow (which is a rich source of many types of precursor cell) contain substantial proportions of young cells, showing a high rate of mitosis. Within these tissues, we found that *M. lepraemurium* multiplied more rapidly. The enhanced growth of the bacillus in cells of the bone marrow and spleen was confirmed by histological examination. Further possible evidence for the favorable environment provided by bone marrow cells was obtained from the preliminary experiment with T900R mice. After lethal irradiation, a proportion of the marrow cells used for reconstitution locate in the spleen (6), and one interpretation of the results presented in Table 5 is that *M. lepraemurium* grows faster in the spleens of reconstituted mice than in the spleens of normal mice because of this relocation of cells. We are examining this possibility further in current experiments and also the effect of tissue infiltration by blood monocytes on doubling time of bacilli in liver and spleen.

Apart from any influence the phagocyte might have on the subsequent development of bacilli, an alternative explanation for the differences in generation time could be that the growth of bacilli was inhibited in the liver, and to a lesser extent in the spleen, compared with the bone marrow. The environment of the tissue per se would not seem to be responsible for this inhibition, because later in infection bacilli grow perfectly well in the liver. A possible cause of inhibition is the development of a transient immune response effective against this obligate intracellular organism. This will be discussed in another communication.

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