THE EFFECT OF BODY TEMPERATURE AND CELL-MEDIATED IMMUNITY ON THE GROWTH OF *MYCOBACTERIUM MARINUM* AND *MYCOBACTERIUM LEPRAE* IN MICE

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SUMMARY. Evidence is presented that the high susceptibility of armadillos to infection with *Mycobacterium leprae* cannot be explained solely in terms of body temperature because mutant mice maintained with a body temperature similar to that of armadillos do not become heavily infected with *M. leprae*. The depression of cell-mediated immunity accompanying the low body temperature is not sufficient to produce an overwhelming infection. The results obtained with *M. marinum* suggest that whereas lack of cell-mediated immunity or a low body temperature result in a moderately enhanced infection in the mouse a combination of both of these factors is required to produce an overwhelming infection involving the internal organs.

INTRODUCTION

The inoculation of *Mycobacterium leprae* into the nine-banded armadillo (*Dasypus novemcinctus*) produces a disseminated infection that closely resembles human lepromatous leprosy (Kirchheimer and Storrs, 1971; Kirchheimer, Storrs and Binford, 1972; Storrs, 1971; Storrs *et al.*, 1974). The particular characteristic that initially stimulated interest in the armadillo was the fact that these animals have a primitive thermoregulatory mechanism which enables them to be maintained with a core temperature of 30–36°C (Wislocki and Enders, 1935; Johansen, 1961; Burns and Waldrip, 1971; Storrs, 1971). For many years *M. leprae* has been considered to have an optimum growth temperature below 37°C (Brand, 1959) and in studies with the mouse-footpad model, Shepard estimated that the optimum growth temperature of the organism was approximately 32°C (Shepard, 1965). The inoculation of small numbers of *M. leprae* into the footpads of mice results in a localised infection with a maximum bacillary yield of approximately $10^{6.3}$ organisms per footpad (Shepard, 1960*a* and *b*, 1965). Non-specific depression of T-cell function, for example by thymectomy and whole-body irradiation (Rees, 1966), results in enhancement of the footpad infection and dissemination of *M. leprae* to superficial tissue sites. Similarly, disseminated *M. leprae* infections in neonatally thymectomised Lewis rats (Fieldsteel and McIntosh, 1971) and congenitally athymic, nude mice (Colston and Hilson, 1976; Kohsaka, Mori and Ito, 1976) have been reported. However, none of these animals appears to develop the overwhelming infection seen in the nine-banded armadillo, from which bacillary yields of 10^{10} – 10^{11} /g can be obtained from internal organs such as liver, spleen and lymph nodes.

The congenitally obese (ob/ob) mouse was first recognised in 1950 (Ingalls, Dickie and Snell, 1950) and has since been shown to have many physiological abnormalities, including hypothermia (Joosten and Van der Kroon, 1974; Yen, Fuller and Pearson, 1974; Ohtake, Bray and Azukizawa, 1977). These studies have shown that the core temperature of obese mice is significantly lower than that of controls when the mice are kept at an ambient temperature of

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20°C. We have maintained obese mice in environmental conditions that ensure a body temperature similar to that reported for the armadillo. These "hypothermic" mice have been challenged with injections of *M. leprae* and bacillary growth was monitored. We have also used ob/ob mice, congenitally athymic (nu/nu) mice and mice homozygous for the "ob" and the "nu" gene to study infection with *M. marinum*. This is a rapidly growing cultivable mycobacterium with a growth temperature optimum similar to that of *M. leprae*, and a growth pattern in normal and immunosuppressed mice similar in many ways to that of the leprosy bacillus.

MATERIALS AND METHODS

Bacteria and preparation of inocula. The strain of *M. leprae* used in this study was originally obtained from a patient with untreated lepromatous leprosy and has been maintained in continuous mouse passage in our laboratory for several years. Inocula were prepared by harvesting bacilli from the footpads of mice by homogenisation in Middlebrook-Dubos medium containing 0.05% Tween 80 and 10% albumin (*Difco*). Counting of *M. leprae* in the inoculum was done by the method of Shepard and McRae (1968).

The strain of *M. marinum* was a variant of the original *M. balnei* strain X (Linell and Norden, 1954) isolated from a skin lesion in 1950. It was maintained on Löwenstein-Jensen medium at 4°C. This culture was used to inoculate Middlebrook-Dubos medium, which was then incubated at 30°C for 4 days. A viable count was done on the inoculum as described below.

Mice. Male C57B1 mice were used as controls. Obese mice were C57B1 mice homozygous for the obese gene. Nude mice and nude-obese (nu/nu-ob/ob) mice were from an outbred stock in which the mutations are maintained, and were supplied by the Medical Research Council Laboratory Animals Centre, Carshalton, Surrey.

Body-temperature measurements. Rectal temperatures of mice were recorded with an electronic digital thermometer fitted with a suitable probe. The probe was inserted 1.5 cm into the rectum of the mouse and the instrument was allowed to stabilise before the temperature was recorded.

Mouse inoculation and monitoring of bacillary growth. The methods of footpad inoculation, harvesting and processing of footpads, and counting of *M. leprae* were those described previously (Rees, 1965; Shepard and McRae, 1968; Holmes and Hilsen, 1972). The spleen was inoculated through a subcostal incision while the mice were under anaesthesia.

Viable counting of *M. marinum* in tissue was done by homogenising the tissue aseptically in Middlebrook-Dubos medium and making dilution plate counts by the method of Miles, Misra and Irwin (1938) on Middlebrook 7H10 agar plates.

RESULTS

Mouse body temperatures

In preliminary studies we found that environmental factors had a profound effect on the body temperature of obese mice; in particular, obese mice housed with more than one animal per cage had body temperatures similar to those of control mice. However, if the mice were caged individually at an ambient temperature of 20°C, there was a significant lowering of obese-mouse temperatures compared with those of control mice.

Fig. 1 illustrates the body temperatures of 10 control and 10 obese mice recorded twice daily for 3 weeks. The fluctuation in ambient temperature throughout the observation period is also shown. The mean temperature of the obese mice was 33.52°C, which is approximately 3.5° lower than that of controls, and similar to that reported for armadillos. The difference between the body temperatures of obese and normal mice was highly significant.

Growth of M. leprae in obese mice

Seven obese and control mice were inoculated into the left hind footpad (LHFP) with 5×10^3 *M. leprae*. The mice were killed at 9–11 months after inoculation and *M. leprae* in the LHFP,

FIG. 1.—Body temperature of control mice (O), mean = 36.9°C, and obese mice (●), mean = 33.5°C. Room temperature (▼), mean = 19.9°C.

TABLE I

Recovery of M. leprae from the tissues of control and obese mice after inoculation of 5×10^3 organisms into the left hind footpad

Days after inoculation	Number of <i>M. leprae</i> harvested per organ from					
	control mice			obese mice		
	LHFP	Spleen	Ears	LHFP	Spleen	Ears
280	...	...	...	3.2×10^6	$< 10^4$	$< 10^4$
308	...	...	...	8.7×10^5	$< 10^4$	$< 10^4$
310	...	...	...	1.1×10^6	$< 10^4$	$< 10^4$
312	...	...	...	2.9×10^6	2.2×10^6	$< 10^4$
312	...	...	...	8.4×10^5	$< 10^4$	$< 10^4$
317	...	...	...	9.0×10^5	$< 10^4$	$< 10^4$
324	5.6×10^5	$< 10^4$	$< 10^4$	...	...	...
356	1.4×10^6	$< 10^4$	$< 10^4$	...	...	...
356	1.0×10^6	$< 10^4$	$< 10^4$	...	...	...
356	1.8×10^6	$< 10^4$	$< 10^4$	...	...	...
356	1.3×10^6	$< 10^4$	$< 10^4$	...	...	...
356	1.1×10^6	$< 10^4$	$< 10^4$	...	...	...
356	2.0×10^6	$< 10^4$	$< 10^4$	...	...	...

LHFP = left hind footpad (which received the inoculum).

... = Not done.

FIG. 2.—Number of viable *M. marinum* harvested per footpad from control, obese, nude and nude-obese mice. ◆=normal mice; ■=obese mice; ▲=nude mice; ●=nude-obese mice.

TABLE II

Recovery of M. leprae from control and obese mice inoculated intrasplenically with 5×10^4 organisms

Days after inoculation	Number of <i>M. leprae</i> harvested per organ from			
	control mice		obese mice	
	Spleen	Ears	Spleen	Ears
123	...	...	2.9×10^4	$< 10^4$
240	...	...	9.5×10^4	$< 10^4$
324	...	...	8.8×10^4	$< 10^4$
339	$< 10^4$	$< 10^4$	1.1×10^4	$< 10^4$
356	$< 10^4$	$< 10^4$	...	...
356	$< 10^4$	$< 10^4$	...	...
356	$< 10^4$	$< 10^4$	...	...
356	$< 10^4$	$< 10^4$	...	...
356	$< 10^4$	$< 10^4$	...	...

... Not done.

spleen and ears were counted. In addition, four obese mice and six controls received inoculation of 5×10^4 *M. leprae* into the spleen: these mice were killed 4–11 months after inoculation and examined for the presence of acidfast bacilli in spleen and ears. These sites were selected to monitor the infection because, in armadillos infected with *M. leprae*, the spleen becomes heavily infected and recovery of organisms from the ears is an early indication of dissemination of the infection, in the armadillo (Kirchheimer *et al.*, 1972) and in the neonatally thymectomised rat (Dr A. H. Fieldsteel, personal communication). The results are shown in tables I and II.

FIG. 3.—Number of viable *M. marinum* harvested per g of tissue from obese, nude and nude-obese mice. ■—■ = obese spleen; □---□ = obese liver; ▲—▲ = nude spleen; △---△ = nude liver; ●—● = nude-obese spleen; ○---○ = nude-obese liver.

The growth pattern in the footpads of obese mice is similar to that in footpads of normal mice (Shepard, 1960*a* and *b*; Rees, 1966). The maximum yield of bacilli was 3.2×10^6 per footpad and in only one mouse were acidfast bacilli detectable in the spleen. In the four obese mice that were inoculated directly into the spleen with *M. leprae*, there was no apparent growth of the inoculum and at no time could *M. leprae* be recovered from the ears.

Thus, despite the low body temperature of the obese mice, there was no enhancement or dissemination of the *M. leprae* infection, and no growth of the organism in internal organs.

Growth of M. marinum in obese, nude and nude-obese mice

Groups of 10 control, obese, nude and nude-obese mice each received an inoculation in the

footpad of 3×10^4 viable *M. marinum*. Mice were killed at approximately 7-day intervals, and viable counts were made of *M. marinum* in footpad, spleen and liver. The results are shown in figs. 2 and 3, each point being the mean of two mice sampled. The results of the footpad harvests (fig. 2) show a distinct difference between the normal mice and the other three groups. In the control mice a peak of 3×10^6 viable *M. marinum* per footpad at 7 days after inoculation was followed by a rapid decline in the number of viable organisms recovered. In nude mice the maximum number of viable organisms was also recovered at 7 days but the peak was approximately tenfold higher than in the normal mice and there was no decline in viable numbers. The growth curve in obese mice was similar to that of nude mice, the numbers levelling off at 3×10^7 viable *M. marinum*, though the appearance of the peak was delayed in comparison with the other groups. The growth of *M. marinum* in the footpads of nude/obese mice was similar to that of nude mice, with a peak of 6×10^7 viable organisms per footpad 7 days after inoculation.

The recovery of viable *M. marinum* from the liver and spleen of footpad-inoculated mice is shown in fig. 3. Viable *M. marinum* could not be detected in the liver of control mice at any time, and at only one harvest, 14 days after inoculation, were they detectable in the spleen. Viable organisms could not be detected 11 days after inoculation in either liver or spleen of obese mice, but by 20 days, 7×10^4 and 3×10^3 organisms were isolated per g of spleen and liver respectively. These levels were maintained 31 days after inoculation. Numbers of viable organisms isolated from the spleen of nude mice reached a peak of 3×10^5 /g at 14 days, with a plateau of approximately 10^5 viable organisms per g of tissue. Similar results were obtained for the livers.

The progress of the infection in the internal organs of the nude-obese mice was clearly different from that in the obese or the nude mice. In liver 6×10^3 organisms were harvested 7 days after inoculation, increasing exponentially, with a doubling time of approximately 24 h, to reach 2×10^9 /g by 34 days. Growth of *M. marinum* in spleen followed the same pattern with 3×10^3 /g at day 7 rising to 2×10^9 /g on day 34.

DISCUSSION

In this study we have demonstrated that *M. leprae* does not produce an enhanced or disseminated infection in obese mice maintained with a body temperature thought to be optimal for the growth of the bacillus. The low body temperature of obese mice is consistent with the hypothermia reported previously for these animals (Yen *et al.*, 1974; Ohtake *et al.*, 1977), and is similar to that of the nine-banded armadillo (Wislocki and Enders, 1935; Burns and Waldrup, 1971; Storrs, 1971). Purtilo (Purtilo *et al.*, 1974, 1975) concluded that the high susceptibility of the armadillo to infection with *M. leprae* is due to a depressed cell-mediated immunity, probably resulting from the animals low body temperature. The obese mouse also appears to have depressed cell-mediated immunity as judged by its prolonged acceptance of heterografts and reduced delayed-type hypersensitivity response to *M. leprae* (Lancaster, unpublished results). This depression of cell-mediated immunity may account for the occasional appearance of *M. leprae* in the spleen of obese mice that have been inoculated in the footpad, but does not result in the very large numbers of organisms that are recoverable from the internal organs of armadillos (Kirchheimer and Storrs, 1971; Kirchheimer *et al.*, 1972; Storrs, 1971; Storrs *et al.*, 1974).

The results with *M. marinum* indicate that a combination of low body temperature and lack of cell-mediated immunity are required for extensive growth of this organism in internal organs. The results in normal and nude mice confirm previously reported data; in the footpad of normal mice, *M. marinum* grows to a peak of approximately 10^6 per footpad and is followed by a rapid decline in viable numbers (Ng, Jacobsen and Levy, 1973; Colston and Hilson, 1976). In nude mice the footpad infection reaches a peak approximately tenfold higher than in controls, and the infection reaches a plateau with no decline in numbers. Viable organisms can be isolated from livers and spleens. In the experiments reported in this study, growth of *M. marinum* in obese mice was similar to that in nude mice although fewer organisms were isolated from the liver. In nude-obese mice, however, the numbers of viable organisms recovered from the livers and spleens was approximately 10 000 times that of the nude or obese animal.

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