The kinetics and quality of acquired resistance in self-healing and metastatic leishmaniasis

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SUMMARY

Quantitative methods for enumerating viable *L. enriettii* in tissues have been used to determine the course of cutaneous leishmaniasis in guinea-pigs. The development and kinetics of acquired resistance have been evaluated in self-healing and chronic metastatic forms of the disease. It is revealed that 3 weeks after a primary local infection, a standard challenge infection is totally eliminated within 7 days. This resistance is as strong in animals with a current infection as it is in those that have fully recovered from such an infection. Animals developing metastatic disease also develop resistance to the standard challenge. This is initially as strong as in animals with only localized disease, but wanes with the progression of the infection. Although the quality of resistance becomes poorer in animals with metastatic infection, it is not lost completely. The relationship between acquired resistance and the resolution of the primary infection is discussed.

INTRODUCTION

Cutaneous leishmaniasis, like leprosy, is a disease that exhibits a clinical spectrum of severity (Garnham & Humphrey, 1969; Turk & Belehu, 1974). This spectrum ranges from a chronic but self-healing localized lesion (cutaneous leishmaniasis) to a disseminated non-healing disease where multiple cutaneous sites are involved (diffuse cutaneous leishmaniasis). It is currently thought that increased clinical severity is related to an impairment of the immune response of the host (Turk & Belehu, 1974; Bryceson *et al.*, 1970; Bryceson, Bray & Dumonde, 1974).

Although many animal models exist for the study of this disease, *L. enriettii* infection of the guinea-pig is unique in that both local and diffuse disease can be induced in this animal simply by varying the route or size of inoculum (Bryceson *et al.*, 1970; 1974; Adler & Halff, 1955). Studies of the immunological response to leishmaniasis in the guinea-pig and other laboratory animals have shown that local self-healing infections are associated with the development of cell-mediated immunological mechanisms (Bryceson *et al.*, 1970; Poulter, 1976a; Bryceson *et al.*, 1972), and that diffuse cutaneous infections result from the suppression of these mechanisms of acquired immunity (Bryceson *et al.*, 1974; Bryceson & Turk, 1971; Bryceson *et al.*, 1972). It is apparent, however, that the situation is not quite so simple. For instance, reports have indicated that resistance to re-infection develops some time before the resolution of the lesion at a primary site of infection (Bryceson *et al.*, 1970; 1972), and even animals that develop chronic metastatic lesions appear capable of resolving the primary lesion (Bryceson *et al.*, 1970). In general, previous work has relied on clinical, morphological and histological criteria to judge the acquisition of resistance (Bryceson *et al.*, 1970; Rezai *et al.*, 1972; Behin, Mauel & Rowe, 1977). The success of the immune response against any infection can only be measured objectively in terms of the ability of the host to eliminate the parasite. It is clearly important, therefore, to be able to quantify changes in the number of viable organisms within primary and metastatic lesions throughout the

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clinical course of the disease and the development of resistance by determining the rate of removal of a standard challenge infection in terms of parasite survival.

This paper uses quantitative methods of enumerating viable leishmania (Poulter, 1979) to record the development and quality of immunity in local and diffuse leishmaniasis.

MATERIALS AND METHODS

Animals. Inbred strain No. 2 guinea-pigs bred at this Institute were used throughout. Animals of either sex weighing 400–500 g were fed guinea-pig diet 6AP137 supplemented with cabbage and were given water *ad libitum*.

Parasites. Leishmania enriettii was maintained in the laboratory by serial passage in guinea-pigs (Belehu, Poulter & Turk, 1976). For experimental use, amastigotes extracted from guinea-pig tissues were cultured at 28°C in Grace's insect medium as described previously (Poulter, 1979), for at least 48 hr before use. This period was sufficient for differentiation into promastigotes to occur.

Inoculation. Promastigotes of *L. enriettii* were harvested from culture, washed twice, and suspended in Earle's balanced salt solution (EBSS) at appropriate dilutions. After counting in a haemocytometer, inocula were prepared in terms of numbers of motile parasites per ml. Normal guinea-pigs were infected with either 5×10^6 promastigotes intradermally in the nose, or with 10^8 promastigotes into the ear. For all inoculations, an equivalent amount of the promastigote suspension was seeded into Grace's medium and cultured for 48 hr so that the number of viable organisms injected could be quantified as described previously (Poulter, 1979). Only rarely did the number of viable organisms differ by more than 20% from the total number of organisms injected, and in such cases the recorded viability is quoted in the results.

Size of the lesion. As the number of viable organisms in the lesions of the nose or ear are related to a known weight of tissue, it was appreciated that this did not give a value for the total number of parasites. Therefore, the gross size of these lesions was also measured at progressive times, using micrometer scaled calipers. The relationship between total parasite load and concentration of parasites per unit of tissue is discussed later.

Quantification of L. enriettii in tissues. At appropriate times after infection a piece of the infected nose or ear was surgically removed. These samples were weighed, and then ground by hand in a glass homogenizer containing 3·0 ml of EBSS as described previously (Poulter, 1979). 1·5 ml of suspension was then added to 13·5 ml of Grace's insect medium supplemented as described (Poulter, 1979), and triplicate 5·0 ml cultures were set up in 25 ml Thompson flasks. These were sealed and incubated at 28°C. Each day thereafter the cultures were examined with an inverted microscope. Samples were taken for enumeration before their density was such as to inhibit growth (Poulter, 1979). If no parasites could be seen under the microscope after 7 days, the triplicate cultures were pooled, spun down and concentrated in 1·0 ml of EBSS before counting.

Challenge infection. At various times during primary infection, animals were given a standard challenge with 2×10^7 viable promastigotes either in the front foot (following primary infection in the nose), or in the nose (following primary infection in the ear). In initial experiments, tissues were removed from the challenge site after 10 min, 1 hr, 3 hr, 1 day, 3 days and 14 days, and the parasites extracted and quantified as described (Poulter, 1979). In subsequent experiments tissues were only sampled from challenge sites 7 days after challenge.

Experimentation. All experiments contained at least three animals at each time point. Following extraction of parasites, three cultures were set up from each animal. Results are expressed as the mean and standard deviation about the mean.

RESULTS

The course of infection

A pool of normal guinea-pigs were infected with 5×10^6 viable *L. enriettii* i.d. in the nose. Each week thereafter the noses were measured and tissue was taken from a group of this pool to quantify the concentration of viable parasites. It was found that the lesion reached a maximum size by week 7. A rapid decrease in size occurred between weeks 7 and 9, and the lesion appeared fully resolved macroscopically by week 13 (Fig. 1).

The concentration of viable organisms within this lesion reached a peak 2 weeks after infection, stayed at a level of 10^5–10^6 organisms/mg tissue up to week 8, and then parasitaemia resolved. No viable parasites were detectable 14 weeks after infection (Fig. 1). These results demonstrate that the progressive increase in the size of the local lesion between weeks 2 and 7 does not reflect an increase in the concentration of parasites per unit of tissue, and secondly that the onset of the resolution of the lesion in terms of size, precedes a reduction in the concentration of viable parsites. Between 8 and 12 weeks, a considerable reduction in parasite concentrations was concomitant with a decrease in lesion size. At 13 weeks, however, some parasites persisted at the site despite the lack of any gross lesion.

This experiment was repeated with animals injected with 10^8 viable parasites into the ear. Although
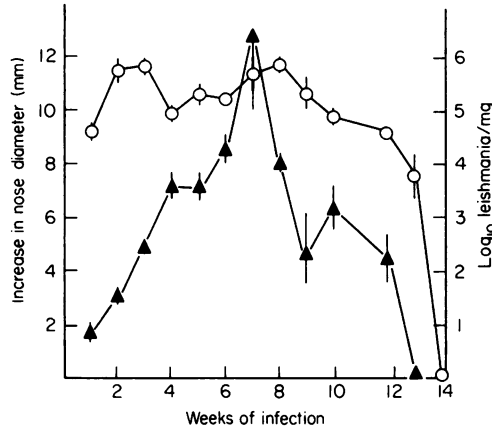
FIG. 1. The relationship between the size of the primary lesion (▲———▲) and the concentration of viable parasites (○———○) at progressive times after the inoculation of 5×10^6 *L. enriettii* in the nose. Mean ± s.d. from three guinea-pigs at each point.
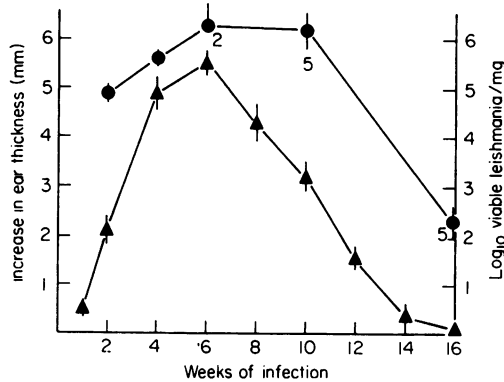

FIG. 2. The relationship between the size of the primary lesions (▲———▲) and the concentration of viable parasites (●———●) at progressive times after the inoculation of 10^8 *L. enriettii* in the ear. Mean ± s.d. from five guinea-pigs at each point. (The numbers of animals out of five showing detectable metastasis are also given.)

these animals were destined to develop metastatic disease, it was found, in agreement with others (Bryceson *et al.*, 1970), that the primary lesion on the ear resolved completely. In these animals however, both the size of the lesion and the concentration of parasites peaked at the same time. Resolution of lesion size however, preceded a reduction in the concentration of viable organisms within the tissue (Fig. 2).

The elimination of a challenge infection

The most appropriate way to measure the development of required resistance is to measure the ability of the host to destroy a standard challenge infection. Initially, guinea-pigs that had recovered from a primary infection of 5×10^6 parasites in either the ear, or the nose were tested. A challenge inoculum of 2×10^7 *L. enriettii* was selected as this has been found to be the largest number of organisms that can be injected at any site without producing metastatic disease in strain No. 2 guinea-pigs.

A group of fifteen guinea-pigs that had been infected in the nose with 5×10^6 *L. enriettii* between 5 months and 1 year previously were challenged with 2×10^7 viable organisms in the front foot. As a control, fifteen normal guinea-pigs were infected in an identical manner. Ten minutes, 1 hr and 3, 7 and
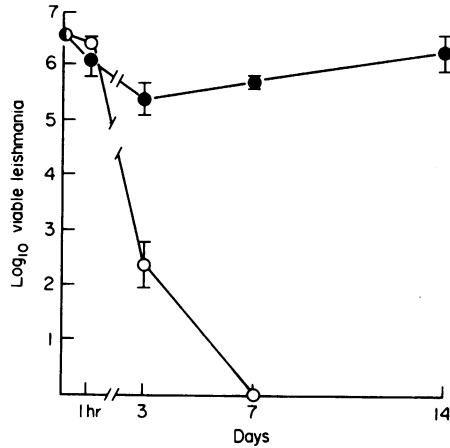
FIG. 3. The number of viable *L. enriettii* extractable from the front foot of animals recovered from a primary infection with *L. enriettii* in the nose (○————○) and normal controls (●————●) at various times after a standard challenge with 2×10^7 viable organisms. Mean ± s.d. of three animals at each point.

14 days later the challenge foot was removed and parasites extracted and cultured to determine the survival of viable organisms. It was found (Fig. 3) that by 7 days no viable organisms could be extracted from the immune animals. In the control animals 80–90% of the inoculum was destroyed over the first 3 days but after this, the number of viable parasites at this site progressively increased.

This experiment was repeated using immune animals that had recovered from a primary infection of 5×10^6 organisms in the ear. These animals received the challenge infection of 2×10^7 viable parasites into the nose as did a control group of normal animals. As in the preceding experiment, the recovered animals eliminated the challenge infection within 7 days, whereas the normal animals were showing progressive growth of the leishmania at this time (Fig. 4). Of some significance is the observation that the kinetics of the parasite was the same whether results were expressed as survival of total viable parasites (Fig. 3) or as concentration of parasites per unit of tissue (Fig. 4).

The development and quality of resistance

It has already been established (Bryceson *et al.*, 1970; Kretzchmar, 1965) that guinea-pigs remain immune to re-infection after they have recovered from *L. enriettii* infection. The experiments above confirmed this by quantifying the kinetics of elimination of viable parasites from a challenge site. In the
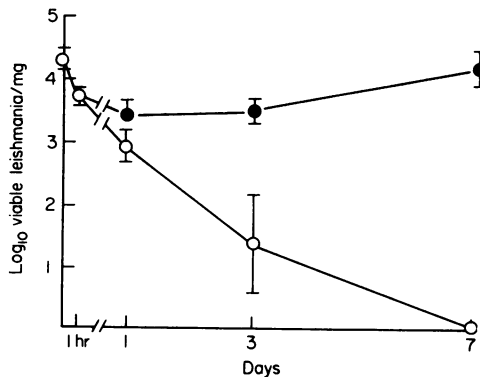

FIG. 4. The concentration of viable *L. enriettii* in the nose of animals recovered from a primary infection with *L. enriettii* in the ear (○————○) and normal controls (●————●) at various times after a standard challenge with 2×10^7 viable organisms. Mean ± s.d. of three animals at each point.
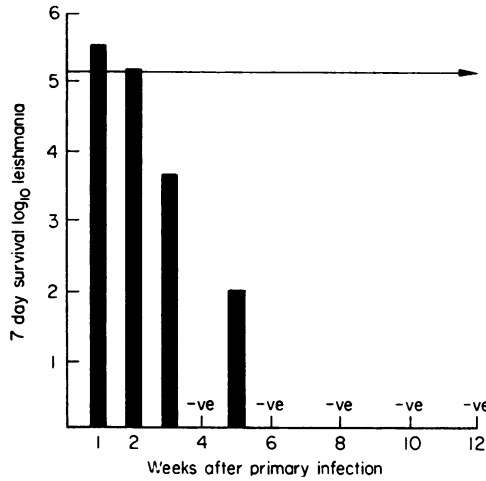
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FIG. 5. The number of viable organisms in the front foot 7 days after challenge with 2×10^7 *L. enriettii* in animals given a primary infection with 5×10^6 *L. enriettii* in the nose at various times previously. The horizontal line represents the mean 7 day survival of a 2×10^7 challenge in normal animals.

following experiments the same procedure was used to measure changes of resistance against time of infection to determine at which point solid immunity was established.

A panel of animals were injected with 5×10^6 *L. enriettii* into the nose. At various times following primary infection, groups of three animals were challenged with 2×10^7 *L. enriettii* in the front foot, and the challenge feet were excised 1 week later to enumerate viable parasites. It was found (Fig. 5) that 4 weeks after primary infection the animals were capable of totally eliminating the challenge within 7 days. Animals taken 5 weeks after primary infection did not completely eliminate the challenge, but from 6 weeks onwards all guinea-pigs appeared solidly immune to re-infection.

This experiment was repeated with animals given a primary infection of 10^8 viable organisms in the ear (a dose and route that results in metastatic disease). Groups of these animals were challenged in the nose with 2×10^7 viable *L. enriettii* at various times after primary infection. One week after each challenge tissues were removed and the number of viable parasites determined.

It was found (Fig. 6) that 3 weeks after infection with 10^8 organisms in the ear, a standard challenge inoculum was eliminated from the nose within 7 days. Animals tested 6 weeks after this infection showed less resistance, and at 10, 12 and 15 weeks significant numbers of viable organisms were per-
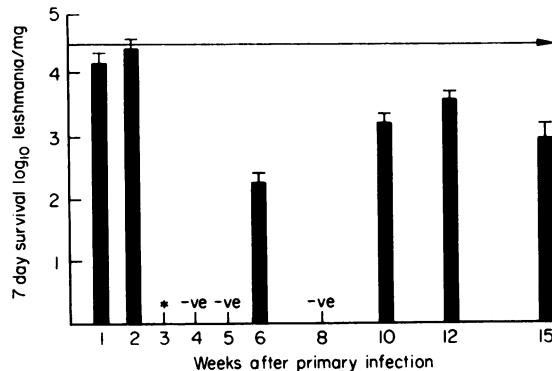

FIG. 6. The concentration of viable organisms in the nose 7 days after challenge with 2×10^7 *L. enriettii* in animals given a primary infection with 10^8 *L. enriettii* in the ear at various times previously. (Mean ± s.d.) The horizontal line represents the mean 7 day survival of a 2×10^7 challenge in normal animals. *Results at this time point were negative but the challenge dose was found to contain only 8.9×10^6 viable *L. enriettii*.
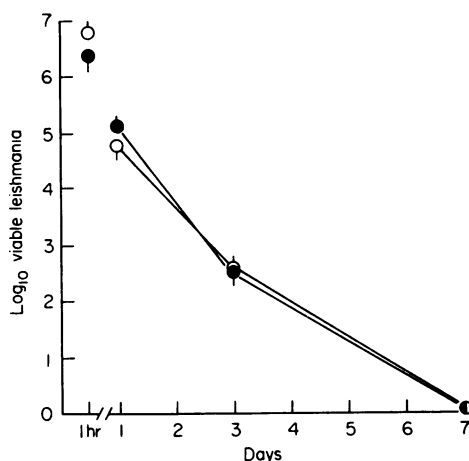
FIG. 7. The kinetics of removal of a challenge infection of 2×10^7 *L. enriettii* from the front foot of animals totally recovered from a primary infection (● · · · · · ●) and animals with an ongoing primary infection given 4 weeks previously (○————○). Mean ± s.d. at each point.

sistently recovered 7 days after challenge. I*t* should be noted however, that when some of these challenged animals were observed over a prolonged period they all failed to develop a gross lesion at the challenge site, even though they were carrying metastatic foci seeded from the primary infection. In this regard, care was taken to avoid using animals that had a secondary lesion on the nose. It was clear, therefore, that resistance did develop during metastatic disease, but was less efficient than that expressed by animals with only localized infection.

To determine whether the resistance generated during a current infection is of the same quality as that expressed after healing, a direct comparison was made between animals recovered from infection and those having the disease. Two groups of guinea-pigs were selected: one that had been given 5×10^6 *L. enriettii* in the ear 4 weeks previously and one that had totally recovered from such an infection. Both groups were challenged with 2×10^7 organisms in the nose and at various times afterwards tissues were removed and the number of surviving viable parasites determined. It was found (Fig. 7) that the kinetics of the removal of the challenge infection was the same in both groups of animals.

To determine whether the development of resistance was dose-dependent, groups of animals were given primary infections in the ear with graded inocula of viable organisms. Four weeks later all groups
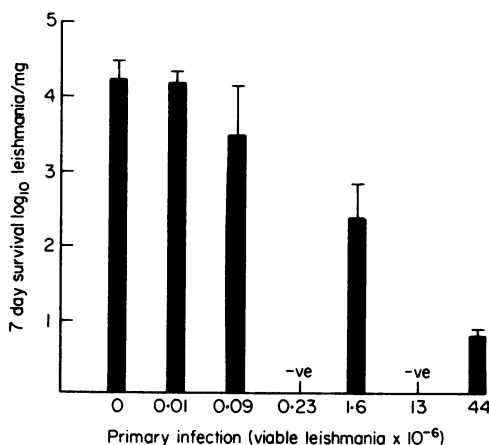

FIG. 8. The 7 day survival of a standard challenge of 2×10^7 *L. enriettii* given in the nose, in groups of animals that received primary infections 4 weeks previously with graded doses of *L. enriettii* in the ear. Mean ± s.d.

were challenged with 2×10^7 leishmania in the nose. After 7 days tissues were sampled and the concentration of parasites determined. The results (Fig. 8) show that strong resistance to challenge developed during the 4 week period, even when the inoculum was as low as $2 \cdot 3 \times 10^5$ organisms. Indeed, these animals were only just developing a gross lesion at the primary site by this time. With 10^5 organisms as a primary infective dose the results were equivocal. Below 10^5, no significant resistance to challenge had developed by this time.

DISCUSSION

Although much work has been done in examining the ability of guinea-pigs to develop acquired immunity against infection with *L. enriettii* (Bryceson *et al.*, 1970; 1972; Rezai *et al.*, 1972; Krettschmar, 1965), determination of resistance has been limited to gross observations of lesion development or histological studies of the challenge sites. It is generally thought that the severe manifestations of this disease result from some impairment of cell-mediated immunological reactivity (Bryceson *et al.*, 1972; Turk & Belehu, 1974; Bryceson *et al.*, 1974). This being the case, it is clearly important that the quality of acquired resistance be quantified precisely in terms of the kinetics of parasite removal, as in this way direct comparisons can be made between local and metastatic disease. By describing the course of infection and development of resistance in terms of concentrations of viable parasites within primary lesions and the elimination of a standard challenge, it was possible to determine quantitatively both the kinetics and efficiency of acquired resistance to leishmaniasis.

After primary infection in the nose, the local concentration of parasites reached a peak 2 weeks after infection, whereas the gross size of the lesion did not reach maximum size until week 7. Subsequently, a considerable decrease in the size of the lesion occurred 7 days before any significant decrease in the concentration of the parasites. From week 8 onwards, the decrease in lesion size was concomitant with a reduction in parasite concentration. The course of the primary infection in animals destined for metastatic disease followed a similar course, the exception being the development of parasitaemia which was temporally related to the increasing gross size of the lesion. Again, however, the resolution of the lesion preceded any decrease in parasitemia. The healing of the primary site of *L. enriettii* infection appears to be initiated by a reduction in the size of the cellular mass, followed by a significant reduction in the concentration of parasites. As it is unlikely that parasitized cells leave the site, it would seem probable that they are destroyed locally, as has been suggested previously (Bryceson *et al.*, 1970). This might well lead to a release of organisms which would promote an immune response to reduce the local parasitaemia. The whole process of resolution appears to require at least 6 weeks, during which time the mechanism suggested above may be repeated over and over again, as the inflammatory response slowly resolves. This is clearly in contrast with the process of elimination induced by the acquired immune response, where large challenge infections are removed within 7 days. It must be emphasized, however, that the ultimate mechanisms of parasite destruction both at the primary site of infection, and at a challenge site in an immune animal, remain unknown.

The suggestion that the removal of the parasite from the primary lesion is, at least in part, dependent on the gradual resolution of the inflammatory response is in keeping with previous observations by others. Firstly, Bryceson *et al.* (1970) and Rezai *et al.* (1972) have reported that the lymphocytic infiltrate which develops at the infected site forms a layer around the outside of a mass of parasitized macrophages and that the contraction of the lesion occurs from the inside outwards, thus implying that even if the lymphocytic infiltrate is a result of the immune response, its effect is to contain the parasite rather than kill it directly. Secondly, Behin *et al.* (1977) observed that the resolution of a local infection can be induced by the development and resolution of an inflammatory response not generated specifically against this parasite.

It has been shown in this paper that within 3–4 weeks after primary infection, a mechanism of protective immunity has developed that will eliminate a standard challenge infection within 7 days. Further experiments (Poulter, unpublished observations) have also shown that these animals will resist a challenge with 10^8 organisms. Indeed, the efficiency of this response is equivalent to that in animals that have

recovered and which have been shown to maintain resistance at least 1 year beyond the resolution of the primary lesion (Kretschmar, 1965; Poulter, 1976b). Guinea-pigs developing metastatic disease also generate resistance to challenge, as has been described previously (Bryceson *et al.*, 1970), but the effectiveness of this response varies, and is not as strong as in animals with self-healing infection.

These results clearly demonstrate that however, strong the mechanisms of acquired immunity may be, they are either not expressed or are actively suppressed, both at the site of primary infection and at metastatic foci. Histological evidence (Bryceson *et al.*, 1970) would indicate that an immunological response is occurring, at least at the site of primary infection, but is prevented from effecting the destruction of the parasites by interference caused by the granulomatous nature of the lesion.

In all, the results presented here demonstrate that the ability to quantify viable organisms from tissues infected with *L. enriettii* allows a more objective examination of the relationship between resistance and healing, and imply that the differences in immunological responsiveness which result in metastasis might be found more at local level than systemically.

Experiments are now in progress both to analyse the immune response using methods of adoptive immunization with cells and serum, and to study the local events in lesions to determine the possible significance of the intramacrophage localization of the parasite.

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