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Regulation of *Leishmania* populations within the host IV. Parasite and host cell kinetics studied by radioisotope labelling

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In memoriam Oscar Felsenfeld

Summary

Tritiated thymidine pulse labelling followed by autoradiography has been used to analyse the kinetics of *Leishmania donovani* infections within the mouse host. Resistant strains of mice show much lower parasite proliferation rates than do susceptible strains. In chronic infections the rate is also reduced. Parasitised mononuclear phagocytes may undergo mitosis. No evidence for selective destruction of parasitised cells in chronic infections was found by labelling methods. It is possible to get parasite labelling in vitro immediately after biopsy to levels comparable to those seen in vivo.

Key words: Leishmania donovani; mouse genetics; cell kinetics; immunity to parasites; autoradiography.

I first met Oscar Felsenfeld at a meeting in Vienna on radioactive tracers in microbial immunology. He was at that time much occupied with improving radioimmunoassay techniques for epidemiological purposes in developing countries. Nevertheless he showed an interest in the quite different uses of labelling involved in our work on leishmaniasis. Then, and in his subsequent visit to Oxford, I began to enjoy his very broad yet profound understanding of infective disease, and his European cultural background. Taking up the subsequent course of the leishmanial labelling brings back memories therefore of Oscar Felsenfeld and the Vienna State Opera at that first meeting, and the debt I owe to his encouragement. May his soul rest in the peace that was so rarely allowed him on the earth.

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Introduction

It is not unusual for animal parasites to provoke in their mammalian hosts an immune response which greatly inhibits the parasite population but fails to completely eradicate it from the host. This phenomenon, called premunition by Sergent (1963), has a clear relevance to the life history strategies of many parasites (Bradley, 1972a). This single ecological strategy is achieved by varied immunological tactics in different host-parasite systems and these have both intrinsic interest and practical importance. Part of the parasite population escapes the immune response in trypanosomiasis by antigenic variation over time (Gray, 1965) and this same mechanism is used by the *Borrelia* of relapsing fever that so interested Oscar Felsenfeld (1965; Schuhardt and Wilkerson, 1951), while antigenic disguise by the uptake of host antigens is involved in schistosomiasis mansoni of the rhesus monkey (Smithers et al., 1969). In the hope of discovering yet other mechanisms of incomplete resistance to infection our studies of mouse visceral leishmaniasis were begun. Stauber (1958) had already shown that when a mouse is inoculated intravenously with *Leishmania donovani* the parasite numbers rise, then fall, but do not completely disappear, persisting at the lower plateau level. Our hypothesis to explain these events was that the parasites divide repeatedly within the macrophages at first, and then burst the cells about the time that an effective immune response is mounted by the mouse. Free parasites were supposed to be largely destroyed, and thereafter a smaller number of parasites persisting within a population of activated macrophages, having a much lower proliferation rate of the parasite. Division of the macrophages could allow an increase in the number of infected cells, without the amastigotes of *Leishmania* being exposed to serum. Labelling methods for the parasite in vivo were developed to test this group of hypotheses and methodological aspects were reviewed in Vienna where Felsenfeld was present (Bradley, 1972b). It was shown that the proliferation rate of *L. donovani* in the mouse could be estimated by determination of the parasite labelling index using tritiated thymidine injection into the host followed by autoradiography to show the proportion of parasites in the DNA-synthesis phase of their life cycle, in a manner analogous to mammalian cell kinetic studies but requiring a much higher dose of tritiated thymidine. Many of the experiments described below were intended to test the hypotheses given above. They were temporarily abandoned when it was found that in inbred mice the course of infection was not as reported by Stauber (Bradley, 1974; Bradley and Kirkley, 1977) and that his results, probably from a genetically heterogeneous group of mice, were overshadowed by the massive genetic variation between strains which has both an innate and an acquired component, the former depending largely upon a single gene (Bradley, 1977) which has now been mapped (Bradley et al., 1979, in press).

The system studied has turned out to be a useful model for studying the

genetic control of resistance to infection rather than premunition. In attempting now to analyse the mechanisms by which genetic variation affects the natural history of infection, the earlier labelling studies are of renewed relevance and they are therefore reported here, even though they were originally intended to study other things. However, results can be interpreted in the light of the genetic observations made subsequently, when the mouse strain used is taken into consideration.

Inbred mice fall into two groups: those in which *L. donovani* multiplies in the liver a hundredfold in the first 15 days after infection, which we call susceptible, and those in which parasite multiplication is around fivefold, which are called resistant (Bradley and Kirkley, 1977). Susceptible strains used in the present study include C57 BL, NMR1 and the outbred white PO strain. Inbred CBA and C3H and outbred 'Ash' are resistant. The acutely susceptible mice vary in their long-term responses. Both NMR1 and C57 BL recover after 3 months of infection and almost all parasites in the liver are destroyed. Many PO strain mice maintain immense parasite populations in the liver, spleen and bone marrow, and the intensity of infection is comparable with that seen in hamsters though the anaemic mouse may sometimes survive a normal lifetime. Most labelling studies were of susceptible PO mice before it was clear that, though infection moves towards a plateau in this strain, partial reduction of the parasite population does not occur first.

Labelling methods allow proliferation of cells or parasites to be determined independently from changes in the total populations. The objectives of the experiments were by this means to determine whether parasite proliferation was inhibited in chronic infection, whether parasitised macrophages proliferate, whether susceptible and resistant mouse strains differ in parasite proliferation rates, and how soon after infection such divergence is manifest, and whether labelling of biopsies in vitro can be used to give results comparable with those from in vivo studies.

Materials and methods

Methods used in more than one of the separate sets of experiments are set out here; design details specific to one experiment are given with the results section.

Mice were infected using amastigotes of an Ethiopian strain of *L. donovani* via the tail vein and parasite numbers followed in liver imprints of mice killed at various time intervals (detailed methods in Bradley and Kirkley, 1977). The standard inoculum was 5×10^7 parasites, but to determine labelling indices in the first few days of infection inocula of 2×10^8 amastigotes were used.

For in vivo radioisotope labelling of parasites, tritiated thymidine was given intravenously at 20 micro Ci/g in a total volume of 0.5 ml of buffered saline and the mouse killed 1 h later. Imprints of the cut surface of the liver were fixed in methanol, extracted with trichloroacetic, and dried.

For labelling of parasites in vitro a 5 mm cube of liver was removed immediately after killing a mouse with ether, placed in a ground glass grinder with 3 ml of tissue culture medium 199, containing 5 μCi/ml of tritiated thymidine, and homogenized gently in a tissue grinder. The homogenate was transferred rapidly to a Leighton tube, gassed to 5% carbon dioxide, and incubated for 1 h at

37° C. The suspension was then mixed, slides prepared both as smears and cytocentrifuge preparations, and fixed in methanol before extraction with trichloracetic acid.

In longer term experiments, macrophages were labelled in vivo using 0.5 μCi/G on each of 3 consecutive days intravenously.

Autoradiographs of imprints and smear preparations were prepared using Ilford K5 dipping emulsion and exposed for 35 days. They were developed in Kodak fine grain developer, fixed and hardened, and stained with dilute aqueous Giemsa (George Gurr) through the emulsion. The parasite labelling index was determined on 500 consecutive parasites. Amastigotes with 5 or more silver grains overlying them were considered as labelled.

Extraction of mononuclear phagocytes from the liver used pronase digestion and from the spleen was by gently grinding the tissue in balanced salt solution and allowing macrophages to adhere to cover slips.

Histology used standard formol-saline fixation and wax embedding. Sections were cut at 4 μm and stained overnight in haematoxylin diluted 1:10 in distilled water before differentiation and eosin counterstaining. Other tissues were fixed with glutaraldehyde and embedded in araldite in the standard manner for electron microscopy but cut at 1 μm and stained with 1% aqueous methylene blue for 3 min.

Experimental objectives and results

Experiment 1: Acute parasite proliferation in susceptible and resistant mice

The dramatic difference in parasite population growth rates between resistant and susceptible mouse strains can be explained in two ways. Either proliferation is equally rapid in each strain but parasites are being continually destroyed in the resistant strain, or the proliferation rates differ markedly between strains, with very slow multiplication of the parasites in the resistant strain. In the former case, proliferation rates of the parasites would be similar in the two types of strain, whereas in the latter the labelling index would be reduced in the resistant mice. Parasite counts give a high variance at low parasite loads and are a poor guide to events in the first few days of infection. Labelling is less subject to these disadvantages.

Mice of the susceptible PO and NMR1 and resistant Ash and CBA strains were killed 1 h after in vivo labelling and 3, 4 or more days after heavy infection, as shown on Fig. 1, which indicates the labelling indices. The immense parasite inoculum needed limits the number of replications.

A clear difference between susceptible and resistant strains is evident from the third day. Since the parasite load is much lower in the resistant animals, any bias due to heavily infected cells having less isotope per parasite will tend to reduce rather than accentuate the observed difference.

In the PO mouse, parasite numbers increase by a factor of 92.4 from day 1 to day 15 of the infection (Bradley and Kirkley, 1977). This gives a generation time of 2.14 days, and using the observed mean labelling index of 13.0% gives the duration of the S phase as around 0.3 days. If we assume an identical duration of S in the resistant Ash mouse with a mean labelling index of 2.1%, a

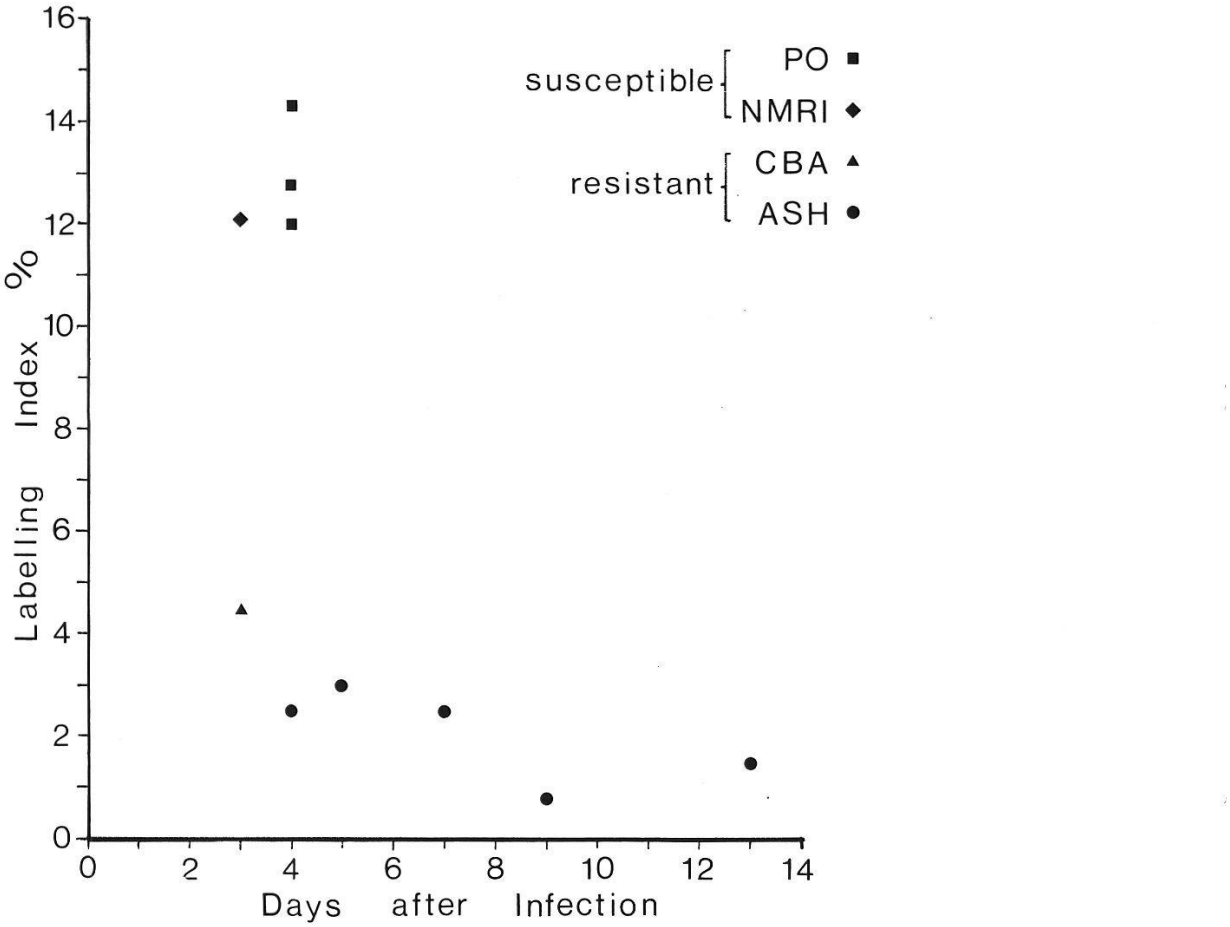

Fig. 1. Labelling indices of *Leishmania donovani* following tritiated thymidine labelling in four mouse strains at varying intervals after infection.

doubling time of 13.5 days is reached. This may be compared with the observed parasite population increase by a factor of 1.5 in the 14 days. Given the uncertainties of each estimate, the difference between resistant and susceptible strains can be explained on the basis of the differential proliferation of the parasites in the two hosts, though some differential parasite destruction cannot be excluded.

Experiment 2: Parasite proliferation in acute and chronic infections

The total parasite population of the PO mouse liver initially rises exponentially after intravenous infection with *L. donovani,* but after two months the rise in total parasites proceeds much more slowly. This could either be due to parasite destruction while the initial rate of multiplication is maintained, or it could result from a fall in the rate of parasite proliferation. In the latter case the parasites' labelling index will be expected to fall in chronic infections, while in the former it will remain at its initial high level. This experiment was therefore to compare the labelling indices in acute and chronic infections.

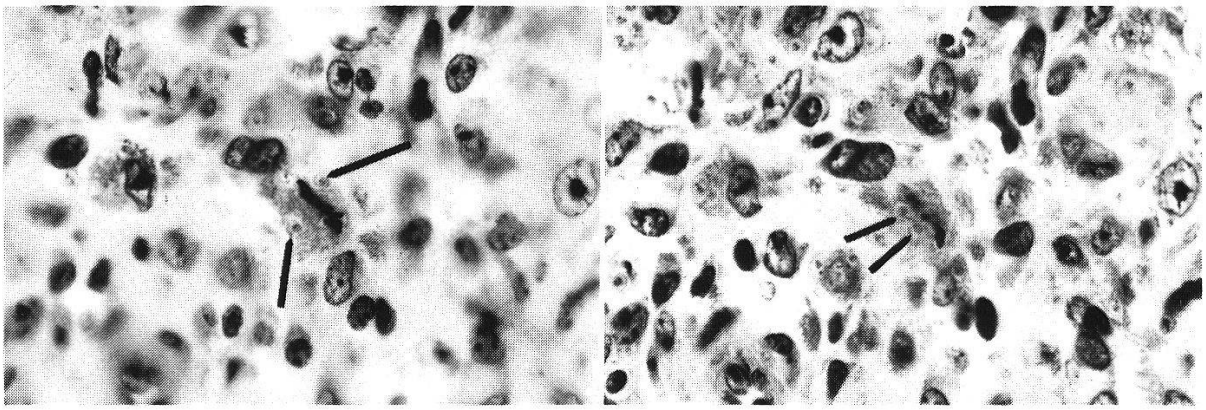

Fig. 2. Contiguous sections of chronic leishmanial granulomas of the PO mouse liver to show mononuclear phagocyte in mitosis and parasites (indicated) on either side of metaphase plate.

PO strain mice were infected with *L. donovani* and 4 mice killed 1 h after labelling on day 6 after infection and another 4 on day 70. Parasite labelling indices were compared between the groups.

On day 6 a mean of 8.25%, with a standard error of ± 0.78, parasites incorporated tritiated thymidine as compared with 2.75% ± 0.39 on day 70, a highly significant reduction. The labelling index in acute infections did not vary inversely with the number of parasites per cell. There was no evidence of a fall-off in labelling levels among very crowded parasites where many infected a single macrophage and on imprints showed as a dense cluster around a single mouse nucleus.

These results indicate that either the parasite proliferation rate is greatly reduced in chronic infections or the duration of the S phase is specifically reduced. The latter is biologically unlikely. An alternative explanation in terms of there being more heavily infected macrophages in which the thymidine does not reach all the parasites in chronic infections also appears inconsistent with the observations on heavily infected cells in acute infections.

It therefore appears that the decreased growth of the total population of parasites in the PO mouse over time is at least partly due to inhibition of parasite proliferation.

Experiment 3: The relation of host cell and parasite proliferation

This experiment was to determine, in qualitative terms, whether parasitised cells can pass into the DNA-synthesis phase, whether this bears any relation to parasite DNA synthesis, and whether the parasitised cells ever go on to divide.

PO strain mice infected with *L. donovani* were killed on the 6th, 30th and 70th day after infection, 1 h after labelling. Imprint preparations and cytocentrifuge preparations of extracted hepatic and splenic mononuclear phagocytes were examined by autoradiography.

Table 1. Attrition of labelled parasitised and unparasitised mononuclear phagocytes from the livers of chronically infected PO mice

Mouse	Day after labelling	Blood monos % label.	Kupffer cells			
			% parasitised	% labelled	% label of unparasit.	% label of parasit.
A	−3	0	17.6	0	0	0
B	−2	2.5	17.0	2.4	2.4	2.4
C	+5	2.0	30.2	4.2	4.6	3.3
D	+10	3.5	26.4	2.0	1.9	2.3
E	+24	0	48.2	2.6	2.7	2.5
F	+31	0	8.8	1.4	1.1	4.5

Tritiated thymidine given on days –2, –1, 0.
Mouse B killed 1 h after first labelling injection.

In both acute and chronic infections it was possible to find labelled parasites, and parasitised cells with labelled nuclei, in both liver and spleen. Occasionally a mononuclear phagocyte in the S phase contained labelled parasites but there was no evidence of synchronicity between the parasite and host cell DNA synthesis phases. Occasionally parasitised cells could be seen in mitosis, and Fig. 2 shows amastigotes lying to each side of chromosomes in metaphase. There is therefore both direct and indirect evidence that division of parasitised macrophages occurs.

Experiment 4: Survival of parasitised cells in chronic infections

Chronically infected PO mice had their proliferating macrophages labelled by tritiated thymidine given on three successive days. Then, after a variable number of days they were killed and mononuclear phagocytes extracted from the liver. The numbers of parasitised and unparasitised, labelled and unlabelled cells were determined, Table 1. Had there been selective destruction of parasitised cells one would have expected selective reduction in the cohort of labelled parasitised cells with time relative to the labelled unparasitised cells. There was no evidence of this selective destruction. While the numbers of mice are limited and the extraction method of low efficiency, the experiment gave no evidence, in these chronically infected PO mice, of the operation of a cytotoxic mechanism of the type described by Bray and Bryceson (1968) in *L. enriettii* in guinea-pigs.

Experiment 5: Comparative observations on labelling in vitro and in vivo

In view of the small amount of DNA in the amastigote of *L. donovani*, autoradiography requires either heavy labelling or very long exposures. In the

latter case separation of labelling from background artefact is a real problem. With the doses of tritiated thymidine suitable for host cell labelling, 0.5 μCi/g, the parasites appear unlabelled, and reliable use of the labelling method for kinetic studies necessitates an in vivo dose of 500 μCi for a typical mouse, so making experiments very expensive.

A comparative study was therefore made of parasites labelled in vivo and those from a comparable acute infection in which the unlabelled host was killed and a small portion of liver ground with tissue culture fluid containing tritiated thymidine and then incubated before making autoradiographs (see Methods section). PO strain mice were used 14 days after infection with *L. donovani.*

The labellling indices were $13.03\% \pm 0.67$ in vivo and $11.93\% \pm 0.77$ in vitro, so that the in vitro method given levels of labelling comparable to the more expensive whole mouse method. These preliminary results suggest that such an approach could be developed, which would be essential for applying labelling methods to human leishmaniasis.

Discussion

These experiments have clarified the role of parasite proliferation in the pathogenesis and course of mouse visceral leishmaniasis. In the early stages of infection in a susceptible mouse strain the labelling index is high and it remains so at least between the 3rd and 14th day after infection.

In innately resistant mice, in which parasite populations do not increase more than about five-fold, the labelling index is much lower (experiment 1), even as early as the 3rd day, suggesting that parasite destruction need not be invoked to explain the strain differences.

The divergence of resistant and susceptible strains as early as the 3rd day after inoculation is indirect evidence against an acquired immune response being responsible for the difference as this is early for so marked a resistance effect to be developed. It is consistent with our unpublished observations that thymus deprivation leaves acute resistance unmodified.

In the course of infection in susceptible PO mice there is again a fall in labelling as the parasite population stabilises (experiment 2). Because of the limited extent of immunity acquired by this strain it would be wrong to generalise to other mice. Nevertheless, this result, along with the parasite kinetic data above, suggests that, in the PO mouse liver, turnover of parasites and cells is limited and parasite reproduction is not of overwhelming significance.

The mechanism by which the mononuclear phagocytes achieve this inhibition of parasite growth is not known, though evidence from our work with other mouse strains suggests a role for acquired immunity as T cell deprivation is associated with continued proliferation of *Leishmania.*

The persistence of parasites in some strains which mount an effective immune response could be partly explained by low grade proliferation within

dividing host cells and experiment 3 shows that this does occur, while experiment 4 does not provide any evidence of selective killing of parasitised liver macrophages.

Labelling approaches are thus able to illuminate the dynamics of the *Leishmania* population and point to key areas for immunological analysis.

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