THE PRESENCE OF SYSTEMIC AND LOCAL ACTIVATED MACROPHAGES IN MICE INFECTED IN THE FOOT PAD WITH MYCOBACTERIUM LEPRAE AND M. MARINUM

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The stationary phase of growth of Mycobacterium leprae in the foot pad of the immunocompetent mouse occurs 3-5 months after injection of a small number of organisms. The growth of the leprosy bacillus plateaus at approximately 10^6 organisms and multiplication essentially ceases (1). That an immune response underlies this cessation of growth and the subsequent death of the leprosy bacillus is suggested by the observation that, concurrent with the establishment of the stationary phase in the foot pad of an immunocompetent mouse, the opposite foot pad becomes resistant to challenge (2).

That this immune response is cell-mediated rather than humoral is suggested by the following evidence: 1) Histopathologic observation of the leprosy infected foot pad during the stationary phase of growth is characterized by a mononuclear cell (lymphocytes and monocytes) infiltrate (3); and 2) Growth occurs beyond 10^6 bacilli per foot pad in T-cell depleted mice (4). The present as well as previous studies from our laboratories are concerned with a further exploration of cell-mediated immunity to M. leprae -- especially the role of monocytes or macrophages.

Previous reports from our laboratory (5, 6) have revealed that mice chronically infected with the obligate intracellular protozoa Toxoplasma gondii or Besnoitia jellisoni are nonspecifically resistant to a variety of phylogenetically unrelated intracellular pathogens, such as Listeria monocytogens, Salmonella typhimurium, Brucella melitensis, Cryptococcus neoformans and Mengo virus. Among these organisms which lend themselves to in vitro study, the effector of this nonspecific resistance appears to be the enhanced microbicidal capacity of macrophages which have become activated as a result of the protozoal infection (7). Antigen persists for life in these protozoal infections in the form of living organisms in virtually all of the tissues. The process of activation of macrophages appears to result from the immunologically specific interaction of antigen (protozoal in the above examples) with T-lymphocytes. Macrophages then appear to become activated by a soluble T-lymphocyte produced lymphokine (8).

Nonspecific Resistance to M. leprae. In previous studies we have reported that mice chronically infected with Toxoplasma or Besnoitia were markedly resistant to foot pad infection with M. leprae. Moreover, a booster injection of Toxoplasma antigen administered into the infected foot pad further enhanced resistance to M. leprae (9). We interpreted these results as nonspecific resistance to M. leprae, indirectly suggesting that activated macrophages from these protozoal-infected mice are capable of killing or inhibiting the growth of M. leprae in the foot pads of infected mice. However, conversely we found no evidence that foot pad infection with M. leprae provided any nonspecific resistance to a test organism, Listeria monocytogenes (10). These studies included the in vitro Listeria challenge of peritoneal macrophage monolayers prepared from M. leprae infected mice -- no detectable resistance was found. We have recently reinvestigated the presence of systemic activated macrophages in M. leprae infected mice by employing the highly sensitive in vitro assay described in Table 1 (11). The results of such an experiment are shown in Fig. 1. Peritoneal macrophage monolayers were prepared from normal BALB/c mice and mice infected in the left rear foot pad with M. leprae 90, 120, 130, 150 and 185 days previously. As additional controls, monolayers of activated macrophages from Toxoplasma-infected and Corynebacterium parvum-stimulated BALB/c mice were employed. All cultures were challenged with EMT-6 adenosarcoma tumor cells (syngeneic for BALB/c) and pulsed with ^{3}H-TdR. Whereas these activated macrophages inhibited the DNA synthesis of the target cells by 99.99%, there was no significant inhibition of target cell DNA synthesis in the presence of any of the macrophage monolayers from M. leprae infected mice. This may be due, at least in part, to the localized nature of M. leprae foot pad infection.

Nonspecific resistance to M. marinum. Because foot pad infection with M. marinum might provide information useful in understanding M. leprae infection, we had previously studied nonspecific resistance in this model (12). In contrast to the ability of Besnoitia infection to induce resistance to M. leprae there was no such nonspecific resistance to the growth of M. marinum in the foot pad (13). However, foot pad infection with M. marinum did induce significant nonspecific resistance to systemic challenge with Listeria (resistance was shown by a lower number of viable Listeria in the spleen 24 and 48 hr after I.V. challenge). This nonspecific resistance was transient, being lost by the fourth week after M. marinum infection. Employing the assay depicted in Table 1 we have recently studied whether foot pad infection with M. marinum activates the macrophages of the peritoneal cavity, As seen in Fig. 2, peritoneal macrophages from mice infected with M. marinum for 5, 12, 16 or 22 days, do not inhibit ^{3}H-TdR uptake by target cells and therefore do not appear to be activated.

Table 2 summarizes our experience and that of others with nonspecific resistance directed to, or induced by, M. leprae and M. marinum. Some of these data appear to be contradictory -- mice infected in the foot pad with M. marinum are resistant to Listeria (as measured in their spleen) but do not possess activated peritoneal macrophages. This apparent paradox might be partially due to distribution of M. marinum antigen in the foot pad-infected mouse. Mackaness has shown that whereas a single relatively low I.V. dose of

Table 1. Assay for macrophage-effected inhibition of DNA synthesis in target cells

Time	Procedure
-5 hr	Harvest peritoneal cells (PC) from appropriate groups of mice.
-4 hr	Plate PC and incubate.
-1/2 hr	Remove nonadherent PC (lymphocytes) by washing.
0 hr (i)	Challenge macrophage monolayers with tumor target cells.
(ii)	Pulse half of cultures with ^{3}H-TdR
+6 hr	Remove extracellular ^{3}H-TdR by washing and freeze.
+18 hr	Pulse remaining cultures with ^{3}H-TdR
+24 hr	Remove extracellular ^{3}H-TdR by washing and freeze.
(later)	Extract DNA from cultures and measure amount of ^{3}H-TdR incorporated by the target cells by liquid scintillation counting.

Table 2. Summary of studies of nonspecific resistance to or induced by *M. leprae* or *M. marinum*.

Mice infected with	Assayed for nonspecific resistance in vivo against			Assayed in vitro for activated peritoneal macrophages
	M. leprae	*M. marinum*	Listeria	
Toxoplasma	+	n.d.	+	+
Besnoitia	+	-	+	+
M. leprae	+	+	-	-
M. marinum	+	+	+	-

\+ = nonspecifically resistant

\- = not resistant

n.d.= not done

Figure 1. The effect of peritoneal macrophages from M. leprae-infected mice on ^{3}H-TdR uptake by tumor target cells. Macrophages (2×10^{6}) were cultured from normal mice (○), mice infected with M. leprae for varying periods (●), mice treated 1 week previously with C. parvum (△) or infected 1 month previously with T. gondii (▲). ^{3}H-TdR uptake by 1×10^{5} EMT-6 target cells was measured during two 6-hr pulse intervals at 0 and 18 hr after challenge. (■) = ^{3}H-TdR uptake by target cells alone.

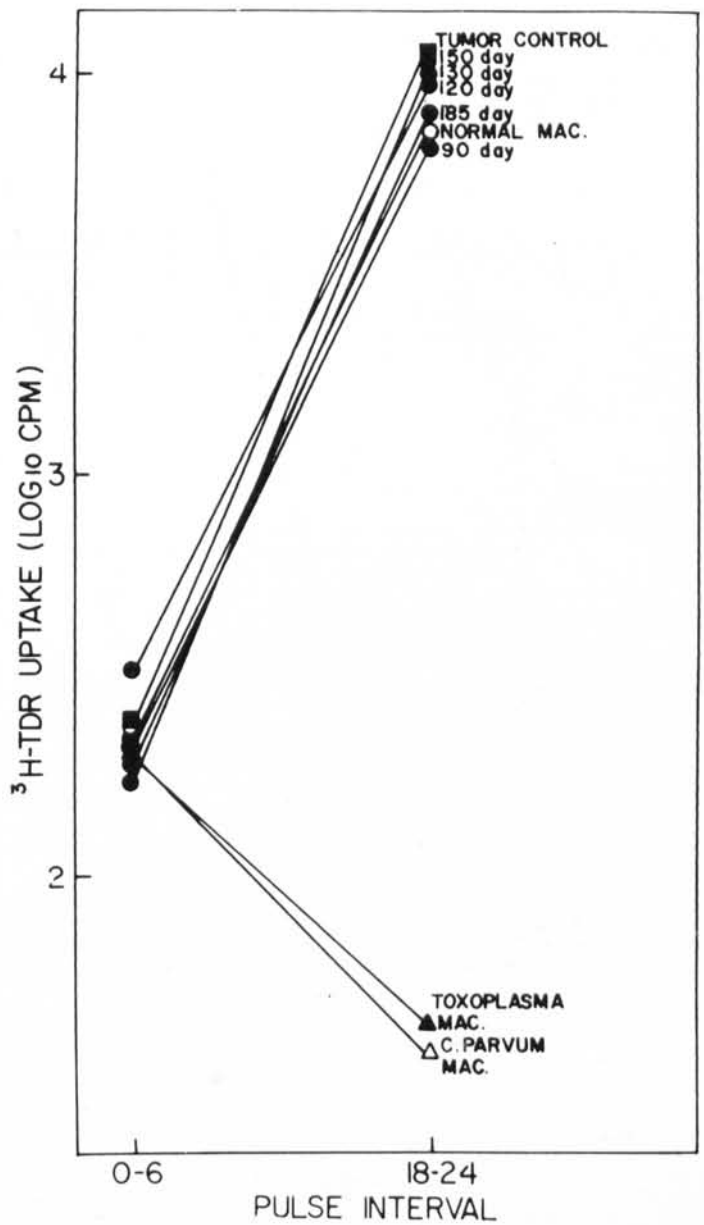
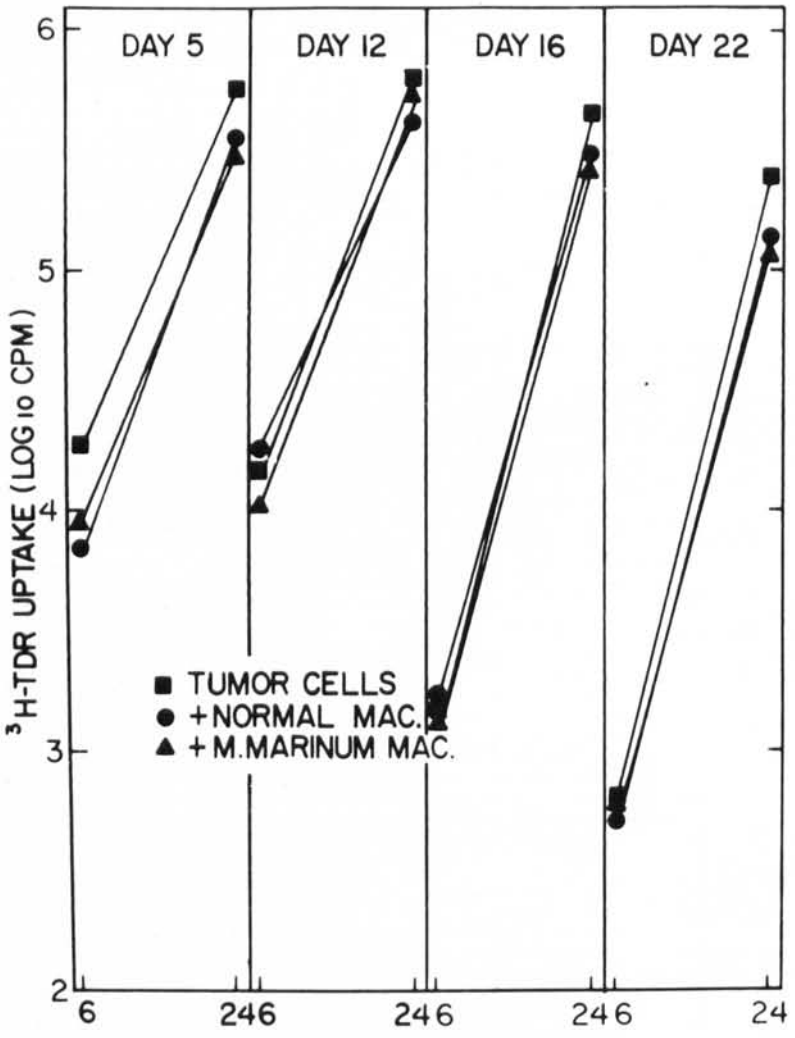


Figure 2. The effect of peritoneal macrophages from M. marinum-infected mice on ^{3}H-TdR uptake by tumor target cells. Macrophages from normal (●) and M. marinum-infected (▲) mice were challenged with EMT-6 target cells at varying days after infection. ^{3}H-TdR uptake was determined during 6-hr pulse intervals at 0 and 18 hr after challenge. (■) = ^{3}H-TdR uptake by target cells alone.



BCG activates the macrophages of the spleen and liver (to become non-specifically resistant to Salmonella typhimurium) a much higher dose (10-100X) is required to activate the macrophages of the peritoneal cavity (14). Foot pad infection with M. marinum is not solely a local infection; viable organisms are recoverable from the spleen and liver (Levy, unpublished). As mentioned above, the activation of macrophages requires the persistence of antigen. Thus, the presence of M. marinum in the spleen and its absence from the peritoneal cavity might explain the presence of (Listericidal) activated macrophages in the spleen and their absence in the peritoneal cavity. These results further emphasize our need to understand more clearly, local cell mediated immunity.

Presence of local activated macrophages in the M. leprae-infected foot pad. An attempt to determine whether activated macrophages could be demonstrated in the M. leprae-infected foot pad tissues was made. The procedures employed the mincing of infected foot pad tissues and the preparation and plating of the resulting cell suspensions in the wells of microtiter plates. Four hours later nonadherent cells were removed by washing, and EMT-6 target cells were added. As in the technique described in Table 1, 6 hr pulse intervals with ^{3}H-TdR were employed. The results of 8 such experiments were inconclusive. The results shown in Figs. 3, 4, and 5 summarize 3 typical experiments in which ^{3}H-TdR uptake by target cells cultured alone (controls) or with foot pad macrophages is expressed as the mean cpm during the 0-6 and 18-24 hr pulse intervals.

In Fig. 3, cells were harvested from the foot pads of M. leprae infected mice 3 days after infection. In comparison to target cell controls, these results suggest that these foot pad cells effected no significant inhibition of ^{3}H-TdR uptake ($p < .4$). In the results shown in Fig. 4, foot pad cells were cultured from 3 groups of M. leprae infected mice: Group A = mice 108 days past 10^{6} bacilli per foot pad (plateau), Group B = 66 days past plateau, Group C = 12 days past plateau. Although all 3 groups of cultures differed significantly from the controls (A, B $p < .001$; C $p < .01$) the greatest level of inhibition of ^{3}H-TdR uptake was less than 10-fold (71%). The data shown in Fig. 5 represents the effects of normal mouse foot pad cells on ^{3}H-TdR uptake by target cells. The results show that macrophages (or adherent foot pad cells) caused a significant (70%, $p < .001$) inhibition of ^{3}H-TdR uptake, suggesting that the inhibition of ^{3}H-TdR uptake observed with foot pad macrophages from M. leprae infected mice (Fig. 4) should probably be attributed to an inhibitory material or substance associated with merely placing the foot pad material into the culture wells.

These results do not disprove the presence of activated macrophages in the foot pads of M. leprae infected mice. Microscopically, mononuclear cells capable of phagocytizing heat-killed Candida albicans or saccharated iron (i.e. macrophages) were cultured from these infected foot pads. However, their number and density would appear to preclude the use of the ^{3}H-TdR inhibition assay in futher attempts to determine if these foot pad macrophages are activated.

Figure 3. Effects of adherent cells from M. leprae-infected mouse footpads on ^{3}H-TdR uptake by target cells. A = group infected 3 days previously with M. leprae. (----) = target cells only.

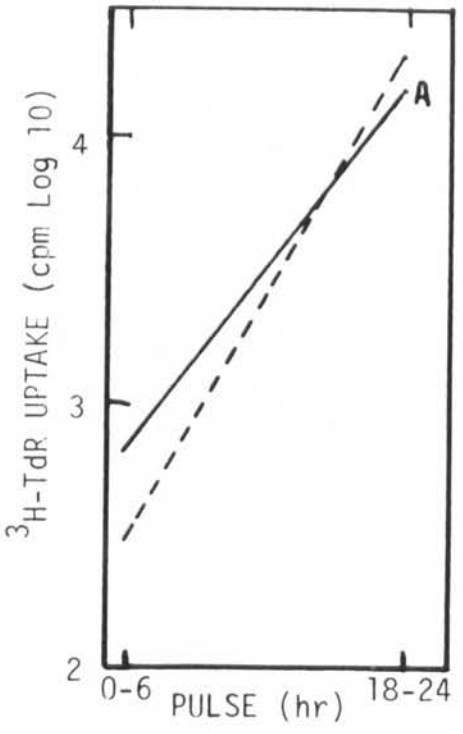


Figure 4. Effects of adherent cells from M. leprae-infected mouse footpads on ^{3}H-TdR uptake by target cells. A = mice 108 days past 10^{6} bacilli per footpad (plateau). B = 66 days past plateau. C = 12 days past plateau. (----) = target cells alone.

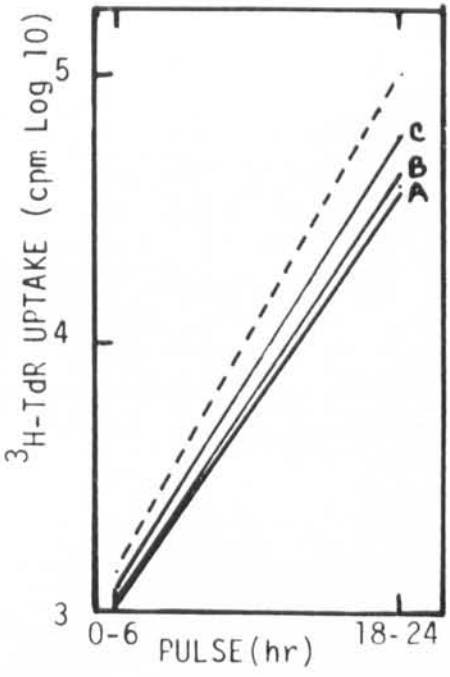
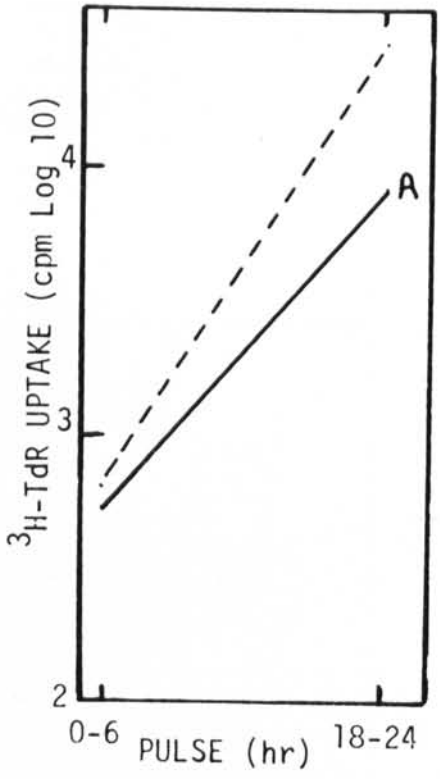


Figure 5. Effects of adherent cells from normal mouse footpads on ^{3}H-TdR uptake by target cells. A = normal mice. (----) = target cells alone.



SUMMARY

A highly sensitive assay for quantitating the presence of activated macrophages was employed to show that foot pad infection of mice with neither M. leprae nor M. marinum induced a population of activated peritoneal macrophages. In vitro attempts to demonstrate local activated macrophages in the foot pads of M. leprae infected mice failed, but, because of the technical problems encountered, do not preclude their presence. Additional studies are being carried out.

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