

PRELIMINARY STUDIES WITH THE LYMPH NODE LYMPHOCYTES
OF M. leprae INFECTED MICE

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Unsuccessful attempts to transfer protective immunity with splenic lymphocytes of mice infected in the foot pad with either M. marinum or M. leprae suggested that either splenic lymphocytes were incapable of transferring protective immunity or that we had not obtained the lymphocytes at the correct time during the infection to effect passive transfer(1). In an attempt to understand these results, we measured the response of these splenic lymphocytes to M. marinum and M. leprae antigens. Whole cell antigens of M. leprae from armadillo elicited only weak stimulation of splenic lymphocytes from M. leprae-infected mice, but bacillary antigens of M. marinum elicited significant stimulation. This stimulation was detected just after growth of M. leprae had reached plateau, and continued for several weeks. At around 150 days after infection, the lymphocyte stimulation ratio (LSR) returned to less than 2(2), the level generally accepted as significant. These findings suggested that killing of M. leprae was initiated by an antigen-specific cell-mediated immune response of the mouse. Our studies of splenic lymphocytes from M. marinum-infected mice yielded similar results, although LSR's were generally higher.

Because of our failure to transfer protection with splenic lymphocytes, it seemed reasonable to look for another source of antigen-reactive lymphocytes for these experiments. By analogy, for example, with the localized lesion of cutaneous leishmaniasis, in which the draining lymph node is the only detectable source of antigen-reactive lymphocytes until after the primary lesions have healed(3), the popliteal nodes appeared to represent a better source of reactive lymphocytes. Therefore, we have examined the popliteal lymph nodes of infected mice, measuring their weight and cellularity and the properties of lymphocytes obtained from these nodes at various times after infection as indicated by lymphocyte stimulation by several mycobacterial antigens.

Because we had found that M. leprae antigens did not elicit significantly elevated LSR's with lymphocytes from the spleens of M. leprae-infected mice, we began with a study of lymphocytes from the popliteal lymph nodes of M. marinum-infected mice to insure that we could detect antigen reactive cells. In a second set of experiments, the expressible lymphocytes of the popliteal lymph nodes of M. leprae-infected mice were examined. In addition to whole bacilli antigens we used cell extracts from M. marinum and M. leprae prepared by Dr. Ram Navalkar(4).

At various times after infection of BALB/c mice with M. leprae or M. marinum, the popliteal lymph nodes were removed and weighed. The expressible lymphocytes were combed from the nodes and spleens of these animals.

Splenic lymphocytes and aliquots of the lymph node lymphocytes were passed over nylon fiber according to the method of Julius et al(5). Lymph node lymphocytes were also cultured without column separation. All cultures were stimulated with PHA to determine the ability of the cells to respond; LPS was used in some experiments. Cells were counted, viability was determined by trypan blue exclusion, and the lymphocytes were cultured at concentrations of $2-5 \times 10^5$ lymphocytes per well in Linbro microtiter tissue culture plates with RPMI 1640 tissue culture medium containing 5% fetal calf serum. After 48 hours, the cultures were pulsed with $1 \mu\text{Ci}$ of tritiated thymidine, reincubated for 16 hours, and then terminated with an automatic cell harvester. Incorporation of isotope was measured in a Packard Tri-Carb liquid scintillation counter.

As shown in Figure 1, the lymph nodes of infected animals were greatly increased in size and weight and the expressible lymphocytes from these nodes had doubled in number as early as 3 days after infection with M. marinum. In contrast, the lymph nodes of M. leprae-infected mice did not increase in weight until after about 100 days of infection. The numbers of expressible lymphocytes are proportional to the lymph node weight; as the weight increases, so do the numbers of lymphocytes.

The results of studies of lymphocytes from M. marinum-infected mice are shown by the histogram in Figure 2. In each case, the solid bars represent the responses to whole-cell M. marinum antigens and the hatched bars the responses to M. marinum cell extract. Antigen concentrations are placed in decreasing order from left to right:

<u>M. marinum</u>	4×10^6 per well
<u>M. marinum</u>	4×10^5
<u>M. marinum</u>	4×10^4
<u>M. marinum</u> cell extract(CE)	1:100, 0.1 ml per well
<u>M. marinum</u> CE	1:1000
<u>M. marinum</u> CE	1:10,000

As early as 3 days after infection, LSR's approach significance, and by 7 days after infection LSR's are significantly raised and remain elevated for as long as 7 weeks after infection. Remember that the pathological process reaches its peak about 2 weeks after infection, and subsides spontaneously thereafter. It has also been shown that mice are protected against reinfection as early as 3 to 5 days after the primary infection(6).

Important differences between the responses to whole-cell antigens and those to cell extracts can also be noted. This is particularly apparent in the splenic lymphocytes at 7 to 21 days after infection and in the lymph node lymphocytes at 21 and 35 days. Perhaps this is a reflection of different sub-populations of lymphocytes, some reacting to one antigen and

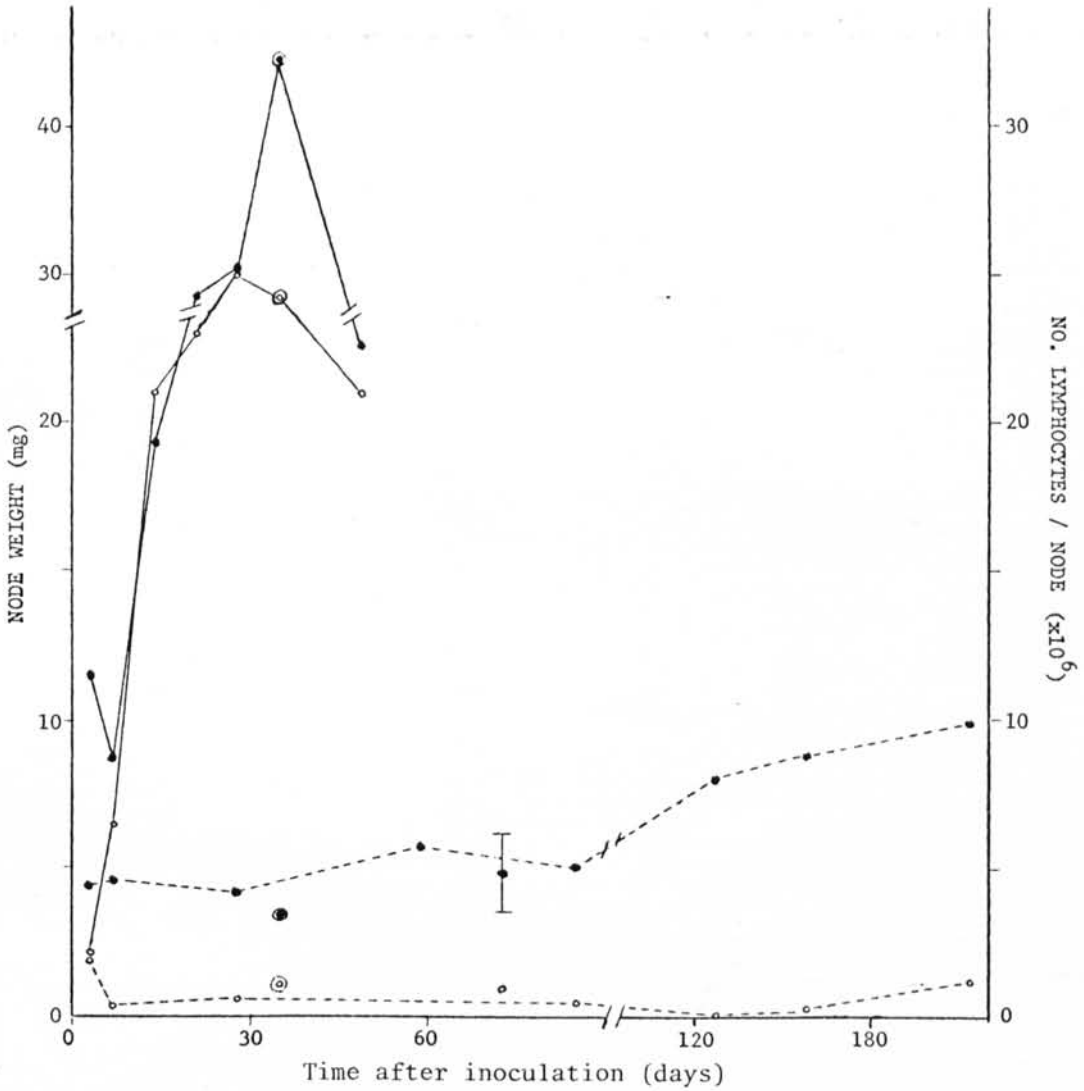


FIG. 1. Changes in popliteal lymph nodes during foot pad infections. Solid lines = *M. marinum* infections; broken lines = *M. leprae* infections; closed circles = lymph node weights; open circles = lymphocytes/node.

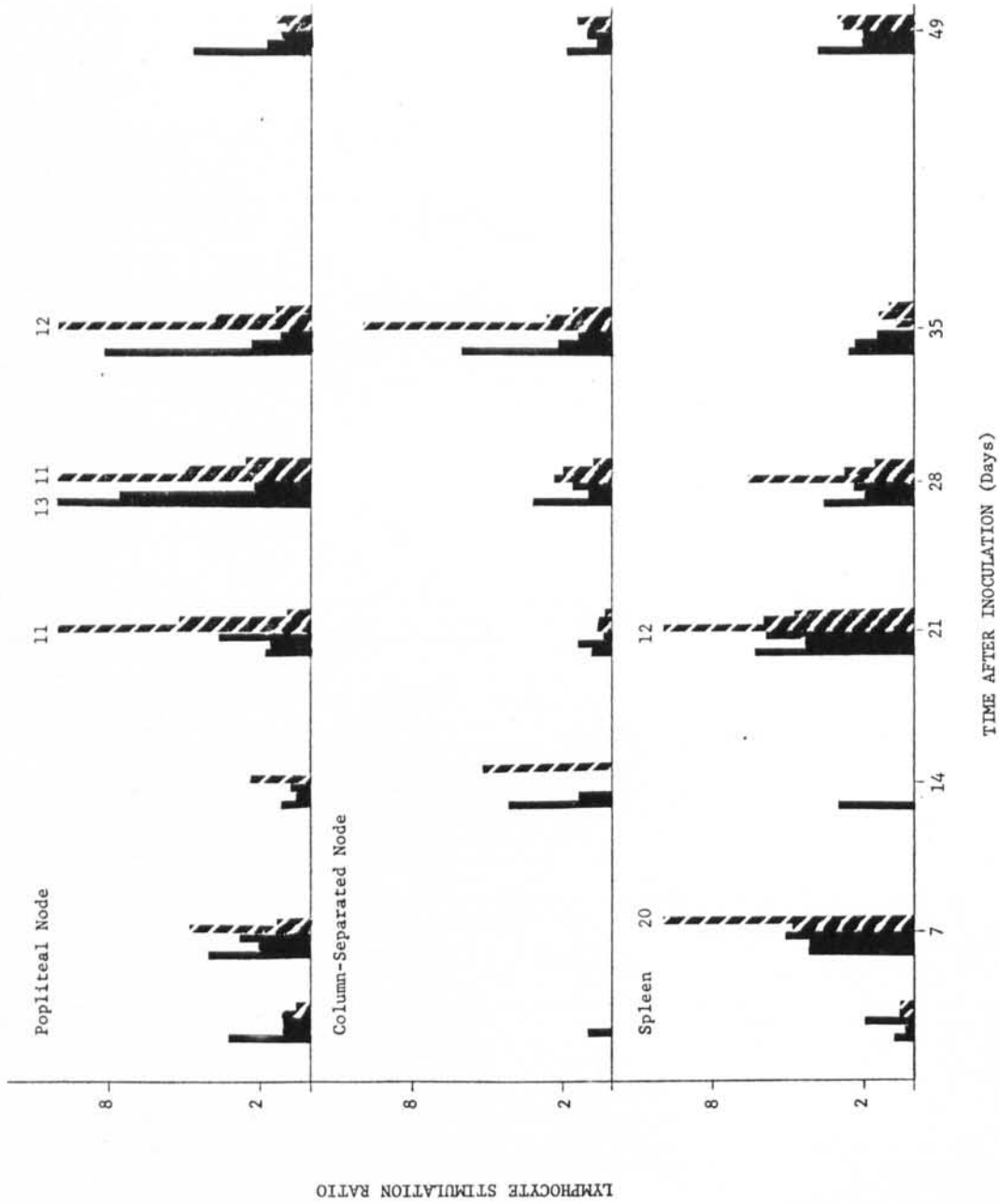


FIG. 2. Lymphocyte stimulation ratios in M. marinum-infected mice.
(See text for legend)

some to another. The column-separated lymph node lymphocytes differ in responsiveness from the unseparated populations. This is particularly evident at 21 and 28 days. Perhaps we are seeing the result of non-specific stimulation of B cells in the unseparated populations. PPD is known to be a non-specific B cell mitogen in mice(7); perhaps some structural antigens also act in this manner. Alternatively, this may represent specific B-cell activation. Studies of specifically depleted B and T cell populations with LPS, PHA and antigenic fractions may clarify this point.

The results of incomplete studies of lymphocytes from M. leprae-infected mice are shown in Figure 3. In each case, the hatched bar on the left represents the response to 4×10^5 M. marinum; the two solid bars represent the responses to 4×10^5 M. leprae on the left and 4×10^4 M. leprae on the right. The hatched bars on the right depict the responses to M. leprae cell extract; the bar on the left represents the largest concentration -- 1:100, the middle bar 1:1000, and the right-hand bar the smallest concentration -- 1:10,000. These results show that, soon after inoculation, lymph node lymphocytes enriched with T cells by column treatment respond to M. leprae bacillary antigens but show little response to cell extracts. Unseparated lymphocytes react to neither antigen. Splenic lymphocytes also respond to bacillary antigens at this time but in addition they respond more vigorously to cell extracts. Sixty days after inoculation, LSR's from infected mice are not different from those obtained from uninfected mice. By 85 days, however, the responses to M. marinum bacillary antigens and M. leprae cell extracts have reappeared in the splenic but not in the lymph node lymphocytes.

These very early data suggest that we may be dealing with two subpopulations of T cells, one responsive to whole-cell M. leprae antigens and the other to M. leprae cell extract. It is interesting that the response to M. marinum resembles more closely the response to M. leprae cell extract than that to M. leprae bacillary antigens.

These studies have progressed slowly because the evolution of M. leprae infection in mice is slow. In order to obtain information later in the infection, we have studied mice inoculated with M. leprae for other experiments. The results of these studies are shown in Table 1.

LSR's to M. leprae bacillary antigens seem elevated only at 106 and 143 days after infection. The lymphocytes respond to cell extracts at these times as well. Perhaps the elevated LSR's reflect the production of antigen-reactive lymphocytes that "home" to the lesions and activate macrophages so that killing and clearing of M. leprae can be accomplished. Alternatively, the elevated LSR's may represent responses to M. leprae already killed in the foot pad, the clearing of which allows new pulses of antigen to drain into the popliteal node.

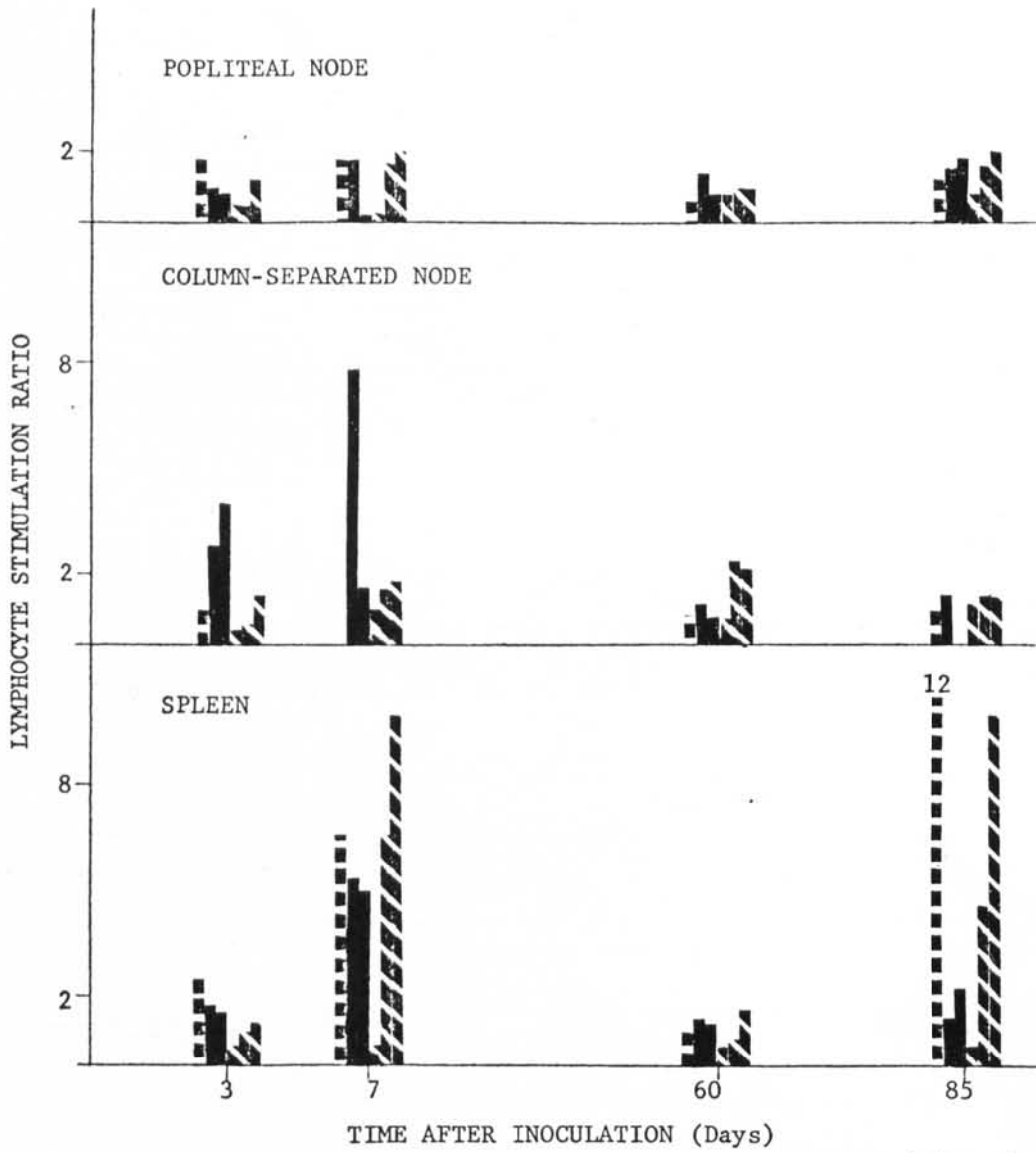


FIG. 3. Lymphocyte stimulation ratios in *M. leprae*-infected mice. (See text for legend)

In conclusion, we have begun studies *in vitro* of the response of *M. marinum*- and *M. leprae*-infected mice to various mycobacterial antigens. Populations of lymphocytes reactive to different mycobacterial antigens have been found. These reactive cells can be demonstrated at different times and in different sites during the infection. Further studies are needed to understand the complex interactions of these lymphocyte populations during the immune responses of mice to *M. marinum* and *M. leprae*.

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TABLE 1. Response of Popliteal Node Lymphocytes from *M. leprae*-Infected Mice,

Antigens	Lymphocyte Stimulation Ratio									
	Time After Inoculation (Days)									
	78	92	106	130	143	156	186	214	230	255
<i>M. leprae</i> 4 x 10 ⁵	1.7	1.1	4.0	1.2	3.6	2.4	0.8	6.0	1.6	5.8
4 x 10 ⁴	1.9	1.9	3.7	0.8	1.1	0.6	0.5	-	1.8	-
<i>M. leprae</i> CE 1:100	0.8	0.9	1.9	0.7	1.2	0.3	0.4	-	0.7	-
1:1000	1.5	1.2	2.2	1.5	15	0.5	0.3	-	2.2	-
1:10,000	2.0	1.2	3.0	1.1	7.6	1.8	0.2	-	3.1	-
<i>M. marinum</i> 4 x 10 ⁵	1.1	1.0	1.8	1.0	1.3	1.2	0.6	-	2.6	-