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Protective Immunity and Delayed-Type Hypersensitivity in C57BL Mice After Immunization with Live *Mycobacterium lepraemurium* and Sonicated Bacilli

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The immunizing effects of live *Mycobacterium lepraemurium* (MLM) and bacillary sonic extract (MLMSon) were compared in C57BL mice. MLMSon-immunized mice developed a delayed-type hypersensitivity (DTH) reaction when tested in the footpad with diluted MLMSon. The ability to develop a DTH reaction was transferable with immune cells but not with serum. Footpad testing with live MLM and MLMSon indicated that the specificity of the DTH response induced by MLMSon was different from that induced by infection with live bacilli. Two weeks after footpad inoculation with live MLM, MLMSon-immunized mice developed a strong local reaction to the bacilli. No local reaction developed in these mice after injection of the same number of heat-killed MLM. Studies of bacillary growth after inoculation with live MLM indicated that the bacilli did not multiply in mice previously inoculated with live MLM, but that they multiplied for about 2 weeks in MLMSon-immunized mice versus 4 weeks in the controls. The results suggest that immunization with MLMSon does not by itself induce a protective immune response, but creates a state in which the development of protective immunity is accelerated.

There is at present no completely satisfactory experimental model in which to study immunity to *Mycobacterium leprae*, but *M. lepraemurium* (MLM) infection in mice creates a model for the study of the immune response to another slowly progressing, intracellular mycobacterial infection. Mice differ in their susceptibility to MLM infection, and the manifestations of the disease may to a certain degree mimic the clinical spectrum of human leprosy. Inbred strains of mice exhibit markedly different levels of resistance, C3H/Tif mice being highly susceptible and C57BL/6 mice being relatively resistant (3, 6).

About 4 weeks after subcutaneous inoculation of live MLM, C57BL/6 mice develop a cell-mediated immune (CMI) reaction which manifests itself as a granulomatous reaction at the inoculation site (7, 13) and as an accelerated reaction to a challenge inoculum of live MLM (4, 7). As the CMI reaction develops, the bacilli cease to multiply (3) and the mice become capable of completely inhibiting the multiplication of a challenge inoculum of live MLM (4).

Because the C57BL/6 mice have been shown to develop what appears to be a protective immune response to MLM infection, this strain was chosen for the immunization experiments described here. The immunizing effect of a single subcutaneous injection of sonicated MLM (MLMSon) in Freund incomplete adjuvant (FIA) was compared with that of live MLM, with regard to both the induction of delayed-type hypersensitivity (DTH) to MLM antigens and protective immunity to MLM infection.

MATERIALS AND METHODS

Animals. Female mice of the inbred strain C57BL/6 were obtained, as specific-pathogen-free animals, directly from the breeder, Gl.Bomholtgård Ltd., Ry, Denmark. The mice were kept in the laboratory for about 2 weeks and had reached a weight of 14 to 18 g before they were used in experiments.

MLM. The Douglas strain of MLM was maintained by repeated passage in C3H/Tif mice. The bacilli were harvested from the spleens of animals infected intraperitoneally with about 10^9 MLM 16 to 24 weeks previously. The spleens were homogenized in a Ten Broeck all-glass homogenizer (Bellco Glass, Inc., Vineland, N.J.), and purified suspensions of bacilli were prepared by differential centrifugation as previously described (3). For inoculation of mice, bacilli were either used directly or stored on liquid nitrogen. The bacilli were counted by a slide technique (3). The preparations were stained for acid fastness with auramine (9) and counted in a Leitz Ortholux microscope equipped with incident illumination for fluorescence microscopy. Heat-killed bacilli (HK MLM) were prepared by heating a suspension of MLM to 100°C for 30 min.

Preparation of MLMSon. MLM bacilli were purified by differential centrifugation, washed twice in 0.9% NaCl, and finally resuspended in 0.9% NaCl at a concentration of 4×10^{10} bacilli/ml. The suspension was sonified for 15 min at a measured effect of 100 W, using a Branson B12 Sonifier (Branson Sonic Power

Co., Danbury, Conn.). Insoluble material was removed by centrifugation at 20,000 × *g* for 20 min. The protein concentration of the supernatant, as determined by the modified Folin-Ciocalteu method (10), was 0.8 mg/ml. The same batch of antigen was used for all subsequent DTH tests. The antigen was stored undiluted and in dilutions of 1:10 and 1:100 at −20°C. Several batches of MLMSon, prepared in the same manner, were used to immunize the mice.

Immunization. Immunization with live MLM was given as a single subcutaneous injection of 5×10^7 MLM in 50 μl of 0.9% NaCl in the right side of the thorax. MLMSon was given in FIA. Equal volumes of MLMSon and FIA were thoroughly mixed and exposed to repeated brief pulses of ultrasound until a thick, stable emulsion was obtained. A single injection of 50 μl of this emulsion was given subcutaneously in the right side of the thorax.

Experimental infection. The multiplication of the bacilli was followed after injection of 10 μl of a suspension containing about 5×10^8 acid-fast bacilli (AFB) per ml in 0.9% NaCl into one of the hind footpads. The injections were made through a 30-gauge needle, and the needle path was sealed immediately afterwards with a plastic spray (Nobecutan, AB Bofors, Nobel-Pharma, Sweden). At various intervals after inoculation, groups of five animals were killed; the infected foot and the popliteal lymph nodes on the infected and contralateral sides were removed. The groups of mice were coded, and the code was not broken until all groups had been counted. The lymph nodes were weighed (Oertling R42, Oertling Ltd., Orpington, Kent, Great Britain). The bacilli were harvested from the footpads and lymph nodes as previously described (3), and the AFB were counted.

Footpad reactions. DTH reactions were measured after injecting 10 μl of MLMSon or bacilli into one of the hind footpads, and the needle path was sealed off immediately afterwards. The thickness of both hind feet was usually measured 4, 24, 48, and 96 h after injection of antigen, using a modified dial gauge caliper (C. E. Johansson AB, Eskilstuna, Sweden). Five successive measurements of each hind foot were recorded, and the swelling was expressed as the difference between the median value on the injected and control sides. In one experiment, antigen was injected into both hind footpads. The swelling was then expressed as the difference in foot thickness before and after injection of antigen.

Cell transfer. Five C57BL mice, immunized 8 weeks previously with MLMSon in FIA, were killed, and their spleens were removed under aseptic precautions. The spleen tissue was distintegrated by pressing it gently against a stainless-steel mesh. Clumps were removed by sedimentation at 1 × *g*, and the cells were washed three times in cold phosphate-buffered saline, pH 7.4 (PBS). More than 95% of the cells were recorded as viable in the trypan blue exclusion test. The cells were suspended at a concentration of 2×10^8 cells per ml in either PBS or PBS containing 8 μg of MLMSon protein per ml. Spleen cells from normal mice were prepared in the same manner. Groups of five normal C57BL mice were injected in the right hind foot with 50 μl of one of the cell suspensions. The cell suspensions were kept on ice until they were injected.

Serum transfer. One group of mice was injected with 50 μl containing 0.4 μg of MLMSon protein in serum collected from C57BL mice 9 weeks after immunization with MLMSon.

Statistical analysis. Wilcoxon's rank sum test for unpaired samples (15) was used to test the significance of differences between groups, and $P = 0.05$ was used as the limit of statistical significance.

RESULTS

C57BL mice were immunized with a single subcutaneous injection of MLMSon in FIA and tested by measuring the footpad reaction after injecting 10 μl of MLMSon containing 0.08 μg of protein into the right hind footpad. Different groups of mice were tested at days 0, 7, 11, 18, 33, and 40 after immunization. A slight increase in the 48-h footpad reaction was observed 11 days after immunization (Fig. 1). The reaction was somewhat stronger in the mice tested at 18 days and then remained at the same level in the groups tested after 33 and 40 days and after 68 days (data not shown), when the experiment was terminated.

The footpad reaction followed a time course typical of a DTH reaction, with a peak 24 to 48 h after injection of the test antigen and a small but significant reaction remaining after 96 h (Fig. 2).

Local transfer experiments were performed by injecting spleen cells from mice immunized with MLMSon or normal donors together with specific antigen into the right hind footpad of normal recipient mice. A significant swelling of the footpad 24 h after transfer was found in the mice

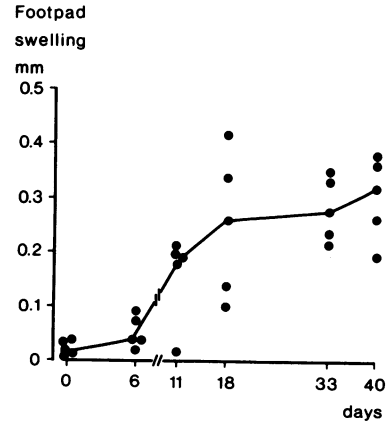

FIG. 1. *C57BL mice immunized with MLMSon in FIA and tested by injecting 10 μl of MLMSon diluted 1:100 into the right hind footpad at various times thereafter. The reaction was measured as the difference in thickness between the right and left foot 48 h after injection of the test antigen. Each point represents the median of five successive measurements of one mouse. Each group of mice was tested only once.*

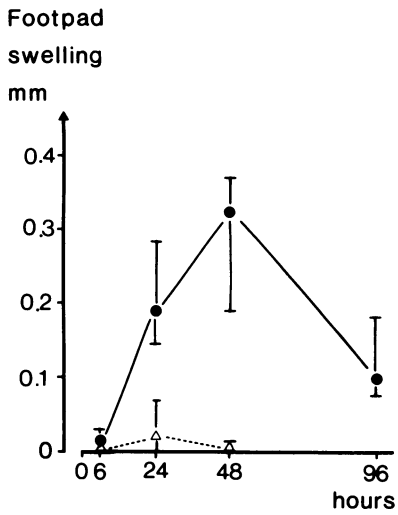

FIG. 2. *Kinetics of the footpad reaction in C57BL mice given 10 μl of MLMSon diluted 1:100 40 days after a single immunizing dose of MLMSon subcutaneously in the thorax. Symbols: ●, immunized mice; △, normal mice. The reaction was measured as the difference in thickness between the injected and the uninjected (hind) foot. Median and range for groups of five mice.*

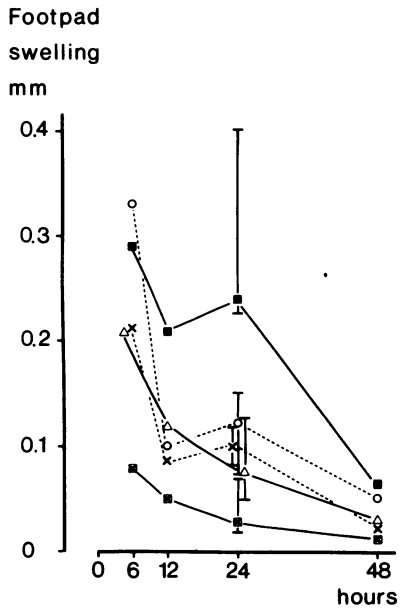

FIG. 3. *Footpad reaction in normal C57BL mice after local transfer of 10^7 syngeneic spleen cells. The mice received either: spleen cells from donors immunized with MLMSon suspended in MLMS (■), spleen cells from normal mice suspended in MLMSon (△), spleen cells from immunized donors suspended in PBS (☒), MLMSon (×), or 50 μl of serum from immunized mice plus MLMSon (○). Each point represents the median of five mice. At 24 h the range is indicated by vertical bars.*

receiving immune cells and antigen. In the mice given immune serum plus antigen, the 6-h reaction was as strong as in the mice given immune cells and antigen, but the reaction 24 and 48 h after transfer was not different from the reaction in the control groups (Fig. 3). Apparently, the immunization with MLMSon had induced a CMI reaction.

The 48-h footpad reaction to various doses of MLMSon and live MLM was tested in mice immunized either with 5×10^7 live MLM or with MLMSon 6 weeks previously. The strongest reaction was found when the mice were tested with the homologous antigen (Fig. 4). MLMSon-immunized mice reacted strongly to MLMSon but only slightly more than normal mice even to a large dose of live MLM (1.25×10^8 bacilli). On the other hand, mice immunized with live MLM had a relatively strong footpad reaction 48 h after injection of a test dose of live MLM, but had only a weak reaction to MLMSon which was not significantly different from that of normal mice. These results indicated that the specificity of the immune response induced by MLMSon was different from that induced during infection with live organisms.

The protective effect of the two immunization procedures was compared by challenging immunized mice with 5×10^6 live MLM in one hind footpad. The host response was monitored by measuring the footpad swelling at regular intervals after inoculation. The reaction appeared earliest in mice immunized with live MLM (Fig. 5). The reaction was relatively weak after 24 h but increased during the following days and reached maximum 12 days after inoculation. In this particular experiment, a second peak in the reaction was seen after 35 days. In the MLMSon-immunized mice, the reaction after 24 h was not significantly different from that in the previous group, but thereafter very little swelling was observed for the next 10 days, until a strong reaction developed about 2 weeks after inoculation. In the normal nonimmunized mice, almost no footpad swelling was observed until between 3 and 4 weeks after inoculation, which was significantly later than in both of the immunized groups.

The popliteal lymph node on the inoculated side was taken out and weighed 1, 3, 5, and 7 weeks after inoculation. One week after inoculation, lymph node enlargement was significantly greater in the group immunized with live MLM than in the other two groups (Fig. 6). Thereafter the nodes of the MLMSon-immunized mice quickly became greatly enlarged and were significantly larger than in the other two groups both 3 and 5 weeks after inoculation. Thus, in the popliteal node as well as in the footpad, the

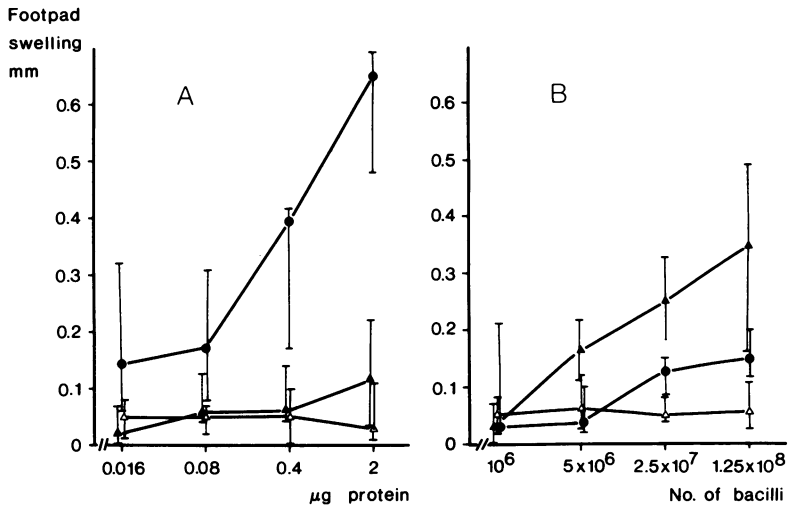

FIG. 4. *Increase in thickness of the hind footpads in C57BL mice 48 h after injection of 10 μl containing varying amounts of (A) MLMSon or (B) live MLM. Symbols: ▲, mice immunized with live MLM (5 × 10^7 AFB); ●, mice immunized with MLMSon; △, normal mice. The median and range of groups of five mice are given at each point.*

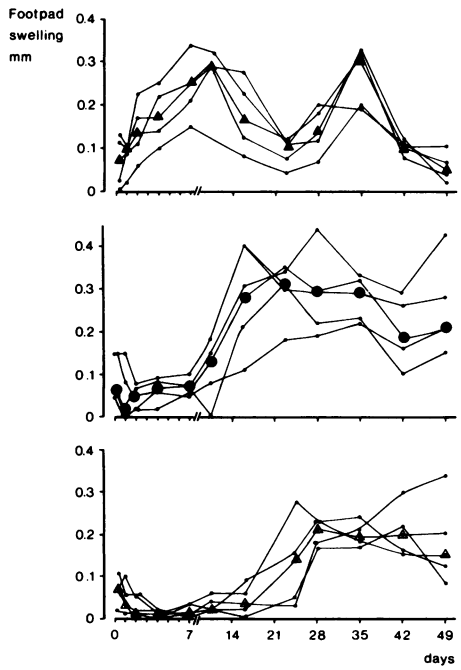

FIG. 5. *Kinetics of the local reaction in response to injecting live MLM (5 × 10^6 AFB) into the right hind footpad of normal or immunized C57BL mice. The mice had been immunized subcutaneously on the thorax 6.5 weeks previously. The reaction was measured as the difference in thickness between the infected and the uninfected foot. Symbols: ▲, mice immunized with live MLM (5 × 10^7 AFB); ●, mice immunized with MLMSon; △, normal mice. The large symbols indicate the median, and the thin lines connect successive measurements in individual mice.*

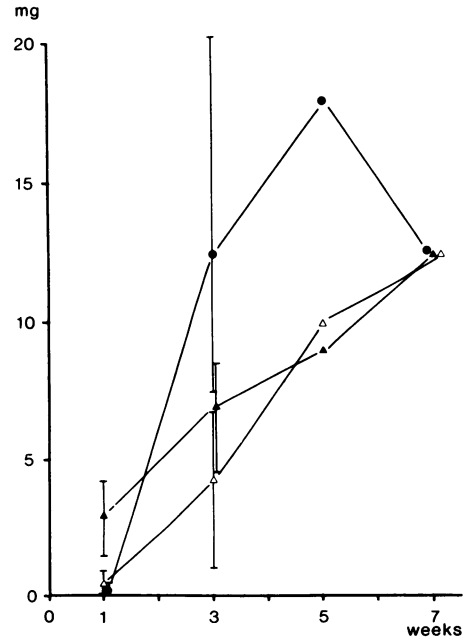

FIG. 6. *Increase in weight of the popliteal lymph node with time in normal and immunized C57BL mice after inoculation of live MLM (5 × 10^6 AFB) into the ipsilateral footpad. Symbols: ▲, mice inoculated with live MLM (5 × 10^7 AFB) subcutaneously in the thorax 6.5 weeks previously; ●, mice identically immunized with MLMSon; △, nonimmunized controls. The values represent the difference between the weight of the ipsilateral and the contralateral lymph nodes. The median for each group is given and at 1 and 3 weeks is shown the range of the observations in five mice.*

reaction started earliest in the mice immunized with live MLM, the reaction in the MLMSon-immunized mice was stronger but more delayed, and in both immunized groups the reaction developed earlier than in the normal mice.

Bacilli were harvested from the footpads 1, 3, 5, and 7 weeks after inoculation. In the group immunized with live MLM, no growth of the bacilli was observed (Fig. 7). In contrast, in the nonimmunized group the number of bacilli increased until 5 weeks after inoculation with a doubling time of approximately 18 days. The same or a slightly lower number of bacilli was found at 7 weeks, indicating that the multiplication of bacilli had stopped and suggesting that some degradation of the bacilli had taken place. The growth pattern in the group immunized with MLMSon was intermediate between the other two groups. The bacilli increased in number between 1 and 3 weeks after inoculation, whereafter the numbers did not change significantly. Five weeks after inoculation, the number of bacilli present in the infected footpad was significantly different in the three groups. This indicated the MLMSon-immunized mice were somewhat more capable of inhibiting the multiplication of MLM than normal mice but that this effect was less than in mice immunized with live MLM.

When MLMSon-immunized mice were given 5×10^6 HK MLM, the footpad reaction was different from that seen after injection of the same number of live bacilli. After 24 and 48 h, the mice showed a significant footpad reaction to a small dose of MLMSon (0.08 μg) but no reaction to HK MLM. Two weeks after injection of HK MLM, a slight footpad swelling was present which remained about the same size until 7 weeks (Fig. 8). This swelling reaction was much weaker than that seen after injection of the same number of live MLM (Fig. 4).

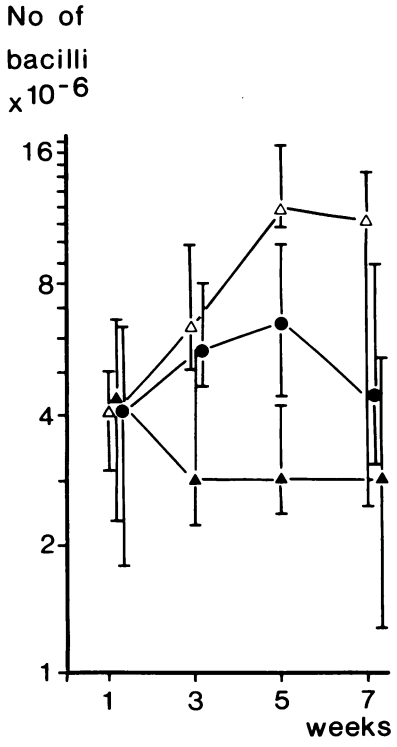

FIG. 7. *Growth of MLM in the footpads of C57BL mice after injection of 5×10^6 AFB into the right hind footpad of three different groups of mice: △, normal mice; ●, mice immunized with MLMSon subcutaneously in the thorax 6.5 weeks previously; ▲, mice identically inoculated with live MLM (5×10^7 AFB). The median and range for groups of five mice are given.*

DISCUSSION

The control of mycobacterial infection depends on the activation of the infected macrophage by antigen-specific T lymphocytes (11). Apart from this basic mechanism, our knowledge of protective immunity to mycobacterial infections is lacking in three major areas: we do not know the nature of the antigens that induce protective immunity, nor the subpopulations of lymphocytes involved in the response, nor the immunological parameters in vivo or in vitro that correlate directly with protective immunity. The relationship between DTH and protective immunity has been much debated, and although the two phenomena tend to occur together, there is ample experimental evidence showing that they can be dissociated (16). The present results indicate that in MLM infection there is no direct relationship between the level of DTH to soluble

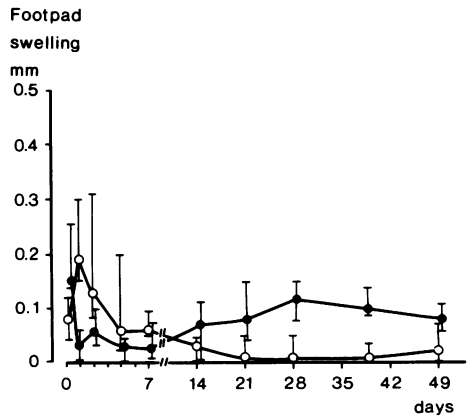

FIG. 8. *Increase in footpad thickness after injection of 5×10^6 heat-killed MLM. Symbols: ●, mice immunized with MLMSon 6 weeks previously; ○, normal mice. The median and range for a group of five mice are given at each point.*

antigens of MLM and the level of protective immunity.

Infection with MLM has been shown to induce protective immunity (4) and a CMI response (13) in C57BL mice. As previously reported, inhibition of multiplication of MLM can be demonstrated in mice immunized by a previous subcutaneous infection, when a small challenge inoculum is given in the footpad (4). In general, demonstration of protective immunity depends on the size of the challenge inoculum. Although most mouse strains tend to be susceptible to a very high and resistant to a very low infecting dose, an intermediate dose may provide good discrimination between resistant and susceptible mouse strains (2). Thus, the failure of others to demonstrate immunity to reinfection (13) may be due to the facts that they used a 50- to 500-times-larger challenge inoculum and that their mice, after primary inoculation, developed disseminated infection which may induce immune paralysis in C57BL mice (4).

In the present study, we compared the immunizing effect of live MLM bacilli with that of an extract of soluble antigens. Sonic extracts of MLM have previously been shown to contain more than 40 antigens (5). Most of these antigens are of cytoplasmic origin, but they also include some solubilized components of the cell wall. Extracts of this kind have been claimed to be superior to tuberculin-type antigens as skin test antigens in humans (12). The polyspecific nature of the sonic extract reagents could allow detection of DTH responses that are absent in more purified preparations of the tuberculin type. On the other hand, the immunogenic or eliciting capacity of certain antigens may be obviated or suppressed in such complex preparations. We have presently shown that the soluble fraction of the MLMSon is capable of inducing a considerable level of DTH reactivity, but the kinetics of the reaction to a challenge injection with live MLM were not the same in such sonic extract-immunized mice and in mice that had become immune after previous infection with live MLM. When challenged with 5×10^6 AFB, MLMSon-immunized mice did not develop any substantial footpad reaction until 2 weeks after inoculation. Almost no footpad reaction developed in MLMSon-immunized mice 2 weeks after injection of the same number of HK MLM. These results suggest that immunization with MLMSon does not by itself induce a protective immune response, but creates a state in which the development of protective immunity is accelerated. The reaction, which developed about 2 weeks after infection, in these mice seems to correlate with inhibition of bacterial multiplication and thus with protective immunity. As in normal mice, the induction of protective immunity in MLMSon-immunized mice seemed to require exposure to live MLM. This agrees with our findings in C3H mice, where DTH to MLMSon can be induced without any effect on protective immunity and where there is no footpad reaction to a challenge injection with live MLM in the immunized mice (M. Løvik and O. Closs, manuscript in preparation).

A difference in the protective effect induced by immunization with MLMSon and that induced by infection with live MLM could be due to a difference either in the specificity or in the type of immune response induced by the two procedures. We found that mice infected with MLM developed increased resistance to reinfection but only weak DTH reactivity to MLMSon. Conversely, mice immunized with MLMSon showed a moderate to weak response initially to live bacilli. Others have found that B6D2F mice develop a substantial DTH reactivity to MLMSon 5 weeks after inoculation with 10^8 live MLM in the contralateral footpad (13), but these workers used the total sonic extract as test antigen, whereas only the soluble fraction was used in the present study. Furthermore, we did two tests simultaneously, using both hind footpads. A weak response to MLMSon may then have become suppressed (1) in the previously infected mice by competition from the stronger reaction to live MLM, and similarly the reaction to live MLM may have become reduced in the MLMSon-immunized mice. Although there is a definite difference in the specificity of the immune responses induced by MLMSon and live MLM infection, the responses may not be as completely dissociated as the present results suggest.

The MLM bacilli used in the present study were prepared from spleens of heavily infected C3H mice and were contaminated with some tissue material. If these contaminants acted as alloantigens in the C57BL mice, it might complicate the interpretation of the results. However, both MLMSon and live MLM were made from similar preparations of bacilli, and the dose-response experiment was done with closely similar doses of the two antigens (2 μg of MLMSon protein equals 10^8 sonicated bacilli). Thus, allogeneic antigens should be expected to have been present to a similar extent in both preparations. A possible DTH reactivity to allogeneic antigens in MLMSon-immunized mice therefore is not a likely explanation for the differing responses we have observed to the live MLM and MLMSon test antigens.

The results of the cell transfer experiment clearly indicate that immunization with MLMSon induced a CMI response. This CMI

was demonstrable as a DTH reactivity to MLMSon. Recently it has been claimed that three types of DTH response may be evoked by mycobacteria; classical tuberculin-type DTH developing 4 weeks after inoculation, the Jones-Mote type, which is present after 1 week, and a third type that shows a peak 10 days after inoculation and then subsides (14). The latter two types of reactions peak at about 24 h after injection of antigen. The DTH developing after MLM infection has the kinetics and histomorphology of a tuberculin-type DTH reaction (8). The reactivity induced by immunization with MLMSon could be of the "day 10" type described by Rook (14) since it was demonstrable at day 11, but instead of decreasing it increased on days 18 and 33, and the reaction tended to peak at 48 rather than at 24 h. The late occurrence of the reaction, its persistence for many weeks, and the kinetics of the footpad response are more in favor of a tuberculin-type than a Jones-Mote-type reaction. Thus we have no evidence suggesting that immunization of C57BL mice with MLMSon induced a type of DTH that is different from that induced by live MLM.

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