INTERNATIONAL JOURNAL OF LEPROSY

Volume 52, Number 3
Printed in the U.S.A.

Immunization of Highly Susceptible C3H Mice with Ultrasonicated *Mycobacterium lepraemurium* (MLM) Bacilli Facilitates the Development of Increased Resistance During MLM Infection[1]

Martinus Løvik, Otto M. Closs, and Olav A. Haugen[2]

The slow-growing intracellular bacterium *Mycobacterium lepraemurium* (MLM) is a natural pathogen for mice and rats ([13, 16, 29, 30]). C3H mice are highly susceptible to progressive infection with MLM ([3]). Subcutaneous immunization of C3H mice with the antigens of ultrasonicated MLM bacilli (MLMSon) or heat-killed MLM bacilli in Freund-type adjuvants induces delayed-type hypersensitivity (DTH) against the water-soluble components of MLMSon (MLMSon-S) but no local reactivity against MLM bacilli and no protective immunity as determined 9 to 11 weeks after challenge with live MLM bacilli ([20]). Immunization with MLMSon-S in Freund's incomplete adjuvant (FIA) in C57BL mice resistant to progressive MLM infection ([3]) fails to induce manifest protective cell-mediated immunity, but accelerates the development of such immunity ([5]). The present experiments were performed to see if a late-onset increase in resistance after immunization with MLMSon could be obtained in C3H mice. Different adjuvants, components of the MLMSon antigen, and cyclophosphamide (CY) treatment ([34]) were tried. Furthermore, we explored the effect of MLM infection on resistance to a challenge infection in C3H mice.

[1] Received for publication on 13 May 1983; accepted for publication in revised form on 9 January 1984.

[2] M. Løvik, M.D., Research Fellow in Immunology; O. Closs, M.D., Ph.D., Senior Lecturer of Immunology, University of Oslo, Institute for Experimental Medical Research, Ullevaal Hospital, Oslo, Norway. O. A. Haugen, M.D., Ph.D., Professor of Pathology, Department of Pathology, University of Trondheim, Trondheim, Norway. Current addresses: Dr. M. Løvik, Department of Immunology, SIFF National Institute of Public Health, Postuttak, Oslo 1, Norway. Dr. O. Closs, Bacteriology Department, National Institute of Public Health, Oslo, Norway.

MATERIALS AND METHODS

Animals. Five- to six-week-old female mice of the inbred strain C3H/Bom (C3H) ([21]) were obtained as specific-pathogen-free animals directly from the breeder (Gl. Bomholtgård Ltd., Ry, Denmark).

MLM bacilli. The Douglas strain of *M. lepraemurium* was maintained by repeated passage in inbred C3H mice. The bacilli were harvested, prepared, stained with auramine ([15]), and counted as previously described ([18, 20]). The viability of MLM bacilli cannot be determined by conventional bacteriological methods, and acid-fast bacilli prepared and stored as described are therefore referred to as live MLM bacilli.

Preparation of MLMSon. MLM bacilli were purified by differential centrifugation, ultrasonicated and stored as previously described ([4, 20]). Insoluble material was removed by centrifugation at 20,000 × *g* for 20 min. The protein concentration of the supernatant was 0.8 mg/ml. The same batch of sonicate was used for all foot pad injections to determine DTH. Separate batches of sonicate (protein concentration 0.7–0.9 mg/ml) were used for immunization. The sonicate to be used for immunization in some experiments was reconstituted with the amount of insoluble material (mainly cell wall fragments) removed by centrifugation, and such reconstituted sonicate is referred to as MLMSon-P. Sonicate containing only the water-soluble material is referred to as MLMSon-S.

Cyclophosphamide (CY) treatment. CY (Sendoxan, Pharmacia AB, Uppsala, Sweden) was dissolved in sterile water and injected intraperitoneally in a single dose of 200 mg/kg three days before immunization unless otherwise specified.

Immunization with MLMSon prepara-

tions. For immunization, equal volumes of antigen and adjuvant 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 emulsion was given subcutaneously in the right side of the thorax after removal of the hairs with Surgex hair remover cream (Cooper Laboratories, Inc., Wayne, New Jersey, U.S.A.). Freund's incomplete adjuvant (FIA) and Freund's complete adjuvant (FCA) were obtained from Behringwerke AG, Marburg/Lahn, West Germany.

Foot pad injection of MLMSon-S and experimental infection. For all foot pad injections a volume of 10 μl of the appropriate material was given through a 30-gauge needle, from a 100 μl syringe (Hamilton Bonaduz, Bonaduz, Switzerland).

At appropriate intervals after the inoculation with bacilli, groups of 5–6 mice were killed and the infected foot pad, the draining popliteal lymph node and, in some experiments, the spleen and liver were removed. The lymph node and spleen were weighed. Preparations were made from the removed organs as previously described ([3]), and the acid-fast bacilli were counted after staining with auramine.

Foot pad reactions. DTH against MLMSon-S and local reactivity against MLM bacilli were determined by the foot pad swelling assay ([9]). The thickness of the hind feet was measured using a modified dial gauge caliper ([5]).

Histological methods. Foot pads and lymph nodes were fixed for 24 hr in 10% formol alcohol containing 5% glacial acetic acid and then transferred to 80% ethanol. Further processing and embedding were performed by standard procedures. Sections were stained with hematoxylin-erythrosin-saffron. For the demonstration of acid-fast bacilli, auramine-rhodamine staining was used ([12]). The preparations were coded before reading.

Statistical methods. The non-parametric Kruskal-Wallis statistic, Wilcoxon signed rank test, and Mann-Whitney U test with the Bonferroni correction for multiple comparisons were used to avoid the assumption that the data were normally distributed. In one case (Fig. 8) the parametric Student's *t* test had to be used because of a low number of individuals in one group ([8, 27, 28]). Differences with $p > 0.05$ were considered as not statistically significant (n.s.).

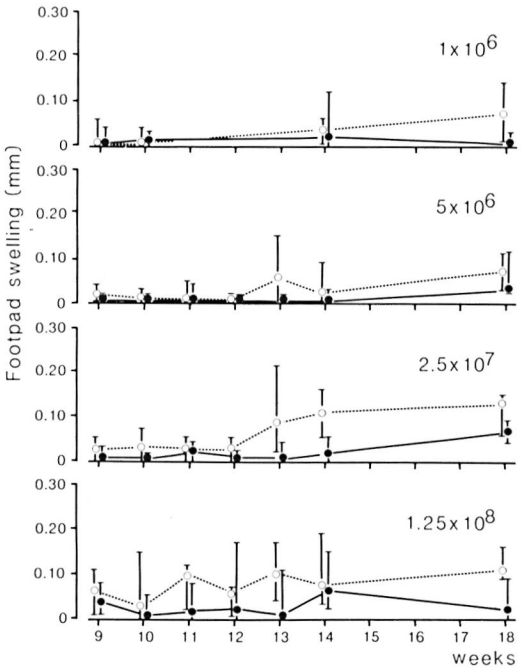

FIG. 1. Foot pad swelling in groups of mice immunized with MLMSon-S in FIA (○) and in normal mice (●) 9–18 weeks after inoculation with various doses of live MLM bacilli. (Median and range for groups of 5–6 mice.)

RESULTS

Local reactivity against MLMSon-S and MLM bacilli. C3H mice were immunized subcutaneously in the thorax with MLMSon-S in FIA. Six weeks after immunization, foot pad testing showed that DTH to MLMSon-S had developed, as previously observed after similar immunization ([17, 20]). At the same time, groups of immunized and normal mice were inoculated in one hind foot pad with a dose of 1×10^6, 5×10^6, 2.5×10^7, or 1.25×10^8 live MLM bacilli. The foot pad swelling in response to the bacilli was measured at intervals for 18 weeks. As in previous experiments ([17, 20]), a dissociation between DTH to MLMSon-S and local reactivity against live bacilli was observed, and no swelling of the infected foot pad developed during the first weeks after inoculation with bacilli (data not shown). However, about nine weeks after inoculation with bacilli the development of a small swelling of the infected foot pad

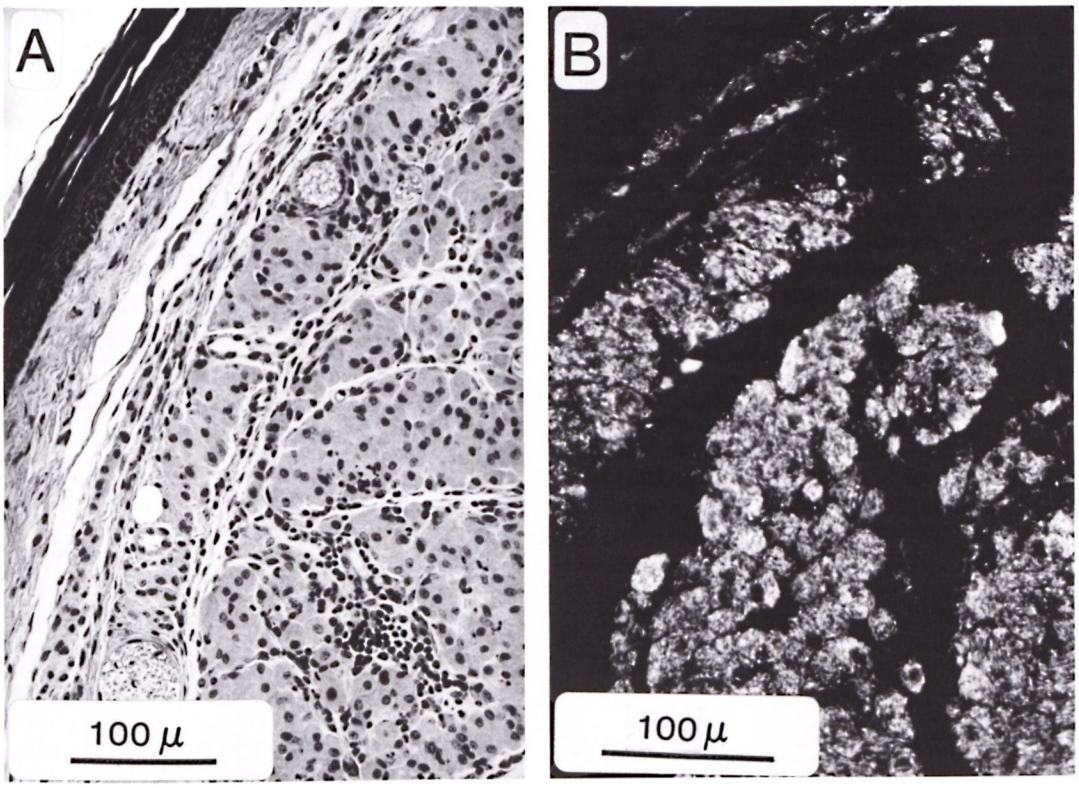

FIG. 2. Histological appearance of foot pad of MLMSon-immunized C3H mice 16 weeks after inoculation with 2.5×10^7 live MLM bacilli. (A) Large subcutaneous infiltrate of macrophages in the foot pad with occasional clusters of lymphocytes/small mononuclear cells (hematoxylin-erythrosin-saffron stain). (B) Neighbor section of (A) showing large numbers of fluorescent intracellular bacteria (auramine-rhodamine stain).

started in mice given the largest doses of bacilli (Fig. 1). The increase in foot pad thickness in normal mice was generally small and was observed in only a few mice. In immunized mice the swelling was somewhat larger, especially in mice given the two highest doses of bacilli. A statistically significant difference between the foot pad swelling in all immunized mice compared to all normal mice was found at 13 and 18 weeks. Mice that had been given an emulsion of saline in FIA were not different from the normal mice (data not shown).

Histological examination. Sixteen weeks after the inoculation with bacilli, normal and immunized mice given the three highest doses of bacilli were sacrificed. Histological examination of the foot pad revealed extensive subcutaneous infiltrates of closely packed enlarged macrophages (Fig. 2A) which contained large numbers of acid-fast bacilli (Fig. 2B). In immunized as well as normal mice the macrophage infiltrates were virtually devoid of small mononuclear cells, and no epithelioid cell transformation was observed. However, in the septa between the macrophage infiltrates occasional clusters of small mononuclear cells, presumably lymphocytes, were seen (Fig. 2A). These infiltrates with small mononuclear cells were more frequent in the mice immunized with MLMSon-S. There was no necrosis or marked infiltration by polymorphonuclear cells. In the popliteal lymph node numerous granulomas were observed, all of which contained large numbers of acid-fast bacilli. The granulomas were measured and counted and found to be larger and more numerous in the non-immunized mice.

Lymph node and spleen enlargement. One and nine weeks after the inoculation, mice given 1×10^6 bacilli were harvested. At nine weeks some lymph node enlargement was observed, and the lymph node in immu-

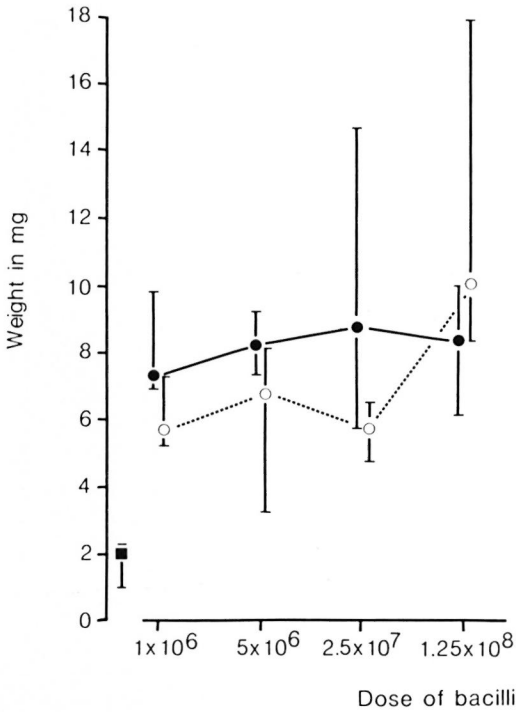

FIG. 3. Weight of popliteal lymph node draining infected foot pad of mice immunized with MLMSon-S in FIA (○) and normal mice (●) 20 weeks after inoculation with various doses of live MLM bacilli. Normal lymph nodes = ■. (Median and range for groups of 5–6 mice.)

nized mice tended to be heavier than the lymph node in the controls (n.s.). At 20 weeks (Fig. 3) mice given 1×10^6, 5×10^6, 2.5×10^7, and 1.25×10^8 MLM were sacrificed. In mice given 1×10^6 bacilli, a further increase in lymph node weight had taken place from 9–20 weeks. Except for mice given the highest challenge dose, the lymph nodes were now smaller in immunized than in non-immunized mice. In immunized as well as in non-immunized mice, the lymph node weight was remarkably similar in the different groups (Fig. 3).

The spleen weight showed only a small increase from one week to 20 weeks. There was no significant difference in spleen weight between mice given the highest and the lowest doses of bacilli. However, there was a tendency (n.s.) toward smaller spleens in immunized mice than in non-immunized animals (data not shown).

Effect of immunization with MLMSon-S on bacillary multiplication. After inoculation with 1×10^6 bacilli, immunized mice, mice given an emulsion of saline in FIA subcutaneously, and normal mice all showed rapid multiplication of bacilli from 1–9 weeks (Fig. 4). In the foot pad the doubling time was 11 days in normal mice. From 9–20 weeks the multiplication in the foot pad in all groups was slower, with a doubling time of 20 days in normal mice and approximately 30 days in immunized mice. In the popliteal lymph node, the doubling time from 9–20 weeks in normal mice was approximately 9 days; in immunized mice it was 13 days. In the spleen, the doubling time from 9–20 weeks was 8–9 days in normal mice and 11 days in immunized mice (determined from group medians).

Bacillary numbers at nine weeks were not different in normal and immunized mice in any of the organs. At 20 weeks immunized mice as compared to normal mice had a 3-, 10-, and 6-fold reduction in bacillary numbers, respectively, in the foot pad, the popliteal lymph node, and the spleen (Fig. 4). In another experiment, immunization was found to reduce bacillary numbers in the liver also. Bacillary numbers at 20 weeks in mice given saline in FIA subcutaneously were not different from normal mice in the liver and the spleen but were significantly higher than the numbers in MLMSon immunized mice. In the foot pad mice given saline in FIA were not different from immunized mice (Fig. 4).

TABLE 1. *Ratio in non-immunized mice between number of acid-fast bacilli harvested from various organs and number of bacilli inoculated into foot pads 20 weeks earlier. (Median and range for 5–6 mice.)*

Inoculum	Foot pad	Popliteal lymph node	Spleen
1×10^6	440 (320–840)	530 (400–1040)	70 (22–190)
5×10^6	52 (18–94)	160 (86–220)	14 (13–110)
2.5×10^7	7 (4–12)	104 (68–144)	200 (80–1160)
1.25×10^8	7 (7–8)	18 (9–65)	17 (5–320)

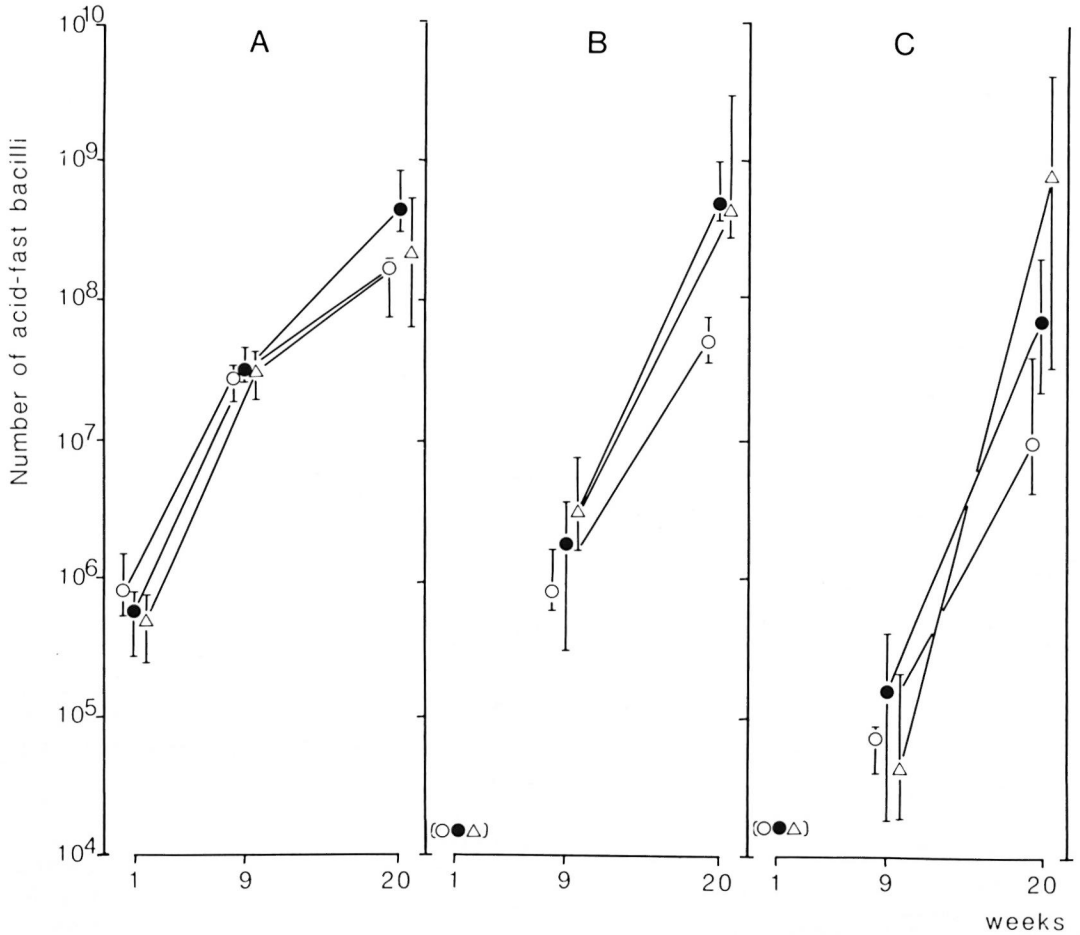

FIG. 4. Number of acid-fast bacilli in the foot pad (A), draining popliteal lymph node (B), and spleen (C) of mice immunized with MLMSon-S in FIA (○), mice given an emulsion of saline in FIA (△), and normal mice (●) 1, 9, and 20 weeks after inoculation in foot pad with 1×10^6 live MLM bacilli. Symbols in parentheses denote that no bacilli were detectable. (Median and range for groups of six mice.)

In a separate experiment (not shown), CY pretreatment did not increase the effect of immunization with MLMSon-S on bacillary multiplication.

Effect of inoculum size on bacillary multiplication. If bacillary multiplication were unhindered, an increase in the number of bacilli inoculated would give a proportional increase in the number of bacilli in the mouse at any time. However, an increase in the inoculum generally gave a smaller than proportional increase in the number of bacilli harvested from the foot pad and the popliteal lymph node and, in some instances, there was no increase at all (Fig. 5). For example, a 125-fold increase of the inoculum gave only a fourfold increase in bacillary numbers in the popliteal lymph node (Fig. 5B). The tendency to reduced bacillary multiplication with higher doses was more pronounced in normal than in immunized mice. Bacillary numbers in the spleen showed a less regular pattern. The relative growth of the bacilli in normal mice with increasing inocula is summarized in Table 1.

Inoculated dose of bacilli and effect of immunization. In the foot pad an effect from immunization was found only with the lowest dose of bacilli (Fig. 5A). In the popliteal lymph node (Fig. 5B), the effect of immunization was larger and again was most pronounced in mice given the lowest doses of bacilli. After the largest dose of bacilli, the

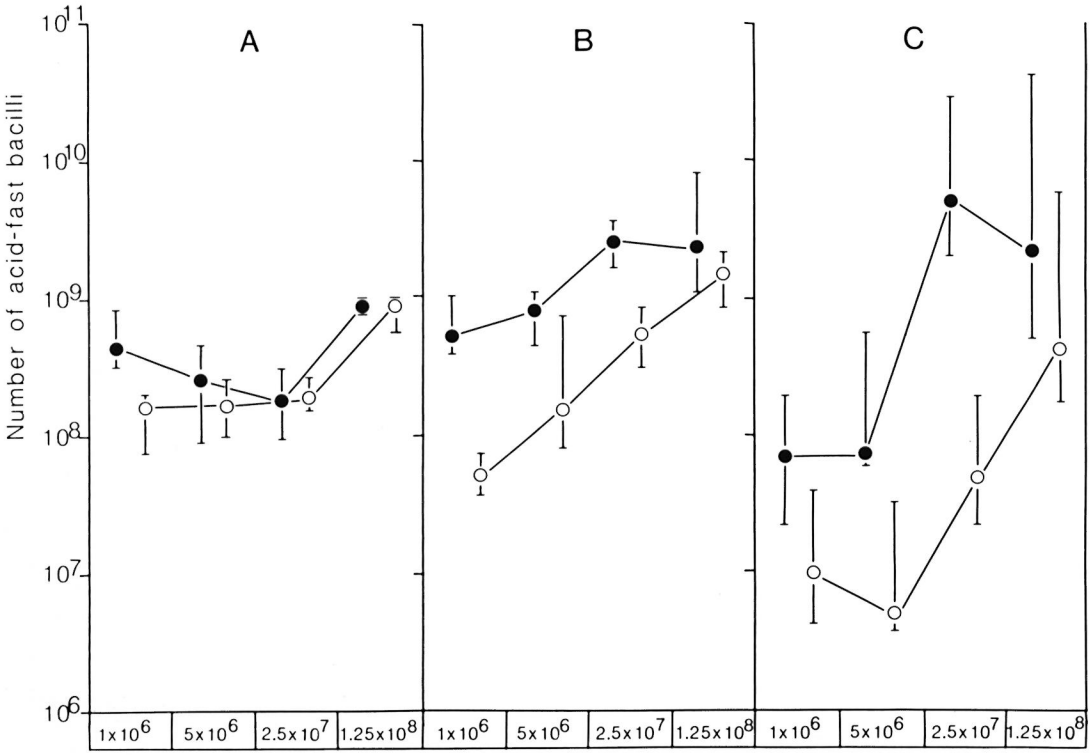

FIG. 5. Number of acid-fast bacilli in foot pad (A), draining popliteal lymph node (B), and spleen (C) of mice immunized with MLMSon-S in FIA (○) and normal mice (●) 20 weeks after inoculation in foot pad with various doses of live MLM bacilli. (Median and range for groups of 5–6 mice.)

difference between normal and immunized mice was small and not statistically significant. The largest differences between normal and immunized groups were seen in the spleen, but there was in this organ no clear relationship between the challenge dose and the effect of immunization (Fig. 5C).

Effect of immunization with insoluble MLMSon material and FCA. C3H mice were immunized subcutaneously in the thorax with MLMSon-P in FIA and MLMSon-P in FCA, and experimental infection with 1×10^6 bacilli was carried out with the same protocol as in the experiment above. Compared with the results in the experiment with MLMSon-S, the experiment with MLMSon-P showed a somewhat reduced effect of immunization in the popliteal lymph node but an increased effect in the foot pad and the spleen (Fig. 6, Table 2). The use of FCA instead of FIA as an adjuvant did not alter the effect of immunization. Note that the effect in the foot pad of immunization with MLMSon in this ex-

TABLE 2. *Relative bacillary numbers in organs of normal and various groups of immunized mice 20 weeks after foot pad inoculation with 1×10^6 MLM. (Calculated from medians of groups of 5–6 mice in two experiments.)*

Organ	Normal mice	Immunized mice		
		MLMSon-S/FIA	MLMSon-P/FIA	MLMSon-P/FCA
Foot pad	1	0.39	0.13	0.14
Popliteal lymph node	1	0.09	0.42	0.34
Spleen	1	0.16	0.01	0.03

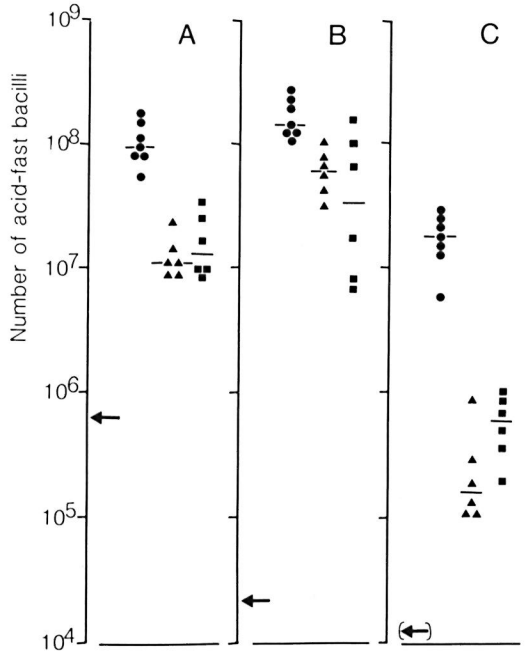

FIG. 6. Number of acid-fast bacilli after 20 weeks in foot pad (A), popliteal lymph node (B), and spleen (C) in normal C3H mice (●), mice immunized with MLMSon-P in FIA (▲), and mice immunized with MLMSon-P in FCA (■). Six weeks after immunization, 1×10^6 bacilli were inoculated into one hind foot pad. Horizontal bars denote medians; arrows denote median bacillary numbers one week after inoculation; parentheses indicate that no bacilli were detected.

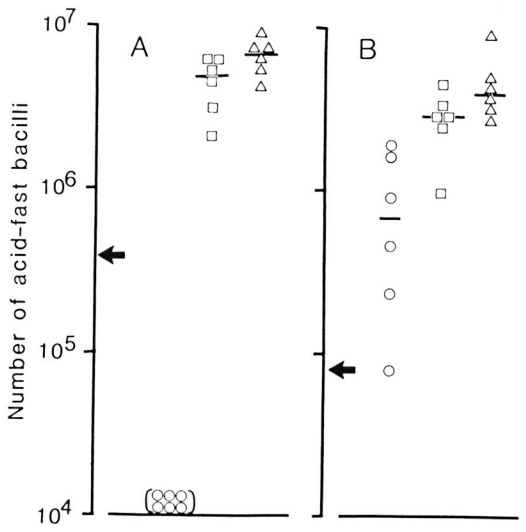

FIG. 7. Number of acid-fast bacilli in left food pad (A) and draining popliteal lymph node (B) 11 weeks after challenge. Individual mice primed in right foot pad with 1×10^7 live MLM bacilli 20 weeks earlier and not challenged in left foot pad (○); mice similarly primed in right foot pad and challenged in left foot pad with 5×10^5 live MLM bacilli 9 weeks later (□), and mice given challenge inoculum only (△). Symbols in parentheses denote mice without detectable bacilli. Horizontal bars denote medians of each group; arrows denote median numbers of bacilli in groups of normal mice one week after challenge inoculum.

periment was larger than the small effect of FIA alone, as observed in the foot pad in the previous experiment (Figs. 5A, 6A).

Effect of MLM infection on susceptibility to reinfection. The late increase in doubling time for bacilli in the foot pad even in normal mice (Fig. 4) could be due to induction of resistance mechanisms by the infection itself. A reinfection experiment was therefore performed.

Two groups of C3H mice were inoculated in the right hind foot pads with 1×10^7 MLM bacilli (priming inoculum). Nine weeks later one of these groups and a group of non-infected mice were inoculated in the left hind foot pads with 5×10^5 MLM bacilli (challenge inoculum). Eleven weeks after challenge (20 weeks after priming), there was no difference in the bacillary numbers in the challenged foot pads between mice given a priming inoculum and normal mice (Fig. 7A). In the popliteal lymph node, there was some dissemination of bacilli from the priming infection, but a tendency towards lower bacillary numbers in the mice given a priming inoculum was found (Fig. 7B). The difference from mice not primed was statistically significant only when dissemination from the priming inoculum had been compensated for by subtracting the median number of bacilli found in the primed, non-challenged mice from the numbers found in the primed and challenged mice. The median bacillary numbers in the two groups then differed by a factor of 1.9.

A previous experiment with a different protocol also had indicated a small reduction in susceptibility to reinfection in C3H mice. In that experiment, C3H mice were given CY and inoculated subcutaneously in the thorax with 5×10^7 live MLM bacilli. Six weeks later the mice were reinfected by foot pad inoculation with 5×10^6 live MLM bacilli. CY alone six weeks before challenge has in other experiments not affected ba-

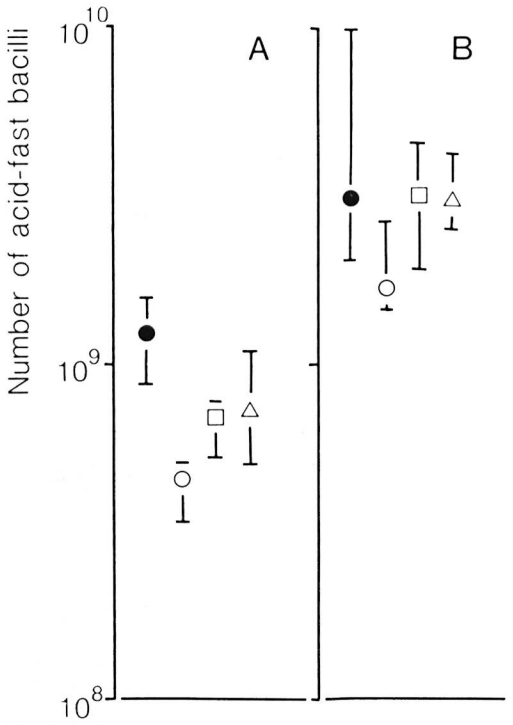

FIG. 8. Number of acid-fast bacilli in foot pad (A) and draining popliteal lymph node (B) 29 weeks after foot pad inoculation with 5×10^6 live MLM bacilli in normal mice (●), CY-pretreated mice inoculated on the thorax with 5×10^7 live MLM bacilli 6 weeks before foot pad inoculation (○) and normal mice given CY, 200 mg/kg, 9 days (□) or 5 weeks (△) after foot pad inoculation. (Median and range for groups of 3–5 mice.)

cillary multiplication. After 29 weeks, the reinfected mice had lower bacillary counts in the foot pads than the control mice (Fig. 8A, circles). The ratio between the medians of the groups was 2.7, which corresponds to a complete stop in bacillary multiplication for 3–4 weeks. The reinfected mice also had a tendency (n.s.) toward lower bacillary numbers in the draining popliteal lymph node (Fig. 8B). However, very high numbers of bacilli in the liver (not shown) indicated extensive dissemination of bacilli from the priming infection.

Effect of CY on primary infection. Immunomodulating agents might enhance the development of increased resistance during MLM infection. Therefore, mice given one foot pad inoculation only (5×10^6 live MLM) were given CY nine days or five weeks after the inoculation with bacilli. Twenty-nine weeks after the inoculation, both CY-treated groups showed a small reduction in bacillary numbers in the foot pads compared to the controls (Fig. 8A). In the draining popliteal lymph node, the CY-treated mice were not different from the controls (Fig. 8B).

DISCUSSION

Protective immunity to MLM is associated with a local granulomatous response ([3]). C3H mice showed extensive macrophage accumulation at the site of infection (Fig. 2A) and thus did not lack the nonspecific component of the cellular response to MLM. Reduced bacillary multiplication in mice immunized with MLMSon preparations became detectable later than nine weeks after the mice had been inoculated with bacilli, and the responsible mechanisms were largely dependent upon some component in MLMSon. This late appearance of the effect and the dependence on MLM antigen indicates that the mechanisms of reduced bacillary multiplication were due to a development of specific immunity rather than to direct stimulation of nonspecific resistance ([22]). Apparently, immunization with MLMSon primed the mice for immunity against live bacilli, but the development of manifest antibacterial activity took place only after exposure of the immune apparatus to live MLM bacilli for several weeks. The experiments with reinfection and CY treatment of non-immunized mice (Figs. 7, 8) lend some support to the theory that C3H mice can develop immunity with some protective capacity in response to live MLM bacilli. This is in agreement with a previous report on superinfection in mice highly susceptible to MLM ([11]), and may also be one reason for the longer doubling time for MLM observed even in non-immunized mice after nine weeks of infection (Fig. 4).

Bacillary growth after increasing inocula (Fig. 5) in normal mice showed a pattern reminiscent of the plateau phenomenon observed after inoculation with *M. leprae* in the mouse foot pad ([28]), said to be a phenomenon peculiar to *M. leprae* infection ([26]). The plateau phenomenon in *M. leprae* infection has been reported to be T cell dependent ([23]), and the poor multiplication of BCG in the mouse after large doses com-

pared to small doses ([6, 10]) has been explained by a more rapid onset of specific cell-mediated immunity after large doses ([14]). However, the plateau phenomenon with MLM infection in C3H mice may have been caused also by a passive microenvironmental limitation of the number of bacilli that could be harbored in the organ and given the necessary conditions for multiplication. Apparently, MLMSon immunization did not lower the "plateau" (Fig. 5B).

Weak immunization effects may become less evident if the challenge dose is large. In our experiment, the largest differences between immunized and normal animals tended to be found with the smallest challenge doses. This was due not to relatively stronger bacillary multiplication in the immunized mice with the higher doses but to relatively weaker multiplication in normal mice (Fig. 5).

The effect of immunization differed in different organs. The stronger effect of immunization on bacillary numbers in the lymph node and spleen may have been due to the shorter doubling time for MLM in these organs. A stop or slow-down of bacillary multiplication for a certain period will cause the greatest difference in bacillary numbers in the organs where bacillary multiplication normally is fastest. Reduced dissemination of bacilli in immunized mice ([18]) may have contributed to the organ differences. However, it should be remembered that cell-mediated immunity to mycobacteria to some extent is of a local nature ([7]). In a weak cell-mediated immune reaction with perhaps incomplete amplification and recruitment mechanisms, immunity may have been better expressed in lymphoid organs than in peripheral tissues.

The small foot pad swelling (Fig. 1) and the histology of the foot pad in immunized C3H mice (Fig. 2) is compatible with a weak and possibly abortive cell-mediated immune reaction. There was no evidence for an antibody-mediated local reaction in the foot pads ([24]). Although lymphocyte accumulation and proliferation may have contributed to the lymph node enlargement also in C3H mice, immunized mice had the smallest lymph nodes. This suggests that the normal mechanisms for amplification of the immune response failed to become activated or were suppressed in immunized C3H mice. Suppressor cells often develop in chronic infections, including murine leprosy ([2, 31, 32]). However, it is not known whether the lack of an effective immune response to MLM in C3H mice is caused mainly by a primary deficiency of the immune response, by the development of suppressor cells, or by other mechanisms. The effect of CY in primary infection (Fig. 8) indicates that CY-sensitive suppressor mechanisms to some extent facilitate bacillary multiplication in C3H mice ([1]). MLMSon immunization may have circumvented some suppressor mechanism triggered by live bacilli.

Whether the effect of immunization would have lasted long enough to increase survival time ([33]) is not known, and the duration of immunity may depend on the adjuvant used for immunization. However, for the immunological analysis of murine leprosy it is important that some increase in resistance was obtained even in the C3H substrain previously found to be the most susceptible to MLM infection ([19, 21]).

SUMMARY

C3H mice were immunized subcutaneously with water soluble antigens of ultrasonicated *Mycobacterium lepraemurium* bacilli (MLMSon-S) in Freund's incomplete adjuvant (FIA) and challenged with 1×10^6–1.25×10^8 live MLM bacilli inoculated into the foot pad. No swelling of the infected foot pad and no differences in bacillary multiplication and dissemination between the immunized mice and the control animals were observed in the first nine weeks. From nine weeks on, a small foot pad swelling developed in the immunized mice. Twenty weeks after inoculation, the number of bacilli in the foot pad, the popliteal lymph node, and the spleen was significantly lower in the immunized mice than in the normal controls after challenge with the lowest bacillary doses. Cyclophosphamide (CY) pretreatment did not increase the effect of immunization. The addition of MLM cell wall fragments to the emulsion used for immunization tended to increase the difference between immunized and normal animals, while no further increase of the immunization effect was obtained by the use of Freund's complete adjuvant (FCA).

A tendency toward a plateau phenome-

non for the multiplication of MLM bacilli was observed in the normal mice. In non-immunized mice, CY treatment caused some reduction in bacillary numbers in the foot pads, and reinfection experiments suggested that a small reduction in susceptibility had been induced by the priming infection. Although unable to prevent a progressive course of the infection, genetically low-resistant C3H mice were able to modify the development of the infection by mechanisms that were activated by the infection itself. Similar mechanisms were facilitated by immunization with MLMSon-S.

RESUMEN

Se inmunizaron (subcutáneamente) ratones C3H con antígenos solubles extraídos por sonicación del *Mycobacterium lepraemurium* (MLMSon-S) emulsificados con adyuvante incompleto de Freund (FIA), y se retaron con 1×10^6–1.25×10^8 bacilos MLM vivos inoculados en el cojinete plantar. Durante las primeras 9 semanas no ocurrió engrosamiento de los cojinetes infectados y no se notaron diferencias en la multiplicación bacilar ni en el grado de diseminación entre los animales inmunizados y los controles. Después de la novena semana se desarrolló un pequeño hinchamiento plantar en los animales inmunizados. Veinte semanas después, el número de bacilos en el cojinete plantar, en los gánglios linfáticos poplíteos y en el bazo, fue significativamente menor en los ratones inmunizados que en los controles cuando el reto se hizo con las dosis de bacilos más bajas. El pretratamiento con ciclofosfamida (CY) no incrementó el efecto de la inmunización. La adición de fragmentos de pared celular del MLM a la preparación usada para inmunizar tendió a incrementar las diferencias entre los animales inmunizados y los controles en tanto que no se observó ningún efecto adicional cuando se utilizó adyuvante completo de Freund (CFA).

En los ratones normales, la multiplicación del MLM progresó hasta alcanzar una meseta. En los animales no inmunizados, el tratamiento con CY causó cierta reducción en los números de bacilos en los cojinetes plantares y los experimentos de reinfección sugirieron que la primoinfección indujo una pequeña reducción en la susceptibilidad. Aunque los ratones C3H genéticamente susceptibles fueron incapaces de evitar el curso progresivo de la infección, sí fueron capaces de modificar su desarrollo gracias a mecanismos activados por la infección misma. La inmunización con MLMSon-S propició mecanismos similares.

RÉSUMÉ

Des souris C3H ont été immunisées par voie souscutanée avec des antigènes solubles dans l'eau provenant de bacilles *Mycobacterium lepraemurium* traités aux ultrasons (MLMSon-S), dans l'adjuvant incomplet de Freund (FIA); ces souris ont alors été stimulées par l'inoculation de 1×10^6–$1, 25 \times 10^8$ bacilles MLM vivants, inoculés dans le coussinet plantaire. Au cours des neuf premières semaines, on n'a constaté aucun gonflement du coussinet plantaire inoculé; de même, aucune différence n'a été constatée entre les souris immunisées et les souris témoins en ce qui concerne la multiplication bacillaire et la dissémination des bacilles. A partir de la neuvième semaine, et ensuite, un léger gonflement du coussinet plantaire est survenu chez les souris immunisées. Vingt semaines après l'inoculation, le nombre de bacilles dans le coussinet plantaire, dans les ganglions lymphatiques poplités, et dans la rate, était significativement plus bas chez les souris immunisées que chez les témoins normaux, lorsqu'on les simulait avec les doses bacillaires les plus basses. Un traitement préalable par la cyclophosphamide (CY) n'a pas accru l'effet de l'immunisation. L'addition de fragments de parois cellulaires de MLM à l'émulsion utilisée pour l'immunisation a tendu à augmenter la différence notéee entre animaux immunisés et normaux, encore qu'aucune augmentation supplémentaire de l'effet de l'immunisation n'ait été obtenue par l'utilisation de l'adjuvant complet de Freund (FCA).

Chez les souris normales, on a observé une tendance à un phénomène de plafonnage dans la multiplication des bacilles MLM. Chez les souris non immunisées, le traitement par la cyclophosphamide a entraîné une certaine réduction dans le nombre de bacilles au niveau des coussinets plantaires, et les expériences de réinfection suggèrent qu'une légère réduction de la susceptibilité peut être induite par l'infection première. Quoiqu'elles soient incapables d'empêcher l'évolution progressive de l'infection, les souris C3H présentant une faible résistance d'origine génétique, pouvaient modifier le développement de l'infection par des mécanismes qui étaient déclenchés par l'infection elle-même. Des mécanismes similaires étaient facilités par l'immunisation par MLMon-S.

Acknowledgments. This work was supported by the Norwegian Research Council for Science and the Humanities, the Heiser Fellowship Program for Research in Leprosy, Professor Carl Semb's Medical Research Fund, and Anders Jahre's Fund for the Promotion of Science. The skillful technical assistance of Ellen S. Karlstrøm, Sidsel Hermansen, and Kahsai Beraki is gratefully acknowledged. We thank Ruth Stupski and Mary Durett for excellent secretarial help with the manuscript.

REFERENCES

1. ALEXANDER, J. Effect of cyclophosphamide treatment on the course of *Mycobacterium lepraemurium* infection and development of delayed-type hypersensitivity reactions in C57Bl and BALB/c mice. Clin. Exp. Immunol. **34** (1978) 52–58.
2. BULLOCK, W. E., CARLSON, E. M. and GERSHON, R. K. The evolution of immunosuppressive cell populations in experimental mycobacterial infection. J. Immunol. **120** (1978) 1709–1716.
3. CLOSS, O. Experimental murine leprosy: Growth

of *Mycobacterium lepraemurium* in C3H and C57/BL mice after footpad inoculation. Infect. Immun. **12** (1975) 480–489.

4. Closs, O., Harboe, M. and Wassum, A. M. Cross-reactions between mycobacteria. I. Crossed immunoelectrophoresis of soluble antigens of *Mycobacterium lepraemurium* and comparison with BCG. Scand. J. Immunol. **4** Suppl. 2 (1975) 173–185.
5. Closs, O. and Løvik, M. Protective immunity and delayed-type hypersensitivity in C57BL mice after immunization with live *Mycobacterium lepraemurium* and sonicated bacilli. Infect. Immun. **29** (1980) 17–23.
6. Collins, F. M. and Mackaness, G. B. The relationship of delayed hypersensitivity to acquired antituberculous immunity. I. Tuberculin sensitivity and resistance to reinfection in BCG-vaccinated mice. Cell Immunol. **1** (1970) 253–265.
7. Dannenberg, A. M., Jr. Cellular hypersensitivity and cellular immunity in the pathogenesis of tuberculosis: Specificity, systemic and local nature, and associated macrophage enzymes. Bacteriol. Rev. **32** (1968) 85–102.
8. Glantz, S. A. *Primer of Biostatistics.* New York: McGraw-Hill, Inc., 1981.
9. Gray, D. F. and Jennings, P. A. Allergy in experimental mouse tuberculosis. Am. Rev. Tuberc. **72** (1955) 171–195.
10. Gros, P., Skamene, E. and Forget, A. Genetic control of natural resistance to *Mycobacterium bovis* (BCG) in mice. J. Immunol. **127** (1981) 2417–2421.
11. Kawaguchi, Y. The relation between the host resistance and the disease type in mouse leprosy. Part 8. Superinfection in malignant mouse leprosy. Jpn. J. Bacteriol. **17** (1962) 355–359.
12. Kuper, S. W. A. and May, J. R. Detection of acid-fast organisms in tissue sections by fluorescence microscopy. J. Pathol. Bacteriol. **79** (1960) 59–68.
13. Lampe, P. H. J. and De Moor, C. E. Ratten-lepra. Geneesk. Tijdschr. v. Nederl.-Indië **75** (1935) 634–654.
14. Lefford, M. J. The effect of inoculum size on the immune response to BCG infection in mice. Immunology **21** (1970) 369–381.
15. Lempert, H. Fluorescence microscopy in detection of tubercle bacilli. Lancet **2** (1944) 818–822.
16. Lowe, J. Rat leprosy. A critical review of the literature. Int. J. Lepr. **5** (1937) 311–328 and 463–481.
17. Løvik, M. and Closs, O. Delayed type hypersensitivity to mycobacterial antigens without protective immunity: A failure to produce the right specificity or the right type of immune reaction? Scand. J. Infect. Dis. **24** Suppl. (1980) 224–227.
18. Løvik, M. and Closs, O. Effect of BCG vaccination on *Mycobacterium lepraemurium* infection in a highly susceptible inbred mouse strain. Acta Pathol. Microbiol. Scand. [C] **89** (1981) 133–138.
19. Løvik, M. and Closs, O. Variation between substrains of C3H mice in resistance to *Mycobacterium lepraemurium* infection. Scand. J. Immunol. **15** (1982) 119.
20. Løvik, M. and Closs, O. Induction of delayed type hypersensitivity against ultrasonicated *Mycobacterium lepraemurium* bacilli without simultaneous local reactivity against live bacilli or protective immunity. Clin. Exp. Immunol. **53** (1983) 319–327.
21. Løvik, M., Collins, F. M. and Closs, O. Inbred C3H mouse substrain differences demonstrated in experimental murine leprosy. Immunogenetics **16** (1982) 607–611.
22. North, R. J. Cell mediated immunity and the response to infection. In: *Mechanisms of Cell-mediated Immunity,* McCluskey, R. T. and Cohen, S., eds. New York: John Wiley and Sons, Inc., 1974, pp. 185–219.
23. Rees, R. J. W. Enhanced susceptibility of thymectomized and irradiated mice to infection with *Mycobacterium leprae.* Nature **211** (1966) 657–658.
24. Ridley, M. J., Marianayagam, Y. and Spector, W. G. Experimental granulomas induced by mycobacterial immune complexes in rats. J. Pathol. **136** (1982) 59–72.
25. Shepard, C. C. The experimental disease that follows the injection of human leprosy bacilli into foot-pads of mice. J. Exp. Med. **112** (1960) 445–454.
26. Shepard, C. C., Walker, L. L. and van Landingham, R. Heat stability of *Mycobacterium leprae* immunogenicity. Infect. Immun. **22** (1978) 87–93. Abstract by Rook, G. A. W. in Lepr. Rev. **50** (1979) 164–166.
27. Siegel, S. *Nonparametric Statistics for the Behavioral Sciences.* New York: McGraw-Hill, Inc., 1956.
28. Snedecor, G. W. and Cochran, W. G. *Statistical Methods.* Ames: Iowa State University Press, 1967.
29. Stefanskij, V. K. Zabolevanija u krys, vyzvannyja kislotoupornoj palotsjkoj. Russkij Vratsj (1902) 1726–1727.
30. Stefansky, W. K. Eine lepraähnliche Erkrankung der Haut und der Lymphdrusen bei Wanderratten. Zbl. Bakt. **33** (1903) 481–487.
31. Turcotte, R. Suppressor cells in experimental murine leprosy. Int. J. Lepr. **46** (1978) 358–363.
32. Turcotte, R. Influence of route of *Mycobacterium lepraemurium* injection on susceptibility to mouse leprosy and on lymphoblastic transformation. Infect. Immun. **28** (1980) 660–668.
33. Turcotte, R. and Lemieux, S. Lack of a sustained protection against murine leprosy in C3H mice vaccinated with extracts of *Mycobacterium lepraemurium* in admixture with *Mycobacterium bovis* (BCG). Int. J. Lepr. **50** (1982) 494–500.
34. Turk, J. L. and Parker, D. Effect of cyclophosphamide on immunological control mechanisms. Immunol. Rev. **65** (1982) 99–113.