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EDITORIALS

Editorial opinions expressed are those of the writers.

Experimental Murine Leprosy and Its Relevance for the Study of Resistance to Mycobacterial Infections in Man*,**

Murine leprosy was first described in 1902 by V. K. Stefanskij[1] who found the disease in rats (*Mus decumanus* at the time, now *Rattus norvegicus*) while working with plague in Odessa, Russia. Within a few years cases were reported from virtually all parts of the world. The first descriptions of the disease had already pointed out a striking similarity between murine and human leprosy, both in terms of the supposedly causative organisms (noncultivable, acid-fast mycobacteria) and in terms of the clinical and histopathological manifestations of the disease.[2, 3]

The notion that human and murine leprosy were closely related and possibly identical infections was supported by early studies showing serological crossreactivity between *Mycobacterium leprae* and the causative agent of murine leprosy, *M. lepraemurium* (MLM); by reports that human leprosy had been transferred from man to mouse;[4] by reports on murine leprosy in man;[5] and by microbiological reports claiming that *M. leprae* and MLM were identical or almost identical. Considerable attention was paid to murine leprosy in clinical journals and public health reports. Gradually

* This review is based on the introduction to Dr. Løvik's Ph.D. thesis submitted to the University of Oslo. The thesis, with an extensive reference list, may be obtained from the author.

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[1] Stefanskij, V. K. Zabolevanija u krys, vyzvannyja kislotoupornoj palotsjkoj. Russkij Vratsj No. 47 (1902) 1726–1727.

[2] Dean, G. Further observations on a leprosy-like disease of the rat. J. Hyg. (Lond.) **5** (1905) 99–112.

[3] Stefansky, W. K. Eine lepraähnliche Erkrankung der Haut und der Lymphdrüsen bei Wanderratten. Centralbl. f. Bakteriol. **33** (1903) 481–487.

[4] Sugai, T. Nachtrag zu gelungenen Übertragungsversuchen mit Lepra bei Säugetieren. Lepra **8** (1909) 203–210.

[5] Marchoux, E. La lèpre du rat et sa transmission probable a l'homme. Paris Medical **49** (1923) 313–316.

however, the idea that murine leprosy was a reservoir for human leprosy was abandoned, and the last studies on a possible causative relationship between human and murine leprosy were published around 1930.

A second important reason to study murine leprosy was the need to create an experimental model for human leprosy. Before experimental infection with *M. leprae* in the mouse foot pad was described by Shepard in 1960, there was no experimental model other than murine leprosy which might elucidate the mechanisms determining host resistance to infection with *M. leprae*. Stefanskij and Dean had already described polar forms of murine leprosy, with a more or less continuous spectrum in between.[2, 3] The value of murine leprosy as a model was greatly strengthened when experimental murine leprosy in inbred strains of mice was established as a model for the spectral aspect of human leprosy by Kawaguchi[6] and by Closs and Haugen.[7] In contrast to *M. leprae*, MLM has the clear advantage for experimental use in the mouse that it is a natural pathogen in this species.[8, 9]

Murine leprosy as a model for human leprosy

Murine leprosy and human leprosy are both naturally occurring chronic granulomatous diseases of mammals caused by acid-fast bacteria. The macroscopic and microscopic similarities between murine and human leprosy are striking, and both diseases are "spectrum diseases." Both *M. leprae* and MLM were until recently considered to be noncultivable; only since 1970[10] has MLM been grown *in vitro*. Both bacteria multiply very slowly in the host with a doubling time of 1–3 weeks; both are found predominantly in an intracellular location; and both appear to be essentially nontoxic to host cells. The two mycobacteria display structural similarities,[11] and show immunological crossreactivity as detected with antibodies[12] and in skin tests.[13] In human as well as in murine leprosy, there appears to be a specific depression of cell-mediated immunity, but no clear depression of humoral immunity.

There are, however, some problems with the use of murine leprosy as a model for human leprosy. MLM and *M. leprae* each show distinct metabolic, morphological, ultrastructural, chemical, and antigenic characteristics, and are clearly different species of bacteria.[14] Both immunological studies[15, 16] and studies of bacterial DNA[17] indicate that *M. leprae* and MLM are as closely related to other mycobacteria as they are to each other. Patients with lepromatous leprosy, anergic to human lepromin, re-

[6] Kawaguchi, Y. Classification of mouse leprosy. Jpn. J. Exp. Med. **29** (1959) 651–663.

[7] Closs, O. and Haugen, O. A. Experimental murine leprosy. 2. Further evidence for varying susceptibility of outbred mice and evaluation of the response of 5 inbred mouse strains to infection with *Mycobacterium lepraemurium*. Acta Pathol. Microbiol. Scand. [A] **82** (1974) 459–474.

[8] Krakower, C. and Gonzalez, L. M. Spontaneous leprosy in a mouse. Science **86** (1937) 617–618.

[9] Lampe, P. H. J. and de Moor, C. E. Ratten-lepra. Eerste mededeeling: De diagnostiek van de rattenlepra. Het voorkomen van deze rattenziekte te Batavia. De geographische distributie. Geneesk. Tijdschr. Nederl.-Indië **75** (1935) 634–654.

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[11] Fukunishi, Y., Okada, S., Nishiura, M. and Kohsaka, K. Ultrastructural features of the multiplication of human and murine leprosy bacilli in macrophages of nude mice. Int. J. Lepr. **50** (1982) 68–75.

[12] Kronvall, G., Closs, O. and Bjune, G. Common antigen of *Mycobacterium leprae*, *M. lepraemurium*, *M. avium*, and *M. fortuitum* in comparative studies using two different types of antisera. Infect. Immun. **16** (1977) 542–546.

[13] Nishimura, S., Yasukawa, T. and Kohsaka, K. Immunological correlation of leprosy and murine leprosy. I. Skin reaction. Medicine and Biology **51** (1959) 129–133. Cited in G. Meissner and A. Schmiedel, editors: *Mykobakterien und Mykobakterielle Krankheiten*. Chapter 9. Human and murine leprosy by S. Sato and S. Nishimura. Jena: Gustav Fischer, 1967, pp. 366–367.

[14] Portaels, F. and Pattyn, S. R. Taxonomy of *Mycobacterium leprae* and *M. lepraemurium*. Ann. Microbiol. (Paris) **133B** (1982) 99–108.

[15] Katoch, V. M., Wayne, L. G. and Diaz, G. A. Characterization of catalase by micro-immunoprecipitation in tissue-derived cells of *Mycobacterium lepraemurium* TMC 1701. Int. J. Syst. Bacteriol. **32** (1982) 416–418.

[16] Stanford, J. L. An immunodiffusion analysis of *Mycobacterium lepraemurium* Marchoux and Sorel. J. Med. Microbiol. **6** (1973) 435–439.

[17] Athwal, R. S., Deo, S. S. and Imaeda, T. Deoxyribonucleic acid relatedness among *Mycobacterium leprae*, *Mycobacterium lepraemurium*, and selected bacteria by dot blot and spectrophotometric deoxyribonucleic acid hybridization assays. Int. J. Syst. Bacteriol. **34** (1984) 371–375.

spond to MLM.[18] Further, the intracellular behavior of MLM and *M. leprae* is different: MLM multiplies within host cell phagolysosomes[19] and a high concentration of lysosomal enzymes appears to facilitate bacterial multiplication;[20] whereas *M. leprae* appear to multiply in the cytoplasmic matrix outside the phagolysosomes.[21] Although formation of globi is a typical feature of *M. leprae*, MLM does not form globi.[22] Finally, MLM does not show affinity for peripheral nerves as does *M. leprae*, and in experimental murine leprosy, the skin involvement so typical for human leprosy is usually absent.[22, 23] It is, therefore, uncertain how similar the mechanisms of pathogenesis are in the diseases caused by *M. leprae* and MLM, and there may also be differences in the mechanisms of protective immunity in human and murine leprosy.

Natural and experimental murine leprosy

A review of the literature describing natural and experimental murine leprosy has revealed few differences between natural and experimental infection with MLM. Skin involvement appears to be more common in natural than in experimental disease, and there appears to be more involvement of the viscera in experimental than in natural disease.[24] These differences could be due to larger inocula in experimental infections.

The way in which murine leprosy is naturally transmitted has not been definitely established. However, it appears that the disease starts in the skin and draining lymph nodes, and the lesions tend to be in the regions most exposed to scratches and bites.[25] Attempts to transmit the disease experimentally with insects[25] have been unsuccessful, and rats fed infected tissue only rarely developed signs of the disease.[25, 26] Bacteria placed on newly shaved skin caused infection,[27] and rats shaved and repeatedly rubbed with mud collected near houses where leprous rats were abundant developed "soil leprosy," which could not be distinguished from ordinary rat leprosy.[28] Experimentally, it has been reported that five MLM bacteria given subcutaneously (s.c.) is enough to start infection.[29]

Natural infections usually go through a stage in which the primary defense barriers are broken, and the initial immune reactions will occur at a peripheral site. It is of great importance to take the natural development of the infection into consideration when setting up an experimental model. Experimental infection with small doses of bacteria given subcutaneously would be expected to closely mimic natural MLM infection.

Why, then, is the skin rarely involved in experimental murine leprosy? Marchoux[25] claimed that the degree of skin involvement was greatly influenced by concomitant or secondary infection, and that he could experimentally produce the musculocutaneous form of the disease by using impure inoculations. In natural murine leprosy, as in human leprosy, the mycobacteria may be accompanied by diphtheroids.[2] Such accompanying bacteria might be lost during passaging and preparation of MLM. A difference between strains of MLM with regard

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[23] Lowe, J. Rat leprosy; a critical review of the literature. Int. J. Lepr. **5** (1937) 311–328, 463–481.

[24] Krakower, C. and Gonzalez, L. M. Mouse leprosy. Arch. Pathol. **30** (1940) 308–329.

[25] Marchoux, E. and Sorel, F. La lèpre des rats. Recherches étiologiques et réflexions qu'elles suggèrent a propos de la lèpre humaine. Ann. Inst. Pasteur **56** (1912) 778–801.

[26] Muir, E. and Henderson, J. M. Rat leprosy. Indian J. Med. Res. **15** (1928) 807–817.

[27] Marchoux, E. and Sorel, F. Recherches sur la lèpre. La lèpre des rats (Lepra murium). Ann. Inst. Pasteur **56** (1912) 675–700.

[28] Lampe, P. H. J., de Moor, C. E. and van Veen, A. G. Ratten-lepra. Vijfde mededeeling: "Modderlepra" van witte ratten. Geneesk. Tijdschr. Nederl.-Indië **76** (1936) 2204–2227.

[29] Marchoux, E. and Chorine, V. Cinq bacilles de Stéfansky suffisent pour infecter le rat blanc. Ann. Inst. Pasteur **61** (1938) 296–299.

to skin involvement has been reported,[30] but there are few data on this point.

There is no evidence that the manifestations of the disease are different in mice as compared to rats. One group of investigators[9] found murine leprosy in 5% of *Mus musculus*, which is about the same percentage that has been found in several studies in rats. Spontaneous musculocutaneous murine leprosy has been described in a specimen of wild *Mus musculus*,[8] and experimental disease in mice and rats with bacteria from this mouse showed only minor differences between the species. Some experimentally inoculated mice developed distant skin lesions resembling those in spontaneous disease.[24] With rats, it has been found that certain rat strains are relatively resistant to visceral infection and develop leprosy more like human leprosy and natural musculocutaneous murine leprosy with skin involvement; other rat strains show little tendency for the skin to be affected.[31] Nude mice[32] and hairless mice[33] develop extensive skin involvement after experimental MLM inoculation, which indicates that the immune system and possibly other genetic factors are important in determining whether or not involvement of the skin develops.

Methodological considerations with regard to our work

Mycobacterium lepraemurium. *M. lepraemurium* (MLM) is a slender, rod-like, acid-fast bacillus with quite characteristic morphological features in a preparation stained for acid-fastness.[14] Because MLM could not be grown *in vitro*, MLM traditionally has been propagated by animal passages after isolation from infected wild rats or mice. Each isolate gave rise to a separate strain of MLM. Some differences between strains of MLM have been reported.[30] For our work, we have used the Douglas strain of MLM, isolated in 1922 from a wild rat at Winchester, England.[34] In our laboratory, the bacteria were passaged in low-resistant, inbred C3H mice which gave high yields of bacteria of uniform and good quality with regard to staining characteristics and infective capacity. No growth of mycobacteria has been seen after seeding of standard microbiological media with MLM. A different group of investigators employing the C3H-C57BL model have used C57BL mice for passaging the bacteria, with experimental results in full agreement with ours.[35]

Measuring protective immunity. Protective immunity in murine leprosy means an acquired, immunologically specific increase of the host's capacity to limit bacterial multiplication and dissemination as compared with a normal nonimmune individual. Protective immunity against MLM infection has been shown to be cell mediated.[36] Protective immunity is not an all-or-none phenomenon. Complete protective immunity, that is, complete eradication of the infection with killing of all the bacteria, probably never occurs in murine leprosy.[37] Partial protective immunity has a range of expressions, from a modest reduction of bacillary growth and dissemination to complete bacteriostasis and even a reduction of bacterial numbers, along with more or less complete inhibition of bacterial dissemination.

Severity of clinical disease and altered survival time are expressions of the overall result of host-parasite interactions, and do not allow for analysis of the immunological mechanisms involved. Therefore, we chose enumeration of bacteria as the best way to

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[31] Hanks, J. H. and Backerman, T. The tissue sites most favorable for the development of murine leprosy in rats and mice. Int. J. Lepr. **18** (1950) 185–207.

[32] Kawaguchi, Y., Matsuoka, M., Kawatsu, K., Homma, J. Y. and Abe, C. Susceptibility to murine leprosy bacilli of nude mice. Jpn. J. Exp. Med. **46** (1976) 167–180.

[33] Packchanian, A., Emery, R., MacDonald, E. M. and Rigdon, R. H. Experimental leprosy with *Mycobacterium lepraemurium* in hairless mice (*Mus musculus*). Trans. R. Soc. Trop. Med. Hyg. **76** (1982) 183–186.

[34] Balfour-Jones, S. E. B. The experimental transmission of rat leprosy to the golden hamster (*Cricetus auratus*). J. Pathol. Bacteriol. **45** (1937) 739–744.

[35] Kawaguchi, Y., Matsuoka, M. and Kawatsu, K. Susceptibility to *M. lepraemurium* of CBA, DBA, and C3H mice. Jpn. J. Lepr. **51** (1982) 57–64.

[36] Alexander, J. Adoptive transfer of immunity and suppression by cells and serum in early *Mycobacterium lepraemurium* infections of mice. Parasite Immunol. **1** (1979) 159–166.

[37] Løvik, M. and Closs, O. Survival of *Mycobacterium lepraemurium* in C57BL mice after acquired protective immunity. Clin. Exp. Immunol. **57** (1984) 115–122.

measure protective immunity. Mycobacteria remain acid-fast long after they are dead and, ideally, one would like to discriminate between live and dead bacteria. MLM cannot be grown quantitatively from small inocula, and the morphology of the bacteria after acid-fast staining has been widely used as a measure of viability. Newer methods include staining with fluorescein diacetate and ethidium bromide[38] and radioactive labeling.[39] However, the reliability of the morphological method has been disputed,[40] and all of these methods are hampered with uncertainties or are impractical for use on a large scale.

We enumerated bacteria by counting acid-fast organisms in tissue homogenates, using a slide technique[41] and acid-fast staining with auramin. Fluorescent dye staining is excellent for study of the morphological details of the microbes,[42] has been found to be specific for acid-fast bacteria,[43] and numerous publications support the notion that auramin staining is the most sensitive and also the most convenient staining technique for the detection of acid-fast bacteria.[44]

For challenge infection, we injected the bacteria into one hind foot pad, which offers the advantage of being an anatomically well-defined site with a well-defined route of lymphatic drainage. Restriction of bacterial dissemination clearly is an important aspect of protective immunity, and the importance of studying bacterial dissemination soon became clear in our experiments. BCG immunization of C3H mice tended to increase bacterial numbers at the site of MLM inoculation, whereas bacterial dissemination was reduced.[45] Further, although there was good agreement between bacterial growth in the inoculated foot pad, the popliteal lymph node, and the liver and spleen in C3H and C57BL mice, some other strains of mice behaved differently. A/Sn mice and mice from other strains of high or low susceptibility were examined. A/Sn mice were found to be the most susceptible strain according to bacterial numbers in the foot pad, of intermediate susceptibility according to bacterial counts in the popliteal lymph node, and the most resistant strain according to bacterial counts in the spleen and liver (Løvik and Closs, unpublished data). Thus, resistance to MLM infection should not be determined by bacterial counts in one organ alone.

Measuring delayed-type hypersensitivity (DTH). Traditionally, DTH is measured by determining the diameter(s) of erythema or swelling in the skin 24 or 48 hr after injection of specific antigen. The foot pad swelling technique makes it possible to measure DTH to soluble antigen, local reactivity to MLM bacteria, and resistance to infection in the same anatomical site. To measure DTH, local reactivity to MLM bacteria, and resistance to infection in the same anatomical site should be considered important in work exploring the relationship between these manifestations of antibacterial immunity.

A number of different instruments and techniques have been used to measure DTH in the mouse foot pad. The type of instrument used to measure DTH and, in particular, the pressure exerted during measurement can markedly influence the result of kinetic studies on DTH reactions.[47] We

[38] Jarnagin, J. L. and Luchsinger, D. W. The use of fluorescein diacetate and ethidium bromide as a stain for evaluating viability of mycobacteria. Stain Technol. **55** (1980) 253–258.

[39] Drutz, D. J. and Cline, M. J. Incorporation of tritiated thymidine by leprosy bacilli in cultures of human lepromatous macrophages. J. Infect. Dis. **125** (1972) 416–419.

[40] Desikan, K. V. Correlation of morphology with viability of *Mycobacterium leprae*. Lepr. India **48** (1976) 391–397.

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[42] Truant, J. P., Brett, W. A. and Thomas, W. Fluorescence microscopy of tubercle bacilli stained with auramine and rhodamine. Henry Ford Hosp. Med. Bull. **10** (1962) 287–296.

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[46] Deleted in proof.

[47] van Loveren, H., Kato, K., Ratzlaff, R. E., Meade, R., Ptak, W. and Askenase, P. W. Use of micrometers and calipers to measure various components of delayed-type hypersensitivity ear swelling reactions in mice. J. Immunol. Methods **67** (1984) 311–319.

measured foot pad swelling with a modified dial-gauge caliper,[41] which exerts an even pressure throughout the range of measurement and causes a slight compression of the swollen foot pad. This, supposedly, favors the detection of tuberculin-type DTH reactions which are more indurated than the softer Jones-Mote DTH reactions.

Summary of our own work. Inbred C3H mice have been found highly susceptible to MLM infection; whereas C57BL mice are relatively resistant to subcutaneous infection.[6, 7] Our aims were to study the immune response to live MLM and noninfective MLM material (dead antigen) in C57BL and C3H mice and to induce protective immunity against MLM in the susceptible C3H strain. Further, we wanted to explore the mechanisms responsible for the apparent absence of cell-mediated immune reactivity against live MLM in C3H mice.

First, we demonstrated that DTH to an ultrasonicate of MLM (MLMSon) under certain conditions is a naturally occurring phenomenon during MLM infection in C57BL mice. The development of DTH to MLMSon after immunization of C3H and C57BL mice with MLMSon in Freund's incomplete adjuvant (FIA) was described, and the conditions for induction of DTH and elicitation of a DTH reaction to MLMSon were explored. DTH induced in C3H mice by MLMSon in FIA was shown to be cell mediated and to conform to common criteria for tuberculin-type DTH. Thus, MLM-reactive lymphocytes, presumably T cells, are present in C3H mice, and a vigorous immune response could be induced in C3H mice as demonstrated by immunization and testing with dead MLM antigen. However, a difference in maturation of DTH in C3H and C57BL mice was suggested, and C57BL mice showed somewhat greater reactivity than C3H mice to MLMSon. This could be due to stronger reactivity to some antigenic components in C57BL mice, reactivity to a broader range of antigens, or a greater capacity for nonspecific inflammation in C57BL mice.[48]

C3H mice were then immunized with MLMSon, and DTH to MLMSon, local reactivity to live MLM, and bacterial growth were studied for 11 weeks after challenge infection with live MLM. Induction of DTH to MLMSon was not accompanied by induction of local reactivity against live bacteria, nor did such reactivity develop during infection. MLM infection neither induced nor suppressed DTH to MLMSon. Bacterial multiplication and dissemination was the same in immunized and normal mice during the first 11 weeks of infection and was, therefore, not affected by immunization with MLMSon. Thus, in C3H mice there was a clear dissociation between DTH to soluble MLM antigens and reactivity to live bacteria, as well as between DTH and protective immunity. Heat-killed, morphologically intact MLM in FIA induced an immune response that appeared to have the same qualities as the immune response induced by MLMSon, i.e., DTH to MLMSon, but no reactivity to live MLM, and no protective immunity was induced. This indicates that the preparations with dead MLM antigen, water-soluble or particulate, induced an immune response against antigenic determinants not exposed by MLM-infected cells.[49, 50]

Prolonged observation (20 weeks) showed that in C3H mice immunized with MLMSon, after several weeks of exposure to live MLM, mechanisms became active that retarded bacterial multiplication and dissemination. The findings were compatible with the development, some weeks after infection, of a weak, protective immune response in immunized mice. Although the effects were small, experiments in nonimmunized mice also suggested that MLM infection induced some reduction of susceptibility to reinfection. Finally, the growth curve for MLM in primary infection in normal C3H mice tended to flatten out after

[48] Løvik, M., Haugen, O. A. and Closs, O. Delayed-type hypersensitivity after immunization with ultrasonicated *Mycobacterium lepraemurium* in C3H and C57BL mice. Scand. J. Immunol. **20** (1984) 227–235.

[49] 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. Suppl. **24** (1980) 224–227.

[50] 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.

some weeks, and it did so at approximately the same level (in terms of bacterial numbers) after inoculation with high and with low doses of MLM. Thus, several pieces of evidence suggest that C3H mice can develop some sort of rudimentary protective immune response after infection with MLM, and MLMSon immunization enhances this response in much the same way (but possibly by different mechanisms) as shown for C57BL mice (see below), clearly resulting in some protection. The protective mechanisms require exposure to live MLM for their induction.[51]

Immunization of C57BL mice with MLMSon in FIA induced DTH that showed characteristics typical of tuberculin-type DTH. Immunization with MLMSon induced no manifest protective immunity and only very weak local reactivity against an inoculum with live bacteria, but the development of protective immunity and strong local reactivity was accelerated in immunized mice. The onset of protective immunity in all experimental situations in C57BL mice was accompanied by local reactivity to live MLM. Live bacteria induced protective immunity and strong local reactivity against live bacteria, but no significant DTH against MLMSon. Apparently, also in C57BL mice live MLM and MLMSon induced immunity with different antigen specificities. Strong reactivity to live MLM and protective immunity developed simultaneously in normal mice as well as in MLMSon-immunized mice, and seemed to require exposure to live MLM for their development.[52] The results of experiments in C57BL mice thus confirmed the results of experiments in C3H mice with regard to the relationship between DTH, local reactivity to live MLM, and protective immunity.

Further studies in C57BL mice confirmed and extended the findings from the previous investigation of the capacity of live MLM and dead MLM antigen (soluble antigen as well as whole bacteria) to induce immunity and elicit immune reactions. Live MLM was found to induce strong local reactivity against live MLM. In contrast, under the experimental conditions used, little or no local reactivity (DTH) against soluble antigen (MLMSon) and whole killed bacteria could be detected in mice immunized with live MLM. Vice versa, MLMSon and killed whole bacteria induced local reactivity against MLMSon and to some extent against killed bacteria; whereas only weak reactivity was detected against live bacteria in mice immunized with MLMSon, and no reactivity at all was seen against live MLM in mice immunized with morphologically intact, killed MLM. Thus, also in these extended experiments live MLM and dead MLM antigen appeared to induce immune responses that had largely different specificities, and these antigens were found to share antigenic specificity only to a barely detectable degree when used to elicit local reactions.[53]

To directly address the question of whether DTH to soluble bacterial antigen is a manifestation of protective immunity, repeated DTH reactions against MLMSon were elicited in the infected foot pads of C3H mice immunized with MLMSon. The multiplication and dissemination of MLM in C3H mice were not affected by repeated DTH reactions against MLM antigens. Thus, the experiments provided further evidence that DTH to MLMSon and protective immunity are separate phenomena.[54]

We then further explored the nature of protective immunity against MLM. By three types of experiments, namely, cortisone treatment of infected mice, transfer of MLM from C57BL to C3H mice, and long-term observation of infected C57BL mice, it was shown that MLM is only slowly inactivated in C57BL mice after the onset of protective immunity, and sterile immunity was not

[51] Løvik, M., Closs, O. and Haugen, O. A. Immunization of highly susceptible C3H mice with ultrasonicated *Mycobacterium lepraemurium* (MLM) bacilli facilitates the development of increased resistance during MLM infection. Int. J. Lepr. **52** (1984) 351–361.

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[53] Løvik, M. and Closs, O. Induction of immunity against live *Mycobacterium lepraemurium*: a requirement for viable bacilli? Immunology **53** (1984) 165–173.

[54] Løvik, M. and Closs, O. Repeated delayed-type hypersensitivity reactions against *Mycobacterium lepraemurium* antigens at the infection site do not affect bacillary multiplication in C3H mice. Infect. Immun. **36** (1982) 768–774.

achieved in any of the experiments. Reactivation of the infection—several months after the onset of protective immunity—was demonstrated in long-term observation experiments. Thus, even a brisk immune reaction must last several days and possibly weeks in order to affect MLM viability to a detectable degree, and persistent alertness of the immune system appears necessary to prevent reactivation of the infection.[37]

Different substrains of C3H and C57BL mice would have to be used for the study of the expression of DTH, local reactivity to MLM, and protective immunity in genetic variants and recombinants of the two strains. Also, there were discrepancies between our experimental results and the results from one other laboratory with regard to which mouse strain was the most susceptible to MLM.[55] Therefore, MLM infection in C3H and C57BL substrains was studied. Two C3H substrains examined were both found to be distinctly more susceptible to MLM infection than two C57BL substrains, so C3H and C57BL substrain differences could not explain the aforementioned discrepancy. However, considerable substrain differences were seen, which would tend to make results with genetic-variant strains and recombinant strains based on two of the substrains less clear than results with our original C3H and C57BL mice. Interestingly, the two C3H substrains showed considerable difference in MLM multiplication, although they both lacked a local reaction in the inoculated foot pad. This indicates that some protection can be effected without concomitant local reactivity, and suggests that there may be more than one mechanism of resistance against MLM.[56]

Studies in C3H and C57BL inbred mouse strains, F1 hybrid and backcross mice, and B×H recombinant inbred strains were then performed. Local reactivity to live MLM could be separated from protective immunity, and local reactivity appeared to be necessary but not sufficient for immunologically mediated resistance to MLM infection. Dominant gene(s) linked to the H-2 complex on chromosome 17 were found to influence the local reaction to live MLM in the mouse strains tested, and the same gene(s) also influenced host restriction of MLM multiplication. This suggests a role for immune-response genes in determining the course of infection after inoculation of C3H and C57BL mice with MLM,[57] and our results represent the first demonstration[58] in an experimental model that major histocompatibility complex (MHC)-linked genes influence resistance to bacterial infection.

Summary of our findings. 1) DTH to soluble MLM antigen could be dissociated from protective immunity against MLM infection. However, protective immunity was always accompanied by local DTH-like reactivity to live MLM. 2) The major difference between C3H and C57BL mice was the greatly different capacities of the two strains to respond to live MLM. The two mouse strains were similar in their response to dead MLM antigen. 3) There appeared to be a fundamental difference in terms of antigen specificity and protective capacity between the immune responses to live MLM and dead MLM antigen. Protective immunity was induced only by live MLM.

Discussion

Relationship between acquired protective immunity and DTH. The question of the relation of DTH to acquired protective immunity has been a controversial one almost since Koch's original observation on tuberculin hypersensitivity. A direct demonstration that DTH can kill the microbes in question would help to bring an end to the controversies. However, the only study of this type with mycobacteria appears to be our own with MLM,[54] which showed that

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repeated DTH reactions against MLM antigen at the site of MLM infection did not affect MLM multiplication, and bacterial dissemination was, if anything, enhanced in mice with DTH reactions. These results are in disagreement with studies on the effect of DTH on growth of *Listeria monocytogenes* in the mouse foot pad.[59, 60] *L. monocytogenes* is, however, exquisitely easily killed by macrophages. A large proportion of an inoculum with *L. monocytogenes* is killed even by normal macrophages,[61] and variations in the natural level of macrophage activation greatly affect growth of the bacterium in nonimmune mice.[62] An influx of normal macrophages to a DTH-reaction site is, therefore, likely to exert some restrictive effect on the growth of *L. monocytogenes* without representing a major mechanism of immune protection. On the other hand, an influx of permissive macrophages might enhance MLM infection, and some of our data[51] can be interpreted as support for this view.

On the cell level, the evidence is conflicting even after the introduction of T-cell surface markers, genetic restriction studies, and T-cell cloning. It has been reported that individual T-cell lines or T-cell clones can mediate DTH, B-cell help, and protective antilisterial immunity,[63] or DTH and antiviral cytotoxicity,[64] or specific allohelp, cytolysis, and DTH.[65] However, the effects with *L. monocytogenes* were small and must be interpreted with caution. Further, it is uncertain to what extent *in vitro*-derived cells correspond to *in vivo*-derived cells in their functions and expression of membrane markers, because cloned T cells and T-cell lines may be deregulated, and clearly are different from *in vivo*-derived cells in some aspects.

A number of recent studies support the contention that DTH against soluble antigen and protective immunity are separable phenomena. The genes that restrict cells mediating DTH and protective immunity in some cases map to different regions of the MHC complex.[66, 67] Studies with tuberculosis[68] and listeriosis in rodents[69, 70] have shown that *in vivo*-derived cells capable of transferring DTH to naive recipients have the helper/inducer cell phenotype and transfer DTH but little or no protection; whereas the cells transferring protective immunity have the suppressor/cytotoxic cell phenotype and apparently do not transfer DTH. It should be noted that DTH to soluble antigen and antibacterial protection in the studies referred to were determined in

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the skin and in the lungs and spleen, respectively, and one cannot from these studies conclude that cells mediating protective immunity do not also mediate DTH-like reactivity against the infecting bacteria. The most recent data indicate that with *L. monocytogenes* protection is critically dependent on granuloma formation mediated by Lyt 2+ H-2 K restricted T cells.[71] Direct macrophage activation is mediated by MHC class II restricted T cells that have little protective capacity, but instead exert an enhancing function on the protective immune response, possibly by promoting the congregation of T cells and macrophages at sites of bacterial invasion.[69] Thus, it appears that viable *L. monocytogenes* and viable BCG, much like influenza virus,[72] excite the formation of at least two distinct populations of mediator T cells—one population apparently mediating DTH to dead microbial antigen but little or no protection, the other population mediating protective immunity and, in some instances, DTH-like reactivity against live microbes. Indeed, it has recently been reported that T-cell hybridomas from mice infected with BCG fall into two populations. Most of the hybridomas react exclusively with either live BCG or with PPD, with only a few cells responding (*in vitro*) to both antigens.[73] This supports our own findings with MLM, that the dissociation between DTH and protective immunity appears to be due to a difference in the antigen specificities of the cell populations responding to live MLM and to dead MLM skin test antigen, and that the DTH reaction elicited by the latter type of antigen by itself has little or no protective capacity.

Our experiments further indicate that MLM is relatively resistant to direct inactivation by macrophages,[37, 54] and granuloma formation may be of utmost importance for lasting, protective immunity. This would be in agreement with our finding that local reactivity to live MLM, i.e., immune granuloma formation, is necessary but not sufficient for protective immunity against MLM.[57] It follows that DTH may be an inappropriate model of events in a focus of infection, because different subsets of T cells with different functional characteristics mediate DTH-like reactivity.

Genetic basis for the difference between C3H and C57BL mice. Our genetic studies[56–58] served two purposes. First, the breeding experiments provided information on whether local reactivity to MLM and protective immunity are separable entities. Second, the experiments provided information on the genetic regulation of the immune response against MLM.

In our work, it is evident that a major difference between C3H and C57BL mice lies in the very different capacity of these strains to respond to live MLM; whereas the immune response to dead MLM antigen in the two mouse strains is quite similar. An analogous situation may exist in *L. tropica* infection. In this infection, macrophages of the susceptible BALB/c phenotype present dead parasite antigen to T lymphocytes, but are unable to present antigen from live, infecting parasites.[74] C3H antigen-presenting cells apparently do present dead MLM antigen, otherwise a cell-mediated immune response could hardly be induced;[48] whereas it is uncertain if antigen from live MLM is presented. Antibody responses in C3H mice during MLM infection[75] may be directed against antigens from dead MLM or against T-independent antigens.

Evidence exists that macrophage function is different in C3H and C57BL mice, if not in antigen presentation, in that the natural

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anti-MLM activity of C3H macrophages is greater than the natural activity of C57BL macrophages. C3H macrophages produce more H_2O_2 in response to live MLM than do C57BL macrophages.[76, 77] Further, after s.c. as well as intravenous (i.v.) inoculation, MLM growth during the pre-immune phase of infection is faster in C57BL mice than in C3H mice.[41, 78] This has been attributed to a generally greater antimicrobial capacity of C3H resident macrophages, associated with the so-called Bcg-gene located on chromosome 1. This gene has been found to influence resident macrophage natural antimicrobial capacity against a number of intracellular parasites.[79] C3H mice have the resistant phenotype and C57BL mice the susceptible phenotype in this system.[80] The general importance of the Bcg-gene remains unclear,[81, 82] because the pattern of resistance is turned upside down if MLM is inoculated into C3H and C57BL mice s.c. instead of i.v.[78] This may be taken as evidence that after i.v. inoculation the course of infection is largely determined by mechanisms of natural (macrophage) resistance; whereas after s.c. inoculation the outcome is to a greater extent determined by the acquired protective immune response. A similar reversal of the pattern of resistance is found with blastomycosis in C3H and DBA mice.[83] An inverse relationship between macrophage activity and acquired immunity may have several different causes.[62, 84]

Genetic factors have been shown to play a limited role in human leprosy. We have found that in murine leprosy local reactivity to live MLM is greatly influenced by genes located in the H-2 complex,[57, 58] which indicates that Ir genes are likely to be involved in immune responsiveness to MLM. As discussed below, some of our results may be taken as evidence that the number of target antigens for local reactivity to live MLM and protective immunity is small. This increases the likelihood that the expression of immune responses to the crucial antigen(s) for protection against MLM infection could be regulated by Ir genes, and we did, indeed, find that antibacterial resistance is also influenced by genes in the H-2 complex.[57, 58] This finding has been confirmed by others.[81, 85] However, this does not mean that protective immunity itself is regulated by Ir genes. Local reactivity to live MLM can occur without protective immunity,[57] and the association between certain H-2 phenotypes and protection against MLM infection may be only a secondary effect of the association between H-2 type and local reactivity, local reactivity being necessary but not sufficient for protection. Protective immunity may, therefore, have its own separate, genetic regulation.

The fact that there is a spectrum of clinical manifestations of murine leprosy suggests that resistance to MLM is polygenic. The results from experiments with C3H

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[86] Deleted in proof.

substrains[56, 87] support this contention by demonstrating clearly different levels of resistance in mouse substrains of the same H-2 type, but with a less pronounced difference than what is shown by C3H and C57BL mice. Our studies with C3H-C57BL backcross mice and recombinant inbred strains corroborate this by demonstrating not only a marked effect of one gene or a closely linked group of genes in the H-2 complex, but also a clear influence from other factors, among them background genes.[57] This is in agreement with results from another laboratory.[81, 88] Finally, our experiments indicate that certain genetic combinations of C3H and C57BL mice are even more susceptible or resistant, respectively, than the progenitor strains[57] (and Løvik and Closs, unpublished data). Thus, it appears that the outcome of MLM infection is determined by interactions between a number of factors, which may influence resistance positively or negatively.

Live bacteria and dead antigen. Resistance to intracellular bacteria can, with the possible exception of *M. leprae,* only be achieved effectively by immunization with viable organisms. With killed bacteria little or no protection is achieved unless very high doses or special antigen preparations and adjuvants are used. Our results are in agreement with the classical findings as far as the inability of dead antigen to induce protective immunity is concerned, and they extend the classical findings by demonstrating that live MLM and dead MLM antigen functionally appear to have different antigenic specificities.

MLM for our experiments was grown in C3H mice, which do not develop a protective immune response of any magnitude. Antigen production by mycobacteria may be influenced by the conditions under which the bacteria grow and, formally, we do not know whether the antigens necessary for the protective immune response are produced by bacteria during growth in C3H mice. However, we must assume that this is the case, and the bacteria clearly elicit a protective immune response in C57BL mice.

The interaction of live and dead antigen with antigen-presenting cells may differ in a number of ways. Live and dead antigen may be taken up by different subsets of antigen-presenting cells, and it appears that antigen-presenting cells may select different subsets of T cells for interaction, or in some other way select the type of T-cell response that is to be triggered.[89] Further, the cellular handling of live MLM and dead antigen may differ. With some microbes, intracellular handling of the microbe by macrophages clearly is different, depending upon whether the microbe is live or dead,[90] but there is no direct evidence on this point for MLM.

The chemical nature of the specific antigens and adjuvant components involved in the generation of protective immunity is not known, and damage or loss of the relevant antigen(s) and adjuvant components during bacterial killing and antigen preparation clearly may be one explanation for the failure of dead antigen to induce protective immunity in our experiments. Live MLM may have some sort of coating, either host-derived or parasite-derived, that permits only a very limited number of protection-relevant antigens to be seen by the host; whereas a number of other antigens, among them the components present in dead antigen preparations, remain hidden from the host. Because of instability and special chemical properties, or special requirements for adjuvant activities, these protection-relevant antigens are not present in an immunologically active form in the sonicate or on killed bacteria. In support of this hypothesis are the following observations: a) the electron-microscopic demonstration of a capsule around MLM in infected cells. This capsule is easily lost during preparation of MLM.[91]

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b) There tends to be a lag period before a local reaction develops after injection of live MLM into immune mice, and the reaction then increases gradually over a period of 10–14 days.[53] This could, in part, be due to a requirement for production of the relevant antigens after injection of the bacteria. This lag period appears to be longer after extensive washing of the bacteria (Løvik, unpublished data). Further, MLM stored on liquid nitrogen show a normal capacity for multiplication, but local reactivity to frozen MLM develops substantially later than reactivity to freshly prepared bacteria (Løvik, manuscript in preparation). c) The dissociation between reactivity to live MLM and dead antigen is most easily explained if one type of antigen displays only one or very few antigenic determinants.

There is a possibility that the target antigen(s) for protective immunity and local reactivity to live MLM are present only during a limited phase of the life cycle of MLM, for example in relation to bacterial cell division. MLM is an intracellular parasite, and a comparison with viral infection may be appropriate. Cytomegalovirus causes a latent infection in the mouse. It has been shown that an unexpectedly high proportion of cytotoxic lymphocytes in this infection are directed against cells that synthesize so-called immediate early proteins, which indicates an immunodominant role of viral nonstructural proteins associated with replication.[92] If an analogous situation exists with MLM, this could explain the virtual absence of the protective antigens in MLMSon,[50, 52, 53] the superior immunogenicity of live MLM and the apparent absence of protection-related antigen from heat-killed MLM,[50, 53] and an apparent lack of antigenicity of dormant, live MLM.[37]

Finally, it should be remembered that with live intracellular microorganisms, microbial antigen is supposedly exposed on the surface of cells other than the professional antigen-presenting cells. The ability to detect and destroy microbes in such cells, carrying MHC class I but not class II antigen, should be important for host protection also in MLM infection. Possibly, the difference between live MLM and dead MLM antigen is not a difference in the nominal antigen(s) involved but, instead, a functional difference in the antigens as seen by T cells caused by a difference in antigen presentation. T cells do not recognize the nominal antigen alone, and respond to antigen only in association with cell surface molecules coded for by MHC genes. It appears that the T lymphocytes mediating DTH to dead antigen in mycobacterial, listerial, and viral infections are restricted largely by MHC class II antigens; whereas the T lymphocytes mediating DTH-like reactivity and/or protective immunity to live microorganisms are restricted by MHC class I antigens.[67, 68] The intriguing possibility exists that an important difference between live and dead antigen in general is a difference in the capacity of these antigens to induce a MHC class I or a MHC class II restricted response, and an increasing amount of experimental data supports this hypothesis.

Clearly, to be able to produce effective nonliving vaccines against intracellular bacteria, a more complete understanding of the questions discussed here is needed. At present, on the background of the findings discussed above, it seems that to obtain the desired protective immune response and not only DTH or a positive lymphocyte proliferation test, it may be necessary to introduce genes coding for protection-relevant antigens of pathogenic mycobacteria into some other intracellular microorganism that can be used live for vaccination.

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