The Ecology of Mycobacteria: Impact on Animal's and Human's Health

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Springer

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Cover image: Mycobacteria (slightly bent, short rods) on the surface of hyalocytes in the grey layer of Sphognum magellanicum (Photo K. Muller)

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Preface to the Second Edition

A decade has passed since the primary literature sources were collected and the first edition of this book written. This period of time seems to be relatively short when one considers that mycobacteria were first reported 100 years ago. On the other hand, the known range of mycobacteria has been greatly extended in recent years. The introduction of molecular biology methods has brought about a remarkable burst in the description of new species. While about 70 mycobacterial species were registered at the time of the first edition, more than 130 of them are known at present. With the discovery of new mycobacterial species, the cases of human and animal immunocompetent and immunosuppressed hosts and the isolation of mycobacteria with the enzymatic potential to cause the degradation of aliphatic organic substances are increasing in numbers almost as rapidly.

In order to be able to cover all of the most significant mycobacterial species, it was necessary to consider the ecology of mycobacteria as a discipline that would not only include the external environment but also the occurrence of mycobacteria in animal and human organisms, where interaction occurs. The environment is neither non-living nor static, but the very opposite. It undergoes periodic and other changes (seasons of the year, changing biotic and abiotic factors), while animal and human organisms have the static tendency towards a status *quo ante*. The classification of mycobacteria into respective disciplines such as epidemiology, epizootiology, immunology and environmental ecology did not contribute to a comprehensive understanding of their significance. Therefore, in this book mycobacteria are presented as a whole, under the general designation of mycobacterial ecology, and without limitation by any particular discipline.

This enabled us to concentrate our attention on the genus *Mycobacterium* in all kinds of environments in which they can live, i.e. in macro-organisms as well as in nature. Special attention was paid to the conditions under which mycobacteria can survive, multiply or exist in a dormant state. Of more than 100 species only a few are obligate pathogens for humans and animals. These are unable to grow in the natural environment, but have developed special strategies for reaching susceptible individuals. Furthermore, potentially pathogenic mycobacteria possess the ability both to multiply in natural environments and to cause diseases. A transitional phenomenon creates mycobacterial species that live in the environment and provoke allergic reactions in animals. The majority of mycobacteria are saprophytic and some of them serve as nutrients for dragonfly larvae.

The phylogeny of mycobacteria indicates that pathogenic species developed from saprophytic ones. There is evidence to suggest that the disturbance of their natural

habitats and the overlapping of these biotopes by humans and animals contributed to the spread of mycobacteria and perhaps to their convergence to pathogenicity.

It was not our intention to present a compendium covering all published results, but rather to issue a “readable” book, which is illustrative and thus focused on the principle facts. The increase in the number of Editors has allowed the sharing of original experiences regarding the ecology of mycobacteria, published here for the first time in some cases. The supplemented edition should serve as a guide to these discoveries and also contribute to an understanding of clinically significant species in human and animal medicine.

Borstel, Germany, January 2009 Jindrich Kazda

Editors' Comments

The editors responsible for the chapters are listed under the title of each chapter. Authors are listed under the titles of subchapters.

The references are listed as they appear in the databases Reference Manager (Thomson Reuters, Philadelphia) as imported from Web of Science (Thomson Reuters, Philadelphia) or PubMed (Medline, NLM Bethesda). A few citations, not indexed, were cited according to the reprints or books available. This principle resulted in minor differences in the titles (not all reference titles are in English, some references have capitalized title words, not all species names are according to the contemporary nomenclature and in italics). Some journals are cited with abbreviated titles, some in full, as available in the source databases. These differences were left in the format of the database.

All photos are collected in Chapter 10, with references to the chapter and subchapter where they are quoted.

To keep the structure of the book some information appears in two or more chapters with respect to the chapter's main field. Readers should not consider this as a duplicity.

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Abbreviations

AFB	acid-fast bacilli, acid-fast bacteria
AFLP	amplified fragment length polymorphism
AFR	acid-fast rods
AIDS	Acquired Immunodeficiency Syndrome
ATCC	The American Type Culture Collection
ATP	adenosine triphosphate
BCG	Bacillus Calmette-Guérin or Bacille Calmette-Guérin
BTEX	aromatic hydrocarbons benzene, toluene, ethyltoluene and xylene
CD	Crohn's disease
CFU	colony forming units
CNS	central nervous system
C/N	carbon and nitrogen ratio
CFTR	cystic fibrosis transmembrane conductance regulator
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
ESD	endosulfan-degrading
ESM	environmental saprophytic mycobacteria
GC-MS	gas chromatography-mass spectrometry
HIV	human immunodeficiency virus
HP	hypersensitivity pneumonitis
HPLC	high-performance liquid chromatography or high pressure liquid chromatography
M.	*Mycobacterium*
MAC	*M. avium* complex
MAI	*M. avium-intracellulare*
MAIC	*M. avium-intracellulare* complex
MAIS	*M. avium-intracellulare-scrofulaceum* complex
MDT	multi drug therapy
MDP	muramyl dipeptide
MPTR	major polymorphic tandem repeat
MTC	*Mycobacterium tuberculosis* complex

MWF	metal-working fluid
NC AFB	non-cultivable acid-fast bacilli
NCTC	National Collection of Type Cultures
NTM	non-tuberculous mycobacteria
NOD	nucleotide-binding oligomerisation domain
OIE	World Organisation for Animal Health
OPM	obligate pathogenic mycobacteria
PAHs	polycyclic aromatic hydrocarbons
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PGL	phenolic glycolipid
PGPR	plant growth-promoting bacteria
PPM	potentially pathogenic mycobacteria
PVC	polyvinylchloride
R.	*Rhodococcus*
rDNA	ribosomal DNA
rRNA	ribosomal RNA
RNA	ribonucleic acid
***rep*-PCR**	repetitive-unit-sequence-based PCR
RFLP	restriction fragment length polymorphism
SIV	simian immunodeficiency virus
TCE	trichloroethylene
TLR	toll-like receptor
TMC	Trudeau Mycobacterial Culture Collection
TNF-α	tumor necrosis factor alfa
USA	United States of America
UK	United Kingdom
US EPA	United States Environmental Protection Agency
UV	ultraviolet
WHO	World Health Organization

Chapter 1

The Chronology of Mycobacteria and the Development of Mycobacterial Ecology

J. Kazda

Introduction

In addition to microbiology, three other sciences, epidemiology, biochemistry and molecular biology, have contributed a great deal to recent advances in our knowledge of the genus *Mycobacterium*. Species from this genus are among the most important micro-organisms and include the causative agents of tuberculosis in humans and animals. The ecology of mycobacteria began to develop early after the discovery of the first pathogenic species and its further development differed from mainstream microbiology.

To illustrate the history of mycobacteria, it is useful to describe the contribution of the aforementioned sciences, especially molecular biology, and to mention several important periods in the development of mycobacterial ecology.

1.1 The Microbiology of Mycobacteria

J. Kazda

1.1.1 The First Pathogenic Mycobacteria

The epoch-making discovery was the description of the causative agent of human tuberculosis by Robert Koch (1882; Photo 1.1). He applied and fulfilled the postulate of Henle, who set criteria under which an isolated micro-organism can be regarded to be the causative agent of a disease (commonly known as "Koch's postulates"). A short time after the discovery of *M. tuberculosis*, further pathogenic mycobacteria were discovered: *M. avium* and *M. bovis* as the causative agents of tuberculosis in birds and cattle, respectively (Lehmann and Neumann, 1896). *M. paratuberculosis* was also found in the lesions of intestinal tuberculosis in livestock (Johne and Frothingham, 1895).

In fact, the first known *Mycobacterium* was not *M. tuberculosis* but *M. leprae,* which was discovered as early as 1873 in Bergen, Norway, by Hansen (1875). Due to the lack of suitable staining methods, Hansen identified this bacterium on a native smear by releasing the intracellular bacilli by treatment with a hypotonic solution. A great hindrance to leprosy research is the fact that *M. leprae* is not cultivable on artificial media. Thus, neither Hansen nor his successors were able to fulfil the Henle postulates until the successful multiplication of *M. leprae* in mouse footpads (Shepard, 1960).

1.1.2 Further Progress in Mycobacteriology

Late in the 19th and at the beginning of the 20th century, further attempts were made to identify mycobacteria as a cause of diseases in humans and animals. An improved staining method using carbolic acid and fuchsin introduced by Ziehl (1882) and Neelsen (1883) enabled the differentiation of acid–alcohol–fast rods from other bacteria. This sometimes led

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to false conclusions as acid-fast bacilli (AFB) were thought to cause syphilis because of their presence in conglomerates of epithelioid cells in patients. They were reclassified by Alvares and Tavel (1885) as belonging to saprophytic mycobacteria and later designated by Lehmann and Neumann as *M. smegmatis* (Lehmann and Neumann, 1896).

The possibility of the occurrence of mycobacteria in food, especially in butter, was a question that interested Robert Koch and was researched in his laboratory by Rabinowitsch (1897). She did not find any contamination with *M. tuberculosis* but found saprophytic mycobacteria in 39.6% of 80 butter samples collected in Berlin and Philadelphia, most of them identified as *M. smegmatis* (Lehmann and Neumann, 1896). The search for further environmental mycobacteria continued and the findings were evaluated by Courmont and Potet (1903), who reviewed the hitherto published contributions concluding that plants and soil can be a source of non-pathogenic mycobacteria. Water was also examined for mycobacteria. Acid-fast rods found in tap water were described as "morphologically similar to the bacilli of Koch" and named *M. aquae* (Galli-Valerio and Bornand, 1927).

1.1.3 The First Potentially Pathogenic Mycobacteria

M. chelonae was the first such *Mycobacterium* discovered at the beginning of the last century (Friedmann, 1903), followed several years later by *M. lepraemurium* (Marchaux and Sorel, 1912).A spontaneous disease resembling tuberculosis described in a ring snake and later in a carp, that was confirmed morphologically and by the isolation of acid-fast bacilli, was named "*M. piscium*" (Bataillon and Dubart, 1897). Aronson found acid-fast bacilli in the granulomatous lesions of a viper and described this strain as *M. thamnopheos* (Aronson, 1929), after describing in 1926 the causative agents of tuberculosis in saltwater fish, which he named *M. marinum* (Aronson, 1926).

In the following decades, there was no remarkable progress in the field apart from the experiments of Haag (1927), who systematically examined 90 samples of soil, grass, litter and skin smears, finding mycobacteria in 72 samples. He designated these as *M. phlei,* "*M. lacticola*", "*M. eos*" and "*M. luteum*". Due to the lack of suitable methods for the differentiation of acid-fast micro-organisms, there were then no remarkable developments in the mycobacteriology field for many years.

The attention paid to environmental mycobacteria considerably increased in the late 1930s following the linking of a "pseudo-primary tuberculous complex" of the skin with an abrasion obtained in a swimming pool (Strandberg, 1937). Similar cases which occurred later were described as "water-borne infections of skin in swimming pools" (Hellerstrom, 1952). The increasing interest that followed resulted in the isolation of environmental mycobacteria as the causative agent of granulomatous skin lesions in humans. The source was found to be the water of swimming pools. The disease was described as swimming pool granuloma and the *Mycobacterium* designated as "*M. balnei*" (Linell and Norden, 1954). A comparison revealed that the strain was identical to *M. marinum* described just in 1926 by Aronson (Bojalil, 1959).

At the same time the skin lesions were observed in Sweden, individual cases of lung tuberculosis caused by mycobacteria different from *M. tuberculosis* were reported in the United States (Beavan and Bayne-Jones, 1931). In 1953 two cases of lung tuberculosis were described from which "yellow bacilli" were repeatedly isolated (Buhler and Pollak, 1953). These strains were later designated as *M. kansasii* (Hauduroy, 1955). Avian mycobacteria isolated from human tuberculosis lesions were described in 1939 (Negre, 1939), followed by *M. intracellulare* (Runyon, 1959) and *M. scrofulaceum* (Masson and Prissick, 1956).

1.1.4 The First Methods for the Differentiation of Mycobacteria

The absence of reliable methods for exact differentiation was the main hindrance in the study of mycobacteria. Interest was focused on three pathogenic species: *M. tuberculosis, M. bovis* and *M. avium.* To differentiate between these, a system of "biological methods" was established using inoculation in three kinds of experimental animals: guinea pigs, rabbits and chickens. Heavy lesions in the lungs, liver and spleen of

the guinea pigs and rabbits indicated *M. bovis*, while *M. tuberculosis* provoked such lesions only in guinea pigs. *M. avium* was pathogenic only in chickens. This overly simplistic method was favoured by many laboratories until the late 1960s and thus resulted in a "low tide" in mycobacterial taxonomy. Only eight new mycobacterial species were characterised in the first 50 years of the last century (Table 1.1).

One of the first attempts to introduce tests, commonly used in the differentiation of bacteria other than mycobacteria, was made by Gordon and Smith (1953). In rapidly growing mycobacteria they included the hydrolysis of starch, gelatine and casein, acid production from carbohydrates, the utilisation of citrate, succinate and malate, nitrate reductase production and NaCl tolerance. The last two tests have shown a good discriminating value and are still used in taxonomy. On the other hand, an improved test for the production of acid from carbohydrates, developed 17 years later, did not come into general use (Bonicke and Kazda, 1970).

Great progress in the differentiation of mycobacteria began in the early 1960s with the keen observation of Ernest Runyon, who proposed a new and simple concept for the differentiation of mycobacteria associated with disease or found in the environment. Besides the obligate pathogenic species, he used the designation of "atypical mycobacteria", which he divided into four groups: photochromogenic, scotochromogenic, nonchromogenic and rapidly growing (Runyon, 1965). The morphological character of mycobacterial cultures was a simple but very useful tool for further orientation. Thus, the interest of microbiologists became focused on this genus and in a short time a variety of mycobacterial strains were isolated and "grouped" in laboratories all over the world.

1.1.5 Biochemical Methods for the Differentiation of Mycobacteria

Compared with other fields of microbiology, biochemical methods for the differentiation of pathogenic mycobacteria were introduced at a relatively late date. Many clinical microbiologists had great difficulty moving on from the simple scheme of biological trials using experimental animals, as mentioned above.

One of the first methods, known as the "niacin test", was developed by Konno et al. (1958). It is based on the selective production of nicotinic acid by *M. tuberculosis* and was used for its confirmation and the differentiation of this species from *M. bovis* and other slow growers, which gave negative results in this test. Due to the progress being made in the eradication of bovine tuberculosis in livestock, there was an urgent need for a simple method differentiating *M. tuberculosis* from *M. bovis.* Such a test was developed by Virtanen (1960). *M. bovis* did not produce nitrate reductase, which is produced by *M. tuberculosis.* Unlike the niacin test, nitrate reductase is not limited to *M. tuberculosis*, but is widely distributed among mycobacteria. A very important system for the differentiation of mycobacteria was introduced in the early 1960s by Bonicke (1962). This method, known as the "amide row", offered a new way of distinguishing newly isolated mycobacterial strains. The results, obtained by the degradation of 10 different amides, enabled the more precise differentiation of mycobacteria and contributed a great deal to the general acceptance of biochemical methods in their microbiology.

Further tests for the differentiation of mycobacteria soon followed, particularly those based on arylsulfatase activity (Kubica and Vestal, 1961), Tween hydrolysis (Wayne, 1962) and the detection of phosphatase activity (Kappler, 1965). These and other tests enabled great progress to be made in the differentiation of mycobacterial isolates from humans, animals and environmental sources. Between 1951 and 1970, 21 new mycobacterial species were isolated and described, many more than before. This trend resulted in more interest in mycobacterial taxonomy. Acid-fast bacilli, which had formerly been isolated but had exhibited different properties to those of already known species, were collected as stock cultures and were examined from 1967 onwards by the cooperative studies organised by Lawrence Wayne in the International Working Group on Mycobacterial Taxonomy. Nearly all laboratories working on the differentiation of mycobacteria joined this group and took part in the evaluation of taxonomic methods and in the testing of selected mycobacterial strains. Their results have become a valuable guide in mycobacterial taxonomy and more than 50 phenotypic properties have been adopted for the evaluation of new species of mycobacteria (Wayne et al., 1996).

Table 1.1 Survey of the development of the genus *Mycobacterium* during the years 1873–2007

Decade	Species and discovery		Origin
	Name	Year	
1871–1880	*M. leprae*	1873	Humans
1881–1890	*M. tuberculosis*	1882	Humans
	M. smegmatis	1889	Environment
1891–1900	*M. avium*[1] [1]	1891	Birds
	M. paratuberculosis[2] [1]	1895	Cattle
	M. bovis	1896	Cattle
	M. phlei	1899	Environment
1901–1910	*M. chelonae*	1903	Poikilotherms
1911–1920	*M. aquae/M. gordonae*	1912	Environment
	M. lepraemurium	1912	Mice
1921–1930	*M. marinum*	1926	Fish
	M. microti	1927	Animals
1931–1940	*M. fortuitum*	1938	Poikilotherms
1941–1950	*M. intracellulare*	1949	Humans
	M. ulcerans	1950	Humans
1951–1960	*M. abscessus*	1953	Humans
	M. kansasii	1955	Humans
	M. scrofulaceum	1956	Humans
	M. paraffinicum	1956	Environment
	M. farcinogenes	1958	Animals
	M. xenopi	1959	Poikilotherms
	M. salmoniphilum	1960	Fish
1961–1970	*M. flavescens*	1962	Environment
	M. peregrinum	1962	Humans
	M. vaccae	1964	Environment
	M. simiae	1965	Animals
	M. diernhoferi	1965	Environment
	M. nonchromogenicum	1965	Environment
	M. parafortuitum	1965	Environment
	M. terrae	1966	Environment
	M. gastri	1966	Humans
	M. triviale	1966	Humans
	M. aurum	1966	Environment
	M. thermoresistibile	1966	Environment
	M. chitae	1967	Environment
	M. africanum	1969	Humans
1971–1980	*M. agri*	1971	Environment
	M. asiaticum	1971	Humans
	M. duvalii	1971	Humans
	M. gadium	1971	Humans
	M. gilvum	1971	Humans
	M. obuense	1971	Environment
	M. rhodesiae	1971	Humans
	M. neoaurum	1972	Environment
	M. szulgai	1972	Humans
	M. aichiense	1973	Environment
	M. chubuense	1973	Environment
	M. senegalense	1973	Animals
	M. tokaiense	1973	Environment
	M. shimoidei	1975	Humans

Table 1.1 (continued)

Decade	Species and discovery		Origin
	Name	Year	
	M. petroleophilum	1975	Environment
	M. malmoense	1977	Humans
	M. haemophilum	1978	Humans
	M. komossense	1979	Environment
	M. sphagni	1980	Environment
1981–1990	*M. fallax*	1983	Environment
	M. porcinum	1983	Animals
	M. austroafricanum	1983	Environment
	M. pulveris	1983	Environment
	M. moriokaense	1986	Environment
	M. chlorophenolicum	1986	Environment
	M. poriferae	1987	Environment
	M. cookii	1990	Environment
	M. avium subsp. *silvaticum*	1990	Birds
1991–2000	*M. alvei*	1992	Environment
	M. madagascariense	1992	Environment
	M. confluentis	1992	Humans
	M. hiberniae	1993	Environment
	M. brumae	1993	Environment
	M. celatum	1993	Humans
	M. genavense	1993	Humans
	M. intermedium	1993	Humans
	M. interjectum	1993	Humans
	M. mucogenicum	1995	Humans
	M. branderi	1995	Humans
	M. conspicuum	1995	Humans
	M. hodleri	1996	Environment
	M. lentiflavum	1996	Humans
	M. triplex	1996	Humans
	M. mageritense	1997	Humans
	M. heidelbergense	1997	Humans
	M. hassiacum	1997	Humans, environment
	M. novocastrense	1997	Humans
	M. canettii	1997	Humans
	M. bohemicum	1998	Humans, animals, environment
	M. tusciae	1999	Environment, humans
	M. wolinskyi	1999	Humans
	M. caprae	1999	Animals
	M. murale	1999	Environment
	M. goodii	1999	Humans
	M. kubicae	2000	Humans
	M. elephantis	2000	Animals, humans
	M. septicum	2000	Humans
	M. botniense	2000	Environment
2001–2007	*M. immunogenum*	2001	Environment, humans
	M. doricum	2001	Humans
	M. heckeshornense	2001	Humans
	M. frederiksbergense	2001	Environment
	M. palustre	2002	Environment, humans, animals
	M. lacus	2002	Environment, humans

Table 1.1 (continued)

Decade	Species and discovery		Origin
	Name	Year	
	M. holsaticum	2002	Humans
	M. vanbaalenii	2002	Environment
	M. shottsii	2003	Environment
	M. pinnipedii	2003	Animals, humans
	M. montefiorense	2003	Animals
	M. chimaera	2004	Humans
	M. cosmeticum	2004	Environment
	M. boenickei	2004	Humans
	M. brisbanense	2004	Humans
	M. canariasense	2004	Humans
	M. houstonense	2004	Humans
	M. neworleansense	2004	Humans
	M. nebraskense	2004	Humans
	M. parascrofulaceum	2004	Humans
	M. parmense	2004	Humans
	M. psychrotolerans	2004	Environment
	M. pyrenivorans	2004	Environment
	M. saskatchewanense	2004	Humans
	M. pseudoshottsii	2005	Animals
	M. florentinum	2005	Humans
	M. colombiense	2006	Animals
	M. aubagnense	2006	Humans
	M. bollettii	2006	Humans
	M. phocaicum	2006	Humans
	M. arupense	2006	Humans
	M. conceptionense	2006	Humans
	M. fluoranthenivorans	2006	Environment
	M. massiliense	2006	Humans
	M. monacense	2006	Humans
	M. kumamotonense	2007	Humans
	M. seoulense	2007	Humans

[1]***MAA*** *M. avium* subsp. *avium.*
[2]***MAP*** *M. avium* subsp. *paratuberculosis.*
[1] Thorel MF, Krichevsky M, Levy-Frebault VV (1990) Int. J. Syst. Bacteriol. 40:254–260.

1.1.6 Molecular, Genetic and Other Methods in Mycobacterial Taxonomy

After the first phenotypic period, the second major trend in mycobacterial taxonomy was focused on genotypic characteristics. In the early 1980s, a method for the estimation of partial sequences of 16S ribosomal RNA was introduced into the taxonomy of mycobacteria (Stackebrandt and Woese, 1981). The genotypic studies confirmed the validity of previous mycobacterial taxa and were effective in the discrimination of variable regions. The gene encoding the 16S rRNA is still the primary target of molecular taxonomic studies (Tortoli, 2006). These techniques resulted in the splitting of some mycobacterial species and in the description of new taxa, also using previously isolated strains or clusters.

On the other hand, the more sophisticated methods in chemotaxonomy achieved new results in the study of mycolic acids, present in the mycobacterial cell wall. The consequence of both of these techniques was an enormous increase in the description of new mycobacterial species over the last few decades. Compared with the "classical area" beginning with the description of the first *Mycobacterium* species, which lasted until the early 1990s, and during which 64 new species were

described, an enormous boom in mycobacterial taxonomy resulted in a total of 67 new species being described between 1991 and 2007. Taking into account the fact that this number also includes about 22 previously isolated strains or clusters, it may represent a source of confusion for diagnostic laboratories and clinicians. These new species of mycobacteria might be associated with specific diseases or occur merely as contaminants. Nearly a quarter of the 67 newly described species originated from the environment or from fauna. The high number of isolates from humans, the most examined tissue samples, does not appear to be in conflict with the claim that human isolates represent only the tip of the iceberg and that the environment is in fact the major source of mycobacteria (Tortoli, 2006). The chronology of the description of the genus *Mycobacterium* is documented in Table 1.1.

1.2 The Ecology of Mycobacteria

J. Kazda

As mentioned above, the ecology of mycobacteria developed parallel to their bacteriology and can be divided into the following stages.

1.2.1 First Steps Towards Mycobacterial Ecology

Occasional examinations of environmental samples, food and poikilotherm, as described above, had an epidemiological or epizootiological character and focused on finding the causative agents responsible for diseases.

The first attempts at ecological studies were made towards the end of the 19th century. Nearly 30 years before the discovery of the causative agent of tuberculosis, new methods of therapy were introduced after the claim was made that the disease was in fact curable (Bremer, 1854). The first sanatorium for the treatment of tuberculosis was founded not in Switzerland but in Germany in Görbersdorf, Schlesien (now Sokolowsko). Its founder, Dr. Bremer, gathered physicians interested in the natural sciences to contribute to his project for the successful treatment of this disease. One of them was Dr. Alfred Moeller, who was also the chief of a bacteriological laboratory. His conviction was that bacteria have a close relation to floral nature. After his unsuccessful search for *M. tuberculosis* he found acid-fast bacilli colonising the surface of *Phleum pratense* (timothy) when incubated in distilled water (Moeller, 1898). It was the first species of saprophytic mycobacteria found in the environment, designated as *M. phlei* (Lehmann and Neumann, 1896).

Moeller extended his examinations not only to other plants but also to crops and the excrements of farm animals and found a broad variety of acid-fast rods. Some of them exhibited pleomorphism and true branching would probably place them in the genus *Nocardia* (Moeller, 1899). Nevertheless, his efforts to search for the causative agent of tuberculosis outside of patients and especially the large spectrum of environmental samples he examined for the presence of mycobacteria can be regarded as the beginning of mycobacterial ecology.

1.2.2 Descriptive or "Statistical" Mycobacterial Ecology

The frequent occurrence of non-specific skin reactivity to mycobacterial antigens in animals and new mycobacterioses caused by environmental mycobacteria further stimulated research into the ecology of mycobacteria. Extensive examinations were carried out by Beerwerth (1967) who developed decontamination with oxalic acid and sodium hydroxide, which made it possible to cultivate mycobacteria even from highly contaminated samples like animal droppings. In almost all samples of cattle droppings, mycobacteria could be isolated. This indicated that fodder and watering places can be sources of mycobacteria. In the latter, mycobacteria were isolated from 44% of the samples (Gerle, 1972).

In a further contribution to the ecology of mycobacteria, Beerwerth and Schurmann (1969) examined a total of 3434 samples of soil, wastewater, fodder and faeces of domestic animals and found mycobacteria in 86.1 and 70.3% of arable and meadow soil samples, respectively. Mycobacteria were isolated from 70.5% of samples of faeces of grazing cattle. This took place

more frequently during the spring when pastures were full of new shoots rather than later in the year when the grass was longer. Furthermore, 36% of arthropods, living in close contact with soil, were positive for mycobacteria (Beerwerth et al., 1979).

Encouraged by more sophisticated methods for the isolation of mycobacteria (Kubica et al., 1963), numerous further attempts were made to find mycobacteria in different kinds of environments. In Japan, several new species of mycobacteria were found in the soil (Tsukamura, 1967). In Australia, the isolation of *M. avium–intracellulare* (*MAI*) from soil, milk and dust and their influence on mycobacterioses in humans were reported (Kovacz, 1962). In South Africa, during an extensive study, the isolation of environmentally derived saprophytic and potentially pathogenic mycobacteria (PPM) was described; they were found in soil, plants and dust, where *M. intracellulare* was particularly prevalent (Kleeberg and Nel, 1973).

1.2.3 Applied Research in Mycobacterial Ecology

Most studies dealing with the isolation of mycobacteria from the environment were based on a high number of examined samples and a statistical evaluation of positive findings. Very little or no attention was paid to biotic and abiotic factors in examined biotopes. This approach often led to a misinterpretation of results, with the result that the impression was created that mycobacteria were ubiquitous in soil and water.

One of the first successful attempts to introduce modern principles to the ecology of mycobacteria can be ascribed to Chapman (1971). To understand the interaction between humans, animals and the environment, he introduced the term "infection as a result of overlapping niches". He showed that environmental mycobacteria are far from being "fastidious" in their nutritional requirements and in their optimum growth temperature and that a variety of them can thrive within a wide pH range (Chapman and Bernard, 1962). They also defined the differences between the niches occupied by obligate pathogenic *M. tuberculosis* and those occupied by potentially pathogenic, environmentally derived mycobacteria and demonstrated that the latter are able to tolerate a variety of metals.

1.2.4 Physiological Mycobacterial Ecology

Detailed studies carried out by J. O. Falkinham and his team included not only the isolation of mycobacteria from environments; their experiments expanded on the conditions under which isolated mycobacteria can thrive. They proposed the term "physiological ecology" to distinguish these experiments from the simple isolation of mycobacteria from the environment. They demonstrated, therefore, that strains of the *M. avium–intracellulare–scrofulaceum* complex (*MAIS*), isolated from water polluted by heavy metals, tolerated higher concentrations of heavy metal salts and oxyanions (Falkinham et al., 1984). The ability to detoxify rather than metabolise metallic and other compounds thus contributes to the colonisation and persistence of mycobacteria in aquatic environments (Falkinham, 1996). This led to an increased interest in the conditions under which mycobacteria can thrive in specific environments.

1.2.5 Molecular Ecology

Similar to epidemiology, the polymerase chain reaction offers a valuable tool for the detection of mycobacteria in the environment, and the DNA fingerprinting technique enabling the distinction of mycobacteria at the single strain level has enabled great progress in the description of their ecology. It has been possible to "follow the routes" of mycobacterial strains not only between habitats but also to determine what kind of vectors contribute to their spreading.

Other sophisticated methods like restriction fragment length polymorphism (RFLP), pulsed field gel electrophoresis (PFGE), repetitive-unit-sequence-based PCR (Rep-PCR) and amplified fragment length polymorphism (AFLP) have allowed the detailed distinction of mycobacteria according to their geographic distribution (Johnson et al., 2000).

1.2.6 The Ecology of Habitat Environments

To understand the dynamics of the distribution of mycobacteria it was necessary to ascertain what types

of environments contribute to their multiplication and/or to the spread of mycobacteria. There are many publications which discuss the isolation of mycobacteria from a variety of different environments, but few describe these biotopes in detail. Furthermore, the examination of such biotopes was very seldom repeated. Such results were not even adequate to distinguish particular biotopes as being either sources or merely vectors of mycobacteria. In addition, the biotic and abiotic factors which enable or exclude the multiplication of mycobacteria in these biotopes must be taken into consideration.

The main biotic factors for heterotrophic mycobacteria include those nutrients that are available or released during the decomposition of complex organic substances or are synthesised by autotrophic micro-organisms. Competition from other organisms, especially those belonging to the family *Enterobacteriaceae*, may result in the rapid consumption of nutrients in heavily polluted aquatic environments. The generation time of *Escherichia coli* is 20 min; it is 4.6 h for *M. fortuitum.* Thus, the "strategy" of mycobacteria results in the colonisation of niches which provide conditions suitable for their multiplication and restrict or exclude other competitive micro-organisms from thriving. Such restrictive factors include the acidity of the environment which benefits the selective multiplication of mycobacteria (Kazda, 2000).

Abiotic factors comprise such physical and chemical conditions as temperature, i.e. the accumulation of solar heat, humidity, pH, adhesion to surfaces (especially tubes) and external capillarity in plants. Such biotopes which harbour favourable conditions for the multiplication and longevity of mycobacteria are regarded as habitat environments for mycobacteria. Sphagnum and bryophyte vegetation belong to this group.

1.3 The Classification of Mycobacteria with Regard to Their Ecology

J. Kazda

In the past, several attempts have been made to divide mycobacteria into groups, first into pathogenic and saprophytic ones and later into anonymous, typical or atypical, opportunist, nontuberculous and others. These designations, useful initially, later brought about confusion due to the different ways in which they were interpreted. If it is generally accepted that the term mycobacteriosis is used to define diseases caused by mycobacteria, then the terms atypical, opportunist or nontuberculous cannot be regarded as appropriate for mycobacterial species causing granulomatous tuberculosis. Thus, it cannot be correct to classify *M. avium* as a nontuberculous *Mycobacterium* because of its ability to cause tuberculosis not only in birds but also in humans.

However, such an immensely large genus as *Mycobacterium*, comprising more than 100 species at present, needs at least a guide for initial orientation. For this purpose, three epithets for dividing mycobacteria are currently proposed.

1.3.1 Obligate Pathogenic Mycobacteria

Obligate pathogenic mycobacteria (OPM) are the most specialised species, causing mycobacteriosis in humans and particular species of animals. They possess a high virulence even in the dormant form for a long time in an infected host. Their survival in the environment is very limited, but they can "descend" to feral animals such as the opossum in New Zealand and the badger in Great Britain and Ireland. The control of tuberculosis in feral animals is very difficult and such animals are the source of *M. bovis* infections in cattle. The airborne transmission of OPM to humans is greatly aided by infected particles, which are less than 5 μ in diameter and small enough to remain suspended in the air over a long period of time. When inhaled, their size allows them to enter the respiratory tract and traverse the ciliated epithelium. Their further development depends on the host–parasite relationship. Possessing high virulence, these infected particles are able to cause disease even with a very limited inoculum.

1.3.2 Potentially Pathogenic Mycobacteria

The main source of potentially pathogenic mycobacteria (PPM) is an environment where they are able to

multiply, but they can also be found in living hosts, where they colonise suitable niches on the mucous membranes. They possess two main properties: the ability to multiply in favourable types of environments and to provoke mycobacterioses in susceptible hosts. They comprise therefore a transitional group between OPM and environmental saprophytic mycobacteria. Their number is increasing due to more sophisticated methods used in their differentiation and especially as a result of severe mycobacterioses in HIV/AIDS patients.

1.3.3 Environmental Saprophytic Mycobacteria

Environmental saprophytic mycobacteria (ESM) form the largest group of mycobacteria. They were isolated originally from several kinds of environments, in particular from sphagnum and moss vegetation, surface and drinking water and soil-containing organic material. Due to their large spread, they are often found in clinical material and were for a long time regarded as contaminants. Their association with *M. leprae*, however, has shown that their presence supports the pathogenicity of the leprosy bacillus when inoculated simultaneously into the footpads of nude mice. Some ESM, particularly *M. cookii* and *M. hiberniae*, can provoke a non-specific sensitisation to tuberculin, used as the test for bovine tuberculosis. These species belong to the group of slow-growing mycobacteria, similar to OPM, but are not pathogenic for experimental animals (Kazda and Cook, 1988; Cooney et al., 1997).

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Chapter 2

Obligate Pathogenic Mycobacteria

J. Kazda and I. Pavlik (Eds.)

Introduction

Obligate pathogenic mycobacteria (OPM), above all *Mycobacterium tuberculosis* complex *(MTC)* species, are most often spread by transmission between a variety of animals and humans. From an aspect of epidemiology, it is important that they can survive outside the host organism for a long time; this "compensates" for their limited ability to multiply outside the host organism. Even though they do not sporulate, they can still be cultured from a damp environment protected from direct sunlight after the lapse of several months or years.

2.1 *M. tuberculosis* Complex Members

I. Pavlik

At present, eight mycobacterial species are classified as *MTC*, seven of which are obligatory pathogenic mycobacteria; the remaining eighth species is *M. bovis* BCG used for vaccination (van Soolingen et al., 1997; Aranaz et al., 2003; Cousins et al., 2003):

M. tuberculosis: causative agent of human tuberculosis throughout the world (Photo 2.1); infections in animals occasional (Photo 2.2).

M. africanum: causative agent of human tuberculosis, above all in the inhabitants of Western Africa; infections in animals rare.

M. canettii: causative agent of infections; diagnosed occasionally in people; this species has not been detected in animals yet.

M. bovis: both animals and people are susceptible hosts (Photos 2.3 and 2.4).

M. caprae: the same host spectrum as for *M. bovis* (Photo 2.5).

M. microti: causative agent of tuberculosis in small terrestrial mammals; occasionally found in other animals and people (Photo 2.6).

M. pinnipedii: causative agent of tuberculosis in pinnipeds; transmissible to people, especially from infected animals kept in captivity (Photos 2.7, 6.38 and 6.39).

M. bovis BCG (vaccine strain): occasionally causes post-vaccination infections in immunosuppressed patients; the strain was used in studies of disinfection effectiveness (Omidbakhsh and Sattar, 2006) or survival of mycobacteria in the environment (Vandonsel and Larkin, 1977).

Multiplication outside the host organisms has not been described for any of these eight *MTC* species. These species are most often shed by infected hosts to the environment relative to the localization of infection; it is usually sputum, faeces and urine in the case of humans, milk from dairy animals (especially cattle) and infected body tissues from other domestic and wild animals. The latter, above all from wild animals, become a part of the food chain for other animals or undergo decomposition on the surface of the earth (e.g. wild ruminants), underground (e.g. small terrestrial mammals) or in water (e.g. pinnipeds).

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Accordingly, information regarding seven obligatory pathogenic species of *MTC* is divided into Sections 2.1.1, 2.1.2 and 2.1.3 with regard to their main hosts.

2.1.1 M. tuberculosis, M. africanum and M. canettii

The primary hosts of these three mycobacterial species are people and human-to-human transmission via contaminated aerosol is viewed as the most important (Section 5.9). However, these species can also be shed into the environment through faeces, urine and other excretions or secretions (Section 5.10). From the point of view of ecology, the most important source of these three mycobacterial species is contaminated sewage water; this was mainly investigated in Europe (in Poland and Germany) in the middle of the 20th century. These studies found that *M. tuberculosis* was occasionally detected in sewage water from cities and hospitals (Section 5.2.7).

The available literature provides little information on the occurrence of these three mycobacterial species in sewage water in developing countries with a high occurrence of these infections in humans. *M. africanum* has been isolated from sewage water from hospitals (Nguematcha and Le, 1978); however, other information on the occurrence of *M. africanum* in the environment is almost non-existent. Accordingly, further research in this area should be focused on the investigation of the environment in different niches with a high prevalence and incidence of human infections caused by the above-mentioned mycobacterial species. In-depth knowledge of the circulation of these mycobacterial species in the environment will definitely help to extend control measures in affected countries.

2.1.2 M. bovis and M. caprae

These two species cause tuberculosis in different organs in their hosts, especially in the lungs (Grange and Yates, 1994; Thoen and Steele, 1995; Grange, 1996; Thoen et al., 2006a). The most common sources of infection are ill animals. The intensity of pathogen shedding via secretions or excretions varies depending on the stage of infection; according to the localization of infection, they are shed mainly by sputum, faeces, uterine excretions, urine, semen, etc. Raw milk and milk products from dairy animals (primarily cattle) are important sources of infection for people. The host organism is usually infected by direct contact with an infected animal. Accordingly, at present, tuberculosis caused by these mycobacterial species and paratuberculosis belong to the most serious chronic diseases in animals held in captivity at high densities (e.g. on farms or in zoological gardens).

The sources of infection are usually purchased animals that are in an early stage of the disease and show negative results to intravital diagnostics (especially bovine tuberculin testing). Another risk factor for the spread of causative agents of these diseases is the presence of reservoir animals such as the European badger (*Meles meles*) in Great Britain and Ireland and the brushtail possum (*Trichosurus vulpecula*) in New Zealand. This factor is generally acknowledged and detailed information can be obtained in the recently published books mentioned above (Grange and Yates, 1994; Thoen and Steele, 1995; Grange, 1996; Thoen et al., 2006b). However, it is not the purpose of this book to perform a detailed analysis of all the information but rather to highlight the ecology of these mycobacterial species.

Various components of the environment can become sources of these causative agents of diseases in animals and humans as well. *M. bovis* was detected in 5 (42%) of 12 samples of manure and in 1 (6%) of 18 samples of scrapings from stable walls (Shindler, 1979). Faeces and urine from infected animals on pastures may be sources of contamination of not only soil but also surface water. Kislenko (1972) reported the survival of *M. bovis* in pasture soil from Novosibirsk in Russia for 23 months (entire monitoring period). However, the time of survival of *M. bovis* in soil was shorter in areas of dry climate and high summer temperatures: only 4 weeks in Australia (Duffield and Young, 1985). *M. bovis* has been detected in drinking water for calves in the areas with bovine tuberculosis occurrence in Great Britain (Little et al., 1982).

It is also well known that *M. bovis* can survive in animal carcasses (Thoen et al., 2006b). However, little is known about the risk of *M. bovis* transmission via vegetation on pastures grazed by infected rumi-

nants and studies focused on this topic are scarce. These risks were highlighted by Kislenko (1972). Kislenko performed experiments on guinea pigs, rabbits and cattle fed with mown grass that originated from pastures contaminated with *M. bovis,* either naturally or artificially (the pasture soil was contaminated artificially with *M. bovis* seven months before the grass was fed to animals). Accordingly, the sources of bovine tuberculosis for different animal species in the endemic regions may be various components of the environment, to which little attention has been paid so far.

The importance of contaminated feedstuffs for the transmission of *M. bovis* between different white-tailed deer animals (*Odocoileus virginianus*) and between white-tailed deer and cattle has been demonstrated in the United States (Palmer et al., 2004a,b). However, considering the ecology of mycobacteria, the question concerning the ability of *M. bovis* to survive in different components of such feeds and its potential ability to multiply remains open. It is well known that if processed fodder (especially green fodder) is left standing for a long time, its temperature may rise and fermentation may occur. The temperatures in such fodder do not usually reach values that would reliably kill mycobacteria. On the contrary, the temperatures reached on the margins of the stacks are often about 37 °C (I. Pavlik and P. Miskovic, unpublished data), which can under particular conditions stimulate the growth of *M. bovis*.

2.1.3 *M. microti* and *M. pinnipedii*

Information on the quality and occurrence of these two causative agents of both animal and human diseases can be drawn from many review articles (Thoen et al., 2006b; Skoric et al., 2007). The hosts of *M. microti* are especially small terrestrial mammals, their predators and other animals and humans (Section 6.7). It was observed as early as the middle of the last century that insectivores and small rodents can encounter *M. microti* via the consumption of food of vegetable and animal origin (Chitty, 1954). The occurrence of *M. microti* in the environment and its ability to survive in different components of the environment remains obscure.

A comparable situation exists for *M. pinnipedii*. This species causes tuberculosis in water mammals and their breeders in zoological gardens and aquatic parks (Thoen et al., 2006b). It can be supposed that the most important transmission route among animals is direct contact with one another. They gather together at the time of rest, periods of mating and parental care. However, the occurrence and ecology of *M. pinnipedii* in the environment also remains obscure.

2.2 *M. leprae:* Obligate or Potentially Pathogenic *Mycobacterium*?

J. Kazda

2.2.1 General Characteristic

The causative agent of leprosy *M. leprae*, was discovered during 1873 in Norway by Armauer Hansen (1875). At that time, leprosy was not restricted to tropical and subtropical countries but also occurred epidemically in Norway. The number of leprosy cases (very high in the tropics during the last century) decreased with the introduction of multi drug therapy (MDT) from 5.2 million in 1985 to 805 000 in 1995 and to 286 000 at the end of 2004 (WHO, 2005).

The target of eliminating leprosy as a public health problem was defined as a prevalence rate of less than 1 case per 10 000 by the year 2000. This target could not be reached worldwide. In Africa, America, South-East Asia, Eastern Mediterranean and Western Pacific, the registered number of leprosy cases stood at 219 826 cases in 2006. Recently, the effectiveness of the WHO programme of leprosy elimination has been questioned (Fine, 2007), because reduction of leprosy cases to 1 per 10 000 population still leaves a region of high population density, such as India, with more than 100 000 leprosy cases. This cannot be regarded as "elimination". The real eradication of leprosy remains an urgent target in the future.

2.2.2 Ecological Aspects

The "traditional" opinion that the sole source of leprosy infection is an untreated patient cannot be generally accepted. Patients treated with MDT are

no longer infectious, but the number of new registered cases is higher than the prevalence. The first hypothesis about the possible role of environmental factors in the spread of leprosy is nearly as old as the discovery of the leprosy bacillus itself. Hansen and Looft (1895) discussed in their book the possibility of the leprosy bacillus occurring outside the human body:

> "Here in Norway where the people often go barefoot, wading in streams and rivers, the back of the feet and the under part of the calves are frequently the seat of the first leprous eruption, not so often in the form of nodules, as of a dense, regular infiltration. . . . although as we stated above, the leprosy bacillus has never been found outside of the human body, this might possibly be dependent on insufficient search."

In fact, the first-known examination for mycobacteria in the environment dates back to 1898 (Moeller, 1898).

At almost the same time, an interesting observation was made in a leprosarium in Reitgjaerdet (Norway) by Sand (1910). In his survey of 1221 leprosy patients, he found that the risk of leprosy was higher in men because they have traditionally more environmental contact than women. In contrast, the transmission of leprosy within families was found to be very low. Of 512 married couples, the transmission occurred in only 3.3% of cases. The transmission to children by a leprous father or mother was noted as occurring in 4.9 and 10.5% of cases, respectively. Furthermore, over the 40 years of his investigation, a transmission of leprosy neither within the leprosarium nor between neighbours occurred. The author concluded that the transmission of leprosy did not generally take place directly between humans but indirectly through a medium. He continued questioning as to what kind of medium was needed for the transmission, whether it was a living organism (parasite) or ground containing decomposing material.

The high occurrence of leprosy in the second half of the 19th and the beginning of the 20th century among the population living on isolated farms in coastal Norway gave rise to the question as to how much the environment could influence the epidemiology of this disease. The National Leprosy Registry of Norway documented those farms where leprosy patients were living. The undisturbed environments with deserted houses and a water supply from that time can even be found (Photo 2.8).

During the 1970s and 1980s of the last century, several expeditions were carried out through coastal Norway including the Naustdal district, formerly with the highest leprosy prevalence and incidence rates (Irgens, 1980). To test whether sphagnum moss vegetation could be linked with the occurrence of leprosy in man, the leprosy status of farms was compared with regard to seven environmental variables. The most critical point was the origin of water supply in sphagnum bogs or other close contact to this vegetation at the time of the 1851–1885 leprosy epidemic. These conditions were found to considerably enhance the risk of leprosy. In the same district, leprosy occurred only on farms situated under the southern slopes, characterized by a high accumulation of solar heat beneath the surface of sphagnum vegetation, over 32 °C for a long time, which can enable the growth of mycobacteria. Another important factor was high humidity in summer months. The average incidence rate of leprosy in districts with a relative humidity over 75% in July was 12.4 compared with 0.7 in another district and the ratio of risk was 17.7.

In the former leprosy-endemic coastal areas of Norway, samples of sphagnum and other moss vegetation were collected and inoculated into footpads of mice, at that time (1976) the only suitable method for finding *M. leprae* in the environment. It was possible to examine 759 footpads, originating from 122 samples of which 20.9% contained non-cultivable acid-fast bacilli (NC AFB). These NC AFB continuously multiplied by factors of up to 10^6 in further footpads, although attempts to culture them on media for mycobacteria failed (Photo 2.9).

The NC AFB were positive in the dopa test with a maximum absorption between 480 and 530 nm and could be destained by pyridine, properties shown by *M. leprae*. After inoculation into nine-banded armadillos, antibody production against antigen 7, the presence of AFB in macrophages and pathological findings were similar to those of *M. leprae* (Kazda, 1981). In attempts to identify NC AFB, further techniques have been used: *M. leprae*-specific monoclonal antibodies against the phenolic glycolipid-I (PGL-I) in the indirect immunofluorescence technique (Kolk et al., 1985). It was found that the NC AFB contained PGL-I on their surface, a property characteristic of *M. leprae* (Kazda et al., 1990). In a third study, the polymerase chain reaction technique modified for the testing of samples containing humic acid was used. The results confirmed

that the NC AFB present in sphagnum possess the same fragment of the gene encoding superoxide dismutase as *M. leprae* (Mostafa et al., 1995).

The examination of environmental sources of *M. leprae* continued in a recent leprosy-endemic region. In the urban district of Bombay, samples of drinking and surface water and soil were collected and tested for cultivable and non-cultivable AFB. In samples originating from wet soil in a house washing area (Photo 2.10), the footpad technique repeatedly revealed NC AFB, which multiplied in the footpads of white and nude mice causing swelling in the latter. Tests for dopaoxidase and pyridine decolouration were positive. The bacilli contained *M. leprae*-specific phenolic glycolipid I. Biological tests (nerve involvement in nude mice and systemic leprosy in one infected, nine-banded armadillo) were also positive (Kazda et al., 1986).

The isolation of environmentally derived *M. leprae* together with *M. intracellulare* of serotype 19 from the same biotope in Bombay raised the question of whether the accompanying microorganisms could play any role in the development of leprosy. This was supported by the fact that other mycobacteria were frequently isolated from human lepromа. Many of these mycobacteria belonged to the *M. avium-intracellulare-scrofulaceum* complex (David, 1984). Furthermore, some authors regarded the "accompanying mycobacteria" as an aetiological factor in leprosy (Kato, 1985). Accordingly, the ecological approach to pathogenicity includes the behaviour of pathogenic species in niches shared with other mycobacteria. Few experiments have been performed until now in which the possible effect of a mixed infection with pathogenic and non-pathogenic mycobacteria has been studied.

In our experiments, *M. leprae* (armadillo-derived) and isolated *M. intracellulare* serotype 19 were inoculated simultaneously into footpads of nude mice. Other nude mice were infected with *M. leprae* and *M. intracellulare* of serotype 19 separately and served as controls (Kazda et al., 1987). It was found that the non-pathogenic *M. intracellulare* of serotype 19 considerably enhanced the pathogenicity of the leprosy bacillus. This supporting effect was demonstrated by an acceleration of footpad swelling beginning just 4 months after inoculation and additionally by the development of cutaneous lepromа on dorsal and lateral body sites of nude mice within 6 months. These lepromа increased in number and size during the 9 months they were under observation (Photo 2.11). Their micromorphological characteristics were similar to those of human lepromа. In animals inoculated with *M. leprae* alone, a swelling of the inoculated footpads was first observed 12 months after infection. Cutaneous lepromа did not develop.

This corresponds with the findings of Lancaster et al. (1984), who described the course of leprosy infection in nude mice. Macroscopic visible cutaneous lepromа have not been described in association with the footpad inoculation of *M. leprae* into nude mice and the swelling of footpads has not been previously seen until 9–12 months after inoculation. *M. intracellulare* of serotype 19 inoculated alone neither provoked lesions in nude mice nor showed pathogenic properties when inoculated into rabbits and guinea pigs and can be regarded as non-pathogenic. Thus, this *Mycobacterium*, found together with *M. leprae* in the environment, enhanced pathogenicity in nude mice.

In contrast to past and recent findings, research into the mode of leprosy transmission is still focused on the patient as the sole source of *M. leprae,* although this has often been questioned. It is well known that even in highly endemic areas, contact with a leprosy patient cannot be regarded as the sole source of infection. In a study covering Indonesia, the Philippines, Hawaii and several countries in Africa, no contact could be established in 30–60% of new leprosy cases (Arnold and Fasal, 1973). In the mainland United States, only 25.8% of new detected leprosy cases had had any known contact with a leprosy patient (Enna et al., 1978). As a result of work done by Blake et al. (1987), evidence that environmental non-human sources are critical to human infection with *M. leprae* has been accumulating. These sources include soil, vegetation, water, arthropods and nine-banded armadillos. Recently, a leprosy infection of a woman in Georgia whose only known exposure was to armadillos was described (Lane et al., 2006).

It is generally claimed that the transmission of *M. leprae* takes place in an airborne manner, similar to human tuberculosis, and additionally by direct skin contact from mother to child. However, the presence of viable leprosy bacilli in the environment of endemic regions and systemic leprosy in feral nine-banded armadillos suggests that the leprosy patient is not the sole source of infection and that *M. leprae*