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Isolation of Non-Cultivable Acid-Fast Bacilli in Sphagnum and Moss Vegetation by Foot Pad Technique in Mice[1]

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Cultivable mycobacteria have been found in 29.6% of 132 samples of sphagnum vegetation from different biotypes on the Atlantic coast of Norway ([9]). While some of these belonged to the habitat microflora of the sphagnum vegetation (*M. chelonei*, *M. sphagni*, sp. nov.), others such as *M. gordonae* and *M. komossense* sp. nov. have as yet only been isolated from intact Scandinavian moors ([7,8]). Direct smear examination revealed a high number of acid-fast bacilli (AFB), especially in sphagnum samples originating from regions with former high leprosy incidence rates ([3]). However, attempts to cultivate these AFB on the conventional media for the cultivation of mycobacteria mostly failed. The intention of the present study was to clarify whether these non-cultivable AFB could be demonstrated to multiply by the mouse foot pad technique.

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MATERIALS AND METHODS

Selection of sphagnum bogs and samples. The selection of biotypes was based on information on the former incidence of leprosy in the coastal area of Norway between Bergen and Trondheim ([3]). Two soligenous moors on islands, two on slopes, and two ombro-soligenous moors were chosen for the collection of samples:

1. Soligenous moor on the island of Sotra (near Bergen) above Fjeldberg (32–45 m above sea level) containing predominantly hydrophilic sphagnum species.
2. Soligenous moor on the island of Hitra (near Trondheim) between Gryta and Skumfossöra, 62–70 m above sea level (more or less hydrophilic sphagnum species), and above Aunet (55–60 m above sea level where samples of *Rhacomitrium lanuginosum* were collected.
3. Soligenous moor above Skadal Dalsfjorden, 310–320 m above sea level on a southeast slope, containing more or less hydrophilic sphagnum species.
4. Soligenous moor above Stavnes Stongfjorden, 90–140 m above sea

level on the west slope, containing hydrophilic sphagnum species.

5. Ombro-soligenous moor above Skadal Dalsfjorden, 405 m above sea level, with hydrophilic sphagnum species.
6. Soligenous moor (with an ombrogenous part) above Gjerde Stongfjord, 95–108 m above sea level, with less hydrophilic sphagnum species and some hummocks with *Rhacomitrium lanuginosum*.

The selection of sphagnum and moss samples was based on the occurrence of the species in the region concerned. A total of 39 specimens of *S. papillosum*, 26 of *S. rubellum*, 10 of *S. cuspidatum*, 7 of *S. compactum*, 8 of *S. imbricatum*, 2 of *S. palustre*, and 1 each of *S. plumolosum*, *S. apiculatum*, *S. fuscum*, and *S. magellanicum*, 23 specimens of moss *Rhacomitrium lanuginosum*, and 3 other moss species were examined. Due to technical reasons, the number of samples differs from the number of samples previously reported ([9]).

Collection, transport, and preparation of the samples. The samples were handled with sterile plastic gloves, transported to the field laboratory in sterile plastic bags, and stored at temperatures between 0 and +4°C until they could be examined. Subsequently, the samples were placed in a 20 ml plastic syringe and moistened with a 50:50 mixture of heat sterilized water and distilled water. After 10 minutes the water was pressed out, and the samples were weighed. The fluid was centrifuged (1255 × g, 20 min) and the sediment resuspended in 2 ml of 0.9% NaCl.

Inoculation into the foot pads of mice. Each suspension (0.03 ml) was injected subcutaneously into the right hind foot of each of 10 female mice of the inbred strain NMRI SPF. The local reaction to the injection was monitored twice a week during the first 6 weeks.

Examination for AFB. To examine the great number of samples (1220 in total) at intervals of 6, 9, 12, 18, and 24 months, a method previously published ([2,13]) was modified, tested in preliminary experiments, and finally applied. The mice were killed with chloroform, the right feet were cut off, claws and skin removed, and the rest homogenized in an Ultra Turrax Type TP 18/2n with shaft 10 N (Janke and Kunkel) in 2 ml of a 0.1% sterile albumin solution for one minute at increasing revolutions (max. 10,000 rpm), yielding a homogeneous suspension of tissue and bone. After two minutes, when the bone parts had settled down, the supernatant was poured off. The homogenate (0.01 ml) was uniformly spread over a 1 cm^2 square on a glass slide (the spot slides were marked by means of an electro diamond stencil [Milli 1500, Shandon]). After drying, the smears were fixed and stained by Ziehl-Neelsen's method. At a 1000-fold magnification, the AFB were counted in 100 microscope fields, and the number of AFB per foot pad was extrapolated. Simultaneously, the undiluted suspension as well as a 10^{-1} and a 10^{-2} dilution were cultured (without decontamination) on Middlebrook 7 H 10 agar containing 4% bovine serum. These were incubated at least 6 weeks at 31°C to determine whether or not the AFB were cultivable.

Passages of non-cultivable AFB in the foot pads of mice. Some suspensions with high counts of solidly stained AFB were injected into additional foot pads. The two hind feet of 5 further mice were inoculated with 0.03 ml of each suspension. The examinations were conducted in the same way and at the same intervals as indicated above. Since these experiments require long incubation periods, only preliminary results are presented in this paper. Additional Lowenstein-Jensen media were inoculated and incubated for three months at 31° and 37°C respectively.

RESULTS

Detection of non-cultivable AFB. A varying number of non-cultivable AFB was found in the foot pads of mice 6, 9, 12, 18, and 24 months after inoculation with suspensions obtained from various species of sphagnum and moss vegetation isolated from different habitats. In the majority of cases (69.8%), the harvests performed yielded 10^5 to 10^6 AFB/foot pad; between 10^6 and 10^7 AFB/foot pad were recovered from 13.2% of the foot pads. The AFB recovered from the foot pads were polymorphous, more or less long, thin rods (0.5–0.8 × 0.8–1.8 μ) without true branching but exhibiting pronounced acid-fastness (Fig. 1).

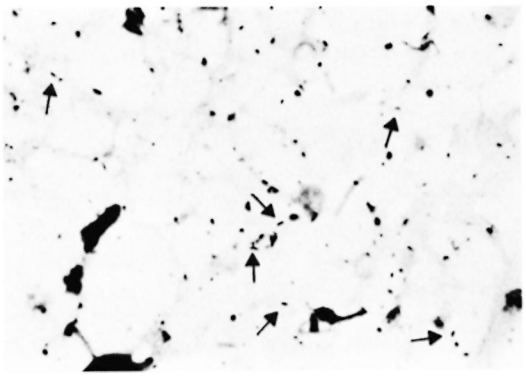

FIG. 1. Non-cultivable acid-fast bacilli (arrows), solidly stained, which multiply in passages in foot pads of mice (foot pad suspension 18 months after inoculation with *S. papillosum* from location 1; stained by Ziehl-Neelsen's method, 1000× magnified).

FIG. 2. *Sphagnum cuspidatum* vegetation—a species with the highest yield of non-cultivable AFB (37.5%).

The highest frequency of non-cultivable AFB (37.5%) was found in *S. cuspidatum* (Table 1). This frequency is significantly higher than in other species (*S. papillosum*, 22.4%; *S. rubellum*, 18.9%; *S. imbricatum*, 15.2%; *S. compactum*, 8.7%). Samples of *S. cuspidatum* (Fig. 2) were only collected from habitat 1 (island of Sotra), exhibiting the highest occurrence of non-cultivable AFB. Accordingly, the maximum yield was obtained from biotype 1 (island of Sotra), positive in 35.3% of the 173 specimens collected. This frequency if significantly higher than in other biotypes, ranging from 10.9 to 23.2%. Of the 759 foot pads examined, 20.9% contained non-cultivable AFB (Table 2).

Apart from a local swelling during the first few days after injection, no clinical changes were observed on the foot pads, nor could any histological changes typical of mycobacterioses be seen in the organs of the killed mice.

Passages of non-cultivable AFB in the foot pads of mice. The AFB recovered from foot pads continued to multiply in further passages whereas attempts to culture them on media for mycobacteria failed. The AFB are solidly stained, which also suggests they are viable. Since these experiments are still under way, Fig. 3 gives only an example of bacterial multiplication. So far, the bacteria multiplied by 2 to 3 powers in two passages in the foot pads of mice.

DISCUSSION

Our studies showed that sphagnum and moss vegetation of former leprosy-endemic regions in Norway contain non-cultivable AFB. The propagation of these AFB in foot pads and their morphology confirm that the bacteria are viable. The method used may be considered suitable for an isolation of *M. leprae* from environmental sources ([11]). Cultivable AFB show a different behavior after foot pad inoculation ([1]). The concentration of *M. tuberculosis*, *M. bovis*, *M. kansasii*, *M. simiae*, and *M. avium* drops steadily, but viable bacteria can still be cultivated. On the other hand, *M. marinum*, *M. scrofulaceum*, *M. nonchromogenicum*, *M. vaccae*, and *M. fortuitum* can no longer be cultivated 6 months after inoculation.

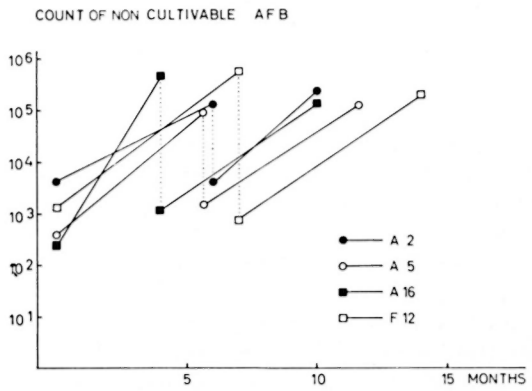

FIG. 3. Multiplication of non-cultivable acid-fast bacilli (100 to 1000-fold increase) during the first and second passage in the foot pads of mice (the vertical broken lines indicate the dilution made prior to the second inoculation).

TABLE 1. *Distribution of non-cultivable AFB (NC-AFB) in the individual species of sphagnum and moss originating from former leprosy-endemic regions of Norway.*

Sphagnum and moss species	No. of specimens examined	No. of mice examined	Foot pads examined	Foot pads AFB-positive	AFB-Level in foot pads				
					10^4–10^5	10^5–10^6	10^6–10^7	10^7–10^8	Not counted
			Number (percent)	Number (percent)	Number (percent)	Number (percent)	Number (percent)	Number (percent)	Number (percent)
S. papillosum	39[a]	390	241 (61.8)	54 (22.4)	1 (1.9)	36 (66.7)	7 (12.9)	0 (0)	10 (18.5)
S. rubellum	26[b]	260	164 (63.1)	31 (18.9)	1 (3.2)	21 (67.7)	3 (9.7)	1 (3.2)	5 (16.1)
S. cuspidatum	10[c]	100	64 (64.0)	24 (37.5)	0 (0)	18 (75.0)	6 (25.0)	0 (0)	0 (0)
S. imbricatum	8[d]	80	46 (57.5)	7 (15.2)	0 (0)	2 (28.6)	1 (14.3)	0 (0)	4 (57.1)
S. compactum	7[e]	70	46 (65.7)	4 (8.7)	0 (0)	4 (100.0)	0 (0)	0 (0)	0 (0)
S. palustre	2[f]	20	12 (60.0)	4 (33.3)	0 (0)	2 (50.0)	1 (25.0)	0 (0)	1 (25.0)
S. magellanicum	1	10	8 (80.0)	1 (12.5)	0 (0)	1 (100.0)	0 (0)	0 (0)	0 (0)
S. plumulosum	1	10	6 (60.0)	1 (16.6)	0 (0)	1 (100.0)	0 (0)	0 (0)	0 (0)
S. fuscum	1	10	7 (70.0)	2 (28.6)	0 (0)	1 (50.0)	0 (0)	0 (0)	1 (50.0)
S. apiculatum	1	10	6 (60.0)	0 (0)	—	—	—	—	—
Rhacomitrium lanuginosum	23[g]	230	148 (64.3)	29 (19.6)	2 (6.9)	24 (82.8)	2 (6.9)	0 (0)	1 (3.4)
Other moss species	3[h]	30	11 (36.7)	2 (18.2)	0 (0)	1 (50.0)	1 (50.0)	0 (0)	0 (0)
Total	122	1220	759 (62.2)	159 (20.9)	4 (2.5)	111 (69.8)	21 (13.2)	1 (0.6)	22 (13.8)

[a] NC-AFB were found in 27 of 39 specimens examined.
[b] NC-AFB were found in 17 of 26 specimens examined.
[c] NC-AFB were found in 8 of 10 specimens examined.
[d] NC-AFB were found in 6 of 8 specimens examined.
[e] NC-AFB were found in 4 of 7 specimens examined.
[f] NC-AFB were found in both of the specimens examined.
[g] NC-AFB were found in 17 of 23 specimens examined.
[h] NC-AFB were found in 2 of 3 specimens examined.

We noted that the same holds true for *M. sphagni* sp. nov., *M. gordonae*, and *M. komossense* sp. nov. isolated from sphagnum vegetation while *M. chelonei* can still be cultivated 6 months after foot pad inoculation.

The demonstration of non-cultivable AFB in sphagnum and moss vegetation multiplying like *M. leprae* after foot pad inoculation raises the question whether this vegetation may be regarded as a possible source of *M. leprae* in the former leprosy-endemic regions of Norway and in other countries in which leprosy is still endemic today. As early as the beginning of this century, environmental sources of *M. leprae* in Norway were suggested ([12]). The recent detection of indigenous leprosy in rural nine-banded armadillos (*Dasypus novemcinctus* L.) indicates that the natural habitat of these animals may be a source of *M. leprae* ([10, 14]). This hypothesis is further supported by the detection of autotrophic enzymatic properties in *M. leprae* which might enable it to maintain its viability in the environment ([5]).

The geographical distribution of leprosy in Norway, with high frequency areas on

TABLE 2. *Incidence of non-cultivable AFB in the foot pads of mice 6 to 24 months after inoculation with suspensions obtained from sphagnum and moss specimens isolated from former leprosy-endemic regions of Norway.*

Locality	Specimens examined	Mice inoculated	Foot pads examined	AFB-positive foot pads	AFB-Level in foot pads				
					10^4–10^5	10^5–10^6	10^6–10^7	10^7–10^8	Not counted
			Number (percent)	Number (percent)	Number (percent)	Number (percent)	Number (percent)	Number (percent)	Number (percent)
1.	26	260	173 (66.5)	61 (35.3)	0 (0)	42 (68.9)	14 (22.9)	0 (0)	5 (8.2)
2.	51	510	327 (64.1)	58 (17.7)	1 (1.7)	42 (72.4)	3 (5.2)	1 (1.7)	11 (19.0)
3.	10	100	58 (58.0)	9 (15.5)	0 (0)	5 (55.6)	2 (22.2)	0 (0)	2 (22.2)
4.	10	100	56 (56.0)	13 (23.2)	0 (0)	9 (69.2)	2 (15.4)	0 (0)	2 (15.4)
5.	10	100	64 (64.0)	7 (10.7)	0 (0)	6 (85.7)	0 (0)	0 (0)	1 (14.3)
6.	15	150	81 (54.0)	11 (13.6)	3 (27.3)	7 (63.6)	0 (0)	0 (0)	1 (9.1)
Total 1–6	122	1220	759 (62.2)	159 (20.9)	4 (2.5)	111 (69.8)	21 (13.2)	1 (0.6)	22 (13.8)

the Atlantic coast where the humidity of the air is high, is consistent with a hypothesis relating the occurrence of leprosy to mycobacterial growth in sphagnum vegetation; such a growth is favored by a high humidity of the air ([6]). Moreover, in a district with formerly high morbidity rates of leprosy in Norway, an association has been demonstrated to exist at the farm level between conditions of mycobacterial growth in the environment today and the previous incidence rates of leprosy ([4]). However, it should be stated that the mechanisms behind these associations are unknown at the present stage.

Thus, it must be further clarified whether the demonstrated non-cultivable AFB represent *M. leprae* or other mycobacteria perhaps influencing susceptibility to leprosy.

SUMMARY

In the former leprosy-endemic coastal area of Norway, 122 samples of sphagnum and moss vegetation were collected from 6 biotopes and examined for non-cultivable AFB by foot pad inoculation. Of the 759 foot pads examined, 20.9% contained non-cultivable AFB. A significantly higher frequency was found in a habitat where *Sphagnum cuspidatum* was preponderant, the sphagnum species from which the maximum yield was obtained. The bacteria were polymorphous, solidly staining AFB, which multiplied in passage in foot pads while they could not be cultivated on the conventional media for mycobacteria. Efforts are continuing to identify these AFB by biochemical methods and by inoculation into nine-banded armadillos.

RESÚMEN

Se colectaron 122 muestras de vegetación musgosa de 6 regiones del área costera de Noruega. Esta área costera es una zona en la que la lepra fue endémica en el pasado. Las muestras se examinaron para buscar bacilos ácido-resistentes no cultivables por el método de la inoculación en los cojinetes plantares del ratón. De los 759 cojinetes plantares que fueron examinados, el 20.9% contuvieron bacilos ácido-resistentes no cultivables. La frecuencia más elevada de aislamientos se encontró en la región donde el *Sphagnum cuspidatum* fue predominante. Las bacterias fueron ácido-resistentes, sólidamente teñidas, pleomórficas y capaces de multiplicarse en los cojinetes plantares del ratón pero no en los medios convencionales para micobacterias. Actualmente se estan haciendo estudios para identificar a estos bacilos ácido-resistentes por métodos bioquímicos y por inoculación en armadillos de nueve bandas.

RÉSUMÉ

Dans la région côtière de la Norvège, jadis endémique pour la lèpre, on a récolté 122 échantillons de

sphaigne et de mousse dans 6 biotopes différents. Ces échantillons ont été examinés afin de mettre éventuellement en évidence des bacilles acido-résistants non cultivables, au moyen de la technique d'inoculation dans le coussinet plantaire de la souris. Sur les 759 coussinets plantaires examinés, 20,9% contenaient des acido-résistants non cultivables. On a noté une fréquence significativement plus élevée de ces isolements dans un habitat où *Sphagnum cuspidatum* était prépondérant. Cette espèce de sphaigne est celle pour laquelle la récolte de bacilles a été la plus abondante. Les bactéries isolées étaient polymorphes et présentaient une forme solide lors de la coloration acido-résistante; ces bactéries se multipliaient par passage dans le coussinet plantaire alors qu'elles ne pouvaient pas être cultivées sur les milieux de culture habituels utilisés pour la croissance des mycobactéries. On poursuit les efforts entrepris pour identifier ces bacilles acido-résistants par des méthodes biochimiques, de même que par l'inoculation chez le tatou à neuf bandes.

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REFERENCES

1. Collins, F. M., Morrison, N. E. and Montaline, V. Immune response to persistent mycobacterial infection in mice. Infect. Immun. **20** (1978) 430–438.
2. Desikan, K. V. A modified method of harvesting *M. leprae* from foot-pads of mice. Lepr. India **48** (1976) 157–162.
3. Irgens, L. M. and Bjerkedal, T. Epidemiology of leprosy in Norway: the history of the national registry of Norway from 1856 until today. Int. J. Epidemiol. **2** (1973) 81–89.
4. Irgens, L. M. and Kazda, J. Leprosy and environmental factors in a high frequency area: epidemiological aspects. Lecture at a Borstel Colloquium, 5 March 1979.
5. Kato, L. Autotrophism of an "intracellular parasite" *Mycobacterium leprae*. Can. J. Public Health **64** (1973) 42–46.
6. Kazda, J. Die Bedeutung der Moorbiotype für die Okologie von Mykobakterien. Zentralbl. Bakt. I. Abt. (Orig. B) **165** (1977) 323–334.
7. Kazda, J. *Mycobacterium sphagni* sp. nov. Int. J. Syst. Bacteriol. (in press).
8. Kazda, J. and Müller, K. *Mycobacterium komossense* sp. nov. Int. J. Syst. Bacteriol (in press).
9. Kazda, J., Müller, K. and Irgens, L. M. Cultivable mycobacteria in sphagnum vegetation of moors in South Sweden and coastal Norway. Acta Pathol. Microbiol. (Scand. B) **87** (1979) 97–101.
10. Kirchheimer, W. F. Leprosy in nature? personal communication, 1978.
11. Rees, R. J. W. personal communication, 1977.
12. Sand, A. *Geschieht die Ansteckung der Lepra durch unmitterbare Übertragung?* Mittl. und Verhandl. d. 2. Leprakonferenz, Leipzig: Fischer Verlag, 1910, pp. 39–46.
13. Shepard, C. C. The experimental disease that follows the injection of human leprosy bacilli into foot pads of mice. J. Exp. Med. **112** (1960) 445–454.
14. Walsh, G. P., Storrs, E. E., Meyers, W. and Binford, C. H. Naturally acquired leprosy-like disease in the nine-banded armadillo (*Dasypus novemcinctus*): recent epizootiologic findings. J. Reticuloendothel. Soc. **22** (1977) 363–367.