Observation on the Host Reaction to the Cultivated *M. lepraemurium* in the Spread Subcutaneous Tissue Preparations of Mice

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Since the first successful cultivation of *M. lepraemurium* was reported by Ogawa⁽¹⁾, it was examined and confirmed by others^(2,3,4). Colonies of *M. lepraemurium* on Ogawa's 1% egg yolk medium are originally rough, but they become smooth, in general, after 10 to 15 cultural passages⁽⁵⁾. Surface of the colonies changes from heaped-up and wrinkled to flat and glistening during serial subcultures. Remarkable ultrastructural differences on the surface substances of the bacilli were recognized between these two colonies by scanning electron microscopic studies^(6,7).

As stated in our previous reports^(8,9,10) on the pathogenicity for mice of cultivated *M. lepraemurium*, bacilli from rough colonies of Hawaiian-Ogawa strain (HO-R) were more virulent than those of original Hawaiian strain maintained only by C3H strain mouse passages (H-M). HO-R bacilli developed malignant type leproma at the inoculation site in male DDD strain mice, in which the subcutaneous lesion with H-M bacilli usually developed benign type leproma. The bacilli from smooth colonies (HO-S) were generally much less virulent for mice than HO-R and H-M bacilli^(11,12). HO-S bacilli did not develop any palpable leproma at the inoculation site even in the highly susceptible C3H mice in long term observation.

Since the spread subcutaneous tissue preparation technique has been found to be reliable and useful for observing the host parasite relation at the early stages of subcutaneous infection with *M. lepraemurium*⁽¹³⁾, this technique was applied to investigate the host reaction and the growth pattern of bacilli at early stages of the infection with HO-R and HO-S, up to 10 weeks.

Materials and Methods

Experiment I. Observation on the growth pattern and the host reaction after subcutaneous infection with HO-R bacilli.

Animals. Five to six week old male mice of C3H, BALB/c, C57BL/6 and DDD strains were used. These mice were bred and raised in our laboratory.

Bacterial material and inoculation. Rough form *M. lepraemurium* was obtained from 8th subculture of Hawaiian-Ogawa strain. This strain was isolated from subcutaneous leproma of C3H mouse previously infected with *M. lepraemurium*, strain Hawaiian and maintained on the Ogawa's 1% egg yolk medium with serial passages at every 6 week cultivation^(1,2,3,4).

The bacterial suspension approximately containing 2×10^8 organisms in 1 ml of saline was prepared by the grinding method. Each mouse was inoculated subcutaneously in the back with 0.25 ml of the suspension.

Preparation and observation. Two or three mice were killed at weekly intervals until 10 weeks after the infection. Four to five pieces of spread subcutaneous tissue preparation (STP) were made from the inoculation site in each mouse by the method described in detail previously⁽¹³⁾. The skin of mouse was cleaned and disinfected with alcohol. An incision was made at the side of abdomen and the skin was inverted. Subcutaneous tissue was grasped with scissors and placed on the glass slide. The tissue was spread out with the tip of scissors and plastered to the slide in an area of about 2 to 4 cm². The specimens were left at room temperature for drying, fixed by passing through a flame and stained by Ziehl-Neelsen method. All specimens were processed for examination by light microscopy (Fig. 1).

Experiment II. Observation on the growth pattern and the host reaction after subcutaneous infection with HO-S bacilli.

Exp. II-1. Male mice of C3H and C57BL/6 strains were inoculated with bacterial suspension of HO-S bacilli prepared as the same manner in Experiment I. The bacterial suspension was prepared from the 36th subculture, 6-week-old, of Hawaiian-Ogawa strain which had changed from rough to smooth at the 21th subculture. Observation was made by the same manner as in Experiment I.

Exp. II-2. Observations were done also on male C3H, BALB/c and DDD mice by the same manner in Experiments I and II-1, except the 42th subculture of the same strain was used for this experiment.

Results

Exp. I. The growth pattern and the host reaction in the host infected with HO-R bacilli.

At 2 days, polymorphonuclears were seen dominantly at the inoculation site as a remain of acute inflammatory reaction. Many macrophages, mononuclears and a few lymphocytes were also seen. Most of the bacilli were ingested by macrophages. There were 10 to 20 bacilli in each macrophage. The form of bacilli was short (2-2.2 μ) and thick as similar to that of initials. No remarkable differences on cellular reaction were recognized at this time among mouse strains.

At one week, the infiltration of polymorphonuclears disappeared in C3H mice, but in the other three strains some polymorphonuclears were still left at the inoculation site.

At 3 weeks, no macroscopic lesions were seen in C3H mice, whereas a benign type nodule developed at the inoculation site in C57BL/6, DDD and BALB/c mice. No good specimens could be obtained from the central part of inoculation site in these three strains of mice, (C57BL/6, DDD and BALB/c), because it is so difficult to strip off subcutaneous tissue and to spread the tissue as a monolayer on the glass slide. In these three strains of mice, many

polymorphonuclears and lymphocytes were observed and the bacilli seen in macrophages at a part of preparation were segmented or beaded. In C3H mice, morphological changes and increase in number of the bacilli were not recognized yet and cellular reactions such as in the other three strains were not seen.

At 5 weeks, cellular reaction and multiplication of the bacilli showed remarkable differences between C3H and other three strains of mice. In C3H mice, cellular infiltration was mainly consisted of macrophages and mononuclears and most of macrophages were heavily loaded with uniformly stained, thick and a little elongated bacilli (Fig. 2, 3). These macrophages, called murine leprosy cell, were diffusely scattered or occasionally accumulated in whole fields of specimens. Bacillary multiplication was clearly revealed from these features. In contrast, severe infiltration with lymphocytes and polymorphonuclears was seen in C57BL/6, BALB/c and DDD mice. The cellular reaction was more severe in DDD mice and no murine leprosy cells were seen in the preparations. In C57BL/6 mice (Fig. 4, 5) and BALB/c mice, a few numbers of murine leprosy cells which contained about 10 bacilli of non-solid form, were surrounded by many lymphocytes and polymorphonuclears.

At 8 weeks, in C3H mice, further increase of murine leprosy cells was observed in the whole fields. Most of these cells were loaded with 50 bacilli or more which were short and uniformly stained. In BALB/c and C57BL/6 mice, the number of murine leprosy cells were much less than at the earlier stages of infection. In these mice the murine leprosy cells were surrounded more heavily by lymphocytes and polymorphonuclears than those observed in 5 weeks and these murine leprosy cells contained a few bacilli. Some bacilli were seen extracellularly, probably resulting from the bursting of bacilli-loaded macrophages. Most of the extracellular bacilli showed a segmented or beaded form. These features indicated that further local spread of the infection was arrested in these three strains. In DDD mice different two features were observed, one DDD mouse showed the same features as BALB/c and C57BL/6 mice but another one showed the features of cellular reaction and bacillary multiplication which were quite different from the former one. In the former mouse, a few extracellular bacilli were found in severe infiltration of lymphocytes and polymorphonuclears. In the latter mouse there were many murine leprosy cells which were heavily loaded with short bacilli, without any cellular reaction of lymphocytes and polymorphonuclears in some parts of the specimen, but in other parts of the same specimen, the same features as observed in C57BL/6 were seen.

At 10 weeks, a small malignant leproma was seen macroscopically at the site of infection in C3H mice and the cellular infiltration was mainly consisted of murine leprosy cells. Notable changes in cellular reaction at this stages were not observed in BALB/c and C57BL/6 mice.

In DDD mice many small masses of murine leprosy cells were found as islands in the specimen (Fig. 6). In this area there were some lymphocytes and polymorphonuclears, but they did not accumulate around the masses of murine leprosy cells. Significant increase of bacillary number was recognized at this stage.

Exp. II. The growth pattern and the host reaction in the host infected with HO-S bacilli.

The findings in C57BL/6 and BALB/c mice were essentially the same with those observed in HO-R infected mice but the remarkable differences were shown in DDD and C3H mice.

At 2 to 3 days, no significant differences in cellular reaction were found among 4 strains of mice. Many polymorphonuclears were observed and this infiltration as the inflammatory reaction to the inoculum was a little severer than that of HO-R infected mice. Most of the bacilli were ingested by macrophages and showed the same form and the same length of about 2 to 3 μ as the inoculums.

At one week, the cellular reaction with many polymorphonuclears disappeared, leaving some polymorphonuclears in BALB/c, DDD and C57BL/6 mice and a few in C3H mice. Any morphological change of the bacilli was not observed and almost all the bacilli were in the macrophages.

At 3 weeks, a small benign type nodule was observed in C57BL/6 but not in the other strains. In C57BL/6 mice, heavy lymphocytic infiltration occurred, surrounding small number of murine leprosy cell and polymorphonuclears were also seen in small number. Most of the murine leprosy cells contained a few bacilli and some of them were bursting. The number of bacilli was already decreased significantly. In C3H mice, murine leprosy cells which ingested many bacilli were diffusely scattered or accumulated in whole fields of the specimens without any cellular infiltration. The bacilli showed a little elongated form and were uniformly stained. Heavy cellular reaction seen in C57BL/6 was not observed in DDD mice, but slight infiltration with lymphocytes and polymorphonuclears was shown.

At 5 weeks, a small benign type nodule appeared in DDD mice and differences in the cellular reaction between C3H and the other 3 strains were augmented. Heavy cellular reactions were developed in C57BL/6 (Fig. 7, 8), DDD and BALB/c. Macrophages contained a small number of bacilli were surrounded by polymorphonuclears and lymphocytes. Bacilli in the murine leprosy cells showed thinner form in these 3 strains of mice. Further increase of murine leprosy cells were recognized only in C3H mice (Fig. 9) without any cellular reaction observed in the other strains.

At 7 to 8 weeks, it was difficult to make good preparations from central part of the infection site except C3H mice, because much heavy infiltration developed than that of the other 3 strains at 5 weeks. In C57BL/6, BALB/c and DDD mice, there were a few bacilli and severe cellular reactions with lymphocytes and polymorphonuclears. At this stage, very remarkable change began to occur in C3H mice (Fig. 10, 11). Murine leprosy cells were decreased distinctly and small masses of these cells were observed in restricted parts of the specimen. Many lymphocytes infiltrated into these masses. These findings looked like those of C57BL/6 mice at 3 weeks. In murine leprosy cells of C3H mice it was difficult to find out solid form bacilli, many bacilli showed elongated, beaded and segmented forms and there were many debris which were considered to be yielded from vanished bacilli.

At 10 weeks, in C3H mice, cellular reaction developed gradually. Many lymphocytes and

polymorphonuclears were observed and some of them accumulated around the small masses of murine leprosy cells. The bacilli decreased in number, furthermore, almost all the bacilli showed non-solid forms.

At 15 to 20 weeks, additional observation was done on C3H mice (Fig. 12). Many murine leprosy cells, which were heavily loaded with somewhat short and well stained bacilli, were observed and the cellular reaction almost disappeared. About 50 murine leprosy cells were accumulated to overlap each other. These features composed of round shaped murine leprosy cells were clearly margined and about $300\ \mu$ in diameter. They were very characteristic and considered as the feature of bacillary multiplication.

Discussion

As stated in our previous reports^(10,11,12) on the pathogenicity of HO-R and HO-S bacilli in mice, HO-R bacilli were found to be more virulent for mice than H-M bacilli. And HO-S bacilli were generally so less virulent for mice than HO-R and H-M that HO-S bacilli did not produce any palpable leproma at the inoculation site in C3H mice even in 40 weeks after the subcutaneous infection.

Differences of the cellular reaction in the subcutaneous lesion in the host infected with HO-R, HO-S and H-M were assumed to be due to their pathogenicity. From this point of view, in this experiment the observations were made by means of the spread subcutaneous tissue preparation technique to clear when the pathological differences begin to occur and how they developed at the inoculation site.

In C3H mice infected with HO-R bacilli, there were no palpable lesions at the inoculation site in the first 10 weeks, but characteristic infiltration mainly composed of bacilli-loaded macrophages (murine leprosy cells) developed gradually. On the contrary, in C57BL/6 mice infected with HO-R bacilli, severe lymphocytic reaction with polymorphonuclears began to occur at the 3rd week and further multiplication of the bacilli was restricted. These differences in cellular reactions between C3H and C57BL/6 mice infected with HO-R were similar to those in our earlier experiments with H-M bacilli infection⁽¹³⁾, so it was considered that the pathogenicity of HO-R bacilli for C3H and C57BL/6 mice was essentially the same with that of H-M bacilli. Closs and Haugen⁽¹⁴⁾ observed a vigorous granulomatous reaction developed in C57/BL mice and arresting of further local spread of infection after subcutaneous inoculation with murine leprosy bacilli. Poulter and Lefford⁽¹⁵⁾ reported that, in B6D2 mice, delayed type hypersensitivity developed 4 weeks after subcutaneous infection with *M. lepraemurium* and concomitant reduction in number of the organisms was observed. Furthermore, we observed typically malignant murine leprosy disease in C3H mice and BALB/c-*nu/nu* mice⁽⁹⁾. Considering these results, it is reasonable to regard that the histopathological changes shown in C57BL/6 and BALB/c mice are due to the cell-mediated immunity elicited and developed after infection with the bacilli, whereas this cell-mediated immunity developed poorly in C3H mice. The findings on spread tissue preparations prepared from experimental mice indicated that the protective immunity to murine leprosy

bacilli and regression of infection began to occur 3 weeks after the infection in resistant mice as regarded by Closs and Haugen⁽¹⁶⁾.

The features observed in DDD mice were of interest. Although lymphocytic reaction was found from the 3rd to the 5th week after infection with HO-R bacilli in DDD mice, one of the DDD mice showed the pathological changes at the 8th week as observed in C3H mice and accumulation of lymphocytes and polymorphonuclears around the murine leprosy cells disappeared in every specimen at 10 weeks. On the other side, it was found that DDD mice infected with H-M bacilli showed heavy cellular reaction with lymphocytes and polymorphonuclears from the 3rd week of infection and multiplication of the bacilli was restricted strongly as observed in C57BL/6 mice⁽¹³⁾. However, it is obvious that these differences between the features obtained from H-M infection and these from HO-R infection are not due to the technical error, because HO-R infected male mice showed some variations of leproma type and most of lepromata were malignant⁽⁷⁾. In this experiment it was indicated that the difference of susceptibility to HO-R and H-M bacilli in DDD mice began to clear at about 8 weeks of infection. The findings in DDD mice infected with HO-R indicated that protective immunity of DDD mice to HO-R bacilli developed in some extent from the 3rd to the 5th week, but these host reactions decreased from the 8th week. However, the mechanisms behind this phenomenon remains to be elucidated.

In C3H mice infected with HO-S bacilli, bacilli-loaded macrophages were found and no host reactions were detected at the inoculation site as in the experiments with H-M and HO-R bacilli until 5 weeks after the infection. At 7 to 8 weeks, polymorphonuclear and lymphocytic infiltration was observed and the bacilli significantly decreased in number. As mentioned repeatedly⁽¹³⁾ such host reaction was not seen at 7 to 8 weeks in C3H mice infected with H-M bacilli and HO-R bacilli. But in 15 to 20 weeks gradual restoration of bacillary multiplication was shown and cellular reaction disappeared. Closs⁽¹⁷⁾ and Lagrange⁽¹⁸⁾ described that killed *M. lepraemurium* did not elicit delayed type hypersensitivity in mice and also in our studies by STP, naturally died bacilli did not show any pathological changes (to be published). Therefore, it is unreasonable to consider that killed bacilli in the inoculum caused the cellular reactions at 7 to 10 weeks. As the cellular reactions at 7 to 10 weeks in C3H mice infected with HO-S bacilli were quite alike with those of C57BL/6 mice infected with HO-R and H-M bacilli, it is reasonable to consider that protective immuno-responses were temporarily evoked in C3H mice at 7 to 10 weeks after infection with HO-S bacilli and vanished after these stages of infection.

As reported before^(11,12), development of mouse leprosy in C3H mice with HO-S bacilli was extremely slow compared with that of HO-R and H-M infection. In the present study it was found that the lag of disease in C3H mice is based not only on the poor *in vivo* growth of the bacilli but also on the host reaction.

In the present studies, the features of the cellular reactions and growth patterns of HO-R and HO-S bacilli in each mouse were well correlated with leproma type in long term observation. By using STP technique the prognosis of mouse leprosy can be predicted, with

special reference to the host reaction against *M. lepraemurium*.

Summary

Cultivated murine leprosy bacilli from rough colonies (HO-R) and smooth colony variant (HO-S) were subcutaneously inoculated into mice of inbred strains (C3H, C57BL/6, BALB/c and DDD). Growth patterns of the bacilli of each strain and cellular reactions of host in subcutaneous tissue at the inoculation site were examined by means of the spread tissue preparation technique.

There were no remarkable differences in cellular reactions at the inoculation site among these inbred strains of mice infected with HO-R bacilli 2 days and 1 week after the infection. An acute inflammatory reaction with accumulation of many polymorphonuclears was observed at 2 days. At one week, it was disappeared and monocytes and macrophages were dominant. A vigorous granulomatous reaction consisted of polymorphonuclears and lymphocytes developed in C57BL/6, BALB/c and DDD mice at 3 weeks. In the course of the following weeks the cellular reaction was augmented in C57BL/6 and BALB/c mice and further local spread of the infection was arrested. On the other hand, in DDD mice the diminution of cellular reaction occurred at 8 weeks and bacilli increased in number. In contrast, no signs of host reactions were observed in C3H mice and many murine leprosy cells were observed at the inoculation site.

C57BL/6 and BALB/c mice infected with HO-S bacilli showed essentially the same host reaction as those observed in the experiment with HO-R bacilli. The diminution of cellular reaction observed in the DDD mice infected with HO-R bacilli could not be found in those infected with HO-S bacilli and the multiplication of the bacilli was not seen. In C3H mice infected with HO-S bacilli no specific host reaction was found until 5 weeks, but cellular reactions happened at 7 to 8 weeks which were never observed in C3H mice infected with HO-R or H-M bacilli. The bacilli decreased in number from the 8th to the 10th week but bacillary multiplication was gradually recovered at 15 weeks. The multiplication of HO-S bacilli was very slow even in C3H mice throughout the observation period.

In this study, we found that slower development of mouse leprosy with HO-S bacilli in C3H mice is due to their poor ability to multiply *in vivo* and the inexplicable host reaction.

The mouse strain differences in response to cultivated *M. lepraemurium* were also discussed on the bases of cell-mediated immunity in the host.

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培養鼠癩菌に対する宿主反応のマウス皮下 組織伸展標本による観察

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培養鼠癩菌の Rough 型菌とその変異株である Smooth 型菌を C3H, C57BL/6, BALB/c, DDD 系の各マウスに皮下接種し、それぞれの菌の接種局所における増殖と宿主反応差を伸展標本を用いて観察した。

Rough 型菌を接種されたマウスでは接種後2日目と1週目には系統間差は認められなかった。即ち2日目には非特異的異物反応として多核白血球が多数認められ、1週目にはこの反応は消失し、ほぼ単核球のみが観察された。3週目において C57BL/6, BALB/c, DDD の各マウスでは多核白血球、リンパ球からなる宿主細胞反応が出現し、C57BL/6, BALB/c マウスではこれらの反応は以後、増強され菌の増殖は抑制された。これらの反応はマウス継代株による伸展標本像と同じであり宿主における免疫反応の発達を反映したものであると推察された。DDD マウスではマウス継代株接種後観察された良性型結節像は8週頃より消失し菌は再び増殖した。DDD マウスはマウス継代株に対しては強い抵抗を示すのに対し、Rough 型菌はこのマウスに対し強い病原性を示すという長期観察実験の結果とよく関連した像が得られ

た。C3H マウスは典型的悪性型結節像を呈し、他の系統のマウスで見られた特異的細胞反応は観察されずマウス継代株による伸展標本像と同様の像を示した。

Smooth 型菌を接種された C57BL/6 マウスおよび BALB/c マウスの宿主反応と菌増殖像は Rough 型菌における経時的変化とほぼ同じであった。一方 DDD マウスでは Rough 型菌接種時観察された宿主抵抗の減退像はなく C57BL/6 マウス, BALB/c マウスとほぼ同じ伸展標本像を示した。C3H マウスは5週目までマウス継代株, Rough 型菌接種による伸展標本像と同じ変化を示したものの7週から8週にかけて宿主細胞反応が出現し、更に8週から10週目においては菌数は著るしく減少した。15週目以後菌の増殖は徐々に回復したもののその増殖像は特徴的な像であった。Smooth 型菌の病原性は他の株に比し著るしく低下していることが示唆された。

それぞれのマウスにおける宿主反応像と宿主の免疫反応の発達との相関についても言及した。

Fig. 1 The gross appearance of Ziehl-Neelsen stained STP.

Fig. 2 C3H mouse, 5 weeks after infection with HO-R bacilli. $\times 200$.

Fig. 3 C3H mouse, 5 weeks after infection with HO-R bacilli. $\times 1,000$.

Fig. 4 C57BL/6 mouse, 5 weeks after infection with HO-R bacilli. $\times 200$.

Fig. 5 C57BL/6 mouse, 5 weeks after infection with HO-R bacilli. $\times 1,000$.

Fig. 6 DDD mouse, 10 weeks after infection with HO-R bacilli. $\times 200$.

Fig. 7 C57BL/6 mouse, 5 weeks after infection with HO-S bacilli. $\times 200$.

Fig. 8 C57BL/6 mouse, 5 weeks after infection with HO-S bacilli. $\times 1,000$.

Fig. 9 C3H mouse, 5 weeks after infection with HO-S bacilli. $\times 200$.

Fig. 10 C3H mouse, 7 weeks after infection with HO-S bacilli. $\times 200$.

Fig. 11 C3H mouse, 7 weeks after infection with HO-S bacilli. $\times 1,000$.

Fig. 12 C3H mouse, 20 weeks after infection with HO-S bacilli. $\times 200$.