Prevention of Diabetes in Non-obese Diabetic Mice by a Single Immunization with *Mycobacterium leprae*

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[Received: 4 Dec 2001/Accepted: 10 Jan 2002]

**Key words: M. leprae, Autoimmune Diseases NODmice, hsp65, 38kD protein**

The incidence of overt diabetes was completely prevented by a single intradermal inoculation of *Mycobacterium leprae* (*M. leprae*) into Non-obese diabetic (NOD) mice as young as 6-7 weeks. Partial prevention was also observed in cases when 65kD heat-shock protein (hsp65) with Freund's incomplete adjuvant (FIA) was injected, and no prevention was observed by 38kD with FIA immunization. Histological examination of pancreata demonstrated that control and *M. leprae*-immunized mice at 24 weeks of age developed the insulitis even though the number of lymphocytes infiltrated in the treated ones were less than the controls. However, later, at 47 weeks of age, even the immunized mice become to develop very severe insulitis. Thus, *M. leprae*-immunization did not prevent the incidences of insulitis. The spontaneous development of serum antibody against hsp65 and 38kD protein preceded the onset of diabetes in NOD mice.

Lymphocytes response, IFN-γ and IL-10 production of splenocytes cultures stimulated with hsp65 were examined to clue the reasons for the prevention of IDDM incidence by *M. leprae* immunization. The spontaneous development of anti-hsp65 T lymphocytes preceded the outbreak of overt IDDM in control NOD mice, but also appeared in *M. leprae* immunized cases in which the IDDM incidence was prevented, and both control and *M. leprae* immunized groups produced IFN-γ and IL-10 by stimulation with hsp65.

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*Mycobacterium leprae* (*M. leprae*) is causative organism responsible for leprosy, which is a chronic inflammatory diseases involving the mononuclear cells and pathogens. One of the most serious problems in leprosy is caused by such as reversal one (Type 1) and erythema nodosum leprosum (ENL, Type 2), but the mechanisms are not well known. Autoimmunity against host cells is also suspected to play a role in the reactions. Therefore, we try to know here the effect of *M. leprae* on autoimmunity using Non-obese diabetic (NOD) mice taking the bacilli as the factor to cause or suppress the autoimmunity.
NOD mice spontaneously develop an autoimmune disease, a diabetic syndrome which, in many respects, resembles to human Type 1 insulin-dependent diabetes mellitus (IDDM)\(^5\). The insulitis, which begins in these female mice at 4-6 weeks of age, progresses to overt IDDM at around 27 - 30 weeks of age due to the cytolyis of \(\beta\)-cells by mainly autoimmune T-lymphocytes\(^2\). The role of the responsible for the autoimmune and peritoneal macrophages has been examined in the disease process\(^2-4\). In this study, we tried to know whether the immunization of \(M.\) leprae can promote or not the outbreak of overt IDDM in NOD mice.

As shown in Table 1, the incidence of overt diabetes up to 47 weeks of age was completely prevented, not promoted, by a single intradermal inoculation of \(M.\) leprae (5 x 10\(^7\) organisms/50 \(\mu\)l PBS) into footpad of NOD female mice (CLEA, Shizuoka Japan) as young as 6 - 7 weeks, and the mice could survive longer without any additional therapy. As control for \(M.\) leprae, 65kD heat-shock protein (hsp65) of \(M.\) leprae and 38kD of H37Rv proteins were used. The hsp65 of mycobacteria, which is evolutionarily highly conserved protein, is highly immunogenic antigen and is produced under a variety of stressed conditions to preserve cellular functions and has been found to be involved in the pathogenesis of NOD/Lt mouse IDDM\(^5-7\). The mycobacterial 38kD is also one of the highly immunogenic protein components of mycobacteria\(^8\). The partial prevention was also observed when hsp65 (10 \(\mu\)g/PBS 25 \(\mu\)l with Freund's incomplete adjuvant 25 \(\mu\)l; hsp65/FIA) was injected, and no prevention of IDDM development was observed by 38kD protein injection (50 \(\mu\)g/PBS 25 \(\mu\)l with Freund's incomplete adjuvant 25 \(\mu\)l; 38kD/FIA)(Table 1).

The \(M.\) leprae (Thai53 strain), derived from footpad of BALB/cA-nu/nu mice, was kindly gift from Dr. M. Matsuoka in Leprosy Research Center, NIID. The hsp65 was purified as described previously\(^9\). The 38kD protein was isolated from the culture fluid of \(M.\) tuberculosis H37Rv and highly purified by biochemical methods, and kindly gift from Dr. S. Nagai, Toneyama Inst. for Tuberculosis Research, Osaka City Univ. Medical School. The cumulative incidences of overt diabetes up to approximately 47 weeks of age were recorded by grading the urinary glucose level with Tes-Tape (Shionogi, Osaka, Japan). Mice showing the grades more than +2 were taken as diabetic.

The autoimmune diabetes process of the NOD mice has been found to be suppressed by a variety of inflammatory and immunological stimuli which ranged from infections with viruses\(^{10,11}\), BCG\(^{12}\), or the Freund's complete adjuvant\(^{13}\). Here we found that the so-called autoimmune diabetes of NOD mice was prevented with \(M.\) leprae inoculation. To clue the reasons why \(M.\) leprae inoculation prevented the IDDM incidence, we examined the intensity of insulitis appearance in islets. And lymphocytes proliferation responses, IFN-\(\gamma\) and IL-10 production of splenocytes cultures comparing the control and immunized groups as followed.

Pancreata were fixed with 10% phosphate-buffered formalin followed by the procedure to make serial paraffin sections, stained with hematoxylin-eosin (H & E) as described previously\(^{14}\), and the lymphocyte infiltration into islets was examined. Both control and \(M.\) leprae-immunized groups at 24 weeks of

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Table 1. Effect of treatments with \(M.\) leprae or others on overt IDDM incidence.

<table>
<thead>
<tr>
<th>Immunized with</th>
<th>Incidence of IDDM</th>
<th>(n)</th>
<th>(p) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(M.)leprae</td>
<td>0/8(^a)</td>
<td>8</td>
<td>0.007054</td>
</tr>
<tr>
<td>38kD/FIA</td>
<td>4/4</td>
<td>4</td>
<td>0.537094</td>
</tr>
<tr>
<td>hsp65/FIA</td>
<td>2/4</td>
<td>4</td>
<td>0.440401</td>
</tr>
<tr>
<td>None (cont.)</td>
<td>7/9</td>
<td>9</td>
<td></td>
</tr>
</tbody>
</table>

\(\text{NOD/Shi Jic female mice (6-7 weeks of age) were immunized with/without } M.\) leprae, 38kD/FIA or hsp65/FIA in the footpad. Up to 47 weeks of age, the incidences of overt IDDM (IDDM/total) were listed.

\(^a\) Significantly differd from control mice \((p < 0.05)\).
age showed the insulitis, but the number of lymphocytes infiltrated in the M. leprae immunized ones were lesser than those in controls (data was not shown), which support the prevention of overt diabetes in these mice. However, at 40 weeks of age, both control (not-yet IDDM) and M. leprae-immunized cases have developed severe insulitis. Thus, M. leprae-immunization has not prevented the incidences, in spite of preventing the incidence of IDDM.

Spontaneous high serum antibody level against the hsp65 has been observed in human autoimmune diseases\(^{15-17}\) and IDDM of NOD mice\(^2\), and the auto-antibody to the 38kD protein of \(\beta\)-cell has preceded the clinical onset of diabetes in BB rats\(^{18}\). Here we investigated the serum antibodies to hsp65 in pre-inoculated NOD mice with M. leprae as shown in Fig.1 using ELISA test with the method as described previously\(^{16}\). The anti-hsp65 appeared at 24 weeks of age of the mice (before the outbreak of overt IDDM) in non-immunized mice, and decreased slightly at 30 weeks as IDDM appeared. On the other hand, M. leprae immunized group, which were prevented from the development of overt IDDM, did not show such reactivity (Fig.1). In addition to anti-hsp65, anti-38kD antibodies were detected in almost parallel to that of anti-hsp65 (Fig.1). Thus, the spontaneous development of antibody to hsp65 and 38kD protein preceded the onset of diabetes in NOD mice.

These results suggest the presence of some common B-cell epitopes between hsp65 and 38kD proteins. We tried to know the common epitopes by western blot assay, but no cross reactivity was found between these proteins. The anti-38kD polyclonal antibody reacted with the 38kD protein, but not with hsp65, and the polyclonal and monoclonal antibodies to hsp65 (MAb-3A, MAb-A5B)\(^{19}\) reacted with hsp65, but not with the 38kD protein (data are not shown). We have still a lot to do our efforts to pursue the relationship of 38kD protein to autoimmune

![Figure 1. Antibody titers in sera of NOD mice to hsp65 and the 38kD protein. Data are mean from two to five mice. SD < 10%. NOD/ml = NOD mouse immunized with M. leprae.](image)
diseases.

When 4 mice had been pre-injected with hsp65/FIA exogenously, 2 mice were protected from IDDM and 2 were not in our experiments (Table 1). In these cases, the antibody titers against hsp65 of these 4 mice (30 weeks of age) were very high in both. The antibody titers are as follows: The average OD of protected ones were OD = 3.18 ± 0.4 and non-protected ones were OD = 3.41 ± 0.46. The OD titer was around 5 times to the spontaneous positive cases as shown in Fig.1. Thus, in the mice pre-injected with hsp65/FIA, the antibody titers to hsp65 did not correlate with the appearance of IDDM.

The onset of beta-cell destruction is associated with hsp65 of the spontaneous development of anti-hsp65 T lymphocytes 2), and the IFN-γ appears to play a role in the development of IDDM, as demonstrated by the prevention of diseases by administration of anti-IFN-γ monoclonal antibody (MAb) to NOD mice 20). Therefore, the lymphocytes proliferation responses to hsp65 and IFN-γ stimulation with hsp65 in splenocytes cultures of NOD mice were tested using the method as previously reported 21).

The lymphocytes proliferation responses to hsp65 (5 μg/ml), in both control and M. leprae immunized cases, became to be higher with age (Table 2), but the responses to M. leprae lysate (5 μg/ml) or the 38kD protein (5 μg/ml) were low or non. Thus, the spontaneous development of anti-hsp65 T lymphocytes preceded the outbreak of overt IDDM in control NOD mice, but also appeared in M. leprae immunized cases in which the IDDM incidence was prevented.

The results for IFN-γ are also shown in Table 2. Splenocytes from both control and immunized groups produced IFN-γ with stimulation of hsp65 or M. leprae lysate. The splenocytes from immunized NOD mice produced high titer of IFN-γ by stimulation with M. leprae lysate.

### Table 2. Lymphocytes proliferation and IFN-γ stimulation with M. leprae lysate, hsp65 or the 38kD protein in splenocytes culture of NOD mice with/without immunized with M. leprae.

<table>
<thead>
<tr>
<th>Mouse age</th>
<th>Restimulate with (in vitro)</th>
<th>Immunized with</th>
<th>M. leprae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm (SI)</td>
<td>IFN γ (IU)</td>
<td>cpm (SI)</td>
</tr>
<tr>
<td>24W</td>
<td>-</td>
<td>4076 ± 99</td>
<td>20 ± 0</td>
</tr>
<tr>
<td></td>
<td>MI</td>
<td>8115 ± 94(2.0)</td>
<td>53 ± 23</td>
</tr>
<tr>
<td></td>
<td>hsp65</td>
<td>68348 ± 414(16.8)</td>
<td>20 ± 0</td>
</tr>
<tr>
<td></td>
<td>38kD</td>
<td>99 ± 266(1.0)</td>
<td>&lt;10</td>
</tr>
<tr>
<td>40W</td>
<td>-</td>
<td>3667 ± 205</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td>MI</td>
<td>6403 ± 84(1.7)</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td>hsp65</td>
<td>58553 ± 1294(15.9)</td>
<td>10 ± 0</td>
</tr>
<tr>
<td></td>
<td>38kD</td>
<td>3822 ± 421(1.04)</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are mean (two to five mice) ± SD.
ISO = stimulation index.
MI = M. leprae lysate.
ND = not done.
IU = International unit. Titers were expressed in the international reference units calibrated against National Institute of Health mouse reference IFN (G002-904-511) in triplicate.
When the cells were cultured with anti-IL10 plus hsp65, the IFN-γ production in 24 weeks of NOD mice became very high. These results may suggest that the IFN-γ induction with hsp65 was suppressed in NOD mice producing cytokine IL-10. Therefore, IL-10 production in vitro was tested. The splenocytes cultures of NOD mice with or without M. leprae immunization (female, 14 weeks of age) produced high titer of IL-10 by stimulation with hsp65, but not by M. leprae lysate and the 38kD protein. On the other hand, BALB/cA mice with or without M. leprae immunization (female, 14 weeks of age) did not produce the cytokine IL-10 even by the stimulation with hsp65 (Table 3).

In results, M. leprae immunization never promoted the onset of IDDM in NOD mice, but completely prevented. The spontaneous development of antibody to hsp65 and 38kD protein preceded the onset of diabetes in NOD mice, even though the reasons are unclear. The reason of immunization to inhibit the onset of IDDM is not yet to know. We speculate as follows: 1) The hsp65 of M. leprae, which will be produced constantly by inoculation of the live bacilli in the footpad, has an ability to induce IL-10 in NOD mice (Table 3), and the IL-10 inhibit the production of Type 1 cytokines, like TNF-α, IFN-γ and IL-12 which probably reflect an ongoing type I autoimmune inflammatory reaction as IDDM. 2) The stress as bacterial infection may induce endogenous mammalian hsp60, and has an ability to inhibit the onset of IDDM, because one of the epitopes of hsp60 has an ability to inhibit that. 3) The PGL-1 of M. leprae inhibit the T lymphocytes responses, and the continuous production of this antigen by live M. leprae may reflect the prevention of IDDM incidence. We would like to continue our efforts to clue the reasons why the IDDM was prevented by M. leprae immunization.

ACKNOWLEDGMENTS

The authors thanks Dr. M. Matsuoka, leprosy Research Center, NIID for the supply of M. leprae, Dr. S. Nagai, for the supply of 38kD protein, and Dr. C.R. Ahsan, STA Fellow in the National Institute for Leprosy Research, for manuscript preparation.

This work was supported in part by the grant from the Sasakawa Memorial Health Foundation.

Table 3. IL-10 production in splenocytes cultures of NOD and BALB/cA mice by stimulation with hsp65 et al (u/ml).

<table>
<thead>
<tr>
<th>Mice</th>
<th>Stimulated with (in vitro)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td>NOD</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>NOD-immun.</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>BALB/c</td>
<td>2.3 ± 0.25</td>
</tr>
<tr>
<td>BALB/cA-immun.</td>
<td>ND</td>
</tr>
</tbody>
</table>

NOD and BALB/cA mice were 14 weeks of age.
Data are mean (two to three mice) ± SD.
ND = not done
<sup>a</sup> = M. leprae lysate
<sup>b</sup> = immunized with M. leprae.
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I型糖尿病自然発症NODマウスの糖尿病発症に対するらい菌感染の予防的効果

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〔受付：2001年12月4日、受理：2002年1月10日〕

キーワード：らい菌、自己免疫疾患、NODマウス I 型糖尿病、hsp65、38kD たんぱく質

NODマウスは加齢により I 型糖尿病を自然発症するマウスである。このマウスに足底1 回のか рай菌投与がどのような免疫応答を示すのかについて検討したところ、糖尿病発症の促進ではなく、完全阻止がみられた。しかし、糖尿病発症に先立つ脳炎発症を阻止することもなかった。この際対照群では、糖尿病発症に先立って、抗 hsp65 および抗 38kD たんぱく質血清抗体がみられたが、らい菌接種マウスではこのような抗体価は検出されなかった。

らい菌接種による発症阻止に占める免疫学的機序解明のため、マウス脾臓培養細胞におけるリンパ球応答反応、ガンマーインターフェロン、IL-10 産生などについて検討したところ、コントロールマウスとらい菌接種マウスとのあいだには特に顕著な差異は認められなかった。