論 文 の 内 容 の 要 旨

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平成 16 年度博士課程　入学
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論文題目　Studies on the intranasal immunization with Leish-111f against *Leishmania amazonensis* infection
(*Leishmania amazonensis* 感染に対する Leish-111f の経鼻免疫効果に関する研究)

Leishmaniasis is a parasitic disease caused by flagellated protozoa in genus *Leishmania spp.* which transmitted by blood sucking female sandfly. Leishmaniasis is considered a zoonosis and humans are generally accidental hosts. Leishmaniasis, a broad spectrum disease varies from simple cutaneous to fatal visceral form depending on the species of parasites. *Leishmania amazonensis* and *L. major* are causative protozoa of cutaneous leishmaniasis in New World and Old World, respectively. Although the severity of cutaneous leishmaniasis is not a life threatening but it makes a chronic disease condition. The lesions of cutaneous leishmaniasis in *L. major* infection mostly heal eventually, usually after several months of infection without treatment. However, the infection with *L. amazonensis* causes developing chronic non-healing skin lesions. Typical form of leishmaniasis caused by *L. amazonensis* infection is associated with a primary lesion which spreads to multiple areas of the skin with the large numbers of parasites in lesions.

The knowledge of pathogenesis and immune response in leishmaniasis comes from mouse models using parasites and inbred mice which serve as the guides for exploration in human leishmaniasis. The mouse model reproduces a range of susceptibility states resembling individual human cases depending on the strain of mouse. BALB/c mice are highly susceptible, they develop skin ulcers which

expand and metastasize, leading to death. C57BL/6 mice are moderate susceptible mouse strain for *L. amazonensis* infection. C57BL/6 mice infected with *L. amazonensis* were not able to control the parasite and developed lesions continuously. In mice, the outcome of infection depends on the polarized activation of two subsets of $CD4^+$ T cells, Th1 or Th2. The subdivision into Th1 and Th2 cells is based on the pattern of cytokines that they produce. Th1 cells produce gamma interferon (IFN-γ) and interleukin-2 (IL-2) whereas Th2 cells produce IL-4, IL-5 and IL-10. Protective immunity depends on the induction of T cells producing Th1 cytokines which activate macrophages to kill the intracellular parasites through nitric oxide-mediated mechanism.

At the present time, a successful of treatment is obstructed by many factors. There is a first line drugs that is available in some countries but it is also limited by the side effect and parasite resistant to drug. Although there is no available vaccine for leishmaniasis, vaccination would be a hopeful strategy to reduce the use of chemotherapy, particularly in the endemic area. Decrease in the severity of the diseases can be also expected in vaccination. However, it still needs many efforts to establish an effective vaccine for using in humans and animals.

Mucosal immunization is alternative antigen delivery system, including intranasal and oral immunization using mucosal membrane to receive the antigen. In leishmaniasis, Pinto et al., reported the successful of intranasal immunization of BALB/c mice with crude *L. amazonensis* antigens which lead to the effective control of lesion growth. However, using a specific recombinant antigen should be a good choice because it is stable, safe, and inducing specific protective immune response without side effect such as induction of autoimmune responses.

Now, there is one recombinant protein, Leish-111f a candidate vaccine for humans against leishmanial infection. Leish-111f, a 111 kDa single recombinant polyprotein comprising the sequences of all three open reading frames genetically linked in tandem (TSA-LmSTI1-LeIF). Successful vaccination by subcutaneous injection with Leish-111f plus MPL-SE adjuvant was reported the protection against *L. major,*

L. amazonensis, and *L. infantum* challenges in BALB/c or C57BL/6 mice.

In our laboratory, we have already shown the protective immune response could be induced by intranasal immunization with leish-111f plus cholera toxin (CT) adjuvant against *L. major* in BALB/c mice. However, the intranasal immunization with leish-111f plus CT adjuvant has not been studied for the other important *Leishmania* strains yet. One of the requirements of an ideal anti-leishmanial vaccination is for it to be effective against more than one *Leishmania* species in order to protect individuals in areas where cutaneous and visceral leishmaniasis coexist.

The aim of this thesis is to evaluate the efficacy of intranasal immunization with leish-111f plus cholera toxin adjuvant in another human pathogenic *Leishmania* species, *L. amazonensis*. To evaluate this efficacy against another parasite by using this model of infection, then the study of the protective immune response after intranasal immunization against *L. amazonensis* infection was studied.

In chapter 1, the intranasal immunization with leish-111f plus cholera toxin (CT) was evaluated for the induction of protective immune response against *L. amazonensis* infection in BALB/c mice. Intranasally immunized BALB/c mice showed the decrease in the lesion growth after *L. amazonensis* infection, when compared with non-immune mice. However, the nodular lesions in immunized mice developed slowly which never disappeared, while in mice treated as the same way and infected with *L. major*, lesions diminished and completely disappeared. The immune responses after intranasal immunization were evaluated by measurement of cytokine production from splenocyte cultures. Intranasally immunized BALB/c mice showed high levels of IFN-γ production but low in interleukin-4 levels. It indicates that intranasal immunization with leish-111f plus CT induced Th1-type response. The protective efficacy was not changed when the number of boosting cycle was changed. In BALB/c mice intranasally immunized 6, 4, or 2 times with leish-111f plus CT, all of immunized mice controlled the lesion growth after infection with *L. amazonensis.* When compared lesion growth to BALB/c mice subcutaneously immunized with leish-111f plus MPL-SE, subcutaneously immunized mice showed the lesion size smaller than

non-treated mice, but larger than intranasally immunized mice.

The results of chapter 1 indicate that intranasal immunization with leish-111f plus cholera toxin induces Th1-type response. The immunized BALB/c mice partially controlled the lesion growth after infection with *L. amazonenesis*. The disease protection may be the result of high IFN-γ production in immunized BALB/c mice. The extent of the protection in intranasally immunized mice was not different when the number of boosting cycles was changed. Intranasal immunization with at least 2 doses was able to induce the protective immune response against *L. amazonensis* infection. In this chapter, the intranasal immunization with leish-111f plus CT was effective to induce the protective immune response against both of *L. major* and *L. amazonensis* infection.

In chapter 2, protective immune responses after intranasal immunization against *L. amazonensis* was evaluated in another susceptible mice with different genetical background, C57BL/6 mice, which is susceptible to *L. amazonensis* but resistant to *L. major*. When C57BL/6 mice were immunized intranasally with Leish-111f plus CT and infected with *L. amazonensis* at the tail base, surprisingly, treated mice failed to control the infection with *L. amazonensis* and showed the progressive lesion development similar to non-immunized C57BL/6 mice. When the cytokine profile after intranasal immunization was evaluated, intranasally immunized C57BL/6 mice showed high production in IFN-γ but low in IL-4, indicating that Th1-type responses were also induced in C57BL/6 mice, as well as shown in BALB/c after intranasal immunization. It indicates that Th1 response after intranasal immunization could not protect C57BL/6 mice from the disease.

In order to compare the “stability” of Th-1 type response induced by intranasal immunization of Leish-111f plus CT between BALB/c (responder) and C57BL/6 (non-responder), cytokine profile was evaluated in those mice after *L. amazonensis* infection. From the result of chapter 1, immunized BALB/c mice showed the lesion sizes smaller than lesions of non-immunized mice. The lesions in BALB/c mice were different clearly, particularly from 7-8 weeks after infection. Thus, I expected the cytokine profiles at

that time should show the difference of Th1 type response between immunized and non-immunized mice. The cytokine levels were checked from supernatants of splenocyte cultures after mice infected with *L. amazonensis*. Leish-111f intranasally immunized BALB/c mice showed high levels of IFN-γ but the levels of IL-4, IL-10 were low when compared to non-immunized mice. High IFN-γ production in immunized BALB/c mice after infection would indicate the stable Th1 induction in BALB/c mice. On the contrary, C57BL/6 mice immunized intranasally with leish-111f plus CT showed fair level of IL-4 and high level of IL-10 productions similarly with those of non-immunized mice, but the levels of IFN-γ in immunized C57BL/6 mice were found lower than non-immunized mice after *L. amazonensis* infection, indicating that Th1-type responses aquired in C57BL/6 by intranasal immunization was altered to Th2-type after *L. amazonensis* infection. It would be possible that the level of Th1 induction might not enough in C57BL/6 mice to control the parasite growth or that the immune mechanism to resist the growth of *L. amazonensis* might differ in BALB/c and C57BL/6. The mechanism why C57BL/6 did not respond to intranasal immunization with Leish-111f plus CT despite of the Th1 induction should be further analyzed.

In chapter 3, I focused on the mechanism of the induction of protective immunity to *L. amazonensis* by intranasal immunization with Leish-111f plus CT. The results in the previous chapters showed both of intranasal and subcutaneous immunizations induced Th1-type response to BALB/c mice but the disease protection after *L. amazonensis* infection was different. It has been reported that the administration of antigen by different antigen delivery system often lead to the different immune responses. Thus, in this chapter, Leish-111f epitope recognition by Th1 cells was compared between BALB/c mice immunized intranasally or subcutaneously with Leish-111f plus CT. Splenocytes were isolated from immunized mice and stimulated with a series of leish-111f peptides (269 peptides) and IFN-γ production after 72 hours were measured by ELISA. The results showed that splenocytes from mice after intranasal immunization with different number of boosting time of 6, 2 and 1 showed similar epitope recognition peaks except that more variety of epitopes seemed to be recognized in a single immunization, which might

demonstrate the selection of T cells with higher affinity epitopes occurring during boost immunization. Most of IFN-γ inducing leish-111f epitopes were found in the middle and final part of all 269 leish-111f peptides. The epitope recognition pattern of Th1 cells from BALB/c mice immunized subcutaneously was similar to that of intranasally immunized mice. These data demonstrates that the antigen presentation by dendritic cells and antigen recognition by Th1 cells did not differ in different antigen delivery routes. However, the mechanism which makes the difference in the extent of protection between intranasal and cutaneous immunization remains to be cleared.

In this thesis, the induction of Th1 responses in BALB/c mice after intranasal immunization with leish-111f is effective not only in *L. major* but also *L. amazonensis* infections. The epitope recognition in intranasal immunization seemed to be similar to those in parenteral immunization. However, as shown in the results of C57BL/6 mice, the unresponsiveness was observed. Further experiments for the stable protection should be performed.