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1．研究テーマ　変異リステル菌による免疫応答制御

2．本年度の研究業績

(1) 学会・研究会等における発表　(有)・無　（学会名・演題）

２００２　日本免疫学学会総会　学術集会
変異リステル菌による免疫応答制御

(2) 学会誌等に発表した論文　有・(無)　（雑誌名・論文名）

3．今後の研究計画

本実験は、弱毒化リステリア菌が動物細胞に遺伝子を導入するためのベクターとして用いて使用できることが明らかになった。今後、リステリア菌の病原性を完全、かつ遺伝子導入の効率を上がる方法を捜索することが必要であると考える。また、今回構築した遺伝子を用いて、感染症のみならず、免疫系の異常でおきる種々の疾患の動物モデルを用いる、治療実験を行う予定である。

4．指導責任者の意見

瀋　華君は、極めて熱心に研究に打ち込み、当初の目的をある程度達成することができたと思う。リステリア菌をベクターとして用いて、宿主の免疫応答を制御する試みでは、特定の病原体に感受性のマウス系統を、感染抵抗性にシフトすることが可能であることを示し、その効率の向上のため種々の試みを継続している。経口感染可能なリステリア菌の作出を試みているが、未だ成功していない。しかしこの過程をとおして多くの分子生物学的手法を学んだと思う。さらに、教室が現在もっとも力を入れて解析している、宿主免疫応答を制御する病原体遺伝子の同定には、積極的に関与し、自らも種々の解析手法等を学んだと思う。この間の研究に対する態度は極めて熱心で、また、研究者としての優れた資質を感じさせます。今後何らかの形で、経済的支援が得られればと考えています。

指導責任者氏名 浅野喜造

5．研 究 報 告 書

別紙「研究報告書の作成について」により、指定の用紙で作成し添付して下さい。

研究発表中または研究中のスナップ写真を添付して下さい。

※研究成果を発表する場合は、発表原稿・抄録集等も添付して下さい。

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-日中医学協会助成事業-

変異リステルア菌による免疫応答制御

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Abstract

We showed that intracytoplasmic infection by *Listeria monocytogenes* (Lm) influences the T cell differentiation process and resulted in dominance of the type 1 T cell subset, and proposed a two-step T cell differentiation pathway during pathogen infection. Base on this study, we established a novel method for Lm-mediated gene transfer into mammalian cells to manipulate the immune response of the host during infection by pathogens. We demonstrate that the gene manipulated Lm containing a self-destroying gene functions as a possible carrier for gene transduction into mammalian host cells.

Key words gene transfer, *Listeria moncytogenes*, type 1 T cell subset, pathogen infection, immune response

Introduction

T helper (Th) cells are divided into two subsets according to the set of cytokines that they produce. Type 1 T cells which produce IFNγ play an important role in the clearance of intracellular pathogens, whereas Type 2 T cells which produce interleukin (IL) –4 eliminate extracellular ones. Shift to one of these two subsets from precursor can determine the outcome of the diseases in many intracellular pathogens infection, such as leishmaniasis. During *Leishmania major* (L. major) infection, resistant C57BL/6 mice develop protective Th1 responses that control infection, on the contrary, susceptible BALB/c mice fail to control infection due to an aberrant Th2 response (1). Cytokines present at the initiation of the immune response at the stage of ligation of the T cell antigen receptor (TCR) determine the type 1 or type 2 T cell differentiation from the precursor. It is known that effective primary immunity against L.major requires IL-12-dependent production of IFN- γ from Th1 cells (2,3). On the other hand, some other cytokines, such as IL-4 and IL-10, act as candidate that induce Th2 cells (4-6). After infected with L. major, the early production of IL-4 in susceptible BALB/c limits the ability to induce a protective Th1 response (5). Treatment with recombinant IL-12 (rIL-12) in the early stage of infection shows protective effect in BALB/c mice infected with L. major (2). Therefore, it is possible to optimize immune response to control pathogens by modification the balance of cytokines at the time of antigen stimulation.

Recently, the technique using intracellular bacteria for DNA transfer into mammalian host cells was performed successfully (6), which provides not only an effective method to study the immune response but also therapy to many kinds of diseases. Attenuated strains of intracellular bacteria are ideal candidates which can deliver plasmids DNA to target cells. *Listeria Monocytogenes* is a Gram-positive intracellular bacterium which invades both phagocytic cells and nonprofessional phagocytic cells. It is now known that various genes and proteins directly involved in the various steps of the infectious process: Internalization by nonprofessional phagocytic cells is often mediated by internalins, of which Internalin A and B are the best characterized (7). Upon entry into the cells, *Listeria* become engulfed within a vacuole. Hemolysin in combination with phospholipases is largely responsed for mediating escape from the vacuole to cytosol (8). Once in the cytosol, bacteria multiply. Intracytoplasmic bactria are immediately surrounded by a cloud composed of actin filaments and use the force generated by actin polimerization to move intracellularly and then spread from cell to cell (9). Taking advantage of this knowledge, attenuated strains of Lm which are able to undergo self-destruction in the cell cytosol by production of a phage endolysin could deliver functional plasmids DNA into the cell (10).

In the present study, we established a Lm-mediated gene transfer system which can transfers IL-12, IL-4, IFNγ, and IL-10 cDNA into mammalian host cells. With this system we investigated the T cell differentiation in the murine model of cutaneous lishmaniasis.

Materials and methods

cDNA: cDNA from murine IL-4 , IFNγ and IL-12 p70 were obtained from Dr. H. Karasuyama, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan and by Dr. H. Yamamoto, Osaka University, Osaka respectively cDNA from *ply118* was kindly provided by Dr. W. Goebel Theodor-Bover-Institut für Biowisenschaften, Würzburg, Germany and Dr. M. J Loessner, Institut Fur Mikrobiologie, Technische Universität Müchen, Freising, Germany.

Mice: OVA-peptide-specific TCR TG mice were originally developed by Dr. D. Loh. Mice and their littermates were reared under specific pathogen free conditions in the animal facility of Ehime University School of Medicine. BALB/c and C57BL/6 mice were purchased from Charles River Japan (Yokohama, Japan). All mice were used in accordance with the institutional guides for animal experimentation.

in vitro stimulation of T cells: T cells of uninfected- and Lm-infected TCR-TG mice were prepared as non-adherent cells as described (11), and were stimulated in vitro with T cell depleted splenic APC from

uninfected- and Lm-infected syngeneic BALB/c mice in the presence of 1μM specific OVA peptides. Cells were collected and washed, and re-stimulated with APCs and homologous antigen for 2 days. Amounts of IL-4 and IFNγ in the culture supernatant were determined by ELISA assay (12-14).

Exprimetal infections in vivo and Pathogens: *Listeria Monocytogenes* (EGD strain) was provided by Dr. P. Cossart, Pasteur Institute, Paris, France and $2x10^3$ of bacteria were inoculated intraperitoneally.

in vitro infection with Lm: For *in vitro* infection of splenic APCs with Lm, T cell depleted spleen cells were infected with Lm at MOI of 5:1 for 1h followed by extensive washing and X-irradiation at 3000 rad. These treated splenic APC cells were cocultured with T cells from TCR-TG mice. For gene transfer by Lm, cultured cell lines were infected with gene manipulated Lm *in vitro* at MOI of 5:1 for 1h and followed by extensive washing and cultivation for 2 days. The expression of the transferred gene was detected either by fluorescence microscopy or ELISA.

Results and Discussion

We used OVA-peptide specific T cell receptor transgenic (TCR-TG) mice (15) and the intracellular infectious pathogen Lm to evaluate the effect of pathogenic infection. When T cells of uninfected TCR-TG mice were stimulated with a specific antigen and antigen presenting cells (APC) *in vitro*, the cells differentiated predominantly into an IL-4-producing type 2 T cell subset (Fig. 1a). In contrast, Lm-infection appeared to stimulate T cells to differentiate into an IFNγ-producing type 1 T cell subset. This effect that infection had on T cell subset differentiation was observed even in RAG-1-deficient mice where T cells are specific exclusively for OVA-peptide and no Lm-specific T cells are present (Fig. 1a). These results suggest that T cells in Lm-infected mice differentiated predominantly into the type 1 subset. In addition, the effect of infection may be mediated by APCs. This possibility was directly tested by stimulating T cells of uninfected TCR-TG mice with APCs from Lm-infected mice (Fig. 1b). When uninfected naïve T cells were stimulated with infected APCs, the T cells shifted to the IFNγ-producing type 1 T cell subset. Therefore, it suggests that Lm-infection influences T cell differentiation through the APC function.

Based on these observations, we thought that the intracellular pathogen Lm functions as an immune response modulator and also as a good candidate for a vector to deliver a gene into mammalian cells to modulate the acquired immune response (16,17). We constructed the plasmid for gene transfer into mammalian cells using Lm. Lm is attenuated by introducing a gene (*ply118*) which functions to lyse Lm (18). The *ply118* gene is inserted with an *actA* gene promoter to lyse the bacteria during infection. Lm

which carries cDNA coding for cytokines (Lm (cytokine)) delivered cDNA into mammalian cells by *in vitro* infection as shown in Fig. 2. Cytokine production by Lm-infected cells was also tested using Cos7 and HepG2 cell lines. Although the amount of cytokine produced by these cells varied, the pattern of cytokine producion was specific for those strains of Lm which were infected *in vitro*. These results demonstrate that the gene manipulate Lm function to deliver gene into mammalian cells and induce expression of the introduced gene.

We next tested whether these manipulated Lm can modulate immune response *in vivo*. To assess this question, BALB/c mice were inoculated with Lm EGD strain (wild type), EGD carrying IL-12 p70 cDNA in the hind leg 10 days prior to inoculation with L. major in the footpad of the same hind leg (Fig. 3). The footpad swelling in the *L. major* susceptible strain BALB/c mice gradually increased, while that in the resistant strain C57BL/6 peaked at 2.5 wk after infection and gradually diminished by 8 wk after infection. The footpad swelling of BALB/c mice infected with EGD was suppressed at 6 wk after infection. The peak magnitude of footpad swelling was suppressed in the susceptible strain BALB/c mice infected with EGD carrying IL-12 cDNA even when compared with the resistant strain C57BL/6. This state of suppression still persisted at 5 wk after *L. major* infection. This result suggests that gene transfer by attenuated Lm infection functions to modulate the host defense system against pathogen infection.

It is known that *Shigella, Listeria, Salmonella* and *Escherichia coli* are able to act as gene delivery vectors. Listeria is one of the most efficient at gene transfer among the facultative intracellular pathogens (19). In this report used self-destroyed Lm as vector, up to 10% of Caco2 cells were transfected after infection (data not shown). Specific cytokines production which were produced by transfected cell lines varied in the different recipient cell and cytokines (Fig 2). In the same cell line, production of IL-4, IFNγ, and IL-10 were more abundant than that of IL-12. In several cell lines that were tested, epithelium cells and hepatocytes were readily transfected, whereas transfection in macrophage cell lines were not efficient (data not shown). This result was consistent with previous report (10,19).

We have shown that we established a gene transfer system with *L. monocytogenes* modulating immune system to control the disease. This system may be used for the development of vaccines and immunotherapy against other intracellular pathogens.

Note

This study was presented as a poster on The 32nd Annual Meeting of The Japanese Society for Immunology

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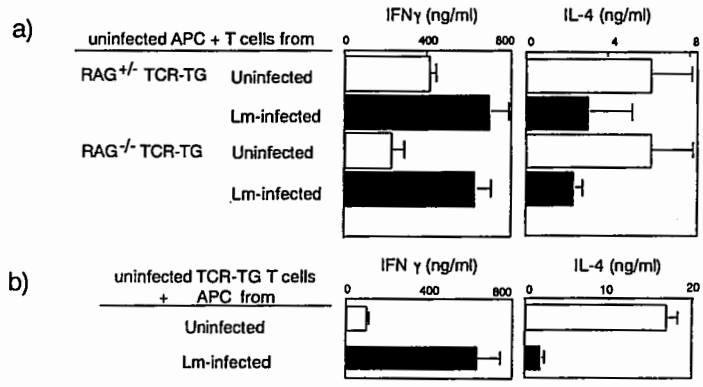

Figure 1

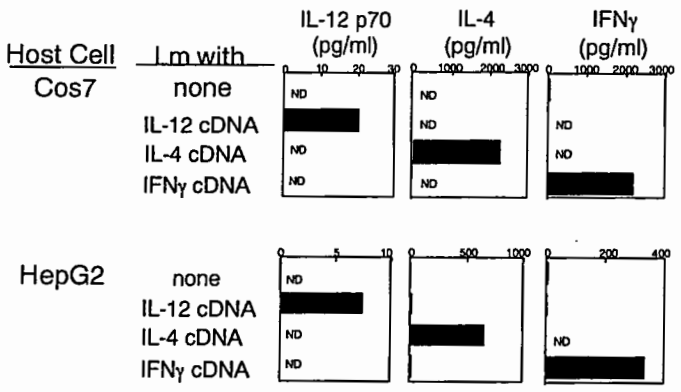

Figure 2

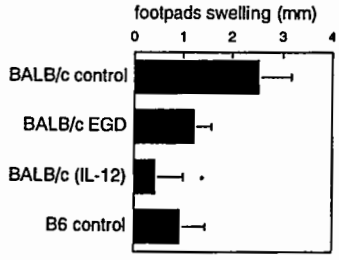

Figure 3

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