Development of a Drug Delivery System using a Model that Mimics Chronic Infection of *Mycobacterium bovis* Calmette-Guérin in Alveolar Macrophages

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Abstract. Macrophages play a dominant role in defense against infective organisms and their regarded abilities can be positioned as their most primitive and important function. On the other hand, tuberculosis, caused by tubercle bacilli which possess the ability to survive in phagosome and grow in cell, poses a serious problem as an intractable disease because the efficacy of drug delivery to the target bacilli is low. We have developed a new approach to therapy against intracellular bacteria using a drug delivery system (DDS), to deliver an effective amount of drug to the target site, based on the phagocytotic ability of macrophages. In this review, the development of an *in vitro* model for chronic infection by tubercle bacilli and therapy against *M. tuberculosis* benefiting from the phagocytotic ability of macrophages, using DDS with microspheres are described.

Macrophages exist in every tissue and are part of the initial defenses against invasive pathogens. They are called Kupffer cells in the liver, intestinal macrophages in the intestine and alveolar macrophages in lung alveoli, and so on. Furthermore, monocytes, macrophages present in the serum, show inflammatory responses and infiltrate into tissues. These cells generally are highly active in phagocytosing foreign substances. This phagocytotic function of the macrophages is phylogenetically conserved in all animals and is a major component of the innate immune system. There are, however, bacilli, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis*, which inhibit the phagocytosis process and escape inside or outside the macrophage to establish their infection. In particular tuberculosis, caused by *M. tuberculosis* which survives in alveolar macrophages as its host (1), has spread over the world and is designated as one of the three major infectious diseases of the world by WHO. One third of the world population is infected with *Mycobacterium tuberculosis*, two million people die of it annually, and eight million newly infected patients appear annually. This review describes first the development of an *in vitro* model for chronic infection by tubercle bacilli and then therapy against *M. tuberculosis* benefiting from the phagocytotic ability of macrophages, using DDS with microspheres.

Survival Strategy of Tubercle Bacillus

In general, after phagocytosing foreign substances such as bacilli, macrophages form phagosomes to eliminate them. The phagosomes maturation through a series of membrane fusion process with endosomes or lysosomes while finally the macrophages can digest and degrade the foreign substances by digestive enzymes derived from lysosomes, often forming...
phagolysosomes (2-5). Bacilli invading lung alveoli are recognized and phagocytosed by alveolar macrophages. Phagocytosed M. tuberculosis bacilli however inhibit the phagosome acidification that is one step in phagosome maturation (6-13). Therefore, the maturation of phagolysosomes is inhibited in the presence of M. tuberculosis bacilli which permits their survival and growth in the macrophages. There are at least two different steps involved in the intracellular survival strategy of M. tuberculosis (1). In the early stage, the period within 24 hours of infection, Toll-like receptor-2 and -4 (14-17), tumor necrosis factor-α (TNF-α) (18,19), interleukin-1 (IL-12) (20) and interferon-gamma (21) have been reported to be host factors relevant to M. tuberculosis survival. It has also been reported that M. tuberculosis inhibits activation of calmodulin and Ca\(^{2+}\)/calmodulin kinase II, which are key molecules responsible for forming bactericidal phagolysosomes (6, 22). These studies have indicated that host factors may be involved in M. tuberculosis survival, but the research has focused almost entirely on the early stage after phagocytosis. In contrast, little work has been done on the late stage of infection, the stage of chronic infection.

Current therapy against pulmonary M. tuberculosis mainly consists of oral administration of therapeutic drugs or antibiotics such as isoniazid (INH), pyrazinamide (PZA) and rifampicin (RFP) (23). However, in order to reach an effective concentration in the alveolar macrophage phagosomes, a large quantity of drug has to be administered long term. Thus, the establishment of a new therapy to kill tuberculosis within alveolar macrophages would be advantageous.

A Model that Mimics a Chronic Infection in Cultured Macrophages

We consider that the most important target for tuberculosis therapy is the functional changes in macrophages occurring in the period of chronic infection. Moreover, the type of host macrophage selected is important in the in vitro study of the function of macrophages infected with tubercle bacillus. Each tissue macrophage possess its distinctive characteristics, for example, peripheral monocytes and alveolar macrophages differ in response to foreign substances. Therefore, there is a strong possibility that the response to tubercle bacillus infection differs depending on macrophage tissue type. However, monocyte cell lines such as J774.1 or THP-1 have often been used as the host cells when testing for candidate molecules and/or mechanisms relevant to the intracellular survival strategy of M. tuberculosis. We consider that since the target of infection is the lung, alveolar macrophages should be used in the development of a new in vitro chronic infection model.

This view was supported by our study of the response to the tubercle bacillus of monocytes and macrophages in terms of TNF-α production (24). TNF-α production was not observed by primary cultured rat alveolar macrophages (AMs) or a rat normal alveolar macrophage cell line, NR8383 after phagocytosis of either live or killed Mycobacterium bovis Calmette-Guérin (BCG) (Figure 1). Conversely, a mouse monocyte cell line, J774.1 produced high amounts of TNF. This result illustrated the possibility of error in observing the response of monocytes and suggested that for experimental targeting of alveolar macrophages a system using monocytes in not appropriate.

Using NR8383 cells as a model of chronic infection, live tubercle bacilli survived while morphological change and complete digestion were observed in the case of dead tubercle bacilli, 7 days and 21 days respectively after phagocytosis. This model therefore mimicked, chronic infection by the tubercle bacillus in cultured cells enabling us to study the morphological changes of macrophage in the period of chronic infection and to development of a new therapy against tubercle bacillus.

Selective Delivery of Drug into Macrophage by DDS

Standard therapy requires the long term administration of large amounts of anti tuberculosis drugs due to the difficulty of attaining effectively high concentrations in the lung, the macrophages and the phagosomes where the bacilli hide,
which leads to side effects or decreased compliance. One of the methods for solving this problem and to enhancing the drug administration to the phagosomes is a drug delivery system (DDS) using microspheres which are phagocytosed by the macrophages.

It is possible that microspheres loaded with anti-tuberculosis drugs could be phagocytosed selectively by macrophages, assuming that alveolar macrophages infected with tubercle bacilli retain phagocytotic capability. We therefore developed microspheres composed of RFP-loaded poly (DL-lactic-co-glycolic acid) (PLGA) which are biodegradable polymers and can be engulfed by macrophages (25, 26). When cells were treated with RFP-PLGA microspheres with a molecular weight of 20,000 and a monomer ratio of 75/25 (lactic acid / glycolic acid) 3.5 Îg RFP / 10^6 cells (3.5 Îg/ml) was recovered (Figure 2). This is about 70 times higher than the minimum inhibitory concentration (MIC) for M. tuberculosis. No RFP was extracted from the cells when an equivalent concentration of free RFP was used. A very low amount of RFP was shown to be introduced to the cells by the addition of 100 Îg/ml free RFP solution (Figure 2). The microsphere DDS using phagocytosis for the drug transport to macrophages was therefore considered to be effective.

Selective Drug Delivery for Macrophages Infected with BCG by Using RFP-PLGA

Following intravenous, peritoneal or oral administration of drug containing microspheres higher transfer rates and longer half-life in the organs such as the lung (27), liver (28, 29), and spleen (30) has been shown in comparison to free drug administration. Microspheres containing INH/RFP administrated intravenously caused an approximately 1.2-fold decrease in colony forming units (CFU) of M. tuberculosis in the tissues (28). However, a considerable number of bacilli were left alive. In other words, an improvement in the method of administration was required. Alveolar macrophages exist in lung alveoli whose surface is said to be about 90-m² in an adult. Administration of drug-containing microspheres directly to lung alveoli is possible and could result in high drug concentration in the lung and a low concentration in the serum. With this in mind, we studied the killing effect of RFP-PLGA on BCG in macrophages using an in vitro model of chronic infection.

The number of bacilli surviving in NR8383 cells with RFP-PLGA microspheres decreased more than 10-times compared to that of the control 7 days after treatment and 100-times 14 days after treatment (Figure 3). In contrast, treatment with a RFP solution of 4 Îg/ml (the same concentration as incorporated in the microspheres) or 15 Îg/ml (maximum serum concentration of current therapy) did not produce a significant killing effect against intracellular bacilli. From this result, RFP-PLGA microsphere was considered to be an...
effective drug when it has direct contact with macrophages infected with tubercle bacilli. We therefore investigated the therapeutic efficacy of intratracheal RFP-PLGA microspheres in rats infected with *M. tuberculosis*, Kurono. Although statistical significance was not apparent, intratracheal administered RFP-PLGA microspheres tended to show a slight decrease in the pulmonary viable bacterial count, while free intratracheal RFP showed no decrease. Moreover, while granulomas were observed in all lobes of the intratracheal free RFP-treated group, granulomas were observed in some but not all lobes of the RFP-PLGA-treated group. From these results also, drug delivery of RFP by PLGA to macrophages infected with BCG using phagocytosis was shown to be very effective.

**Conclusion**

PLGA microspheres incorporating RFP can effectively kill *M. tuberculosis* in alveolar macrophages. The advantage of this therapy is that it enables short term treatment with less RFP compared to current therapies because selective drug delivery to macrophages is possible. Furthermore, the basic concept of this method could be developed for other diseases caused by intracellular parasitic bacteria in macrophages such as legionellosis or chlamydial pneumonia.

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**References**


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