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RESEARCH

DIFFERENCES OF LYMPHOCYTE PROLIFERATION INDEX AFTER CULTURE FILTRATE PROTEIN 10 (CFP-10) STIMULATION IN PATIENTS WITH ACTIVE AND LATENT TUBERCULOSIS AND HEALTHY INDIVIDUALS

(Perbedaan Indeks Proliferasi Limfosit Pascastimulasi Culture Filtrate Protein 10 (CFP-10) di Pasien Tuberkulosis Aktif, Laten dan Orang Sehat)

Binar R. Utami, Betty Agustina T, Suprpto Ma'at

ABSTRAK

Angka kejadian tuberkulosis di dunia semakin meningkat, akibat rendahnya ketepatan penggunaan vaksin BCG untuk pencegahan infeksi TB. Saat ini telah dikembangkan vaksin DNA dari protein *M.tuberculosis* yaitu Culture filtrate protein-10 (CFP-10) yang dapat merangsang respons imun seluler. Tujuan penelitian ini adalah mengetahui salah satu kemampuan antigenik antigen CFP-10 pasca stimulasi CFP-10 di pasien TB aktif, TB laten dan orang sehat. Penelitian bersifat eksperimen semu (quasi experimental). Sampel penelitian adalah Peripheral blood mononuclear cell (PBMC) dari 10 pasien TB paru aktif baru, 10 TB laten RS Khusus Paru Surabaya dan 10 orang sehat. Perlakuan kultur PBMC tanpa stimulasi (pembanding), dengan Mitogen (PHA) sebagai pembanding positif dan dengan stimulasi antigen CFP-10 diinkubasi pada CO₂ 5% selama 5 hari. Uji proliferasi limfosit dilakukan dengan menambahkan MTT. Indeks proliferasi limfosit adalah rasio antara absorbansi pembanding dan absorbansi dengan stimulasi pada λ 560 nm. Terdapat perbedaan bermakna indeks uji proliferasi limfosit pascastimulasi CFP-10 antara pasien TB paru aktif dan orang sehat ($p=0,019$) dan di pasien TB aktif, TB laten dan orang sehat ($p=0,0356$). Antigen CFP 10 mampu merangsang respons imun protektif pada pasien TB aktif, TB laten dan orang sehat. Aktivitas imunogenik antigen CFP-10 dapat dipengaruhi oleh status imun seseorang dan kemampuan antigen CFP menginduksi respons imun protektif.

Kata kunci: CFP-10, indeks proliferasi limfosit, TB paru aktif, TB laten, orang sehat

ABSTRACT

The incidence of tuberculosis in the world is increasing, due to the low effectiveness of the BCG vaccine for the prevention of TB infection. Currently, a DNA vaccine from *M.tuberculosis* proteins has been development that is a Culture filtrate Protein 10 (CFP-10) which can stimulate cellular immune response. The purpose of this study was to determine one of the abilities of CFP-10 antigen to stimulate an immune response in patients with active TB, latent TB and healthy people. This study was a Quasi-experimental study. Samples were Peripheral Blood Mononuclear Cell (PBMC) from 10 patients with active pulmonary tuberculosis, 10 latent TB at the Karang Tembok Pulmonary Hospital, Surabaya and 10 healthy people. Peripheral blood mononuclear cell cultures without stimulation (control), with mitogen (PHA) as a positive control and with CFP-10 antigen, incubated at 5% CO₂ for 5 days. Lymphocyte proliferation test was conducted by adding MTT. Proliferation index measured by comparing the absorbant with antigen stimulation control and absorbant λ 560 nm. There was a significant difference in lymphocyte proliferation index post stimulation CFP-10 between active pulmonary tuberculosis patients and healthy individuals ($p=0.019$) and in patients with active TB, latent TB and healthy individuals ($p=0.0356$). CFP 10 antigen had the ability of inducing a protective immune response in patients with active TB new, latent TB and healthy people. Immunogenic antigens CFP-10 activities can be influenced by a person's immune status and the ability to induce a protective immune response.

Key words: CFP-10, lymphocytes proliferation index, active pulmonary TB, latent TB, healthy people

INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*. World Health Organization (WHO) in 2012 reported that there were 8.7 million new TB cases or 125 cases per 100,000 population. Indonesia even has been categorized in the 22 High Burden Countries (HBCs) with the prevalence of 489/100,000 population, moreover, the incidence of TB in Indonesia was 222/100,000 population with a mortality rate of 48/100,000 population.¹

Many researches actually have revealed that BCG vaccine has various and great effectiveness. BCG vaccine is effective in preventing miliary tuberculosis and meningitis tuberculosis in children, but not for pulmonary tuberculosis in adults. A research conducted at mass in Chingelput India, involving 360 thousand people who received the BCG vaccine, concluded that BCG acquired in childhood apparently does not provide protection against TB at the age of adults.²

However, the effectiveness of BCG vaccine has been studied in many researches as an attempt to get a better vaccine in order to modify the existing BCG vaccine. A modified strategy of the BCG vaccine is to insert a number of genes in BCG vaccine strains that encodes a protein (antigen) stimulating a strong immune response.³

A research with recombinant BCG vaccine in mice, furthermore, reported that recombinant BCG can improve the response of T lymphocyte cells. One of the antigens stimulating protection against *M.tuberculosis* is Cultur filtrate protein - 10 (CFP-10), a protein that is responsible for cellular immune response.⁴

CFP-10 is also called as ESAT-6 like protein esxB or as secreted antigenic protein MTSA-10 or as 10 kDa Culture Filtrate Protein (CFP-10) secreted by *M.tb*. CFP-10 together with ESAT-6 is secreted in a ratio of 1: 1 in a heterodimer complex. Both genes then are expressed from RD 1 (regio differentiation), part of the bacterial genome that has an important role in the virulence of *M.tuberculosis* infection. The use of CFP-10 and ESAT-6 proteins as vaccine candidates, nevertheless, is still controversial. The controversy is allegedly due to lack of the antigen potency in stimulating Cell Mediated Immunity (CMI).^{5,6}

Therefore, the ability of T cell proliferation in response to an antigen can be used to assess the specific immune response of T cells to an antigen. Measurement of lymphocyte proliferation index and IFN γ levels is used to measure the ability of T cells to antigen response against *M.tuberculosis* in vitro. Lymphocyte proliferation index can also be used as a marker of immunological trials of vaccines,

especially for phase I and phase II of *M.tuberculosis* vaccine trial. However, measurement of lymphocyte proliferation capabilities will be better if conducted on cell culture incubated longer (5–6 days) since with longer incubation, expansion of specific antigen secreting memory T cells will occur. Measurement of lymphocyte T cells' functions in culture for 5–6 days is also considered to be more sensitive to assess the ability of the vaccine in the form of a recombinant of specific TB antigens.⁷

Many researches actually have been conducted on the administration of recombinant BCG in mice. Nevertheless, the number of researches on stimulation of recombinant BCG, especially CFP-10 in vitro in human lymphocyte cells is still small. Consequently, further research to determine differences in lymphocyte T cell proliferation index after CFP-10 stimulation is needed.

Therefore, this research aimed to analyze one of immunogenic activities of CFP-10 antigen as vaccine candidates by measuring T lymphocyte cell proliferation index after stimulation of CFP-10 antigen in PBMC cultures from patients with active TB and latent TB as well as healthy individuals.

METHODS

This research used a quasi-experimental design in the laboratory in vitro with randomized post test only controlled group design in cultured Peripheral Blood Mononuclear Cell (PBMC) classified into three sample groups (patients with active TB, patients with latent TB, and healthy individuals) that had been stimulated with protein CFP-10 of MTB.

Subjects of this research, furthermore, were both patients with active pulmonary tuberculosis hospitalized in BP4/Karang Tembok Hospital, Surabaya diagnosed by a physician based on the examination of BTA (+) and radiographics appropriate with the description of pulmonary tuberculosis, as well as patients with latent TB infection (nurses in BP4/Karang Tembok Hospital, Surabaya) with a positive tuberculin test and chest X-ray within normal limits. Healthy control, on the other hand, were healthy individuals aged >16 years with a negative tuberculin results and chest X-ray within normal limits. The number of the research subjects for each group was 10, obtained from a numerical comparative formula, no more than paired two groups

Moreover, serum separation, isolation and culture of PBMC with CO2 incubator, and optical density measurements of lymphocyte proliferation by UV VIS

Data of lymphocyte proliferation index with PHA stimulation in all the three groups were normally distributed (Shapiro-Wilk, $p>0.05$). The results of the independent t-test then showed that there was no significant difference in lymphocyte proliferation index after PHA stimulation between patients with

active pulmonary tuberculosis and healthy individuals ($p=0.351$), between patients with latent TB and patients with active TB ($p=0.597$) and between patients with latent TB and healthy individuals ($p=0.545$). Similarly, the results of the Kruskal Wallis tests showed that there was no significant difference in

Note:

(a) Index Proliferation (IP) between latent TB and active TB, (b) IP between active TB and healthy individuals, (c) IP between latent TB infection and healthy individuals, (d) IP between the healthy individuals, active TB patients and latent TB patients (significance level $p<0.05$).

Figure 1. Lymphocyte proliferation index after PHA stimulation in active TB patients, latent TB patients and healthy individuals.

Note:

a) Index Proliferation (IP) between latent TB and active TB, (b) IP between active TB and healthy individuals, (c) IP between latent TB infection and healthy individuals, (d) IP between the healthy individuals, active TB patients and latent TB patients (significance level $p<0.05$).

Figure 2. Lymphocyte proliferation index post- CFP-10 stimulation in active TB patients, latent TB patients and healthy individuals.

Table 3. The differences in the mean lymphocyte proliferation index after CFP-10 stimulation in active TB patients, latent TB patients and healthy individuals

Group	Mean lymphocyte proliferation index±SD post- CFP-10 stimulation
Latent TB	1,183±0.160
Active TB	1,211±0.122
Healthy individuals	1,093±0.593

lymphocyte proliferation index after PHA stimulation between latent TB patients, active TB patients and healthy individuals ($p=0.537$).

Differences in the mean lymphocyte proliferation index after CFP-10 stimulation in active TB patients, latent TB patients and healthy individuals are shown in Table.3.

Lymphocyte proliferation index data after CFP-10 stimulation in patients with active pulmonary TB, patients with latent TB and healthy people were normally distributed (Shapiro-Wilk, $p>0.05$). Analysis by independent t-test showed that there was no significant difference in lymphocyte proliferation index after CFP-10 stimulation between patients with latent TB and healthy individuals ($p=0.121$). Similarly, the analysis by the Mann-Whitney test showed that there was no difference between patients with latent TB and patients with active pulmonary tuberculosis ($p=0.668$). There was also no significant difference in lymphocyte proliferation index between active pulmonary tuberculosis patients and healthy individuals ($p=0.019$).

The results of the analysis using Kruskal Wallis, moreover, showed that there was no significant difference in lymphocyte proliferation index after CFP-10 stimulation between patients with active pulmonary TB, patients with latent TB, and healthy individuals ($p=0.035$).

Mycobacterium tuberculosis is an intracellular pathogen that stimulates cellular immunity in the body. T cells play an important role in defense against this microorganism. The function of T cells can be assessed by its ability to proliferate mitogen or recombinant antigen. Interferon gamma is a major cytokine in controlling tuberculosis infection, so the proliferation of T cell and IFN- γ can be used as parameters in a test for lymphocyte's function.⁷

The results showed that there was an increase in Optical Density (OD) between the control group (without mitogen) and the three sample groups with mitogen stimulation (PHA). This shows the ability of lymphocytes to proliferate with the mitogen PHA administration on all the three sample groups.

Furthermore, there was no significant difference in lymphocyte proliferation index between the group with active TB, the group with latent TB, and the group of healthy individuals. Similarly, a research conducted by Chuenchira⁸ showed that mitogen stimulation will activate multiple pathways, including CD2⁺ and CD3⁺, as well as some receptors on the cell surface. Mitogen then will activate almost the entire population of mononuclear cells without the need of process, cell presentation and memory T cells function to stimulate proliferation response. In contrary, antigen is heavily influenced by the presence of functional memory T cell response in stimulating lymphocyte cell proliferation.⁸

The highest lymphocyte proliferation index was found in the group with latent TB infection, followed by the group of healthy individuals and the group of active TB. Nevertheless, the differences were not statistically significant. Similarly, a research conducted by Cesar⁹ showed that there was a decrease in lymphocyte proliferation response and IFN- γ production in patients with active tuberculosis compared to healthy patients with a positive TST. Lymphocyte proliferation response to mitogen stimulation, however, may be impaired in certain conditions, such as bacterial infections, benign tumors, burns, severe depression, autoimmune, viral infections, and malignancies. Some researches even also suggested a decrease or loss of lymphocyte proliferation response in patients infected with HIV, but there is still no research focused on the effects of proliferation response on age and sex.^{9,10}

In addition, statistical analysis showed that there was no significant difference in lymphocyte proliferation index after stimulation of CFP-10 antigen in all of the three groups of research subjects. This demonstrates the ability of CFP-10 antigen that is strong enough to induce an immune response. Similarly, a research conducted by Wu *et al.*¹¹ showed that the administration of DNA CFP-10 vaccine in BALB/c mice was able to stimulate an immune response due to an increase in T lymphocytes' function assessed by elevated levels of IFN- γ and lymphocyte proliferation index. Wu *et al.*¹¹ also suggested that the

capacity of CFP-10 antigen in inducing an immune response was influenced by background of the host's immune.¹¹

Proliferation index in healthy individuals, moreover, was lower than in both patients with active pulmonary tuberculosis patients and patients with latent TB. This was consistent with the statement of Sillah *et al.*¹¹ that the levels of CFP-10 antigen in healthy individuals was lower than in patients with active pulmonary tuberculosis. Similarly, a research conducted by Khan *et al.*¹² using experimental animals, macaques, showed that in the early stage of infection, antibodies against CFP-10 can be detected 4–12 weeks after exposure.^{11,12}

CFP-10 antigen is a DNA vaccine that can stimulate an immune response in the host cell (the human body), thus, after the vaccination, it will resemble the production of antigen during infection with microorganisms naturally. As a result, the immune response that occurs is the same with the immune response induced by pathogenic microorganisms.¹¹

Next, patients with latent TB and active TB will form memory T cells due to a prior exposure to *M.tuberculosis*. Therefore, their proliferative responses are higher than healthy individuals'. Healthy individuals are considered to have never had any exposure to *M.tuberculosis* bacteria. Exposure to CFP-10 antigen in healthy individuals is only considered as the initial exposure. Thus, the immune response that occurs is still low. Low immune response is indicated by lymphocyte proliferation index as a result of low T cell function as well as memory T cells that are still little or even still not formed.¹²

Based on the results of this research, lymphocyte proliferation index, furthermore, was higher in both of the latent and active TB groups than in the healthy individual group. Similarly, a research conducted by Wu *et al.*¹¹ showed that in BALB/c mice infected with *M.tuberculosis* and induced with vaccine CFP-10 in vivo, there was a significant increase in CFP-10-specific CD8⁺ T cells, in particular memory T CD8⁺ cells, so if there was a second exposure, it will induce memory T cells and effector T cells inducing higher immune response.¹¹

Redford *et al.*¹³ also suggested that there was a significant difference between patients with active pulmonary tuberculosis and patients with latent pulmonary tuberculosis. Proliferative response of patients with active pulmonary TB was usually lower than patients with latent TB. It may be due to the lower immune status of patients with active TB triggered by suppressive factors produced by monocytes and

lymphocytes causing a shift in cytokine responses from Th1 to Th2. Similarly, Khan *et al.*¹² suggested that there was a higher proliferation response on patients with latent TB indicated by an increase in proliferation index and IFN- γ levels. This condition was related to the protection of the immune response to *M.tuberculosis*. Interferon gamma (IFN- γ) was a strong activator of macrophages in responding to *M.tuberculosis* elimination depicting cellular immune response.^{12,13}

In contrast, the results of this research showed that the lymphocyte proliferation index in patients with active pulmonary tuberculosis was higher than in patients with latent TB. It may be because patients with active pulmonary TB who had been early exposed to *M.tuberculosis* in the long term still did not show clinical symptoms due to an immune response that was capable of neutralizing *M.tuberculosis*. Thus, memory T cells had already been formed although clinically diagnosed as active tuberculosis lung.

The results of data analysis in this research, furthermore, that there was no significant difference in the lymphocyte proliferation index after CFP-10 stimulation between patients with latent TB and healthy individuals. It could be caused due to a negative tuberculin test in healthy individuals but does not mean free of *M.tuberculosis* infection since Indonesia as a country with the largest TB incidence rates in the world, thus making healthy individuals difficult to be free from contact with *M.tuberculosis*. As a result, a good immune system and lack of exposure to *M.tuberculosis* can make few antibodies formed, so tuberculin skin test have negative results.

For those reasons, the immune response on those patients with latent TB was still good and able to neutralize *M.tuberculosis*. Those patients with latent *M.tuberculosis* produced enough antibodies, thus resulting in positive results for tuberculin skin test. Therefore, there was no significant difference in the lymphocyte proliferation index between patients with latent TB and healthy individuals.

In addition, Bennekov *et al.*¹⁴ argued that there are several factors affecting the success or failure of various vaccine strategies that have been developed. The vaccine is able to stimulate an immune response because it can generate specific T-cell response against the antigen. Antigen-specific T cells then will induce a protective immune response to *M.tuberculosis* because the epitopes recognized by T cells induced by a vaccine antigen are presented by cells infected with *M.tuberculosis*. Thus, the vaccine-induced T cells is capable of recognizing infected cells.¹⁴

In other words, vaccine may also fail to stimulate an immune response since it does not induce specific T-cell responses to antigens, or it can induce specific T cell responses to an antigen, but not capable of inducing a protective immune response, called as immunological failure. One of factors that causes the failure of antigen-specific T cells in inducing a protective immune response to *M.tuberculosis* is that the epitopes recognized by T cells induced by a vaccine antigen is not presented by cells infected with *M.tuberculosis*, or it is presented yet not effective. Thus, it can be understood that the epitopes which stimulate T cells after vaccination are different from T-cell epitopes after infection of *M. tuberculosis*. Consequently, the vaccine-induced T cells is not able to recognize cells that are infected, so it requires a deeper analysis of the epitopes to understand about vaccine-induced immunity correlated with protective immune responses in the host.¹⁴

CONCLUSION AND SUGGESTION

Based on the results of this research, it can be concluded that there were differences in lymphocyte proliferation index post-CFP-10 stimulation between patients with active pulmonary TB, patients with latent TB and healthy individuals. The lymphocyte proliferation index in patients with active pulmonary was higher than patients with latent TB and healthy people. Lymphocyte proliferation index as one of the parameters for immunogenic activities of CFP-10 antigen increased in patients with active pulmonary TB and latent TB as a result of memory T cells formed due to early exposure to *M.tuberculosis*. Nevertheless, further researches on proinflammatory cytokines (IFN γ , TNF- α , IL-2, IL-4, IL-6, IL-12, IL-17 and IL-23) in response to T cell effector after stimulation of CFP-10 antigen are needed to investigate the immunogenic activities of CFP-10 antigen as a TB vaccine candidate.

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