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Research Report

LYMPHOCYTE RESPONSE TO *Mycobacterium leprae* ANTIGENS IN REVERSAL REACTION STATE OF LEPROSY

An in vitro study of Lymphocyte Stimulation Index using MTT method

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ABSTRACT

Reversal Reaction (RR) in Leprosy is a sudden inflammatory episode in the chronic course of the disease due to rapid change of cellular immunological status. The aim of the study is to measure the in vitro results of Lymphocyte Stimulation Index (LSI) RR leprosy derived lymphocytes after challenged with M.leprae antigens. Twenty three Borderline Leprosy with RR and 11 Borderline Leprosy patients without RR were included in the study. Peripheral Blood Mononuclear Cells (PBMC) were separated from peripheral blood of these patients using Ficol-Hypaque column and cultured in laboratory. Using the colorimetric tetrazole (MTT) method these lymphocytes were challenged with PHA, Dharmendra antigen (1/100 and 1/10 dilutions), LAM (50 and 100 nanograms). Stimulation Index were calculated and superanatans were collected for measuring the IFN-γ and IL-10 production (ELISA). All of lymphocytes from RR patients showed higher Stimulation Index after challenged with the five M.leprae antigens compared to lymphocytes from non RR patients ($p < 0.05$) . IFN-γ and IL-10 also increased but not significant ($p > 0.05$). It is concluded that lymphocytes of leprosy patients during RR state are more sensitive to antigenic stimuli compared to non-RR leprosy patients. Further extended studies are needed to determine the "cut off" value of lymphocyte Stimulation Index that is useful for clinicians in the field in the prediction of RR before starting anti leprotic treatment.

Key words: Leprosy, reversal reaction, lymphocyte stimulation index, MTT, borderline type

ABSTRAK

Reversal reaction (RR) dalam kusta adalah periode inflamasi mendadak pada jalur kronis penyakit akibat perubahan status imunologi selular yang cepat. Tujuan penelitian ini untuk mengukur secara in vitro hasil indeks stimulasi limfosit (ISL) RR kusta berasal dari limfosit setelah menantang dengan M.leprae antigen. Dua puluh tiga batas kusta dengan RR dan sebelas batas penderita kusta tanpa RR dimasukkan dalam studi. Sel mononuklear darah perifer (SLDP) dipisahkan dari darah perifer dari para pasien menggunakan kolom ficol-hypaque dan dibiakkan di laboratorium. Menggunakan metode colorimetric tetrazole (mtt), limfosit ini ditantang dengan PHA, antigen dharmendra (1/100 dan 1/10 pengenceran), LAM (50 dan 100 nanograms). Indeks stimulasi dihitung dan supernatan dikumpulkan untuk mengukur IFN-γ dan produksi IL-10 (ELISA). Semua limfosit dari RR pasien menunjukkan indeks stimulasi lebih tinggi setelah ditantang dengan lima antigen M.leprae dibandingkan dengan limfosit yang berasal dari pasien non RR ($p < 0.05$). IFN–γ dan IL-10 juga meningkat tapi tidak signifikan ($p > 0.05$). Hal ini disimpulkan bahwa limfosit pada pasien lepra selama masa RR lebih sensitive terhadap rangsangan antigen dibandingkan dengan pasien lepra non RR. Penelitian selanjutnya diperlukan untuk menentukan nilai cut off dari indeks stimulasi limfosityang berguna untuk klinisi dalam memprediksi RR sebelum memulai perawatan anti lepra.

Kata Kunci: *Lepra, reversal reaction, indeks stimulasi limfosit, MTT, batas kusta*

INTRODUCTION

Leprosy is still a public health problem in Indonesia, especially in the eastern part of the country.[1] One of the problems in the field is the Reversal Reaction (RR), which often occurred during the Multi-drugs Therapy (MDT) course for leprosy. It is an acute inflammatory episode that occurred during the chronic course of the disease and sometimes cause disability to the patient. Clinically it is manifested by acute inflammatory skin lesions that previously relative "silent" and acute neuritis can occurred that led to disability.[2] By histopathological examination of the skin lesions in RR, inflammatory process could be found in the granuloma with more lymphocytic cells due to influx of lymphocytes from surrounding tissue came to the granuloma with its inflammatory mediators.[3] Activation of these lymphocytes could be a result of lymphocyte stimulation by many substances, including several antigens originated from *Mycobacterium leprae,* the cause of the disease. The aim of this study is to conduct *an in vitro* study on the response of lymphocytes from leprosy patients during the RR episode.

Figure 1. Type 1 Leprosy Reaction (Reversal Reaction)

MATERIAL AND METHODS

Thirty four blood samples obtained from 23 Borderline Leprosy patients with RR and 11 blood samples from Borderline Leprosy patients without RR were included for in *vitro study*. The Peripheral Blood Mono Nuclear Cells (PBMC) were separated using Ficoll Hypaque column and lymphocyte culture were performed.[4] Phyto-haemagglutinine (PHA) were used as nonspecific stimulans, Dharmendra 1/10 and Dharmendra 1/100 as specific protein stimuli from *M.leprae* , Lipoarabinomannan (LAM) 50 nanogram and 100 nanogram as specific carbohydrates stimuli were also used to the lymphocyte cultures. Lymphocyte Proliferation Test (LTT) were performed using the the colorimetric tetrazole (MTT) procedures as recommended by Mosmann in 1983.[5] Stimulation Index (SI) is ratio between stimulation index result and threshold. SI was read by computer and regarded as positive results if the value >1. The level of IFN-gamma and IL-10 from supernatant were measured by ELISA procedure using appropriate kits. Statistical Significant differences between the RR and non RR group were analyzed using Mann Whitney U test and Fisher's Exact test. Spearmans rho test was also used for calculating Correlation Coefficient.

RESULTS & DISCUSSION

Stimulation with non-specific mitogen (PHA) resulted SI positive in 20/23 RR patients compared to 7/11 in non RR patients ($p<0.05$). All of specific *M.leprae* antigens using for lymphocytes stimulation showed significant statistical differences between the the RR and non RR group.

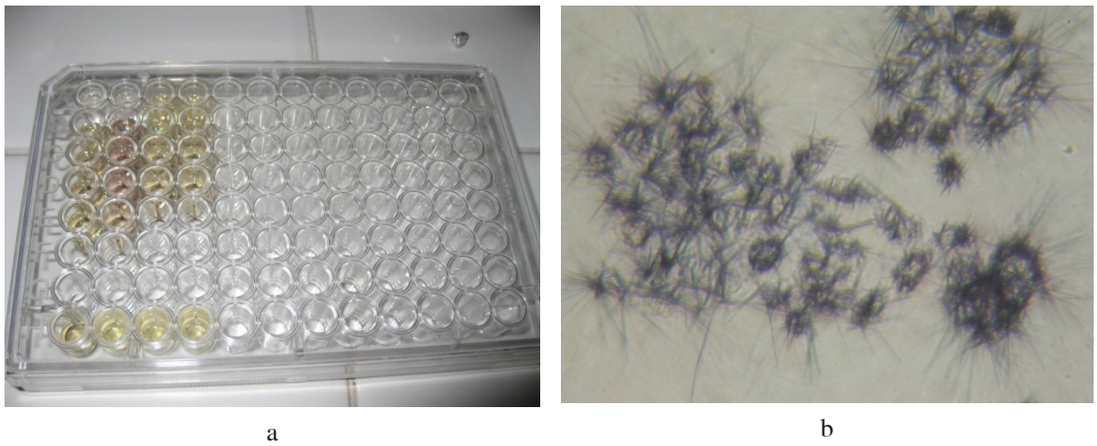

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Figure 2. (a) Lymphocyte culture, (b) Formazan coated lymphocytes

Table 1. Results of Stimulation Index with non-specific and specific antigens of *M.leprae*

Antigen	Positive SI in RR patients	Positive SI in non-RR patients	p
PHA	20 / 23	7 / 11	0.013
Dharmendra 1/100	22 / 23	6 / 11	0.002
Dharmendra 1/10	22 / 23	3 / 11	0.000
LAM 50	22 / 23	5 / 11	0.000
LAM 100	21 / 23	5 / 11	0.000

Table 2. Mean of IFN-gamma level in supernatans after stimulation with non- specific and specific antigens of *M.leprae*

Antigen	Mean level of IFN-^{1} in RR patients	Mean level of IFN-^{1} in non-RR patients	p
PHA	22.98*	24.27	0.699
Dharmendra 1/100	23.97	21.18	0.299
Dharmendra 1/10	25.86	23.40	0.522
LAM 50	20.26	20.46	0.839
LAM 100	28.05	24.06	0.368

*Unit/ml (ELISA)

Table 3. Mean of IL-10 level in the supernatan after stimulation with non- specific and specific antigens of *M.leprae*

Antigen	Mean level of IL-10 in RR patients	Mean level of IL-10 in non-RR patients	p
PHA	29.41	29.70	0.593
Dharmendra 1/100	30.28	30.26	0.974
Dharmendra 1/10	29.59	29.55	0.934
LAM 50	29.44	29.47	0.942
LAM 100	29.36	29.91	0.259

*Unit/ml (ELISA)

Acute inflammatory process in reaction state in leprosy is a result of sudden changes in immunological response stability during the chronic course of the disease. Treatment with MDT drugs will kill a big amount of lepra bacilli inside the host body and many new antigens including protein and carbohydrates antigens from dead *M.leprae* spread to the surrounding tissues and circulation. These antigens will stimulate T-lymphocytes, especially in areas surrounding the location of lepra bacilli. Some of these lymphocytes are already sensitized previously by the same antigen (T-memory cells).[6] The result of lymphocyte re-activation is the proliferation, differentiation and production of interleukin as IL-2, IFN-gamma, IL-10 and other interleukins. In this study the proliferation of lymphocytes was performed in-vitro using the MTT method that requiring a fresh peripheral blood from patients. Previously Lymphocyte Transformation Test (LTT) with radioactive labeled Thymidine were often used for measuring lymphocyte activation. Recently this procedure has been changed to MTT technique that relatively save and accurate. This colorimetric technique is based on the enzyme utilities that needed during proliferation of lymphocytes, which can be labeled by certain dyes.[7] The amount of labeled enzyme used by cells indicated the amount of cell's proliferation. During the reaction state in Reversal Reaction of leprosy, many T-memory cells are activated and proliferation occurred. As a result of proliferation, the number lymphocytes increase including the T-memory cells. Subsequent stimulations by antigens from lepra bacilli will stimulate memory T-lymphocytes that already accumulate during granuloma formation in the pathogenesis of leprosy.[8] Stimulation Index in this study showed that lymphocytes from RR patients gave a higher proliferation process compared to non-RR patients. Not only stimulation by protein antigens, carbohydrate antigens were also showed higher results. Proliferation of lymphocytes is always followed by release of many inflammatory mediators and resulted an acute inflammatory reaction of the skin lesions.[9,10] In this study, the production of interleukins were not significant difference between RR and non-RR patients. This results need to be studied further to find the reason, it might be due to technical or time of harvesting the lymphocytes after stimulation. The results of this study showed that not only a single antigen involved in RR process, but many antigens can stimulate lymphocytes of leprosy patients. How the lymphocytes can be stimulated by carbohydrate antigens from *M.leprae* need to be investigated and which antigen is predominated the lymphocyte stimulation in RR still a question.

CONCLUSION

Lymphocytes from leprosy with RR patients showed significant higher Stimulation Index compared with non RR patients. Both protein and carbohydrate antigens from *M.leprae* can stimulate lymphocytes in leprosy patients. More investigations are needed to clarify the true mechanism of RR.

REFFERENCES

1. Depkes R.I. Buku Pedoman Pemberantasan Kusta (2009). Subdit P2Kusta RI.

2. Agusni I (2011). Clinical Manifestation of Leprosy. In (Makino M, Matsuoka M, Goto M Eds) Leprosy. From
3. Ridley DS (1988). Pathogenesis of Leprosy and Related Diseases. 1st Edition. Butterworth & Co. Publ.
4. Abbas AK, Lichtman AH, Pillai S (2011). Chapter 4. Cell-Mediated Immune Responses. Basic Immunology. Saunders, Philadelphia. pp. 89–112.,
5. Mosmann D (1983). MTT assay, a colorimetric technique for the study of Lymphocyte Stimulation . J Immunol 92, 247–252.
6. Hussain RF, Nouri AME, Olver RTD (1993). A new approach for Measurement of cytotoxicity using colorimetric assay, J of Immunol Method; 160(1993) 89–96.
7. Britton WJ (1998). The management of leprosy reversal reaction. Lepr Rev, 74
8. Bjune GB, Ridley DS, Kronvall G (1976). Lymphocyte Transformation Test in Leprosy; Correlation of the response with clinical inflammation of lesions. Clin exp immunol 25, 85–94.
9. Kawahita IP, Walker SL, Lockwood DNJ. (2008). Leprosy type1 reactions and Erythema Nodosum Leprosum. An Bras Dermatol, 83(1),75–82.
10. Walker SL & Lockwood DNJ (2008). Leprosy type 1 (reversal) reactions and their management. Lep Rev 78, 372–386.