Distribution of iNOS expressions and TNF neutrophil cells as well as PGE2 and S100 Schwann cell dermal nerves in the erythema nodosum leprosum patients

I Gusti Nyoman Darmaputra,1* Nanny Herwanto,2 Luh Mas Rusyati,1 Wibi Riawan,3 Anang Endaryanto,4 Cita Rosita Sigit Prakoeswa2

ABSTRACT

Background: The increase of neutrophil infiltration in the Erythema Nodulosum Leprosum (ENL) is thought to have a role in releasing the free radicals and TNFα which causes dermal nerve damage. ENL occurs in leprosy patient with LL and BL type, which has lipid droplets as a synthesis site of Prostaglandin E2 that plays a role in suppressing nerve inflammation. This study investigates neutrophil distributions that express TNFα and iNOS as well as PGE2 expressions of the S100b distributions on ENL patient’s Schwann cells.

Methods: This cross-sectional study used 23 samples of pedis skin biopsy from patients with MB leprosy type (10 ENL samples and 13 as control samples), from the Skin Polyclinic Sanglah Hospital, Denpasar, Indonesia. Punch biopsy of 4 mm in diameter is performed. Hematoxylin-Eosin standard staining was used to confirm the cell structure, type of leprosy, and distribution of neutrophil cells. Observation of TNFα, iNOS, PGE2, and s100b was conducted with immunohistochemistry techniques. Expression analysis was performed with immune ratio software (freeware). The PGE2, s100b, TNFα, and iNOS expression percentage were put on a list, then the T-test was performed using IBM-SPSS 21 for Windows to figure out the relationships between groups test.

Results: The distributions of neutrophil were increased in the ENL group compared to the ones in the control group. The increased neutrophils appeared in the dermis area around the blood vessels. iNOS and TNFα distributions on neutrophil cells increased significantly in the ENL. In line with that, the s100b expressions on the ENL were significantly lower.

Conclusions: The significant increase of the iNOS and TNFα neutrophil cells in the ENL may play a role in the dermal nerve damage. This study digs into the possibility of the role of Prostaglandin E2 in maintaining the peripheral nervous system in leprosy with ENL. Hence the understanding of PGE2 as the main mediator in the inflammatory process especially in the Schwann cell damage is very important.

Keywords: TNFα, iNOS, PGE2, S100b, ENL
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INTRODUCTION

At present, leprosy is still a fearsome disease and inflicting stigma in the society as this disease can cause ulceration, mutilation, and deformity due to nerve damage. Nerve damage in leprosy patients is most often caused by leprosy reactions. The incidence rate of leprosy reaction has not yet declined despite the use of WHO Multi-Drug Therapy (WHO-MDT) program that has succeeded in reducing the number of leprosy cases. Leprosy reaction is an outcome of the dynamic nature of the body’s immune response against M. leprae which most commonly occurred following the initiation of MDT therapy (Andrade, 2016). Type 2 leprosy reactions are often called by the term Erythema Nodulosum Leprosum (ENL). ENL generally occurs in leprosy patient with Borderline Lepromatous (BL) and Lepromatous Lepromatous (LL) type. The prevalence of ENL cases varies greatly depending on the geographical variations. The ENL cases in Asia remained high, between 19-26% of the entire cases of BL and LL (Walker, 2008). The ENL cases also remained high in Indonesia. Based on the Leprosy Division data of the Outpatient Unit of Dermatology and Venereology Health Sciences, Dr. Soetomo Regional Public Hospital Surabaya, in the year 2011-2013 it was discovered that 24% of all new leprosy patients were suffering from ENL. ENL is generally chronic and recurrent. On Voorend’s research in India, it was found that reoccurred/ countless ENL episodes occurred in 39 - 77% of ENL patients (Voorend, 2013). Heftier and wider nerve damage mostly occurs in chronic or recurrent ENL (Andrade, 2016). The role of neutrophils in the current ENL pathogenesis is getting more attention. The recruitment of neutrophils to the inflammation site will release free radicals and proteases thus causing damage to the local tissues (Lee, 2010). The role of free radicals that causes dermal nerve damage was found in Schon’s research, wherein nitric oxide (NO) and peroxynitrates are...
involved in dermal neural damage of the borderline leprosy patients (Schon, 2004). There is a significant increase of TNFα in the blood and tissues of patient suffered from ENL. TNFα is proven to cause nerve damage either individually or together with other mediators. In lepromatous leprosy the patient’s lipid accumulation is found in the stratum corneum, therefore giving the “foamy” impression. These lipids are stored in the cytoplasmic organelle which is unrelated to the membrane, called body fat or lipid droplets (LDs). Mattos’ research proves that M. leprae can induce LDs biogenesis in stratum corneum as well as an active catalysis site for Prostaglandin-E2 synthesis (PGE2) and Interleukin 10 (IL 10) which play a role in suppressing inflammation in the nerves (Mattos, 2011). This study will examine the neutrophil distributions that express TNFα and iNOS as well as an increase in PGE2 and S100b distributions on damaged Schwann cell from tissue biopsy of leprosy patients with ENL reactions.

METHODS

This study used 23 samples consisted of 10 ENL samples and 13 MB leprosy patients without ENL as controls. The samples were taken by consecutive sampling from October 2016 - February 2017 from the Polyclinic of Dermatology and Venereology of Sanglah Hospital, Denpasar, Bali. The age of the patients ranged from 20-50 years old and had signed the informed consent forms according to the standards from the Research Ethics Institute of Udayana University. Skin biopsy sampling was performed in the dorsum pedis area without skin lesions. Disinfection was carried out with 70% alcohol, then local anesthesia was performed with Lidocaine. The biopsy was performed with a 4 mm diameter punch method. The biopsy results were inserted in a tube containing 10% formalin solution. The biopsy sample was then sent to the Biochemistry-Biomolecular Laboratory, Faculty of Medicine, Universitas Brawijaya, Malang. Tissue processing (fixation and paraffin) was performed and slide preparations were made with 4um cutting thickness using a rotary microtome. The slides were stained with Hematoxylin-Eosin standard and slide preparations were made with 4um cutting thickness using a microscope with 1000x magnification, statistically, the neutrophils showed are distributed in each leprosy patient on the samples with ENL reactions. Neutrophils performed a neutrophil distribution examination in the occurrence of ENL. There is evidence that neutrophils are thought to play an important role in dermal nerve damage of the borderline leprosy. Twenty three samples from MB type leprosy patients were obtained. The patients were classified as follows: (1) 10 ENL samples and (2) 13 samples without ENL. Then the number of neutrophil cells was determined on samples and controls, as shown below.

Neutrophil count was performed in the inflammatory area of the biopsy sample in the control and ENL groups. Based on Hematoxylin staining, under a microscope with 1000x magnification, a number of neutrophil cells was encountered in all the study samples. Statistically, the neutrophil cells in the ENL group increased significantly (p <0.05) compared to the control group. TNFα has been shown to increase in ENL group, but the increase of TNFα in relation to dermal nerve damage in ENL is still unknown. This study showed that statistically, TNFα appeared to increase significantly in the ENL group compared to the control group.

In Schwann cells, there was a defense mechanism by lipid droplets which generated PGE2. Dermal nerve damage was measured using the S100b examination to perceive the structures and branches of Schwann cells as shown below.

The expressions of S100b and PGE2 in this study were seen in each biopsy sample preparation. Based on the immunohistochemical staining, under a microscope with 1000x magnification, statistically, the number of cells expressing s100b and PGE2 in the ENL group decreased significantly (p <0.05) compared to the control group.

DISCUSSION

The recruitment of neutrophils is very important as an immune response in fighting against the microbial infections, but it also contributes to the immunopathology of the disease. At present, neutrophils are thought to play an important role in the occurrence of ENL. There is evidence that neutrophils are the main cell infiltrate of inflammation in the dermis and subcutis with the increase in IL 17 and E-selectin in ENL. In order to see the role of neutrophils in the ENL leprosy reaction, we performed a neutrophil distribution examination on the samples with ENL reactions. Neutrophils appeared to be distributed in each leprosy patient biopsy sample. Statistically, the neutrophils showed a significant increase in the ENL group compared to the control group. It provides information that
there is neutrophil involvement in the ENL process. Many researches had been performed on iNOS involvement in the ENL leprosy reaction. The results of our study showed that *M. leprae* infection may increase iNOS expressions on the neutrophils of lepromatous leprosy. By taking our research into account, iNOS can be considered as part of a repository anti mycobacterial molecules which is expressed in the leprosy lesions. However, the regulated iNOS expressions may not be sufficient to eliminate the pathogens in *lepromatous leprosy* (Lee, 2010; Pereira-Suárez, 2015). In addition to free radicals, neutrophils also produce pro-inflammatory cytokines that are TNFα. There was a significant increase of TNFα in the blood as well as in the tissues of ENL patients. TNFα is proven to cause nerve damage either individually or together with other mediators (Andrade, 2016). Evidence (or you can also say study found) found that TNFα in the spinal mouse nerve culture can cause demyelination and oligodendrocyte necrosis (Selmaj, 1988). TNFα together with TGFβ are shown to have toxic effects

**Figure 1** The number of neutrophil cells in leprosy with ENL reaction
and cause rapid Schwann cell lysis (Skoff, 1998). In this research, TNFαs showed a significant increase in ENL patients compared to the control patients. TNFαs as a proinflammatory cytokine plays an important role in the ENL pathogenesis. T cells and macrophages activation induce the production of TNF-α in large quantities. The additional sources of TNF-α are severe leukocytosis and the presence of intense neutrophil infiltration on the ENL lesions.

Generally, nerve damage in lepromatous leprosy patients has not happened although there are many *M. lepra* in the Schwann cells. Nerve damage in lepromatous leprosy just occurred in advanced lepromatous leprosy where the fibrosis of the nerves starts from the peripheral extremities areas (gloves and stocking anesthesia). Nerve damage in lepromatous leprosy patients occurred faster because of the ENL leprosy reaction, while in chronic and recurrent ENL more severe nerve damage are found. Nerve damage that occurred during reactions at various degrees initially occurs in cutaneous nerve endings, the second at the cutaneous nerve level and the third at the nervous system level. Damage to the cutaneous and subcutaneous nerves will expand the area experiencing the loss of sensation and loss of autonomic nervous function such as sweating (Kar HK, 2010). In leprosy patients, routine nerve damage detection such as the mono-filament test (MFT) tends to be too late in detecting nerve damage. Skin biopsy examination with S100 staining is proven to detect Schwann cell damage at an early stage because it can visualize the Schwann cells and the branches. A skin biopsy can provide diagnostic value even before the neuropathy symptoms develop on the clinical development of a disease. This procedure is safe, minimally invasive, and inexpensive (Singh, 1994). The sensitivity of the skin biopsy in detecting peripheral neuropathy is 90% with a specificity of 95% (Lefford, 1991). The sampling technique for skin biopsies is commonly carried out by using a punch or excisional biopsy (Singh, 1994). The punch biopsy is a standard diagnostic method for the diagnosis of neuropathy in leprosy patients because it can indicate the presence of innervation in the epidermis (Lefford, 1991). Another advantage of the punch biopsy is that the examiner may choose the location for the biopsy in accordance with the area complained by the patient. It can also be done where the nerve conduction test cannot be performed (Kahn, 1983; Singh, 1994). With the S-100 staining, we can easily identify the branches and fragments of the dermis nerves. S-100 is an acidic binding protein calcium expressed in stratum corneum (Singh, 1994). By identifying these neural branches, it can help identify the inflammatory process and nerve damage.

The research conducted showed that S-100 staining is superior to HE routine coloring in identifying the destruction of the dermis nerves of leprosy patients (Kahn, 1983). In this study, biopsies and S100 staining were performed in areas where anesthesia generally occurs first in lepromatous leprosy patients namely in the area of the inferior extremities, not on the ENL nodules. Samples were taken on the dorsum pedis area of ENL and controls patients. The s100b expressions obtained in the ENL group were significantly lower than the ones in the control group. Mattos’ research proves that M. lepra can induce LDs biogenesis in stratum corneum as well as an active catalysis site for Prostaglandin-E2 synthesis (PGE2) and Interleukin 10 (IL 10), which plays a role in suppressing inflammation in the nerves (Mattos, 2011). Inflammatory suppression of the nerves by PGE2 plays a role in maintaining Schwann cells from the damage despite the inflammatory process by the inclusion of M. lepra into the Schwann cells. In this study, PGE2 expressions on the dermis of the biopsy tissue of the ENL patients showed a decrease in the ENL group compared to the control group. This corresponds to the PGE2's role in suppressing inflammation of the nerves in ENL. This is in line with the s100 expression results as a marker of the nerve damage where the s100b expressions obtained in the ENL group were significantly lower than the ones in the control group. The present study explored the possibility of Prostaglandin E2 role in maintaining the peripheral nervous system (Schwann cells) in leprosy patients with ENL reaction conditions. Therefore, the understanding of PGE2 as the main mediator in the inflammatory process particularly the Schwann cell damage is very important.

CONCLUSION

In the ENL cases, an increase in infiltration of neutrophils is thought to play a role in the release of free radical and production of TNFαs which causes dermal nerve damage on the extremities of ENL patients. ENL occurs in the LL and BL leprosy types; in which nerve damage is not found in these types of leprosy although there are many *M. lepra* in them. This condition is caused by the presence of lipid droplets which is the place of Prostaglandin E2 synthesis that plays a role in suppressing nerve inflammation. This study perceives the distribution of neutrophils which expresses TNFα, iNOS as well as PGE2 expressions to the distribution of S100b in the Schwann cells as a marker of dermal nerve damage in ENL patients. This research used cross-sectional method using 23 samples of pedis skin biopsy of patients with MB leprosy (10 ENL
samples and 13 as control samples), from the Skin Polyclinic Sanglah Hospital, Denpasar, Indonesia. Punch biopsy of 4 mm diameter was performed. Hematoxylin-Eosin standard staining was used to confirm the cells structure, type of leprosy and distribution of neutrophil cells. Observation of TNFα, iNOS, PGE2, and s100b was conducted with immunohistochemistry techniques. Expressions analysis was performed with immune ratio software (freeware). The PGE2, s100b, TNFα, and iNOS expression percentage were put on a list, then the T-test was performed using IBM-SPSS 21 for Windows to identify the relationships between groups test.

In this study, it was found that the distributions of neutrophils increased in the ENL compared to the ones in the control. The increased neutrophils appeared in the dermis area around the blood vessels. INOS and TNFa distributions on neutrophil cells increased significantly in the ENL compared to the ones in the control. In line with that, the s100b expressions on the ENL were significantly lower. The significant increase of the iNOS and TNFa neutrophil cells in the ENL played a role in the dermal nerve damage. This study digs into the possibility of the role of Prostaglandin E2 in maintaining the peripheral nervous system on leprosy with ENL. Hence, the understanding of PGE2 as the main mediator in the inflammatory process, especially in the Schwann cell damage, is very important.

**BIBLIOGRAPHY**


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