JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2010. Vol. 4(2), p. 701-707

Extraction and Purification of N-acetyl Muramyl-L-alanyl-D-isoglutamine (MDP) from the Cell Wall of *Mycobacterium tuberculosis*

G. Revathi, P. Suvarnalatha Devi, N.D. Prasanna, P. Jayalakshmi and S.K. Shaheen

Department of Applied Microbiology, SPMVV, Tirupati - 517 502, India.

(Received: 05 April 2010; accepted: 26 May 2010)

N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) is the peptidoglycans obtained from the *Mycobacterium tuberculosis*. The isolate *Mycobacterium tuberculosis* (MT1) was obtained from the sputum of the tuberculosis patients. The MT1 isolates undergone for the morphological, biochemical and staining technique for the conformation of *Mycobacterium tuberculosis*. The mid log phase MT1 isolates were used for the extraction of MDP by using HPLC. The muropeptides were eluted from the HPLC with 2 to 30% linear gradient of aceto nitrile containing 0.5% formic acid at 320 L/min. The purified MDP was eluted with in the retention time of standard Muramyldipeptide.

Key words: *Mycobacterium tuberculosis* (MT1), Muramyldipeptide, Peptidoglycans, High Performance Liquid Chromatography.

N-acetyl muramyl-L-alanyl-D-isoglutamine (MDP) is a class of muramyldipeptide and it is a minimum structural unit of peptidoglycans in acid fast Gram positive bacteria. MDP is responsible for immuno-potentiating ability[1,2]. *Mycobacterium* produces large mycolic acids that distinguish their cell wall from those of other bacteria. Its unusual cell wall, rich in lipids (e.g. mycolic acid), consisting of a highly complex array of distinctive lipids, glycolipid, proteins, and polymers, of which the mycolyl-arabinogalactan-peptidoglycan complex (MAPc) is the major structural component. *Mycobacterium* cell wall constituents: mycolic acid, arabinogalactan and peptidoglycan. Among these constituents, peptidoglycan is responsible for adjuvant activities, causing increased serum antibody levels. Peptidoglycan (PG) is a highly complex and essential macromolecule of bacterial cell walls (except in mycoplasma) that enables bacteria to resist osmotic pressure. Although it was often regarded as an inert structure surrounding bacteria, the PG layer is a highly dynamic and tightly regulated macromolecule that is constantly remodeled to allow cell growth and division. Despite a rather simple building block (the PG precursor) consisting of a disaccharide N-acetyl-D-glucosamine-(b-1,4)-N acetylmuramic acid (GlcNAc-MurNAc). Although the peptides vary extensively in composition from species to species. In Gram-positive bacteria it is very frequent to find cross-bridges linking two distinct stem peptides, increasing even more the complexity of the PG structure.

* To whom all correspondence should be addressed.
E-mail: microbiologyteam4@gmail.com

MDP and its derivatives have important biological activities like adjuvant activity, Stimulation of non- specific resistance against bacterial, viral and parasite infections and against tumors, Somnogenic activity, i.e., increase of the duration of slow wave sleep[3]. MDP interact with both B and T cells. MDP activates macrophages by both direct and indirect effects (via lymphokines). Indirect activation is more effective than direct effect. In *In Vivo*, MDP has immediate effect at the cellular level [4]. MDP influences a variety of macrophage functions such as the production of prostaglandins, motality, enhanced O_2-generating capacity, proliferation in response to lymphokines, increased cytolytic activity against tumor cell and an increase in spreading, adherence and by elevated synthesis of an enzyme (collagenase). This increase in the collagenase production is modulated by enhanced production of prostaglandins that influences intracellular levels of cAMP. These induced macrophages produce biologically active mediators that triggers quiescent fibroblast into active proliferation, which then participate a multiplicity of other reactions resulting in enhanced immune phenomena[5]. It initiates Nod2 expression in macrophages and leads to activation of NF-κ B. Nod2- mediated NF κ B leads to the induction of defensives. MDP induces release of cytokines (IL-6) in the presence of Nod2 containing a caspase activation and recruitment domine.

The present paper deals with the extraction and purification of Muramyldipeptide by High Performance Liquid Chromatography (HPLC).

MATERIAL AND METHODS

Isolation and culturing of *Mycobacterium tuberculosis*

Sputum of TB (Tuberculosis) patients was collected in a sterile, wide mouthed and leak proof, disposable plastic containers (50 ml capacity). Isolates were cultured in the Lowenstein-Jenson medium (LJ medium) and incubated in BOD incubator for at 37ºC for six weeks.

Morphological characterization

Identification of the isolates, initially, was carried out by studying the morphological characters after incubating the culturing in LJ medium plates for 6 weeks at 37°C. The colony morphology, pigment production and its diffusibility on the LJ medium were observed and isolated colonies were selected for staining techniques.

Staining of *Mycobacterium.tuberculosis*

Three weeks old LJ broth cultures was smeared in a thin film on clean grease free glass slides and heat fixed. The Ziehl-Neelsen acid-fast staining was performed by using the stains, carbol fuchsin stain and methylene blue counter stain. Spore staining was performed by using the stains, 5% Malachite green and 0.5% saffranine[6].

Specific Biochemical tests for *Mycobacterium tuberculosis*

Mycobacterium tuberculosis was conformed by performing the specific biochemical tests like Nitrate Reduction test, Catalase tests, Niacin test, Urea test, Thiophene 2-carboxylic acid hydrazide (TCH), Tween 80 hydrolysis[7].

Separation of PeptidoGlycan (PG) from *M. tuberculosis*

M. tuberculosis cells at mid-log phase were harvested by centrifugation and washed with phosphate buffered saline (PBS) to remove growth medium the isolation of PG. The bacilli were resuspended in 10 mM NH_4HCO_3 containing 1 mM phenylmethylsulfonyl fluoride and disrupted by intermittent probe sonication with an MSE Soniprep 150 (MSE-Sanyo; Integrated Services) for 30 cycles (60-s bursts separated by 60 s of cooling). The sonicate was digested with10 g each of DNase and RNase/ml for 1 hr at 4°C. A cell wall-enriched fraction was obtained by centrifugation at 27,000g for 30 min. The pellet containing cell walls was resuspended in PBS containing 2% sodium dodecyl sulfate (SDS), the suspension was incubated for 1 hr at 50°C with constant stirring and recentrifuged at 27,000 g for 30 min, and the supernatant was discarded. This process was repeated twice. The resulting pellet was resuspended in PBS containing 1% SDS and 0.1 mg of self-digested proteinase K/ml, and the suspension was incubated at 45°C for 1 hr with constant stirring. The mixture was then heated at 90°C for 1 hr before centrifugation at 27,000g for 30 min. The supernatant was discarded, and the 1% SDS extraction procedure was repeated twice to remove proteinase K. The

pelleted material was washed twice with PBS and four times with deionized water to remove SDS. The resulting Mycolyl-arabinogalactan-peptidoglycan complex (MAPc) was extracted with ethanol-diethyl ether (1:1) and dried under a vacuum. In order to hydrolyze the mycolic acids, the MAPc was resuspended in 0.5% KOH in methanol and stirred at 37°C for 4 days. The mixture was centrifuged, and the pellet was washed twice with methanol and twice with diethyl ether and dried under a vacuum.

The resulting arabinogalactan-PG was digested with 0.05 N H_2SO_4 at 37°C for 5 days to remove the arabinogalactan. The resulting insoluble PG was washed four times by centrifugation with deionized water and dried under a vacuum[8].

Solubilization of PeptidoGlycan (PG)

The purified PG (2 mg) was suspended in 0.5 ml of 10 mM sodium acetate (pH 5.0) containing 25 g of purified muramidase and the suspension was incubated at 37°C for 16 hr with stirring. Digests were centrifuged at 27,000 *g* for 30 min, and the supernatant was filtered through a 10-kDa-cutoff ultrafiltration membrane (Millipore) to remove muramidase and dried under a vacuum. The muropeptides were resuspended in 0.5 M sodium-borate buffer (pH 9.0), and sodium borohydride was added to achieve a final concentration of 8 mg/ml. The mixture was incubated for 30 min at room temperature to reduce the sugar moieties. The reaction was stopped by the addition of orthophosphoric acid, and the pH was adjusted to 4.0 prior to fractionation by size exclusion chromatography on a Superdex peptide 10/300 GL column with a model 600 controller connected to a model 600 pump and a model 2487 UV detector. The column was equilibrated and eluted with 30% acetonitrile containing 0.1% trifluoroacetic acid with a flow rate of 0.5 ml/ min. The absorbance of the effluent at 214 nm was monitored [8].

Purification of Muramyldipeptide by High Pressure Liquid Chromatography (HPLC)

The fractions containing muropeptides were dried under a vacuum and resuspended in high-performance liquid chromatography (HPLC)-grade water at an approximate concentration of 10 M. An aliquot (20µl) was applied to a 2-by-150-mm Hypersil octyldecyl silane (C18) column connected to Shimadzu CLASS-VP V6.14 HPLC system. The muropeptides were eluted with a 2 to 30% linear gradient of aceto nitrile containing 0.5% formic acid at 320 L/min[8].

RESULTS AND DISCUSSION

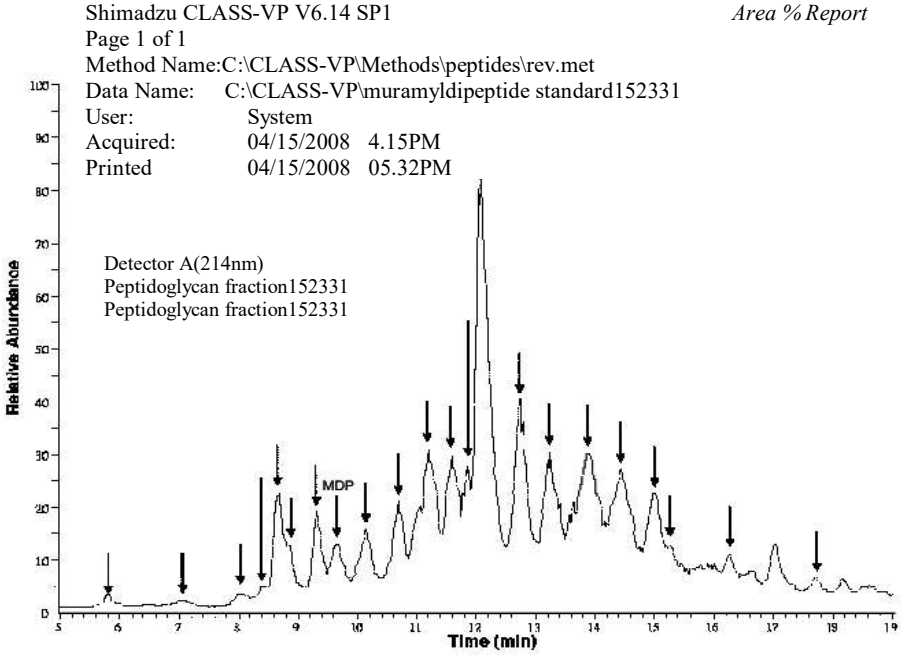
Among the five isolates MT1 showing dry, rough, raised, thick with wrinkled surface and an irregular thin margin on L-J solid medium. They were creamy white, becoming yellowish or buff colored on further incubation. The MT1 isolate is a straight or slightly curved rods, about 3µm x 0.3µm, occurring singly, in pairs or as small clumps were observed after the Ziehl-Neelsen acid-fast staining. The pink vegetative cells with green colored spores were observed under the microscope. These MT1 isolates showing positive reaction for the biochemical tests which were very specific to the *Mycobacterium tuberculosis* Table 1. The isolated MT1 isolates were cultured in Lowenstein-Jenson medium (LJ medium)[9].

By the above morphological and biochemical tests as compared with Bergey's Manual[10], the organism was identified as *M.tuberculosis* and incubated in BOD incubator for at 37ºC for six weeks.

The Muramyldipeptide was extracted and separated from the cell wall of *M.tuberculosis*. The resulting Mycolyl-arabinogalactan-peptidoglycan complex (MAPc) was subjected for purification. The fraction containing muropeptides were dried under a vacuum and resuspended in high-performance liquid chromatography (HPLC) grade water at an approximate concentration of 10 µM. An aliquot (20µl) was applied to a 2 –by-150mm Hypersil octyldecyl silane (C18) column connected to Shimadzu CLASS-VP V6.14 HPLC system. 99% pure muramyldipeptide which was obtained from Sigma PVT Ltd used as standard along with the sample. The muropeptides were eluted with a 2 to 30% linear gradient of acetonitrile containing 0.5% formic acid at 320µl/min Fig. 1. The peptides were separated with in retention times of standard MDP Fig-2. The collected MDP was used for further studies like adjuvant activity and

also the antitumor activity.

Muramyl peptides have a powerful effect on the immune system by way of macrophage and monocyte activation, and by stimulation other cells of the immune system which play a crucial role in cytokine production [11,12,13,14].According to Ikuo Saiki *et al.*, [15] Normal noncytotoxic blood monocytes (or) tissue macrophages can become activated to the tumoricidal state be interation with lymphokines such as MAF (macrophage



Detector A (214nm)

Pk #	Retention Time	Area	Area %	Height	Height %
1	5.84	1192	0.01	36	0.01
2	7.17	3828	0.03	397	0.09
3	8.25	145239	0.98	8381	1.93
4	8.64	125765	0.84	8433	1.94
5	8.85	130646	0.88	10597	2.43
6	8.88	229162	1.54	10703	2.46
7	9.47	685649	4.6	19869	4.56
8	9.76	315431	2.12	17063	3.92
9	10.25	431269	2.89	21169	4.86
10	10.9	304471	2.04	21146	4.86
11	11.32	304176	2.04	19649	4.51
12	11.51	464898	3.12	16700	3.84
13	11.94	109738	0.74	11159	2.56
14	12.22	890802	5.98	73986	17
15	12.73	8636423	57.95	187912	43.17
16	13.1	2123509	14.25	7978	1.83
17	13.13	135239	0.87	7659	1.72
18	13.91	149065	0.95	9833	2
19	14.58	126921	0.92	10789	2.99
20	15.11	232369	1.93	11417	2.74
21	15.27	85649	3.6	19971	5.58
22	16.28	343	0	146	0.03
23	17.79	81	0	7	0
Total		15631865	100	495000	100

Fig. 1. Chromatogram of Muropeptides of *M.tuberculosis*

activating factor) or recombinant γ-interaferon(r-IFN-γ) MDP, the minimal active cell wall component of Mycobacterium that is responsible for the immunostimulatory activity. MDP or its analogues can readily activate human macrophages to become cytotoxic to microorganisms.

The MDP binding sites are located within the intracellular compartment. Nod2 is an intracellular sensor of MDP. Nod2 expression is restricted to monocytes/macrophages under normal conditions, a major cell production was stimulated by MDP. Nod2 -dependent activation of the NF-kB pro inflammatory cascade is modulated by PG [16]. NF-kB activation functions have two distinct regulatory pathways. The classical pathway is typically triggered by ligand binding Tumor Necrosis Factor1/2 receptors(TNFR1/2), T-cell receptor (TCR), B-cell receptor (BCR), or the toll-like receptor (TLR)-interleukin-1 receptor(IL-1R) super family members. In contrast, the alternative pathway is triggered by the activation of certain TNF receptor family members, including lymphotoxin β receptor (LT βR), B cell -activating factor belonging to the TNF family receptor (BAFF-R), CD40 and CD 30 [17]. MDP influences macrophage to enhance O_2-generating capacity which is an important factor for TNF activity in DNA fragmentation[18]. TNF proliferation in response to

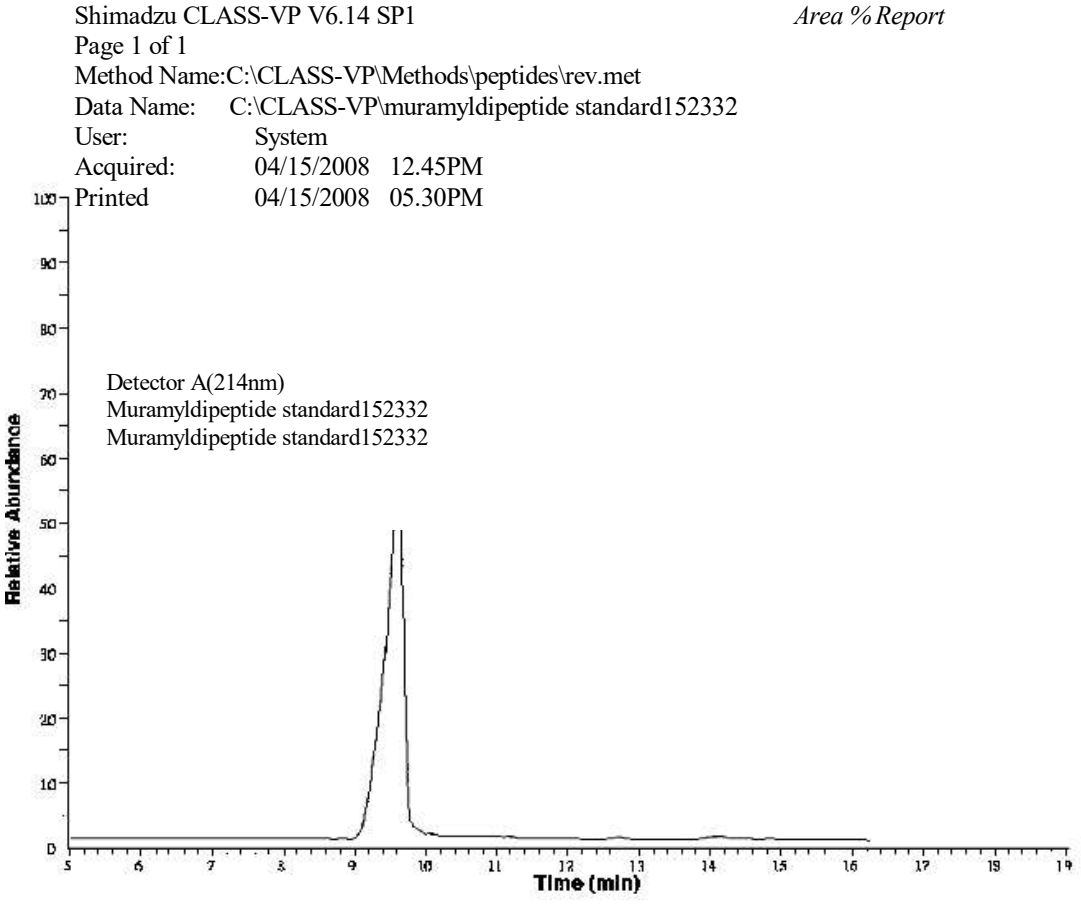


Fig. 2. Chromatogram of standard MDP

Table 1. Morphological and biochemical tests for identification of MT1 isolates

Test	*Mycobacterium tuberculosis*
Colony morphology	
Configuration	Dry, rough, raised
Margins	Irregular thin
Surface	Thick with wrinkled
Pigmentation	Light Yellowish buff(long period)
Turbidity	+
Opacity	Translucent
Gram's reaction	Negative
Cell shape	Rods
Size(ìm)	1 -3 ìm in length, width 0.3 ìm in width
Arrangement	Singles
Spores	+
Motility	+
Physiological tests	Growth at temperature (^{0}C)
4^0C	-
37^0C	+
41^0C	-
45^0C	-
Growth at pH	
4	-
5	-
6	W
7	+
8	-
Growth under anaerobic condition	-
Biochemical tests	
Nitrate reduction	+
Catalase test	-
Niacin accumulation test	+
Urease test	+
Tween 80 hydrolysis	+
Thiophene 2-carboxylic acid hydrazide(TCH)	+

Note: (+) Positive, (-) Negative, (W)-Weak

lymphokines leads to increase the cytolytic activity against tumor cell. MDP also increases TNF- α mRNA. The mechanism of antitumor activity of MDP yet to be not understood. But the cytolytic activity and Production TNF α is might be the direct process involved in anti tumor activity of MDP.

REFERENCES

1. Ellouz, F., Adam, A., Ciobaru, R., Lederer, E: Minimal structural requirements for adjuvants activity of bacterial peptidolycan derivatives. *Biochem Biopys Res Commuun*., 1974; **59**(4): 1317-1325.
2. Kotani, S., Watanable, Y., Kinosita, F., Simono, T., Morisaki, I: Immunoadjuvant activities of synthetic N-acetyl-muramyl-peptides or -aminoacids. *Biken J*., 1975; **18**(2): 105-111.
3. Lefrancier, P., Lederer, E: Muramyl-peptides. *Pure & Appl, Chem*., 1987; **59**(3): 449-454.
4. Chedid, L., Parant, M., Parant, F., Audibert, Lefrancier,F., Choay,J., .Sela, M: Biological Activities of MuramylDipeptide derivatives after conjugation to a multi- poly (DL-alanine)—poly(L- Lysine) carrier. *Proc. Natl. Acad. Sci. USA.,* 1979; **76**(12): 6557-6561.
5. Wal, S.M., Wa,l L.M., McCarthy, J.B., Chedid, L., Mergenagen, S.E: Macrophage activation by Mycobacterial Water Soluble Compounds and Syntetic Muramyldipeptide. *The Journal of Immunology.*, 1979; **122**: 2226-2231.
6. Mackie and Mc Cartney Practical Medical Microbiology. (Collee, G., Dugid, J.P., Fraser, A.G. and Marmion B.P. Eds.). 13th Edition. Churcchill Livingstone, Edinburgh, London, Melbourne and New York: 1989; **2**: 43-160.
7. Wayne, L.G., Engel, H.W.B., Grassi, C., Gross, W., Hawkins, J., Jenkins, P.A., Kappler W K., Kleeberg, H.H., Krasnow, I., Nel, E.E., Pattyn, S.R., Richards, P.A., Showalter, S., Slosarek, M., Szabo, I., Tarnok, I., Tsukamura, M., Vergmann, B., Wolinsky, E: High Reproducible Techniques for use in systematic bacteriology in the genus *Mycobacterium*: tests for Niacin and catalase and for resistance to Isoniazid, Thiophene 2-Carboxylic Acid Hydrazide, Hydroxylamine, and *p*-Nitrobenzoate. International *Journal of Systematic Bacteriology*. 1976; **26(3)**: 311-318.
8. Sebabrata, Mahapatra., Dean, C., Crick, Michael., R, McNeil., Patrick, J., Brennan:Unique Structural Features of the peptidoglycan of *Mycobacterium leprae*. *Journal of Bacteriology*, 2008; **190**: 655-661.
9. Mandira, Varma-Basil, Sujeet Kumar., Jitender Yadav., Nalini Kumar., Mridula Bose: A simple method to differentiate between Mycobacterium tuberculosis and Non-Mycobacterium tuberculosis directly on clinical specimen. *Southeast Asian J Trop Med Public Health.,* 2007; **38(1):**111-114.

10. Bergey's Manual of Determinative bacteriology. http://www.uiweb.uidaho.edu/ micro_biology/ 250/IDFlowcharts. pdf.
11. Mackova, NO., Fedorocko, P: Pre- and postirradiation hemopoietic effects of liposomal muramyl tripeptide phosphatidylethanolamine (MTP-PE) administered to C57/Bl/6 mice before irradiation. *Neoplasma.,* 1993; **40:** 379-385.
12. Mackova, NO., Fedorocko P: Effects of immunomodulators on postirradiation recovery in the thymus. *Physiol Res.,*1997; **46:** 193-197.
13. Namba, K., Nitanai, H., Otani, T., Azuma, I: Romurtide, a synthetic muramyl dipeptide derivative, accelerates peripheral platelet recovery in nonhuman primate chemotherapy model. *Vaccine.,* 1996; **14:** 1322-1326.
14. Pabst, MJ., Beranova, Giorgianni, S., Krueger, J.M: Effects of muramylpeptides on macrophages monokines and sleep. *Neuro-immunomodulation.,* 1999; **6(4)**: 261-283.
15. Ikuo, Saiki., Saburo, Sone., William, E., Fogler, Eugenie S,Kleinerman., Gabriel, Lopez-Berestein, Isaiah J.Fidler:Synergism between human recombinant β-interferon and Muramyldipeptide Encapsulated in liposomes for activation of antitumor properties in human blood monocytes. *Cancer research.,* 1985; **45**: 6188-6193.
16. Stephan E, Girardin., Ivo G, Boneca., Jerome, Viala., Mathias, Chamaillard., Agnes, Labigne.,Gilles, Thomas., Dana J, Philpott., Philippe, J., Sansonetti Nod2 is a general sensor of peptidoglycan through MuramylDipeptide (MDP) detection. *The Journal of Biological chemistry*. 2003
17. Monoko, Nisikori: Classical and alternative NF-êsB activation pathways and their roles in lymphoid malignancies. *J.Clin.Exp. Hematopathol*. 2005; **45**(1): 15-24.
18. Scheringa, M., Marquet, R. L: TNF: A brief review with emphasis on its antitumor activity. *Biotherapy*. 1990; **2**(3): 275-281.