Effects of Lipopolysaccharides of *Pseudomonas Aeruginosa* and Aqueous Extract of *Ginkgo Biloba*, Ginkgoaceae, on Cellular Immune Response in Mice Balb/c

Pseudomonas Aeruginosa ve Ginkgo Biloba, Ginkgoaceae Sulu Özü Lipopolisakkaritlerin Balb/c Farelerde Hücresel Bağışıklık Yanıtı Üzerindeki Etkileri

Najeeb Mohammed Hussein[1], Mustafa Nadhim Owaid[2,3], Ban Hamid Khalaf[4]

[1] Department of Biology, College of Science, University of Anbar, Ramadi, Anbar, Iraq
[2]Department of Heet Education, General Directorate of Education in Anbar, Ministry of Education, Hit, Anbar, Iraq
[3] Department of Ecology, College of Applied Sciences, University of Anbar, Hit, Anbar, Iraq
[4] College of Dentistry, University of Anbar, Ramadi, Anbar, Iraq

ABSTRACT

In this study, lipopolysaccharides (LPS) of *Pseudomonas aeruginosa* IAN5 isolate and aqueous extract of *Ginkgo biloba* leaves were used to investigate the cellular immunity in mice Balb/c (*in vivo*). Some parameters were used to achieve this study, are percentages of polymorphonuclear neutrophils (PMNs), phagocytosis coefficient of PMNs at different time periods 30, 60, 90 and 120 minutes, Formazan granules formation in them, and migration inhibition factor (MIF). The immunization of mice with the LPS antigen affected delay-type hypersensitivity and increased activity of PMNs in *Candida* sp. and reduced NBT, while inhibited migration of PMNs. The immunization did not affect the macrophages of PMNs. The injected treatment with LPS and *Ginkgo biloba* extract showed the best results significantly (p<0.05) compared to LPS and control treatments, whereas the *Ginkgo biloba* extract alone showed no significant differences (p<0.05).

Key Words: LPS, immunology, herbal extract, *in vivo*, maidenhair tree.

Received: 01.20.2018 **Accepted:**06.03.2018

ÖZET

Bu çalışmada, *Pseudomonas aeruginosa* IAN5 izolatının lipopolisakkaridleri (LPS) ve Ginkgo biloba yapraklarının sulu özütü, Balb/c farelerde (*in vivo*) hücresel bağışıklığı araştırmak için kullanıldı. Bu çalışmanın gerçekleştirilmesinde bazı parametreler kullanıldı; polimorfonükleer nötrofil (PMN'ler) yüzdeleri, 30, 60, 90 ve 120 dakika gibi farklı zaman aralıklarında PMN'lerin fagositoz katsayısı, içerlerinde Formazan granül oluşumu ve göç önleme faktörü (MIF). Farelerin LPS antijeni ile immünizasyonu, gecikmiş tip aşırı duyarlılığı etkilemiş, *Candida* sp.deki PMN aktivitesini atırmış, NBT'yi azaltmış ve PMN'lerin göçünü inhibe etmiştir. Bağışıklama, PMN'lerin makrofajlarını etkilemedi. LPS ve *Ginkgo biloba* ekstresi ile enjekte edilen tedavi, LPS ve kontrol tedavileri ile karşılaştırıldığında en iyi sonuçları (p <0.05) gösterirken, *Ginkgo biloba* ekstresi tek başına anlamlı farklılık göstermedi (p <0.05).

Anahtar Sözcükler: LPS, immünoloji, bitkisel öz*, in vivo*, baldırıkara ağacı.

Geliş Tarihi: 20.01.2018 **Kabul Tarihi:**03.06.208

Address for Correspondence / Yazışma Adresi: Mustafa Nadhim Owaid, Department of Heet Education, General Directorate of Education in Anbar, Ministry of Education, Hit, Anbar 31007, Iraq E-mail: mustafanowaid@gmail.com

doi:http://dx.doi.org/10.12996/gmj.2018.57

INTRODUCTION

Many medical and experimental studies declared the importance of cellular immunity induction (1-2). Tang *et al*. (2017) referred that murine acid-rich LPS induces monocytes to produce a high level of INF and IL-1 which increase the adherence of basophils with endothelium vessels then inhibition their migration to the infection place. The results referred that the purified LPS inhibits migration of phagocytes then inhibition of specific immunity responses (4). Lipopolysaccharides induce allergic interactions which lead to filtered acidophil and Th2 cells into respiratory canals. Th2 secretes IL-4 which causes B lymphocytes to produce IgE and IL-5 and to accumulate acidophil (2). Helper T-cells has differentiation CD4+ antigen on their outer surfaces that differentiated LPS and lead to induce helper T-cells for proliferation and to release Lymphokines. Lipopolysaccharides induce macrophages and monocytes to secrete TNF-α, IL-6, and IL-1 through their linking with Lipopolysaccharide Binding Protein to form Lipopolysaccharide Binding Protein Complex which links with the receptors CD14 or TLR-4 (5).

Ginkgo tree is one of the most popular drugs and herbal supplements in Europe and the rest of the world (6). *Ginkgo biloba* is the sole surviving member of the Ginkgoaceae family. Its common name is maidenhair tree (7). Some specimens are over 30–40 meters high and several hundred years of age, and nowadays it is cultivated in many countries (8). The name "ginkgo" derived from the Chinese word for silver apricot and this name is called to the tree because of the size and coloring of the fruit (9). Leaves of *Ginkgo biloba* have been extensively studied for their potential therapeutic properties. Ginkgo leaves contain two primary active ingredients, flavonoids and terpenoids (10).

Studies on mammalian cells indicate that *Ginkgo biloba* extract can scavenge nitric oxide and may prevent its production, consequently protecting mammalian cells against nitric oxide reactivity (11). Traditionally, *Ginkgo biloba* leaves had been used as a drug for bronchial asthma and as wound-plasters (8). Some studies suggested that *Ginkgo biloba* extract is conserving mitochondrial metabolism and adenosine triphosphate (ATP) production in tissues, thus partially inhibiting morphologic distortion and signs of oxidative damages due to mitochondrial aging (12). Therefore, this work investigates the induction of the response of cellular immunity *in vivo* by *Ginkgo biloba* extract and Lipopolysaccharides of *Pseudomonas aeruginosa* IAN5 (local isolate).

MATERIALS and METHODS

Preparing migration medium

This medium was prepared by dissolving 1.5 g agarose in 100 ml D.W and then sterilized using the boiling. Afterwards, the medium is cooled to 45 ºC and added Hanks balanced Salt (pH 7.2) (prepared from Flow Laboratories by v:v) and inactive sera to obtain the final concentration 10% in the medium. All ingredients were wholly mixed and stored in 4 ºC until the use.

Phosphate buffer saline (pH 7.2)

This buffer is prepared according to method of Hudson and Hay (1980). Only 0.85 g KH_2PO_4, 0.795 g Na_2HPO_4, and 9 g NaCl are dissolved in 1000 ml D.W and sterilized using Autoclave for 15 min. this puffer used as a control in this test.

Nitroblue tetrazolium (NBT) Preparation

Only 0.1 g of stain powder was dissolved in 2 ml methanol and then added 50 ml PBS solution. This stain solution is stored in 4 ºC to use in NBT test.

Giemsa Stain

This stain was prepared and ready from British Drug Houses (BDH).

Growth of the bacterial isolate

The bacterium *Pseudomonas aeruginosa* IAN5 was obtained from College of Science, University of Iraq. This isolate was subcultured in 10 ml Nutrient broth and incubated for 48 hr at 37 ºC. Then the inoculation of bacteria was achieved by taking 4 ml of the culture broth in 1000 ml of sterilized nutrient broth and incubated in the same conditions in a shaker incubator at 100 cycle/min for 48 hr.

Centrifugation of culture broth at 3000 cycle/min for 15 min at four Celsius and the sediment was suspended in Phosphate buffer saline (pH 7.2) and centrifuged by same condition three times until getting the pure sediment and stored in 4 ºC.

Extraction and purification of LPS

LPS of *P. aeruginosa* IAN5 was extracted using Westphal and Jann method (1965) and purified using the modified Vogel's method (Hirschfeld *et al.*, 2000).

Laboratory animals

Males of Swiss white mice Balb/c with old 6-7 weeks in this study which obtained from Medicative Control Center, Ministry of Health, Baghdad, Iraq.

RESULTS and DISCUSSION

Effect of the immunization by using lipopolysaccharides (LPS) of *Pseudomonas aeruginosa* and extract of Ginkgo (*Ginkgo biloba*) on the survival of basophile is reported in table 1. LPS of *P. aeruginosa* and Ginkgo biloba extract did not exhibit any significant differences (p<0.05) in mice. The higher percentage is 93.6±0.84% by LPS antigen and *Ginkgo biloba* extract compared with the control (phosphate buffer) 93.2±0.42%. *Ginkgo biloba* extract and LPS isolated from bacteria individually show lower percentages reach to 93.2±0.42% and 93.0±0.71% respectively. The rate of survival of basophile is height percentage that never provides the effect of antigens toward it. This result agrees with results of Hoffbrand *et al*. (2004).

Effect of immunization by LPS and *Ginkgo biloba* extract on reducing Nitro blue tetrazolium dye using Nitro blue tetrazolium reduction test (NBT blood test). However, table 1 shows that LPS of *P. aeruginosa* and *Ginkgo biloba* extract exhibit higher significant differences (p<0.05) in the percentage of PMNs formed Formazan at percent 37.5±1.12% in comparison with the control (32.5±1.00%), followed 35.4±1.00% by LPS treatment alone. The lower percentage is 32.5±1.00% by Ginkgo extract individually. Also, in an apparent form, figure 1 shows PMNs formed Formazan under compound microscope X100. That is agreeing with Clark (1999) and Al-Kubaissi (2002). NBT is used to test phagocytosis of microbes by PMNs through their ability to produce superoxide anion O_2 which reduces the soluble yellow stain (NBT) to precipitate dark blue granules (formazan) in the cytoplasm of PMNs (19-21). These granules appear under a light microscope as in figure 1. This test showed the enzymatic activity of PMNs in their vacuoles after induction of NADPH oxidase to oxidize NADPH to NADP+ (17,22).

Table 1 Effect of immunization of mice using LPS antigen of *Pseudomonas aeruginosa* and *Ginkgo biloba* extract on PMNs

Treatments	Percentage of PMNs	Percentage of PMNs formed Formazan
LPS of *P. aeruginosa*	93.0±0.71a	35.4±1.00b
***Ginkgo biloba* extract**	93.2±0.42a	32.5±1.00c
LPS & *Ginkgo biloba*	±0.84a 93.6	37.5±1.12a
Phosphate buffer (control)	93.2±0.42a	32.5±1.00c

The different letters in the same column refers to the significant differences (p<0.05).

Effect of LPS and Ginkgo extract on phagocytosis of the killed *Candida albicans*, as exhibited in table 2. Generally, LPS and Ginkgo showed best phagocytosis coefficient with significant differences (p<0.05) reach to 73.9, followed 69.6 by LPS in comparison with the control (64.7). Ginkgo extract alone gave lower phagocytosis coefficient 64.7. Figure 2 presented killing *C. albicans* was devoured by MNPs. The differed time periods from 30 to 120 minutes for each treatment were investigated on phagocytosis coefficient. After 30 and 60 minutes, LPS and the plant extract exhibited together higher phagocytosis coefficient 75.3±0.36 and 74.5±0.31 significantly (p<0.05) and then decreased with increasing the time after 90 and 120 min to 73.7±0.33 and 72.1±0.37 respectively. The bacterial LPS exhibited higher phagocytosis coefficient after 30 min (70.7±0.34) then declined to 67.7±0.43 after 120 min. Ginkgo extract showed coefficients 66.1±0.62, 66.0±0.66 and 64.1±0.57 after 30, 60 and 90 min respectively, then dropped significantly (p<0.05) to 62.6±0.63 after 120 min. The control one showed 66.1±0.62 and 66.0±0.66 after 30 and 60 min respectively. Afterwards, phagocytosis coefficient declined to 64.1±0.57 and 62.6±0.63 respectively.

Macrophages are induced by microbes and their byproducts, immune complexes, inflammation cases, and wounds to produce many lysozymes which are leading to raise the organism temperature or to activate lymphocytes for releasing cytokines or producing nitric oxide as an antibiotic according to types of macrophages and microbes (21). Furthermore, Cortes *et al*. (2002) referred to the reason of increasing phagocytosis coefficient in the treated cells of mice with LPS of *Klebsiella pneumoniae* returns to the ability of these antigens to prompt completion system compounds especially C5a and C3b which contribute in Opsonization and attracting basophils to infection site respectively.

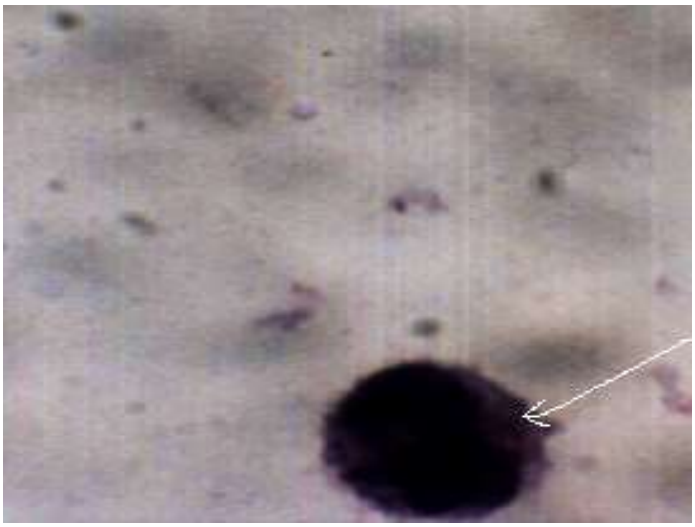

Figure 1 PMNs have dark Formazan granules (Giemsa 100 X)

Table 2 Study influence of injection using LPS of *P. aeruginosa* and Ginkgo extract on phagocytosis of *Candida albicans* killed by the heat

Treatments	Phagocytosis coefficient of PMNs at different time periods (minutes)				Mean
	30	60	90	120	
LPS of *P. aeruginosa*	70.7±0.34a	70.0±0.13a	70.0±0.38a	67.7±0.43b	**69.6B**
***Ginkgo biloba* extract**	66.1±0.62a	66±0.66a	64.1±0.57b	62.6±0.63c	**64.7C**
LPS & *Ginkgo biloba*	75.3±0.36a	74.5±0.31a	73.7±0.33a	72.1±0.37b	**73.9A**
Phosphate buffer (control)	66.1±0.62a	66.0±0.66a	64.1±0.57b	62.6±0.63c	**64.7C**

The different small letters in the same row refers to significant differences (p<0.05) in the period for each treatment. The different capital letters in the last column indicate significant differences (p<0.05) among averages of treatments.

LPS and *Ginkgo biloba* were used to investigate zone of migration (figure 3) and migration inhibition factor (MIF) of PMNs, table 3. LPS and Ginkgo extract jointly showed a lower zone of PMNs migration reached to 7.15±0.12 mm significantly (p<0.05), while LPS antigen individually exhibited migration zone of 11.4±0.10 mm compared with the control (16.44±0.04 mm). Also, LPS and *Ginkgo biloba* extract showed higher migration inhibition factor (MIF) 0.43, followed 0.69 by LPS individually compared with the control which reached to 1.00. Using *Ginkgo biloba* separately did not record any significant differences (p<0.05).

Kulseng *et al*. (1996) referred to that injecting the purified alginate from *Pseudomonas aeruginosa* in mice is inducing macrophage to produce high levels of IL-1 and TNF which leads to adherence of PMNs on endothelium layer of blood vessels then inhibition of migration to the infection site. This study agrees with Al-Kubaissi (2002) who mentioned that *Entamoeba histolytica* inhibits migration of PMNs.

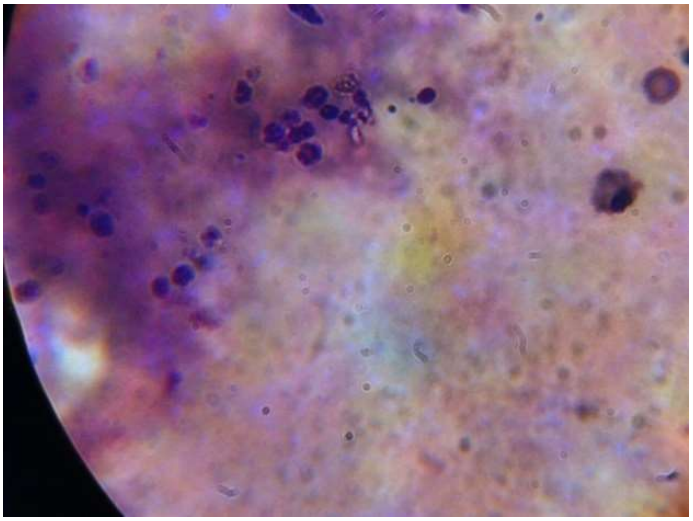

Figure 2 PMNs devour *Candida albicans* killed by the heat (X 100) Giemsa

Table 3 Influence of injection of mice using LPS and Ginkgo extract on the migration of PMNs

Treatments	Zone of migration (mm)	Migration inhibition factor (MIF)
LPS of *P. aeruginosa*	11.4 ±0.10 c	0.69
***Ginkgo biloba* extract**	16.44±0.04 a	1.00
LPS & *Ginkgo biloba*	7.15±0.12 d	0.43
Phosphate buffer (control)	16.44±0.04 a	1.00

Treatment of LPS of *P. aeruginosa* and Ginkgo extract showed the delayed type hypersensitivity (DTH) by an increase of foot thickness of mice 2.51±0.101 mm, 2.44±0.113 mm and 1.55±0.084 mm respectively in comparison with the mice before the treating (1.19±0.034 mm) significantly (p<0.05), as shown in figure 4. Furthermore, the delayed type hypersensitivity of *Ginkgo biloba* extract individually reached to 1.18±0.027 mm before the treating, and that declined to 0.34±0.028 mm after 72 hr in which is similar to the control (0.34), table 4.

In table 4, *Ginkgo biloba* extract and phosphate buffer did not exhibit any irritation area in the treated mice. LPS of *P. aeruginosa* and *Ginkgo biloba* extract jointly showed significant (p<0.05) area of irritation reached to 0.55±0.016 mm^2, 0.44±0.016 mm^2, and 0.36±0.016 mm^2 after 24 hr, 48 hr and 72 hr respectively. The reactions of the delayed type hypersensitivity are considering an indicative of the cellular immunity without the effect of antibodies (22,25,26). Results of this study show changing in the delayed-type hypersensitivity because of influence antigen type on this reaction as mentioned by Abbas *et al*., (2000) and Benschop *et al*., (1999). The responsible cells of the immune response are CD4 T-cells (TDTH) by producing Cytokines which effect on the activity of macrophage (27,28). The increase in thickness of mice's foot may belong to the ability of the injected antigens to enhance lymphokines which lead to migration of WMCs selectively.

Figure 3 Zone of migration of neutrophils

Table 4 The delayed type hypersensitivity test after various periods (mm) and area of irritation after different periods (mm^2)

The injected antigens	Foot thickness after the treating (mm)				Area of irritation after the treating (mm^2)		
	before the treating	After 24 hr	After 48 hr	After 72 hr	After 24 hr	After 48 hr	After 72 hr
Phosphate buffer	1.1±0.024a	1.28±0.023b	1.11±0.025b	0.33±0.024b	0.0±0b	0.0±0b	0.0±0b
G.b	1.18±0.027a	1.23±0.028b	1.18±0.026b	0.34±0.028b	0.0±0b	0.0±0b	0.0±0b
LPS and G.b	1.19±0.034a	2.51±0.101a	2.44±0.113a	1.55 0.084a	0.55±0.016a	0.44±0.016a	0.336±0.016 a

Figure 4 The delayed type hypersensitivity

Conflict of interest

No conflict of interest was declared by the authors.

REFERENCES

1.Mottahedin A, Smith PLP, Hagberg H, Ek CJ, Mallard C. TLR2-mediated leukocyte trafficking to the developing brain. Journal of Leukocyte Biology. 2017; 101: 297-305.

2.Hu P, Xiao H, Alba MA, Falk RJ, Jennette JC. WS6_1 Anti-MPO antibodies cause granulomatosis in mice: an animal model of GPA. Rheumatology. 2017; 56: 29-31.

3.Tang Q-F, Fang Z-Y, Shi C-H. The protective effect and mechanism of sevoflurane on LPS-induced acute lung injury in mice. American Journal of Translational Research. 2017; 9: 1732-1742.

4.Stienstra R, Netea-Maier RT, Riksen NP, Joosten LA, Netea MG. Specific and complex reprogramming of cellular metabolism in myeloid cells during innate immune responses. Cell Metabolism. 2017; 26: 142-56.

5.Gisterå A, Hermansson A, Strodthoff D, Klement ML, Hedin U, Fredrikson GN, Nilsson J, Hansson GK, Ketelhuth DF. Vaccination against T-cell epitopes of native ApoB100 reduces vascular inflammation and disease in a humanized mouse model of atherosclerosis. Journal of Internal Medicine. 2017; 281: 383-97.

6.Van Dongen MCJM, van Rossum E, Kessels AGH, Sielhorst HJG, Knipschild PG. The efficacy of ginkgo for elderly people with dementia and age-associated memory impairment: New results of a randomized clinical trial. Journal of the American Geriatrics Society. 2000; 48: 1183-94.

7.Taylor TN, Taylor EL. The Biology and Evolution of Fossil Plants. Prentice-Hall, Englewood Cliffs, NJ, USA; 1993.

8.Jiratchariyakul W, Mahady GB. Overview of Botanical Status in EU, USA, and Thailand. Evidence-Based Complementary and Alternative Medicine. 2013; 2013: ID 480128, http://dx.doi.org/10.1155/2013/480128

9.Mahadevan S, Park Y. Multifaceted therapeutic benefits of Ginkgo biloba L.: chemistry, efficacy, safety, and uses. Journal of Food Science. 2008; 73: R14-19.

10. Janssens D, Delaive E, Remacle J, Michiels, C. Protection by bilobalide of the ischaemia-induced alterations of the mitochondrial respiratory activity. Fundamental and Clinical Pharmacology. 2000; 14: 193–201.

11. Marcocci L, Maguire JJ, Droy-Lefaix MT, Packer L. The nitric oxide-scavenging properties of Ginkgo biloba extract EGb 761. Biochemical and Biophysical Research Communications. 1994; 201: 748–55.

12. Sastre J, Lloret A, Borrás C, Pereda J, García-Sala D, Droy-Lefaix MT, Pallardó FV, Viña J. Ginkgo biloba extract EGb 761 protects against mitochondrial aging in the brain and in the liver. Cell and Molecular Biology. 2002 48: 685–92.

13. Hudson L, Hay FC. Practical Immunology. 2nd ed. Blackwell Scientific Publicaitons, London, U.K. pp. 229; 1980.

14. Westphal O, Jann K. Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure, p. 83-91. In Roy L. Whistler (ed.), Methods in carbohydrate chemistry vol. 5. Academic Press, Inc., New York; 1965.

15. Hirschfeld M, Ma Y, Weis JH, Vogel SN, Weis, JJ. Cutting Edge: Repurification of Lipopolysaccharide Eliminates Signaling Through Both Human and Murine Toll-Like Receptor 2. J Immunol. 2000; 165: 618-22.

16. Hoffbrand AV, Petit JE, Moss PAH. Essential Haematology. (4th ed) Blackwell, Oxford. pp. 252-253; 2004.

17. Clark RA. Activation of The Neutrophil Respiratory Burst. Oxidase. J. Infect. Dis. 1999; 179: S 309

18. Al- Kubaissi A B. Immunological Epidemiological Study Of Patients Infected With *Entamoeba histolytica*. PhD Thesis, College Of Science, Almustansiriya University; 2002.

19. Al-Hayani HSAS. Experimental Resistance To Tick Species *Hyalomma anatolicum*. PhD Thesis, College of Science, University of Anbar; 2003.

20. Brooks GF, Butel JS, Morse SA. *Staphylococci*. In: Medical Microbiology.22thed. Lange Medical Publication. 2001; pp. 197-203.

21. Stites DP, Terr AL, Parslow TG. Basic and Clinical Immunology, 9th Ed., Appleton and Lange. 1997; pp. 125-132.

22. Roitt IM, Brostoff J, Male D. Immunology. 6th Ed Mosby, 2001; pp.1-16.

23. Cortes G, Borrell N, De Astorza B, Gomez C, Sauleda J, Alberti S. Molecular Analysis of The Contribution of The Capsular Polysaccharide and The Lipopolysaccharide O Side Chain to the Virulence of *Klebsiella Pneumoniae* In A Murine Model of Pneumonia. Infect. Immun. 2002; 70: 2583–90.

24. Kulseng B, Skjak-Brack G, Folling I, Espevik T. TNF Production From Peripheral Blood Mononuclear Cells In Diabetic Patients After Stimulation With Alginate and Lipopolysaccharide. Scard. J. Immunol. 1996; 42: 335-40.

25. Klimas N. Delayed Hypersensitivity Skin Testing In Manual of Clinical Lab. Immunol. 5th Ed., Washington, ASM Press, 1997; pp.276- 80.

26. Parham P. The Immune System. Garland, Elsevier, 2000; pp. 127- 32.

27. Abbas A, Lichtman AH, Pober J S. Cellular and Molecular Immunology. 4th Ed., Saunders, 2000; pp. 109-15.

28. Benschop RJ, Cambier JC. B Cell Development: Signal Transduction by Antigen Receptors and Their Surrogates Curr. Opin. Immunol. 1999; 11: 143.