

# THE EFFECT OF THE SUPERNATANT OF COLEY'S MIXED BACTERIAL TOXIN (MBT) AND BACTERIAL LIPOPOLYSACCHARIDE (LPS) ON SERUM LEVELS OF TNF- $\alpha$ , IL-12, AND VEGF IN MICE

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## ABSTRACT

Coley's Mixed Bacterial Toxin (MBT) used to treat cancer patients, is thought to be a modulator of a number of cytokines. In this study the effects of the MBT supernatant and bacterial lipopolysaccharide (LPS) on serum levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-12 (IL-12), and vascular endothelial growth factor (VEGF) were determined.

MBT was prepared, centrifuged and the supernatant was dialyzed. The protein content of the dialysate was determined by the Bradford assay and SDS-PAGE. A portion of the dialysate was heated to denature proteins. Groups of mice were given intraperitoneal injections of MBT, dialysate or heated dialysate, LPS, RPMI, or were left untreated. Serum levels of TNF- $\alpha$ , IL-12 and VEGF were determined at 2, 4, and 6 hours post-injection by ELISA.

The dialysate contained 9% protein and SDS-PAGE revealed only one band (17KDa). Unheated or heated dialysate increased the levels of TNF- $\alpha$  and IL-12. Heated dialysate decreased VEGF levels. LPS caused an increase in TNF- $\alpha$  and IL-12, and a decrease in VEGF levels.

Activity was present in the MBT and both heated and unheated dialysate. Heating of fractions enhanced activity, suggesting that active sites were exposed or inhibitory factors were inactivated by heat. It appears that LPS could be one of the active constituents of MBT.

**Keywords -** *Streptococcus pyogenes*, *Serratia marcescens*, Lipopolysaccharide

## I. INTRODUCTION

In early 1900, Coley noticed that cancer patients who developed bacterial infections after sarcoma surgery had a better prognosis than those who did not develop any sort of infection post-surgery. Coley associated the regression of certain large inoperable sarcoma of the neck with erysipelas, caused by *Streptococcus pyogenes*. He then developed what is known as the mixed bacterial toxins (MBT) which consists of heat killed Gram-positive *Streptococcus pyogenes* and heat killed Gram-negative *Serratia marcescens* and used it to treat his cancer patients [1, 2, 3].

MBT is thought to have several immunomodulatory properties. These include activation of macrophages, dendritic cells, T-helper cells, T-cytotoxic and NK cells [4, 5], resulting in the production of a number of cytokines including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and Interleukin-1 (IL-1). It is also thought to have anti-angiogenic properties [6, 7].

MBT can induce its effects through its Pathogen Associated Molecular Pattern (PAMP) that engages Pattern Recognition Receptors (PRR) such as Toll-Like receptors (TLR) expressed on macrophages and dendritic cells leading to the activation of the MyD88 dependent and/or independent pathways which eventually results in the

production of cytokines that are involved in the generation of an immune response [8].

To date, it is still unknown what the active component(s) of the MBT is. Some reports indicate that it is lipopolysaccharide (LPS), a constituent of the cell wall of *Serratia marcescens* [9], others indicate that it might be streptokinase produced by *Streptococcus pyogenes*. The aim of this study was to separate the supernatant from the MBT and study its activity and that of LPS by testing the effect of the supernatant and LPS on the levels of serum TNF- $\alpha$ , Interleukin-12 (IL-12) and vascular endothelial growth factor (VEGF) in mice.

## II. MATERIALS AND METHODS

### 2.1 Preparation of MBT

*Streptococcus pyogenes* (strain ATCC 19615) and *Serratia marcescens* (strain Rlab 810040.2 from wound exudates), were used for the preparation of the MBT and were obtained from the stock collection of the department of Experimental Pathology, Immunology and Microbiology at the American University of Beirut. The method described by Martin [10] with some modifications was used. A loop full from a fresh culture of *Streptococcus pyogenes* was suspended in 500ml of RPMI-1640 containing L-glutamine and 25mm Hepes (Bio Whittaker, Lonza, Belgium) and incubated at 37°C for 10 days. The bacterial suspension was then seeded with live *Serratia marcescens* and incubated at 24°C for another 10 days, when the suspension turned pink

in color. Benzyl alcohol (0,3ml, Sigma-Aldrich, MO, USA) was then added to the suspension to prevent fungal growth.

**2.2 Preparation of supernatant**

The bacterial suspension was centrifuged at 3500 rpm for 20 minutes. The supernatant was separated from the pellet and filtered using a 0.2 µm filter unit (Nalgene, Nalge Nunc International, NY, USA) to ensure removal of bacterial cells and their cellular debris. It was then dialyzed for 3 consecutive days against distilled water; water was changed twice daily. The dialysate was re-filtered to ensure complete sterility. Both the MBT and the dialysate were each stored at -70°C in suspension. A portion of the dialysate was heated at 100°C for 5 min before freeze drying to denature the proteins.

**2.3 Bacterial LPS**

LPS extracted from *Salmonella minnesota* was used. It was obtained from Sigma, St. Louis, MO, USA.

**2.4 Bradford assay**

The protein concentration was determined using the Bradford assay. The dialysate was mixed with Bradford reagent and its absorbance was measured using a wavelength of 595nm. The value was determined by extrapolating from a standard curve prepared using different concentrations of bovine serum albumin and their absorbance values [11]

**2.5 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

The dialysate was subjected to SDS- PAGE. The methodology used was that of BioRad (Hecules, CA, USA); 10% SDS, running gel 7.5% pH 8.8 and stacking gel 4% pH 6.8%. Bands were visualized using the Coomassie stain.

**2.6 Limulus Amebocyte Lysate Assay**

The presence of LPS in the heated and unheated dialysate was determined by the limulus amebocyte lysate assay (LAL) (Pyrotell, Cape Cod incorporated, MA, USA). The procedure described by the manufacturer was followed.

**2.7 Injection of mice**

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the Faculty of Medicine at the American University of Beirut. Lyophilized preparations of dialysate (0.1g), heated dialysate (0.1g), bacterial suspension (MBT) (0.5g) and LPS were each suspended in 10 ml of dialyzed RPMI-1640.

Fifty four female BALB/c mice, (6 - 8 wk old), were purchased from the Animal Care Facility at the American University of Beirut. They were divided into 6 groups of 9 mice each and received different intraperitoneal injections. The doses and injection protocol are given in Table 1. At 2, 4 and 6 hours post injection 3 mice from each group were anesthetized with a 0.5 ml of a mixture of 0.12 ml ketamine (final concentration 12mg/ml), 0.03 ml xylazine (final concentration 1.2mg/ml), and 0.35 ml sterile distilled water. The thoracic cavity was opened and blood was collected by cardiac puncture. Blood from each group was pooled; the

serum was separated and used for the determination of TNF-α, Il-12 and VEGF.

**2.8 Enzyme-Linked Immunosorbant Assay (ELISA)**

The single analyte ELISArray kits for TNF-α and IL-12 (SABiosciences, MD, USA) and VEGF mouse ELISA kit (Abcam, USA) were used to determine serum TNF-α, Il-12 and VEGF respectively. Procedures were performed according to the manufacturer’s protocol.

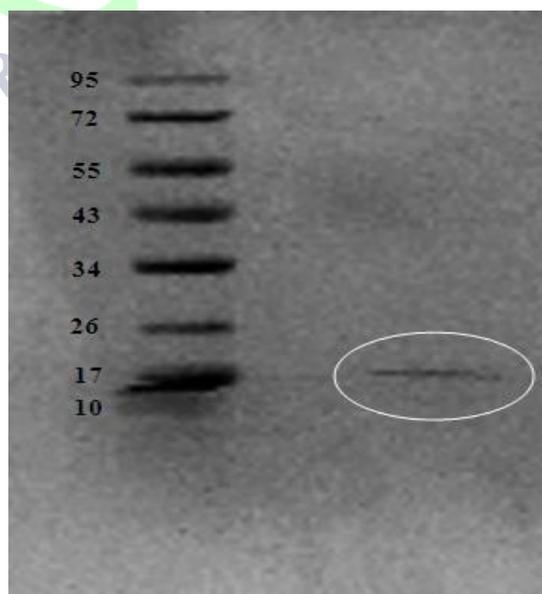
**Table1: Protocol followed for the treatment of mice**

Group	Group Description	Treatment
1	Negative Control	No injection
2	Negative Control	RPMI-1640
3	Positive Control	Mixed Bacterial Toxin (5×10 <sup>7</sup> g/ml)
4	Test Group	Dialysate (5×10 <sup>7</sup> g/ml)
5	Test Group	Heated dialysate (5×10 <sup>7</sup> g/ml, 256 EU)
6	Test Group	Lipopolysaccharide (2.56×10 <sup>-13</sup> g/ml, 256 E U)

**III. RESULTS**

**3.1 Protein Concentration and SDS-PAGE Pattern**

The Protein concentration of the lyophilized dialysate was 0.09 g/1 g (9%). One band appeared in SDS-PAGE having a molecular weight of about 17 KDa. The electrophoretic pattern obtained is shown in Fig1.



**Figure 1:** SDS-PAGE electrophoresis of dialysate. The protein ladder is the left lane, and the single protein band in the dialysate is indicated to the right of the ladder (circled in white). The molecular weight of this protein is approximately 17 KDa.

### 3.2 Heated Dialysate

A portion of the dialysate was heated to inactivate heat labile substances including proteins. LPS, a constituent of the cell wall of *Serratia marcescens* is heat stable. This was confirmed by LAL where both heated and unheated LPS had the same activity (Table 2).

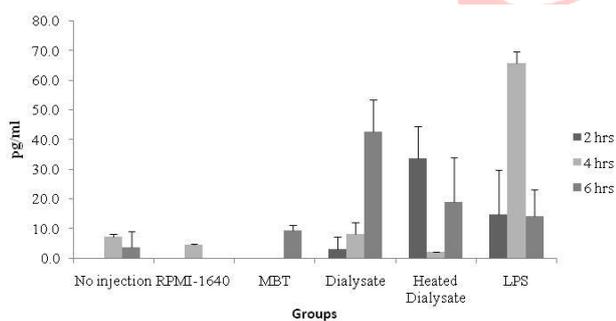
**Table 2: Detection of LPS in heated and unheated dialysate by the Limulus Amebocyte Lysate Assay**

Dilution	Un-heated	Heated
Initial (0.256 mg/ml)	+	+++
1/2	+	+
1/4	+/-	+
1/8	+	+++
1/16	+++	+++
1/32	+++	+++
1/64	+++	+++
1/128	+	-
Negative control	-	-

+ = moderate reaction  
 +/- = mild reaction  
 +++ = strong reaction  
 - = absence of LPS

### 3.3 TNF- $\alpha$ Serum Levels

The levels of TNF- $\alpha$  (Fig 2) increased at 6 hours post-injection in mice treated with dialysate (42.6 pg/ml), and at 2 hours post-injection in mice treated with heated dialysate (33.6 pg/ml) as compared to the levels in untreated or RPMI-1640 treated controls. At 4 hours post injection there was a profound increase in TNF- $\alpha$  level in mice treated with LPS (65.8pg/ml).

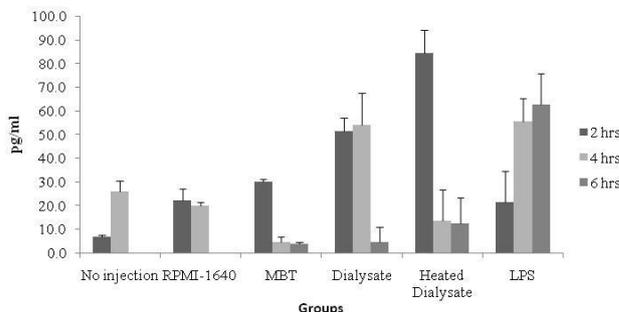


**Figure 2:** Serum levels of TNF- $\alpha$  in the different groups of mice as detected by ELISA. Results show the average of two measurements of TNF- $\alpha$  concentration in pg/ml at 2, 4, and 6 hours post-injection, standard deviations are also shown in the graph. MBT; is the mixed bacterial toxin, and LPS is the bacterial lipopolysaccharide.

### 3.4 IL-12 Serum Levels

Referring to Fig 3; the levels of IL-12 in mice serum increased at 2 and 4 hours post-injection in mice treated with dialysate (51.4pg/ml and 53.9pg/ml respectively) as compared to mice treated with MBT, untreated and RPMI-1640 treated control groups. A more pronounced increase was observed in mice treated with heated dialysate 2 hours

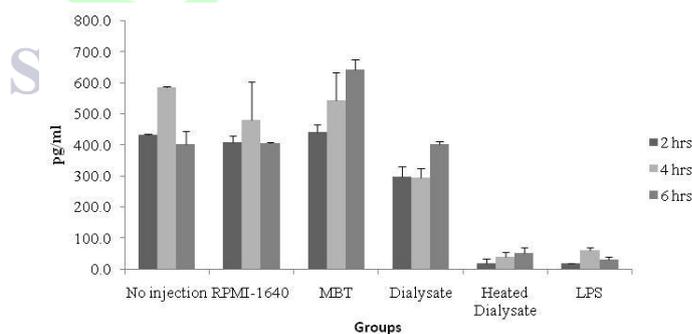
post-injection compared to all of the other groups (84.3pg/ml). LPS administration resulted with an increase in IL-12 levels at 4 and 6 hours post injection (55.4pg/ml and 62.7pg/ml respectively) as compared to all control and MBT groups.



**Figure 3:** Serum levels of IL-12 in the different groups of mice as detected by ELISA. Results show the average of two measurements of the IL-12 concentration in pg/ml at 2, 4, and 6 hours post-injection, standard deviations are also shown in the graph. MBT; is the mixed bacterial toxin, and LPS is the bacterial lipopolysaccharide.

### 3.5 VEGF Serum Levels

The levels of serum VEGF in mice are shown in Fig 4. MBT and dialysate did not appear to significantly affect the physiological levels (levels in RPMI-1640 treated or untreated groups) of VEGF. However, VEGF levels decreased profoundly in mice injected with heated dialysate or LPS at 2, 4 and 6 hours post injection as compared to control groups (19.5pg/ml, 39.1pg/ml and 50 pg/ml respectively for mice injected with heated dialysate and 19.5pg/ml, 59.9pg/ml and 30.4pg/ml respectively for groups injected with LPS).



**Figure 4:** Serum levels of VEGF in the different groups of mice as detected by ELISA. Results show the average of two measurements of VEGF in pg/ml at 2, 4, and 6 hours post-injection, standard deviations are also shown in the graph. MBT; is the mixed bacterial toxin, and LPS is the bacterial lipopolysaccharide.

## IV. DISCUSSION

Coley [12] observed a relationship between infection and tumor regression. Based on the work of Roger [13], Coley used a combination of *Streptococcus pyogenes* and *Serratia marcescens* to treat his cancer patients and reported a certain degree of success [14]. Coley's preparation (MBT) was used for the treatment of a number of different cancers. In 1963 the Food and Drug Administration (FDA) labeled it

as a “new drug” and forbade its use outside of clinical trials [15].

LPS is known to induce the production of a number of cytokines including TNF- $\alpha$  and IL-12 [16, 17,18]. Since LPS is a constituent of the cell wall of *Serratia marcescens*, the aim of this study was to compare the cytokine-modulating effects of LPS and the supernatant of MBT.

The heated and non-heated dialyzed supernatant (dialysate) and LPS induced the production of TNF- $\alpha$  and IL-12. Time intervals post-injection whereby increases in cytokine production were detected varied. If LPS is an active component of the dialysate differences between the LPS concentration of the dialysate and the amount of pure LPS injected might account for the different time intervals where increased cytokine levels were detected. Moreover, *Serratia marcescens* LPS was not available and *Salmonella minnesota* LPS was used instead. It has been reported that LPS from different sources might vary in their biological potency [19].

Tsung and Norton [18] reported that the pro-inflammatory cytokine implicated in the mechanism of action of MBT is IL-12 rather than TNF- $\alpha$ , a cytokine that favors a Th1 response [18]. It is worth noting that one of the agents that drive dendritic cells and other APCs to produce IL-12 is TNF- $\alpha$  [20]. It was expected that rises in TNF- $\alpha$  levels would precede rises in IL-12, but this was not the case. A reasonable explanation would be that other cytokines such as IFN- $\gamma$  were involved in the production of IL-12. Therefore, it appears that both TNF- $\alpha$  and IL-12 are mediators involved in the mechanism of action of MBT.

The dialysate contained 9% protein and appeared as 1 band in SDS PAGE. It was also LAL-positive indicating that it contained LPS. Streptokinase, a product of *Streptococcus pyogenes* along with the patients plasminogen has been reported to be the active ingredient of MBT [21]

The 17 KDa protein band that appeared on the SDS-PAGE gel pattern was not streptokinase or M protein, since they have a molecular weight of approximately 47 KDa and 50 KDa respectively. It could be a breakdown product of one of the 2 proteins, or another factor.

It was assumed that boiling the dialysate would inactivate proteins such as streptokinase. If this assumption is correct than streptokinase and other proteins probably play a minor role in inducing the production of cytokines because heated dialysate was capable of doing so. The dialysate and MBT did not appear to affect VEGF levels. It is of interest to note that heated-dialysate suppressed the physiological level of VEGF, a factor needed for angiogenesis. The production of new blood vessels is needed for survival of tumors [6]. The inactivation of factor(s) that promote VEGF production would probably improve the anti-tumor effect of MBT or dialysate.

LPS also suppressed the physiological levels of VEGF. This finding is contrary to in vitro studies that report that LPS enhances the production of VEGF [22, 23]. This is one of several situations where results of in vivo and in vitro results do not concur.

## V. CONCLUSION

In conclusion, on the one hand MBT contains substances such as LPS that promotes anti-tumor effect by inducing the production of cytokines such as TNF- $\alpha$  and IL-12 and suppresses VEGF production. On the other hand, it contains heat-labile substances that do not affect physiological levels of VEGF, and probably allow its production.

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## Declaration of Interest

The authors report no declarations of interest.

## REFERENCES

- [1] S.A Hoption Cann, J.P. van Netten, C. van Netten, and D.W. Glover, Spontaneous regression: a hidden treasure buried in time, *Med Hypotheses*, 58(2), 2002, 115-119.
- [2] W.K Decker, and A. Safdar, Bioimmunoadjuvants for the treatment of neoplastic and infectious disease: Coley's legacy revisited, *Cytokine Growth Factor Rev*, 20(4), 2009, 271-281.
- [3] H. C Nauts, G.A. Fowler, and F.H. Bogatko, A review of the influence of bacterial infection and of bacterial products (Coley's toxins) on malignant tumors in man; a critical analysis of 30 inoperable cases treated by Coley's mixed toxins, in which diagnosis was confirmed by microscopic examination selected for special study, *Acta Med Scand Suppl*, 276, 1953, 1-103.
- [4] U. Hobohm, Fever therapy revisited, *Br J Cancer*, 92(3), 2005, 421-425.
- [5] U. Hobohm, Fever and cancer in perspective. *Cancer Immunol Immunother*, 50(8), 2001, 391-396.
- [6] S. Minuzzo, L. Moserle, S. Indraccolo, and A. Amadori, Angiogenesis meets immunology: cytokine gene therapy of cancer, *Mol Aspects Med*, 28(1), 2007, 59-86.
- [7] J. Haux, Infection and cancer, *Lancet*, 358(9276), 2001, 155-156.
- [8] U. Hobohm, J.L. Stanford, and J.M. Grange, Pathogen-associated molecular pattern in cancer immunotherapy, *Crit Rev Immunol*, 28(2), 2008, 95-107.
- [9] C.L. Kurz, S. Chauvet, E. Andres, M. Aurouze, I. Vallet, G.P. Michel, M. Uh, J. Celli, A. Filloux, S. De Bentzmann, I. Steinmetz, J.A. Hoffmann, B.B. Finlay, J.P. Gorvel, D. Ferrandon, and J.J. Ewbank, Virulence factors of the human opportunistic pathogen *Serratia marcescens* identified by in vivo screening, *EMBO J*, 22(7), 2003, 1451-1460.
- [10] W. Martin, Coley's Toxins: A Cancer Treatment History, *Townsend letter for Doctors and Patients*, 2006.
- [11] M. Bradford, Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal Biochem*, 72, 1976, 248-254.
- [12] W.B. Coley, II. Contribution to the Knowledge of Sarcoma, *Ann Surg*, 14(3), 1891, 199-220.
- [13] G. Roger, Séance et Mem Soc, *De Biol Paris*, 2, 1890, 573-580.
- [14] W.B. Coley, The treatment of malignant tumors by repeated inoculations of erysipelas. With a report of ten original cases. 1893. *Clin Orthop Relat Res*, (262), 1991, 3-11.
- [15] Coley's Toxins. *Townsend letter for Doctors and Patients*, 2004.
- [16] B. Wiemann, and C.O. Starnes, Coley's toxins, tumor necrosis factor and cancer research: a historical perspective. *Pharmacol Ther*, 64(3), 1994, 529-564.
- [17] P. W. Szlosarek, and F.R. Balkwill, Tumour necrosis factor alpha: a potential target for the therapy of solid tumours, *Lancet Oncol*, 4(9), 2003, 565-573.
- [18] K. Tsung, and J.A. Norton, Lessons from Coley's Toxin, *Surg Oncol*, 15(1), 2006, 25-28.
- [19] A. Nowotny, Relationship of structure and biological activity of bacterial endotoxins, *Naturwissenschaften*, 58(8), 1971, 397-409.
- [20] J.G., K., Targeting the IL-12/1L-23 Th17 pathway in psoriasis. *Interventional Immunology, FOCIS*, 2011.
- [21] E.A. Hussein, and A. el Dorri, Intra-arterial streptokinase as adjuvant therapy for complicated 13Buerger's disease: early trials, *Int Surg*, 78(1), 1993, 54-58.
- [22] Y. Sugishita, T. Shimizu, A. Yao, K. Kinugawa, T. Nojiri, K. Harada, H. Matsui, R. Nagai, T. Takahashi, Lipopolysaccharide augments expression and secretion of vascular endothelial growth factor in rat ventricular myocytes. *Biochem Biophys Res Commun*, 268(2), 2000, 657-662.
- [23] K. Matsushita, R. Motani, T. Sakuta, S. Nagaoka, T. Matsuyama, K. Abeyama, I. Maruyama, H. Takada, M. Torili, Lipopolysaccharide enhances the production of vascular endothelial growth factor by human pulp cells in culture. *Infection and Immunity*, 67(4), 1999, 1633-1639.