World Journal of Pharmaceutical research

Volume 2, Issue 6, 2959-2973. Research Article ISSN 2277 – 7105

THE EFFECT OF THE SUPERNATANT OF COLEY'S MIXED BACTERIAL TOXIN (MBT) ON SURVIVAL AND SERUM CYTOKINE LEVELS IN TUMOR-BEARING MICE

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Article Received on
23 August 2013,

Revised on 29 Sept. 2013,
Accepted on 30 October 2013

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ABSTRACT

The anti-tumor activity of Coley's Mixed Bacterial Toxin (MBT), its dialyzed supernatant (DS) and bacterial lipopolysaccharide (LPS) were assessed by studying their effects on the serum levels of immunosuppressive and angiogenic cytokines in tumor-bearing mice, and their effects on their survival. MBT was prepared and its supernatant was separated and dialyzed (DS). Groups of C57BL/6 mice challenged with B16F10 melanoma cells were treated with either one or 3 injections at 2 day intervals of MBT, DS, LPS or RPMI-1640 (control). The serum levels of Interleukin-10 (IL-10), Transforming Growth Factor-β (TGF- β) and Vascular Endothelial Growth Factor (VEGF) were determined by ELISA at 2, 4 and 6 hours post- injection in 9 mice in each group. The rest of the mice in each group were monitored for ten days for survival rate assessment. The levels of IL-10 and TGF-β decreased after one injection of MBT, LPS or DS. Yet, a significant decrease was induced mainly by three injections of DS. The levels of VEGF significantly decreased after one or three injections of DS. Twenty per cent of the tumor-bearing mice treated with one injection of MBT survived, whereas after three injections of MBT, LPS, or DS there was 20%, 20% and 40% survivals, respectively. DS appears to contain the active agent(s) responsible for the anti-tumor activity. LPS could be one of the active components. Improved protective and therapeutic results probably could be achieved by increasing the number of multiple doses over a longer period of time.

KEYWORDS: B16F10 tumor cells, anti tumor agents, Lipopolysaccharide, IL-10, TGF-β, VEGF.

1. INTRODUCTION

W. B. Coley, a New York surgeon observed that sarcomas regressed in some of his patients after acquiring a streptococcal infection.[1-3] He later developed what is to be known as Coley's Mixed Bacterial Toxin (MBT), a preparation containing both killed Gram positive *Streptococcus pyogenes* and Gram-negative *Serratia marcescens.*[2-6]

Coley's preparation and therapeutic strategy went through several modifications over the years and proved to be effective, mainly in the treatment of soft tissue sarcoma, breast cancer and malignant melanoma.[7-9] Although its mechanism of action is not yet defined, a number of mechanisms were proposed. It was suggested that the anti-tumor effects of Coley's MBT were due to an enhanced immune response induced by exogenous pyrogens such as bacterial lipopolysaccharide (LPS), a constituent of the cell wall of Gram negative *Serratia marcescens*, that binds to Toll-like receptor 4 (TLR-4) and activates the MyD88-dependent or the MyD88-independent pathways.[10, 11] It was also suggested that tumor regression, observed after the administration of Coley's toxin, was attributed to the activity of a plasminogen activator, mainly Streptokinase which is an enzyme secreted by the Gram positive *Streptococcus pyogenes.*[12, 13]

According to the "cancer immunoediting" concept, the immune system is involved in defending the host against tumors, as well as in promoting tumor growth. Therefore, tumor elimination or development is a direct outcome of the interactions taking place between the tumor/tumor microenvironment and the immune system. [14, 15] Tumor progression in a host is mediated by multiple immunosuppressive strategies employed by the tumor itself in order to evade effective immune responses and induce tumor tolerance.[16] These strategies include: impairment of the T cell receptor proximal signals leading to T cell anergy or apoptosis,[17] impairment of the antigen presentation machinery,[16] stimulation of T regulatory cells,[17-19] impairment of the dendritic cell system,[20- 23] activation of indolamine 2,3-dioxygenase (IDO) [24-26] and the elaboration of immunosuppressive cytokines such IL-10, TGF-β [27,28] and angiogenic factors such as VEGF. [29, 30]

In an attempt to describe the mechanism of action and the location of the active component(s) responsible for the anti-tumor activity observed in Coley's MBT, an earlier study was conducted in our laboratories, where the activities of MBT, dialyzed supernatant (DS), heated DS and LPS were assessed by testing their effect on the serum levels of the pro-inflammatory cytokines, Interleukin-12 (IL-12) and Tumor Necrosis Factor-α (TNF-α), and the angiogenic

protein VEGF, in normal mice. This study revealed that most of the activity of Coley's MBT was present in its DS. [31] The current study was carried out, in an effort to determine the effect of MBT and its DS on tumor growth and on the serum levels of the immunosuppressive cytokines, IL-10 and TGF-β, and the pro-angiogenic cytokine VEGF in cancer-bearing mice. Since LPS thought to be one of the active ingredients of MBT, its activity was also investigated.

2. MATERIALS AND METHODS

2.1 Preparation of Coley's MBT and supernatant

A fresh culture of *Streptococcus pyogenes* (strain ATCC 19615) was added to 500ml of RPMI-1640 (sterile for cell culture with L-Glutamine and 25mm Hepes, from Lonza, B-4800 Verviers, Belgium) and incubated at 37°C for 10 days. When the suspension turned pink in color, it was seeded with a fresh culture of *Serratia marcescens* (strain Rlab 810040.2) and incubated at 25°C. Ten days later, the suspension was heated for 2 hours at 65°C. In order to inhibit fungal growth, 0.3ml of benzyl alcohol (Sigma-Aldrich, St. Louis, MO 63103, USA) was added to the mixture. The bacterial suspension was centrifuged at 3500rpm for 20 min. The supernatant was then collected and filtered twice using a 0.45µm and a 0.2 µm filter units (Nalgene sterilization filter unit, Rochester, New York 14602 USA), respectively, to ensure elimination of heat killed bacterial cells and cellular debris. The filtrate was dialyzed against distilled water for three consecutive days, changing the water three times daily. The dialyzed supernatant (DS) was then filtered through a 0.2µm filter unit. Both the MBT and DS were lyophilized and stored for later use.

2.2 Preparation of B16F10 melanoma cells

B16F10 metastatic melanoma cells, syngeneic with the C57BL/6 mice, were obtained from Dr. Marwan El Saban, Department of Anatomy, Cell Biology and Physiological Sciences at The American University of Beirut. These adherent cells were maintained as monolayers *in vitro* in RPMI-1640 supplemented with 1% L-Glutamine, 1% Penicillin-Streptomycin and 10% Fetal Bovine Serum (Lonza,B-4800 Verviers, Belgium) and incubated at 37°C in a 5% CO_2 incubator (Thermo scientific, Forma, series II water jacket, CO_2 incubator). When needed for administration into mice, cells were washed with PBS, detached with 1x Trypsin (2.5% trypsin 10x in HBSS without calcium or magnesium, Lonza, B-4800 Verviers, Belgium) and re-suspended in RPMI-1640. Trypan blue was used to determine viability. Cell

counts were performed and a 0.4ml suspension containing 3.4million cells/kg was injected intraperitoneally to each mouse.

2.3 Challenge of mice with tumor cells and treatment

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the Faculty of Medicine at the American University of Beirut. One hundred and twelve C57BL/6 male mice, four to six weeks old, were obtained from the Animal Care Facility at the American University of Beirut. All mice were challenged with the melanoma cells on day 0 and were then divided into two groups; Group I and Group II. Each group was further divided into four subgroups. Each subgroup was composed of 14 mice. The injection protocol is given in **Table1**. Mice received the treatments or the control either as one time injection, or a triple injection with a two days interval. Injections per mouse were as follows: MBT ($7x10^{-6}$g/kg in 0.4ml), LPS ($3.4x10^{-9}$ mg/kg in 0.4ml) or DS ($7x10^{-6}$g/kg in 0.4ml) all prepared in RPMI. The bacterial LPS used in this study was extracted from the bacterium *Salmonella minnesota* (Sigma-Aldrich, St. Louis, MO 63103, USA).

Table 1: Injection protocol followed for the treatment of tumor challenged mice

	Group I				Group II			
Sub Group	Ia	Ib	Ic	Id	IIa	IIb	IIc	IId
Day 0	Challenge with B16F10 melanoma cells 3.4million cells/kg in 0.4ml				Challenge with B16F10 melanoma cells 3.4million cells/kg in 0.4ml			
Day 7	RPMI-1640	MBT	LPS	DS	RPMI-1640	MBT	LPS	DS
Day 9	-	-	-	-	RPMI-1640	MBT	LPS	DS
Day 11	-	-	-	-	RPMI-1640	MBT	LPS	DS
Injections/mouse= MBT ($7x10^{-6}$g/kg in 0.4ml); LPS ($3.4x10^{-9}$ mg/kg in 0.4ml); DS ($7x10^{-6}$g/kg in 0.4ml) all prepared in RPMI-1640.								

MBT = Mixed Bacterial Toxin; LPS = Lipopolysaccharide; DS = Dialyzed Supernatant

2.4 Procurement of specimens

At 2, 4 and 6 hours post-injection (after the first and only injection for group I (Day 7), and after third injection for Group II (Day 11)), 3 mice from each subgroup were anesthetized each with a 0.5 ml mixture of 0.12 ml ketamine (final concentration 12 mg/ml), 0.03 ml xylazine (final concentration 1.2 mg/ml) and 0.35 ml sterile saline. Blood was collected by cardiac puncture. Blood from each of 3 mice from the same subgroup at different time intervals was pooled. The serum was separated and used for the determination of IL-10, TGF-β and VEGF levels.

Five mice from each subgroup were monitored for ten days, time of death was noted and survival percentage was assessed. Dead mice were dissected to confirm that death was due to the tumor.

2.5 Determination of serum cytokine levels by ELISA

The single analyte ELISArray kits for IL-10 and TGF-β (QIAGEN Sciences, Maryland 20874, USA) and VEGF mouse ELISA kit (Abcam, ab100751, USA) were used to determine the serum levels of IL-10, TGF-β and VEGF respectively. Procedures were performed according to the manufacturer's protocol.

2.6 Statistical analysis

Whenever applicable, data were expressed as Mean ± SD. Mice survival was evaluated by generating kaplan–meier survival curves. P-values <0.05 were considered statistically significant.

3. RESULTS

3.1 IL-10 serum levels

When compared to the control RPMI-treated group, a remarkable decrease in serum IL-10 levels was observed at all time intervals in mice that received one injection of MBT, LPS or DS post- tumor challenge by day 7 **(Table 2)**. Likewise, groups of mice given three injection treatments **(Table 2)**, showed lower serum IL-10 levels compared to those in RPMI-treated control, when tested after the third injection on day 11.

Table 2: IL-10 serum levels and their corresponding standard deviations, of different groups of mice, at 2, 4 and 6 hours after receiving a single injection on day 7 or triple treatments by day 11

IL- 10	Single Treatment (pg/ml)				Triple treatment (pg/ml)			
	RPMI-1640	MBT	LPS	DS	RPMI-1640	MBT	LPS	DS
2 hours	1114 ±0	268.4 ±17.3	637.4 ±3.5	318.5 ±39.7	2020.7 ±0	300.2 ±0	248.9 ±10.4	280.6 ±58.8
4 hours	686.3 ±0	261.1 ±0	333.2 ±1.7	269.6 ±36.3	1118.9 ±0	588.6 ±0	303.8 ±3.5	329.5 ±55.3
6 hours	1786.1 ±0	222 ±6.9	235.4 ±8.6	318.5 ±43.2	1131.1 ±0	289.2 ±22.5	394.3 ±67.4	285.5 ±27.6

MBT = Mixed Bacterial Toxin; LPS = Lipopolysaccharide; DS = Dialyzed Supernatant

3.2 TGF-β serum levels

TGF-β serum levels were measured for mice that received the treatment only once on day 7 post tumor challenge and were compared to control RPMI-treated group **(Table 3)** MBT treatment resulted in a significant decrease in TGF-β serum levels at 2, 4 and 6 hours post injection. LPS decreased their levels at 4 and 6 hours post injection, and treatment with DS decreased TGF-β levels at 2 and 4 hours. Yet a considerable increase was observed at 6 hours post injection with DS treatment.

Table 3: TGF-β serum levels and their corresponding standard deviations, of different groups of mice, at 2, 4 and 6 hours after receiving a single injection on day 7 or triple treatments by day 11

TGF-β	Single Treatment (pg/ml)				Triple treatment (pg/ml)			
	RPMI-1640	MBT	LPS	DS	RPMI-1640	MBT	LPS	DS
2 hours	774.6 ±56.8	535.2 ±16.6	752 ±17.8	664.1 ±30.8	438.1 ±35.5	360.3 ±22.5	323.5 ±53.3	323.5 ±24.9
4 hours	690.9 ±0	479.1 ±20.1	572.9 ±8.3	516 ±185.8	292.5 ±26	281.6 ±15.4	267.4 ±18.9	152.8 ±13
6 hours	766.2 ±52.1	555.3 ±9.5	500.1 ±7.1	1181.3 ±7.1	418.1 ±73.4	379.6 ±11.8	246.5 ±46.2	376.2 ±21.3

MBT = Mixed Bacterial Toxin; LPS = Lipopolysaccharide; DS = Dialyzed Supernatant

By day 11, when TGF-B serum levels were measured at 2, 4 and 6 hours post third injection-treatment, a decrease in TGF-β serum levels was observed in all the groups of mice at different time intervals when compared to control RPMI-treated group **(Table 3)**.

3.3 VEGF serum levels

Serum levels of VEGF in mice receiving one time treatment on day 7 post-tumor challenge **(Table 4),** compared to those in RPMI-treated control radically increased at 2, 4 and 6 hours post-injection, with MBT and LPS treatment. DS treatment also caused an increase in TGF-β serum levels at 2 and 6 hours post-injection, yet at 4 hours post-injection levels drastically decreased.

By day 11, when compared to control RPMI-treated group, triple treatment of mice with MBT or LPS post-tumor challenge increased VEGF levels at 2 and 6 hours post- third injection. However both treatments with MBT and LPS caused a decrease in VEGF serum levels at 4 hours post-third injection. Finally, treatment with DS decreased VEGF levels at 2, 4 and 6 hours post- third injection **(Table 4).**

Table 4: VEGF serum levels and their corresponding standard deviations, of different groups of mice, at 2, 4 and 6 hours after receiving a single injection on day 7 or triple treatments by day 11

VEGF	Single Treatment (pg/ml)				Triple treatment (pg/ml)			
	RPMI-1640	MBT	LPS	DS	RPMI-1640	MBT	LPS	DS
2 hours	195.6 ±11.5	580.4 ±19	557.4 ±13.6	476.9 ±20.2	1052.5 ±0	1196.8 ±23.9	1275 ±31.3	397 ±23.5
4 hours	343.4 ±8.7	483.6 ±0	503.5 ±9.1	158.8 ±10.7	1263.6 ±54.8	1162.1 ±40	1003.5 ±34.2	1232.7 ±50.7
6 hours	158.8 ±1.6	439 ±3.7	431.4 ±11.1	635.3 ±13.2	504.6 ±46.2	591.2 ±11.1	1107.9 ±32.6	316.6 ±21

MBT = Mixed Bacterial Toxin; LPS = Lipopolysaccharide; DS = Dialyzed Supernatant

3.4 Mice survival rates

Five mice from each group were not sacrificed and were left for assessment of survival rates for 10 days after administration of the treatments. Single treatment administration produced low survival rate **"Fig.1".** Only Mice receiving a single shot of MBT had a survival rate of

20% by day 10. None of the mice in the control RPMI-treated group survived beyond the 8th day. Both LPS and DS treated groups had no surviving mice by day 10.

Triple injections of either treatment produced a higher survival rate as compared to the control RPMI treated group **"Fig.2".** Mice treated with MBT or LPS had a 20% survival rate by day 10. Triple treatment with and DS increased mice survival rate to 40%.

The survival results were further evaluated by generating the Kaplan meier survival curves, showing the probability of survival in a given period of time "**Fig. 3 and Fig. 4**". The p-values were calculated to assess the statistical significance of the results obtained. P-values ≤ 0.05 were considered statistically significant (**Table 5**). In group I, given one injection of treatment, the survival results obtained from MBT, LPS and DS-treated subgroups, as compared to the RPMI-treated control, showed no statistical significance. However, in group II given three injections of treatments, all the subgroups, mainly the DS-treated subgroup, showed statistically significant survival results when compared to the RPMI-treated control.

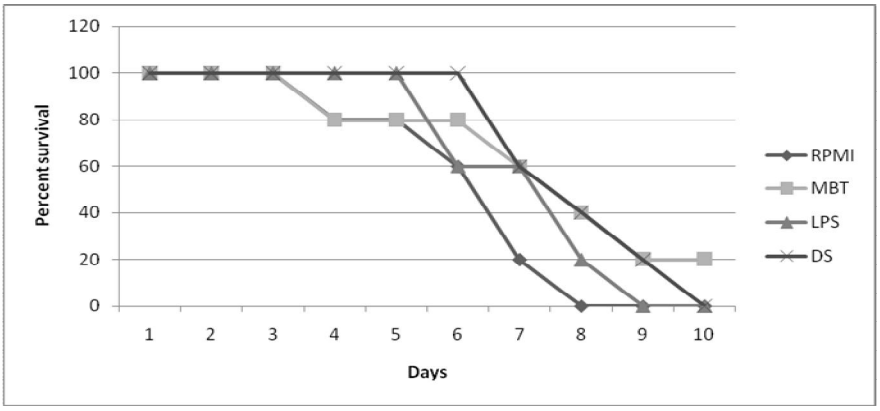

Figure 1: Survival curve for Group I, after one injection of treatment.

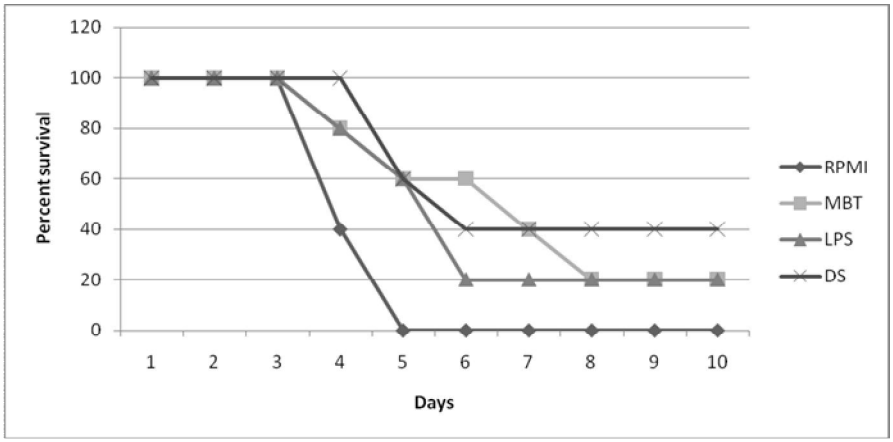

Figure 2: Survival curve for Group II, after three injection treatment.

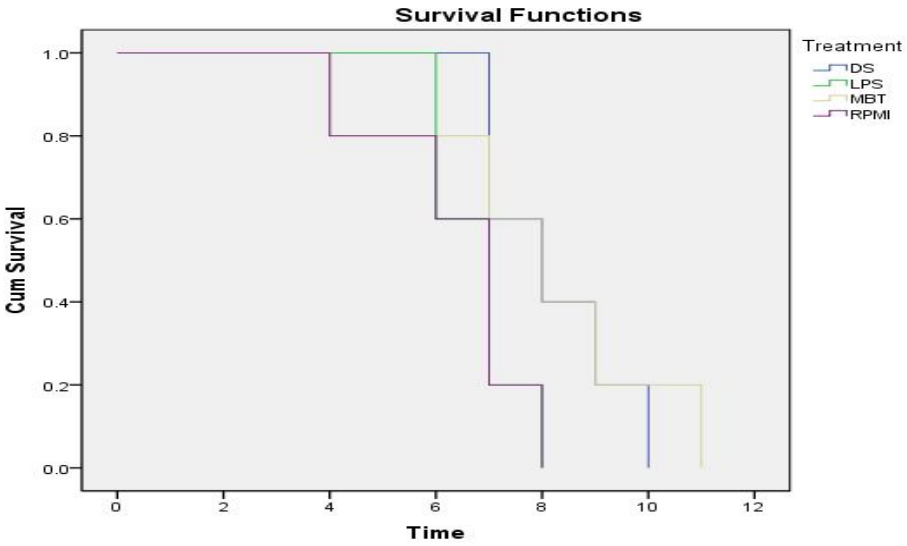

Figure 3: Kaplan–Meier survival curve of the mice treated with one injection of either MBT, LPS or DS. Control RPMI group had a no survival probability beyond day 8 after tumor challenge. Mice survival rate was prolonged when treated with one injection of MBT post tumor challenge.

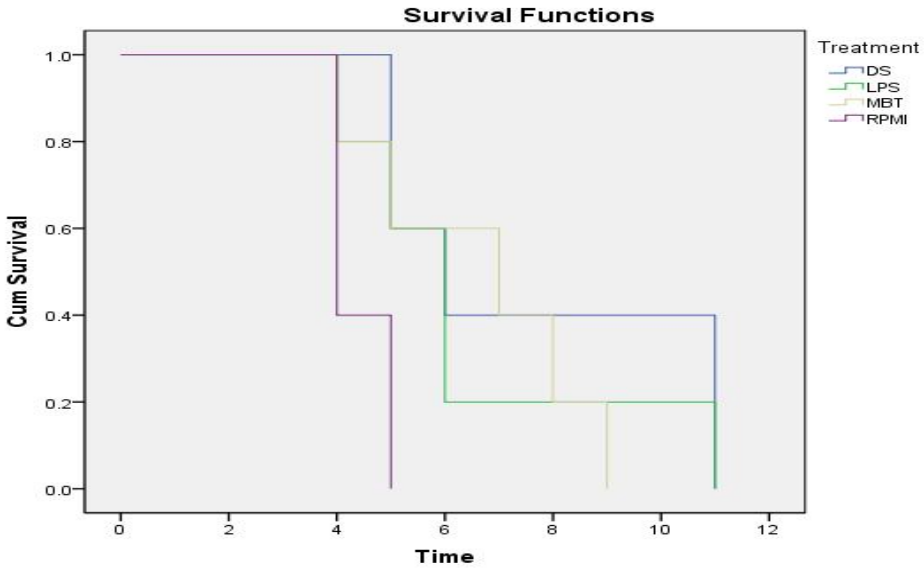

Figure 4: Kaplan–Meier survival curve of the mice receiving triple injections of either treatment, MBT, LPS or DS. Control RPMI group had a no survival probability beyond day 5 after tumor challenge. Mice survival rate was prolonged when treated with one injection of MBT or LPS post tumor challenge. Mice treated with DS had the highest probability of survuval by day 10.

Table 5: Significance of mice survival rates as compared to control groups, shown as P values after the generation of the Kaplan meier curve.

	P -Value		P-Value
Single treatment	**RPMI-1640**	**Triple treatment**	**RPMI-1640**
MBT	0.152	**MBT**	0.053*
LPS	0.879	**LPS**	0.053*
DS	0.7	**DS**	0.018*

* Significant at p-value ≤ 0.05

MBT = Mixed Bacterial Toxin; LPS = Lipopolysaccharide; DS = Dialyzed Supernatant

4. DISCUSSION

Influenced by several cases of tumor regression observed after encountering an infection, Coley developed a preparation comprising *S. pyogenes* and *S. marcescens*. For several years, Coley's preparation, referred to as Coley's Mixed Bacterial Toxin (MBT,) was used to treat cancer and a number of successes was reported. [3,4]

In an attempt to describe the mechanism of action and the location of the active component(s) responsible for the anti-tumor activity observed, a number of studies were performed, one of which was conducted at the Department of Experimental Pathology, Immunology and Microbiology at the American University of Beirut. In this study, the activities of MBT, dialyzed supernatant (DS), heated DS and LPS were assessed by testing their effect on the serum levels of the pro-inflammatory cytokines, Interleukin-12 (IL-12) and Tumor Necrosis Factor-α (TNF-α), and the angiogenic protein VEGF. This study revealed that most of the activity of Coley's MBT was present in its DS. Both heated and non-heated MBT and DS increased TNF-α and IL-12 serum levels. Additionally, LPS increased TNF-α and IL-12 levels and decreased VEGF levels. It was suggested that LPS could be one of the active ingredients of MBT.[31] However, this study did not assess the effect of Coley's MBT and its ingredients in a cancer model. Therefore, the current study was carried out, in an effort to determine the effect of MBT, its DS and LPS on tumor growth and on the serum levels of the immunosuppressive cytokines, IL-10 and TGF-β, and the pro-angiogenic cytokine VEGF in mice. Earlier, it was determined that the DS contained both LPS and protein.[31] Streptokinase had been proposed to be one of the active components of MBT. [12, 13] However, the heated DS in which the proteins were denatured was as active as unheated DS.

Based on these results, the effect of LPS, a heat stable component of the cell wall of Gram negative bacteria (*S. marcescens*) was assessed along with that of MBT and DS. The dose of LPS used was equivalent to the amount present in the DS. It is worth noting that the LPS used was extracted from *S. minnesota* because of its availability. However, LPS from *S. marcsecens* and *S. minnesota* have similar biological properties (Abdelnoor, personal communication).

IL-10 and TGF-β are known to be immunosuppressive cytokines and have been shown to be highly expressed and produced in different types of cancer such as, human gastric carcinoma, human Papilloma virus-Transformed Cervical Cancer and human gliomas.[32-34] Therefore, the efficacy of a certain treatment was thought to be associated with the decrease in the levels of these cytokines. When tumor-bearing mice were treated with one dose of MBT, there appeared to be an association between a decrease in the serum levels of IL-10 and TGF-β and 20% survival of mice. Although both cytokine levels decreased in tumor-bearing mice treated with one dose of either DS or LPS, an association with the survival rates did not exist. On the other hand, when multiple doses of treatment were used, an association existed between decreased levels and survivals in all types of treatment, in particular DS. This probably could be explained in terms of specific activity whereby the active ingredient(s) are used in higher doses. It can be noted that TGF-β levels increased rather than decreased at 6 hours in mice given one injection of DS. A possible explanation might be that this is a case of what is known as the oscillating effect of a biological response after drug administration. [35] However, after three injections treatment a substantial decrease in the levels of TGF-β was induced by the DS.

Angiogenesis is vital for tumor growth and metastasis. Consequently, tumors produce large amounts of the pro-angiogenic protein VEGF which mediates the development of a vascular network, which in turn provides the tumor with the blood needed for survival and dissemination. Therefore, targeting the production of VEGF is a crucial step towards an effective cancer treatment. [30] The anti-angiogenic (anti-VEGF) activity of MBT appeared to be present in the DS. After one injection of MBT, LPS and DS the levels of VEGF increased at 2 and 6 hours post-injection, however a significant decrease was induced by DS at 4 hours post-injection. Interestingly, after three injections treatment, the DS induced a substantial decrease in the levels of VEGF especially at 2 and 6 hours post-injection. These results were consistent with those obtained by Al Akl et al. [31] showing that the DS, but not MBT,

suppressed the production of VEGF. Probably MBT contains inhibitors of VEGF suppressors.

5. CONCLUSION

In conclusion, as shown by the ELISA results and the survival percentages, it appears that the DS contains some of the active agent(s) responsible for the anti-tumor activity exerted by MBT and that LPS could be one of the active components found in the DS. Moreover, it appears that better results were obtained when multiple dose treatment was used. Therefore, improved protective and therapeutic results probably could be achieved by increasing the number of multiple doses over a longer length of time

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