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RESEARCH ARTICLE

THE EFFECT OF GENTAMICIN ON THE GROWTH OF B16F10 MELANOMA CELLS IN C57BL/6 MICE

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ABSTRACT

The anti-tumor effect of bacteria/bacterial products have been observed and reported for over a hundred years. Yet, the increased use of antibacterial agents might alter the normal microbial flora, resulting in the eradication of microorganisms that might be controlling tumor growth and /or affect levels of tumor promoting factors. In this study, the effect of gentamicin on the survival of C57BL/6 mice bearing the B16F10 melanoma cells was assessed and the serum levels of Nitric Oxide (NO) and Vascular Endothelial Growth Factor (VEGF) were determined. Groups of C57BL/6 mice were challenged with the B16F10 melanoma cells and were given a daily dose of pyrogen-free saline (control) or Gentamicin administered intraperitoneally (IP) for a period of 10 days. At day 10 post-treatment, mice from each group were bled; the serum was collected and used to determine NO levels using the Griess reagent system and VEGF levels using the enzyme-linked immunosorbent assay (ELISA). Remaining mice in each group were monitored for ten days for survival rate assessment. After receiving gentamicin intraperitoneally (IP), the serum levels of NO significantly decreased 1 hour after the last dose of gentamicin then increased 3 hours after the last dose of gentamicin was given. The levels of VEGF significantly increased after IP doses of gentamicin. In the saline control group, 66.66% of the tumor-bearing mice survived, yet none of the mice in the group treated with IP gentamicin survived (0% survival). Gentamicin appears to promote growth of the B16F10 tumor in mice. Probably because it modifies the normal microbial flora, of which some members are involved in preventing the establishment of the tumor, and/or its effect on the serum levels of NO and VEGF.

Key words: B16F10 melanoma cells, Gentamicin, Nitric Oxide, VEGF

INTRODUCTION:

Spontaneous tumor regression following bacterial infections has been observed and reported around a hundred years ago. The German physicians, W. Busch and F. Fehleisen, reported that *Streptococcus pyogenes*, the causative agent of erysipelas, caused tumor regression in several cancer patients [1, 2]. Influenced by these observations and moved by the death of his first sarcoma patient, the American surgeon W. B. Coley in1891 started the first systematic study of immunotherapy and injected his patients with bacteria in order to stimulate the immune system to induce an anti-tumor response. Later, Coley developed a preparation containing the extracts of killed Gram-positive *Streptococcus pyogenes* and killed Gram-negative *Serratia marcescens,* known as "Coley's Toxins or Coley's Mixed Bacterial Toxin (MBT)". This preparation was used to treat cancer patients and a number of successes had been reported [3-6].

Additional evidence supporting the anti-tumor effect of bacteria was the use of the Bacillus Calmette-Guerin (BCG) vaccine in the treatment of bladder cancer. BCG, a live attenuated form of *Mycobacterium bovis*, was first developed as a vaccine against childhood Tuberculosis (TB) in 1921. Yet, in the early 1970s, BCG was found to cause regression of a number of solid tumors. These anti-tumor effects were later described by Morales et al. in 1976 [7]. Nowadays, BCG is the only accepted bacterial treatment in use and it is considered the most effective treatment for high grade non-muscle-invasive transitional cell carcinoma of the bladder and carcinoma in situ. Its mechanism of action is yet to be determined, however, BCG is thought to induce inflammatory (innate) and adaptive anti-tumor immune responses [8-10]. Furthermore, it has been shown that certain bacterial products, such as bacterial lipopolysaccharide (LPS), can exhibit anti-tumor effects probably by exerting its potent inflammatory effect. LPS is a ligand for Toll-Like Receptor-4 (TLR-4). When it engages its receptor expressed on a number of cell types, including macrophages and dendritic cells, intracellular signals are transmitted that eventually lead to production of pro-inflammatory

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cytokines and nitric oxide by these different cell types [11-13].

NO is a free radical gas synthesized via a reaction that converts L-arginine into L-citrulline and is catalyzed by a family of isoenzymes known as Nitric Oxide Synthase (NOS) [14]. NO is known to act as a crucial cell signaling molecule in numerous physiological and pathological processes. Under physiological conditions, NO is synthesized by the constitutive NOS, endothelial and neuronal NOS (eNOS and nNOS), and acts as a vasodilator and a neurotransmitter. However, in the presence of inflammatory factors such as bacterial LPS, NO is produced by the inducible NOS (iNOS) and is implicated in several immunological functions [15-17]. NO was first reported to exhibit anti-tumor effects by Hibbs et al. [18]. Later, a number of reports indicated that NO was involved in defending the body against tumors [19-22]. Interestingly, Mei et al [23] reported that NO derived from endothelial nitric oxide synthase inhibited tumor growth by regulating angiogenesis, which is a critical process in tumor progression and metastasis and is mainly promoted and regulated by the Vascular Endothelial Growth Factor (VEGF) [24, 25].

Treatment with antimicrobial agents eradicates infectious agents and might modify the normal microbial flora. However, in doing so microorganisms that might be controlling tumor growth would be eradicated [26-28] Based on reports indicating that therapeutic use of antimicrobial agents would promote growth of tumors [28-31], the current study was carried out in an effort to determine the effect of gentamicin therapy on tumor growth in mice, by comparing survival rates of B16F10 melanoma-bearing mice treated with gentamicin with those of control (saline-treated) B16F10 melanoma-bearing mice. Moreover, the effect of gentamicin therapy on the serum levels of two factors, NO and VEGF, both of which affect the growth of tumors, were determined.

MATERIALS AND METHODS:

1) Preparation of B16F10 melanoma cells

The tumor cells used were the B16F10 melanoma cells, which are syngeneic with the C57BL/6 mice. These adherent cells were maintained *in vitro* as monolayers 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% CO2 incubator (Thermo scientific, Forma, series II water jacket, CO2 incubator). When needed for administration into mice, the cells were detached using trypsin (2.5% trypsin in 10x in HBSS without calcium or magnesium, Lonza, B-4800 Verviers, Belgium), counted and then re-suspended in RPMI-1640.Trypan blue was used to determine viability. The amount injected IP was 10^5 B16F10 melanoma cells/mouse.

2) 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. Twenty four C57BL/6 female mice, 6 to 8 weeks old, were injected IP with the melanoma cells at day 0 and divided into two groups (twelve mice in each). The injection protocol is given in **Table1**. The control group (Group 1) was given pyrogen-free saline IP (0.5mL/mouse), while the other group (Group 2) was given gentamicin IP (60 µg/0.5mL saline/mouse), daily for a period of ten days.

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

	Group 1	Group 2
Day 0	Challenge with B16F10 melanoma cells (10^5 B16F10 melanoma cells/mouse in 0.4ml of RPMI-1640)	
Day 1	Pyrogen-free saline*	Gentamicin*
Day 2	Pyrogen-free saline*	Gentamicin*
Day 3	Pyrogen-free saline*	Gentamicin*
Day 4	Pyrogen-free saline*	Gentamicin*
Day 5	Pyrogen-free saline*	Gentamicin*
Day 6	Pyrogen-free saline*	Gentamicin*
Day 7	Pyrogen-free saline*	Gentamicin*
Day 8	Pyrogen-free saline*	Gentamicin*
Day 9	Pyrogen-free saline*	Gentamicin*
Day 10	Pyrogen-free saline*	Gentamicin*

Injections/mouse: Saline (0.5mL/mouse), Gentamicin (60 µg/0.5mL saline/mouse). *: Intraperitoneal injection (IP)

3) Procurement of specimens

At 1 and 3 hours after the last gentamicin dose (day 10) , three mice from each group were anesthetized each with a 0.5 mL mixture of 0.12 mL ketamine (50 mg/mL), 0.03 mL xylazine (20 mg/mL), and 0.35 mL pyrogen-free saline. Blood from each group was collected and pooled; serum was separated and used for NO and VEGF quantification.

The remaining mice from each group (6 mice/group) were monitored for ten days to determine the rate of tumor growth and number of survivals. Upon death, the mice were dissected to confirm that death was caused by the tumor.

4) Nitric Oxide (NO) quantification

Griess Reagent system (Fluka Nitrate/Nitrite assay kit, Sigma-Aldrich, USA) was used to measure the levels of NO in the mice sera. The procedure provided by the manufacturer and described by Barsoumian et al. (32) was followed.

5) Vascular Endothelial Growth Factor (VEGF) quantification

VEGF mouse ELISA kit (Abcam, ab100751, USA) was used to determine the levels of Vascular Endothelium Growth Factor (VEGF) in the mice sera. The procedure was performed according to the manufacturer's protocol.

6) Statistical analysis

Whenever applicable, data was expressed as Mean ± SD. Mice survival was evaluated by generating Kaplan–Meier survival curves. Statistical analysis was performed using the PASW statistics 18 for windows; P-values ≤0.05 were considered statistically significant.

RESULTS:

1. NO serum levels

When compared to the control saline-treated group, the serum levels of NO significantly decreased at 1 hour after the last gentamicin dose (p-value=0.0008), then increased at 3 hours after the last gentamicin dose (p-value=0.0128), in the group that received a daily IP dose of gentamicin (Figure 1).

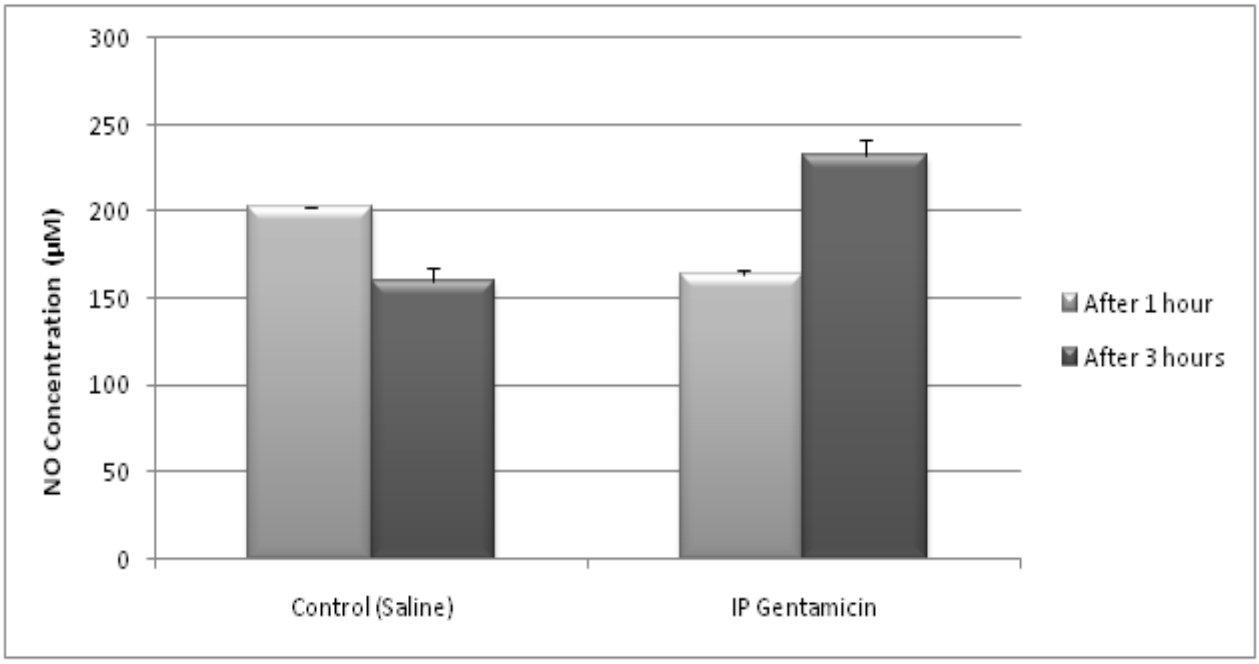

Figure 1: Concentrations of NO (µM) obtained by the Griess Reagent System.

The NO serum levels were measured 1 and 3 hours after the last dose of gentamicin. The results are presented as the mean ± S.D. *: statistically significant at p-value < 0.05 as compared to the control group. (S.D: standard deviation).

2. VEGF serum levels

When compared to the control saline-treated group, the serum levels of VEGF significantly increased at 1 and 3 hours after the last gentamicin dose (p-values= 0.0041 and 0.0063 respectively), in the group that received a daily IP dose of gentamicin (Figure 2).

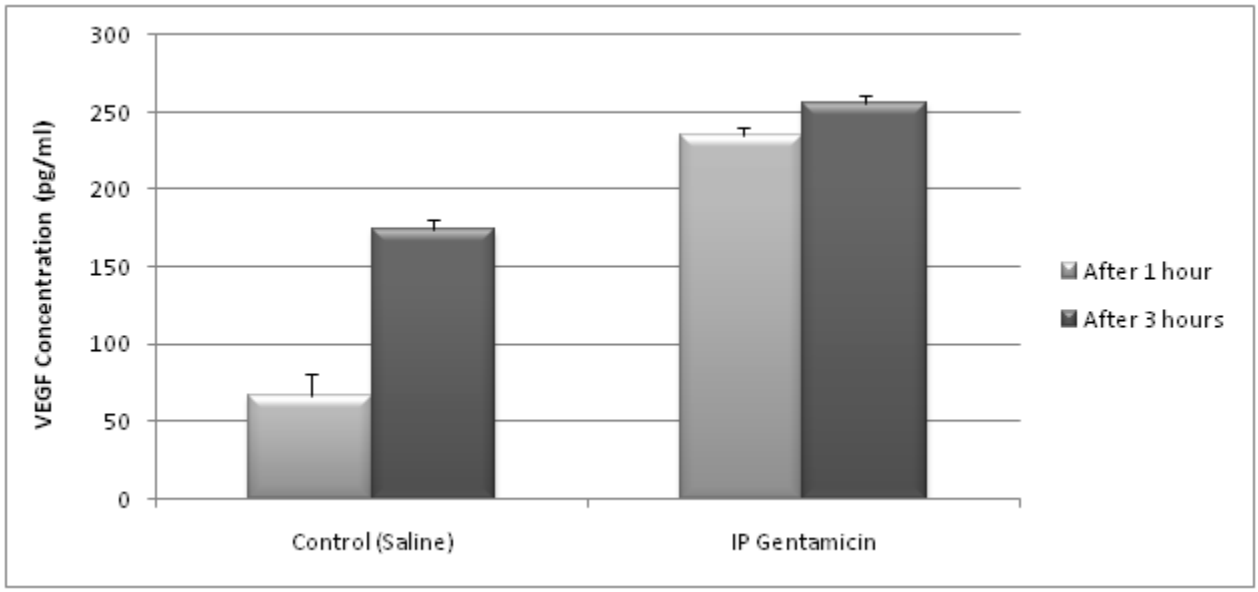

Figure 2: Concentrations of VEGF (pg/ml) obtained by ELISA.

The VEGF serum levels were measured 1 and 3 hours after the last dose of gentamicin. The results are presented as the mean ± S.D. *: statistically significant at p-value < 0.05 as compared to the control group. (S.D: standard deviation).

3. Mice survivals

By day 10, 2 of 6 mice (66.7% survived) in the control group that received saline were dead. However, none of the mice that received daily IP injections of gentamicin survived beyond day 8 (0% survival) (Figure 3).The survival results were further evaluated by generating the Kaplan-Meier survival curves showing the probability of survival in a given period of time (Figure 4).

The statistical significance of the results obtained was assessed by determining the p-values; P-values ≤ 0.05 were considered statistically significant. The group receiving daily IP injection of gentamicin showed statistically significant survival results when compared to the saline-treated control (p- value=0.032) (Table 2).

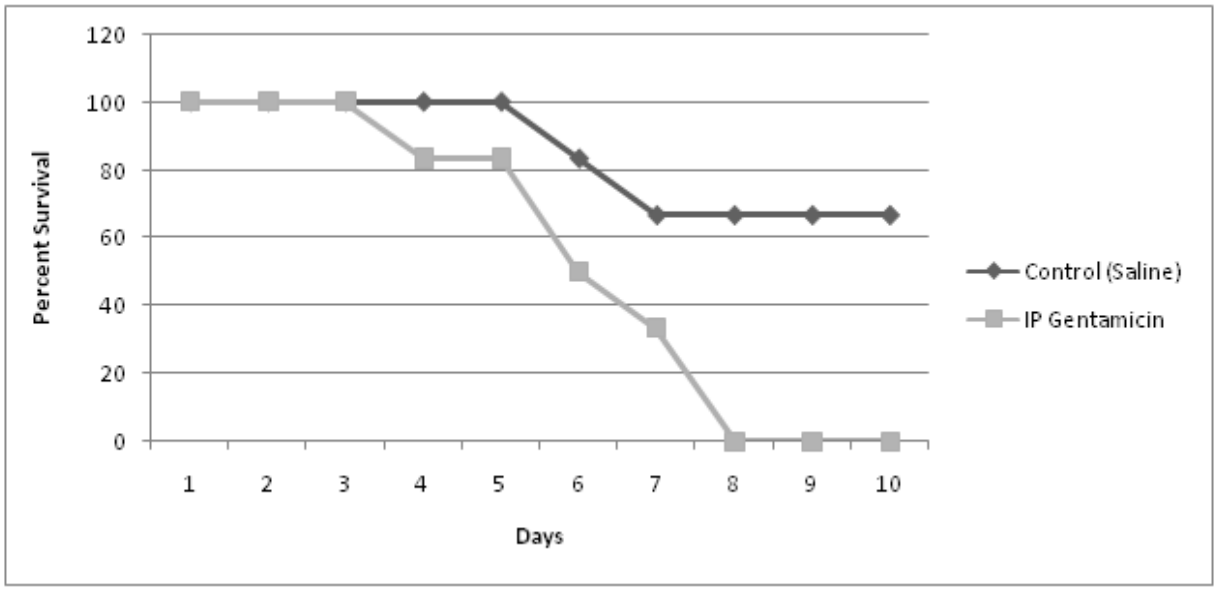

Figure 3: Survival curve for Groups 1 and 2 after ten days monitoring.

Group 1: mice receiving IP injection of saline. Group 2: mice receiving IP injection of gentamicin.

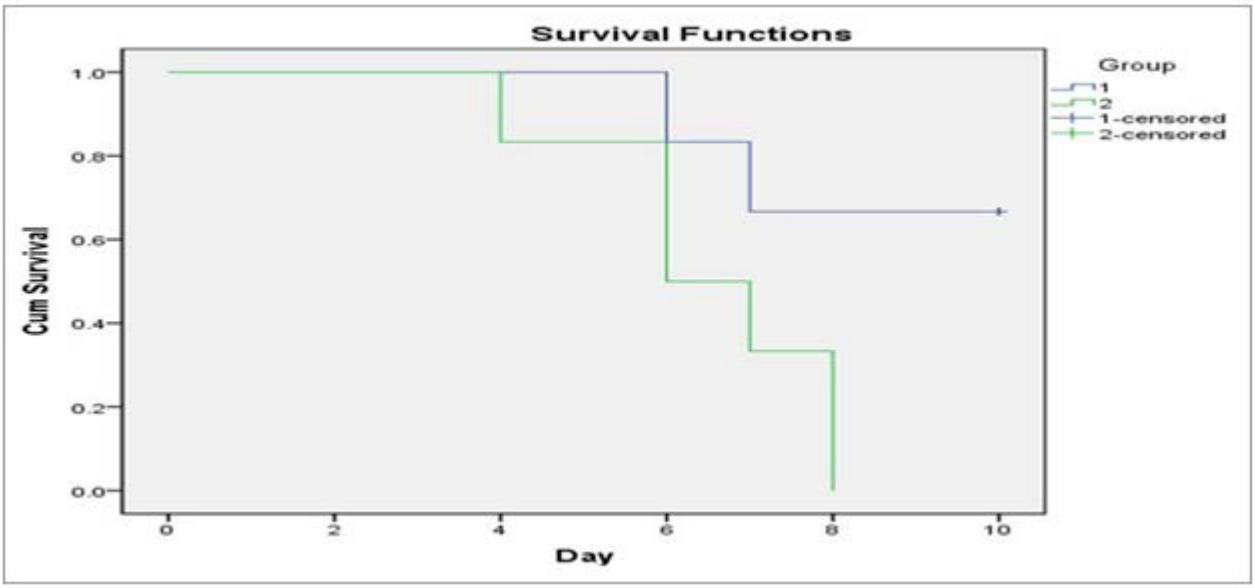

Figure 4: Kaplan-Meier survival curve, for Group 1 and Group 2.

Group 1: mice receiving IP injection of saline. Group 2: mice receiving IP injection of gentamicin.

Table 2: Significance of mice survival rates as compared to control group.

Gentamicin Treatment	p-Value (compared to control group)
IP Gentamicin (Group 2)	0.032*

IP: Intraperitoneally. The significance of the survival results are shown as p-values after the generation of the Kaplan Meier curves. *: Statistically significant at p-value ≤ 0.05.

DISCUSSION:

Evidence supporting the anti-tumor effects of bacteria/bacterial products influenced the development of several bacteria-based cancer treatments, including Coley's Mixed Bacterial Toxin (MBT) and Bacillus Calmette-Guerin (BCG) vaccine [5-8]. Nevertheless, for over two decades, the extensive use of antimicrobial agents has been proposed as a risk factor for cancer. Biological mechanisms have not been clearly established but it has been suggested that antimicrobial agents disrupt the normal microbial flora and eradicate infectious agents that might be controlling tumor growth [26]. Results of two studies done in our laboratory indicated that a number of antimicrobial agents, including gentamicin, significantly decreased the physiological as well as the LPS-induced levels of NO in tumor-free mice [32, 33]. Owing to controversies regarding the role of NO in cancer development, we assessed the effect of gentamicin administration on NO levels in B16F10 melanoma–bearing mice. Moreover, VEGF levels were assessed and the survival of mice was monitored.

The serum levels of NO significantly decreased 1 hour after the last IP injection of gentamicin (18.71% decrease). On the other hand, three hours after the last gentamicin dose, an increase in the level of NO was noted (46.1% increase). The change in serum NO level between the 1 and 3 hour specimens might be explained by the fact that the mean half-life of gentamicin in mouse serum has been estimated to be 1 hour [34]. Earlier it had been reported that treatment of non-tumor bearing mice with gentamicin resulted in a decrease in NO levels at 1 and less so at 3 hour post-treatment [32, 33]. The apparent discrepancy might be attributed to the fact that the current study dealt with tumor-bearing and not normal mice. Nitric Oxide Synthases are extensively expressed in tumor tissues resulting in the production of higher levels of NO [23, 35].

Tumor survival relies heavily on a proper blood supply. A major means by which tumors get their blood is through the production of new blood vessels (angiogenesis) and VEGF promotes angiogenesis. There was a significant increase in VEGF levels at 1 and 3 hour specimens in the IP treated groups.

The role of NO in tumor angiogenesis is not fully understood. However, it has been proposed that the effect of NO on Tumorigenesis is dose-dependent; NO exhibit inhibitory effects when produced in high concentrations, whereas low concentrations of NO seem to promote tumor angiogenesis [23, 36]. In the current study, NO seemed to exhibit inhibitory effects since after IP gentamicin administration, a decrease in NO levels was followed by an increase in VEGF levels, and a less prominent increase in VEGF levels was reported once NO levels increased.

In respect to mouse survivals with respect to gentamicin-induced increased VEGF levels, it appears that increases promoted tumor growth. This result is expected since angiogenesis is needed for tumor survival.

CONCLUSION:

It can be concluded that gentamicin appears to promote growth of the B16F10 tumor in mice. Probably because it modifies the normal microbial flora, of which some members are involved in preventing the establishment of the tumor by mechanism(s) yet to be determined. Moreover, the effect of gentamicin on NO and VEGF serum levels can also be considered in relation to promoting tumor growth.

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