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ORIGINAL ARTICLE

Synergistic effects between Staphylococcal enterotoxin type B and Monophosphoryl lipid A against mouse fibrosarcoma

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Summary

Purpose: *Staphylococcal enterotoxin B (SEB) is a potent inducer of cytotoxic T-cell activity, cytokine production and necrosis induction in vivo. Monophosphoryl lipid A (MPL) is an adjuvant derived from the lipopolysaccharide of E. coli, Salmonella Minnesota Re595 and other gram negative bacteria. We investigated the possibility of the therapeutic application of SEB+ MPL in mice with fibrosarcoma.*

Methods: *The antitumor effect of SEB+MPL, SEB and MPL in mice with inoculated fibrosarcoma tumor (WEHI-164) was examined by intravenous (i.v.) and intratumoral (i.t.) injection and the sizes of the inoculated tumors, IFN-γ production, and $CD4^+$/$CD8^+$ T cell infiltration were determined. The inoculated tumors were also examined histologically.*

Results: *In the i.v.-injected group of mice with SEB+ MPL, reduction of tumor size showed a significant difference compared with mice in the i.t., the i.v. (MPL)-injected groups and the negative control group ($p < 0.02$). Moreover, the mice in the i.v. (SEB+ MPL)-injected group showed significantly higher levels of IFN-γ ($p < 0.009$) and $CD4^+$/$CD8^+$ T cell infiltration when compared with the other groups ($p < 0.02$). A significantly higher frequency of necrosis in tumor tissues was also observed in mice in the i.v. (SEB+ MPL)-injected group in comparison with other groups ($p < 0.009$).*

Conclusion: *Our findings suggest that tumor cell death is caused by increased cytotoxic T-cell activity, cytokine levels, in response to IV injection of SEB+MPL. They also suggest that tumor cell death by synergistic effect of one of the strongest bacterial superantigens (SEB) with monophosphoryl lipid A and SEB+MPL may be a good option for use as a novel therapy in patients with fibrosarcoma.*

Key words: antitumor effect, cytokine, mouse fibrosarcoma, monophosphoryl lipid A (MPL), staphylococcal enterotoxin B (SEB)

Introduction

Staphylococcal enterotoxins, especially type B, are classical models of superantigens (SAgs) [1]. SAg is one of the most powerful T cell mitogens ever discovered; concentrations of less than 0.1 pg/ml of bacterial SAg are sufficient to stimulate T lymphocytes [2]. SEB forms complex with MHC class II molecules on antigen-presenting cells, binds to outside of the peptide-binding groove and then sequentially binds the T cell receptor (TCR) via the variable region of TCR β-chain [3,4]. SEB activates all T cells expressing a defined set of b-chain variable region (Vb)-TCR, irrespective of their actual antigen specificity. As a consequence, up to 25% of the total peripheral T cell pool becomes activated [5]. Both $CD4^+$ and $CD8^+$ T cells have been shown to proliferate in response to this SAg. In addition, this high activation of T cells 1 is accompanied by an increased production of T helper cells 1 (Th1) cytokines such as interferon-γ (IFN-γ), interleukin-2 (IL2), and tumor necrosis factor-α (TNF-α) [6]. This hyperactivation of T cells usually occurs within 48 h after SAg exposure. T cells have a crucial role in eliminating host cells that contain intercellular pathogens and those which underwent malignant transformation [7]. One of the major goals of tumor immunotherapy is

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to generate tumor-specific response that contributes to the eradication of tumor. Because fibrosarcoma often has a very poor capability to present its own antigens to T cells, it is usually resistant to natural immune system [8,9]. MPL is an adjuvant derived from the lipopolysaccharide of *E. coli*, *Salmonella Minnesota* Re595 and other Gram negative bacteria. The lipid A portion of the Gram negative endotoxin has long been known to be a potent adjuvant and T cell activator but unacceptable toxicity has limited its clinical use. Removal of a phosphate and fatty acid group from lipid A produced a molecule, MPL, that retained the adjuvant and T cell activator properties of lipid A but with significantly reduced toxicity [10,11]. The adjuvant activity of MPL investigated in murine studies with viral and bacterial antigens suggests that it promotes primarily a Th-1 response characterized by increased interferon-γ production and induction of IgG2a antibody isotype [12-16]. In human studies with adult volunteers receiving trial vaccines, MPL alone or in combination with other adjuvants has been shown to be well tolerated and to enhance both humoral and cellular immune responses [17,18].

Since MPL causes enhancement of humoral and cellular immune responses and is employed as a proper adjuvant in vaccines, leads certainly to a more sustained immune response. Contrary to other adjuvants, MPL causes enhancement of Th1, their proliferation and secretion of cytokines caused by activation of Th1 including IFN-γ, IL-2 and etc. Therefore MPL may be a qualified candidate for examination of its antitumor activity together with SEB. In this research we examined the antitumor effect related to SEB and MPL separately and in combination in lymphocytes.

This study focused on the direct (i.t. injection) and indirect (i.v. injection) antitumor effect of SEB, MPL, and SEB+MPL against mouse fibrosarcoma in *in vitro* and *in vivo* conditions.

Methods

Cell culture

WEHI-164, a BALB/c-origin methylcolanthrene-induced mouse fibrosarcoma cell line, was purchased from the Pasteur Institute (Tehran, Iran). The WEHI-164 cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), penicillin (100 U/mL) and streptomycin (100 µg/mL) (Sigma-Aldrich, Munich, Germany) and incubated at 37° C in 5% CO_2 with appropriate humidity.

Mice and tumor models

Female inbred BALB/c mice (6-7 weeks old) were purchased from the Pasteur Institute (Tehran, Iran). A single aliquot of WEHI-164 (1×10^6 cells/100 µl phosphate buffered saline/PBS) was injected subcutaneously into the right flank of mice to establish tumors [19]. Palpable tumors usually developed on day 7. Tumor diameters were measured every 48 h, and each tumor volume in mm^3 was calculated by the following formula: $V=0.5 \times D \times d^2$ (V, volume; D, longitudinal diameter; d, latitudinal diameter) [20].

Preparation of SEB and MPL

SEB and MPL were purchased from Sigma-Aldrich (Münich, Germany) and diluted with sterile PBS. Mice were divided into 2 groups: group I: intratumoral injection (i.t.) group, which contained 4 subgroups, SEB, MPL, SEB+MPL and PBS); and group II: intravenous injection (i.v.) group, which contained 4 subgroups, SEB, MPL, SEB+MPL and PBS). Each subgroup consisted of 6 mice. SEB, MPL, SEB+MPL and PBS were injected from day 10, and SEB, MPL, SEB+MPL and PBS challenge was performed every 72 h for 2 weeks. Mice were treated with 10 ng of SEB, MPL, SEB+MPL (test group), 10 ng SEB+MPL in mice without tumor (i.v. injection control group), and PBS (both control groups), respectively [21].

Flow cytometric analysis of T- lymphocytes subpopulations

Animals were killed 24h after the last treatment, and the tumors were removed and cut into small pieces, rinsed twice with PBS and minced with forceps and scalpel. The suspensions were passed through a 100-µm stainless steel mesh and, then, the cells were washed twice and labeled with monoclonal antibodies FITC-conjugated anti-CD4 and anti-CD8 (Becton Dickinson Labware, Franklin Lakes, and NJ) separately. All the stainings were performed in washing buffer consisting of PBS supplemented with 1% heat-inactivated FBS (Invitrogen, Carlsbad, CA, USA), 0.1% sodium azide (Sigma-Aldrich, Munich, Germany), and 2 mM EDTA (Sigma-Aldrich, Munich, Germany). After determining the viability of the cells by trypan blue exclusion, the cells were washed twice in washing buffer. Each sample was immunostained with anti-CD4 and anti-CD8 for 45 min at 4° C. The cells were washed in washing buffer and fixed with 2% paraformaldehyde. Flow cytometric analysis was performed using an EPICS flow cytometer (Beckman Coulter, Fullerton, CA).

The analysis was focused on the lymphoid areas of the forward and side scatters. Single stained cells were analyzed using Beckman Coulter software.

Enzyme-linked immunosorbent assay (ELISA) for measuring cytokines

Spleen from each mouse was isolated and cut into small pieces, rinsed twice with PBS and minced with forceps and scalpel. The suspensions were passed through a 100-μm stainless steel mesh to obtain a single-cell suspension, and erythrocytes were lysed at room temperature using ACK lysis buffer (NH_4Cl, $KHCO_3$, and Na_2-EDTA). The cells were washed and resuspended in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA). The cells at a concentration of 1×10^5 cells/100 μL were cultured with SEB 10 ng/well. After 3 days of incubation, the supernatants were collected and tested for cytokines (IFN-γ and IL-4) by commercially available sandwich based ELISA kits (R&D, Minneapolis, MN. USA) according to the manufacturer's instructions.

Histopathology experiments

Tumor tissues of approximately 0.5 cm ×0.5 cm in size were isolated, and transferred in automatically processing machine for 12 h, and then embedded in paraffin. Five μm-thick sections were then stained with hematoxylin and eosin (HE) for histopathological examinations. Necrosis was also determined.

TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay

Apoptosis was studied by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labelling (the TUNEL method) of fragmented DNAs using In Situ Cell Death Detection Kit AP from Roche Diagnostics GmbH (Mannheim, Germany) according to the manufacturer's recommendations. The sections were then incubated with anti-fluorescein-alkaline phosphatase (Vector Red Alkaline Phosphatase Substrate Kit, Axxora, San Diego, CA, USA) for 60 min at 37° C in a humidified chamber, rinsed in PBS, and incubated with Fast Red TR/Naphthol AS-MX (Sigma-Aldrich, Munich, Germany) for 10 minutes. Cells were counterstained with hematoxylin, mounted, and analyzed by bright-field microscopy. TUNEL-positive cells appeared red (cell shrinkage, nuclear margination, chromatin condensation, and nucleolar disintegration), whereas TUNEL-negative nuclei appeared blue. About 2000 tumor cells were counted, and the count was expressed as a percentage of "apoptotic" positive cells [22]. One negative and one positive controls were included in each experiment. Negative control was incubated without TdT, and positive control was incubated with DNase for 10 min at 15-25° C to induce DNA strand breaks.

Statistical analysis

Quadricate experiments were performed and the mean±SD was calculated. Statistical analysis was performed using the two-tailed Mann Whitney nonparametric test, and p-value of $p < 0.05$ was considered as statistically significant difference. All statistical analyses were conducted with SPSS 13.0 software (SPSS Inc., Chicago, IL, USA).

Results

Tumor size after SEB, MPL, SEB+MPL injection

SEB, MPL, SEB+MPL and PBS were challenged in the groups of mice on day 1, and changes of the tumor volumes on day 17 were observed. The summary of tumor sizes is illustrated in Figure 1. Tumors of i.v.-SEB+MPL group grew more slowly than those in the other i.v. subgroups (SEB, MPL, and PBS). Treatment of mice with the SEB+MPL significantly ($p < 0.009$) suppressed tumor growth comparing to the control (i.v.-PBS and IT-PBS), i.v.-MPL and i.t. injection groups. Results obtained in the i.t.-test groups (SEB, MPL and SEM+MPL) showed no significant difference with the control (PBS) group.

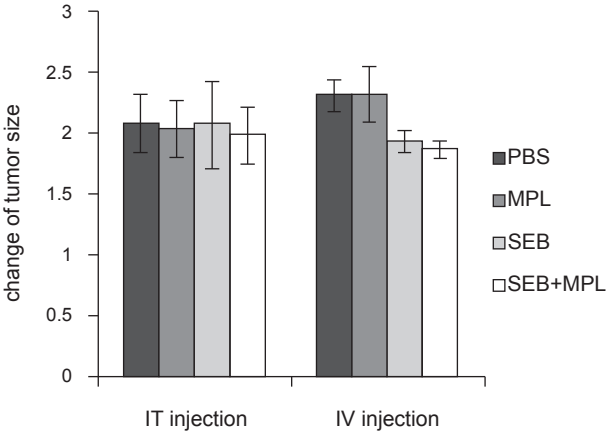

Figure 1. Change in tumor size. Treatment of the mice with the SEB+MPL significantly ($p < 0.009$) suppressed the tumor growth compared with the control (i.v.-PBS and i.t.-PBS), i.v.-MPL and i.t. injection groups.

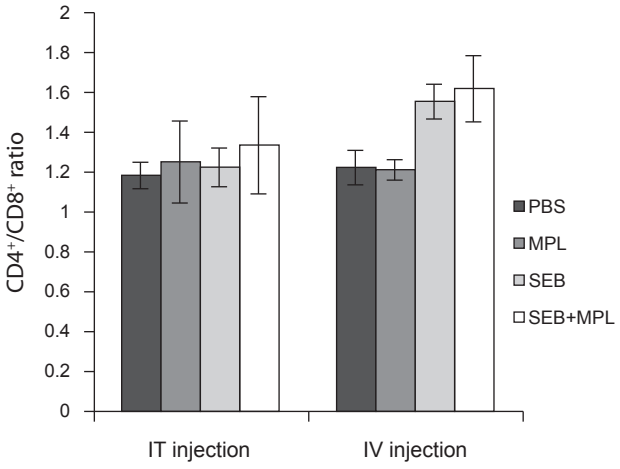

Figure 2. The $CD4^+/CD8^+$ ratio in infiltrating lymphocytes. These results indicate that there was a significant increase in the $CD4^+/CD8^+$ ratio in i.v.-SEB+MPL group ($p < 0.016$) when compared with the i.v.-PBS, i.v.-MPL and i.t. groups by Mann Whitney nonparametric test. No other significant differences were observed.

Lymphocytes subpopulation after SEB, MPL and SEB+MPL injection

In order to assess the effect of the SEB, MPL and SEB+MPL on the tumor-infiltrated lymphocytes, $CD8^+$ and $CD4^+$ subpopulations and $CD4^+/CD8^+$ ratio in tumors were measured on day 17. Results shown in Figure 2 indicate the significant increase in the $CD4^+/CD8^+$ ratio in i.v. SEB+MPL group when compared with i.v.-MPL, i.v.-PBS and i.t. groups ($p < 0.016$). No other significant differences were observed.

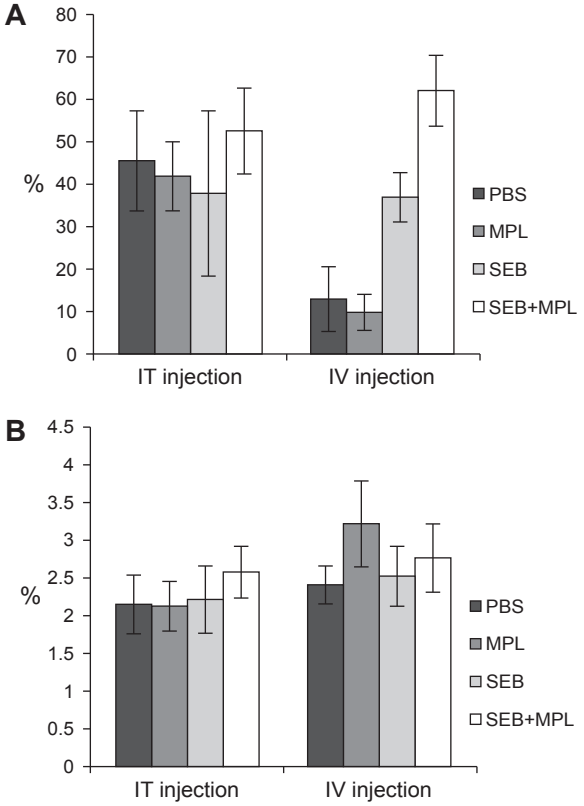

Figure 4. A: The percentage of necrotic tumor cells after treatment with SEB+MPL. These results indicate that tumor cells in mice in the i.v.-SEB+MPL group showed an increased rate of necrosis ($p < 0.001$) when compared with the control (i.v.-PBS) group by Mann Whitney nonparametric test. On the other hand, mice in the i.t.-SEB+MPL group did not show any difference ($p > 0.05$). **B:** The percentage of apoptotic tumor cells after treatment with SEB+MPL. No significant differences were observed by Mann Whitney nonparametric test ($p > 0.05$).

Level of IFN-γ and IL-4 after SEB injection

Splenocytes were challenged and their ability for production of IFN-γ and IL-4 was evaluated. Results are shown in Figure 3A, 3B; the i.v.-SEB+MPL group showed a significant very high level of IFN-γ producing ability ($p < 0.009$). On the other hand, no significant difference in the IL-4 producing ability was observed.

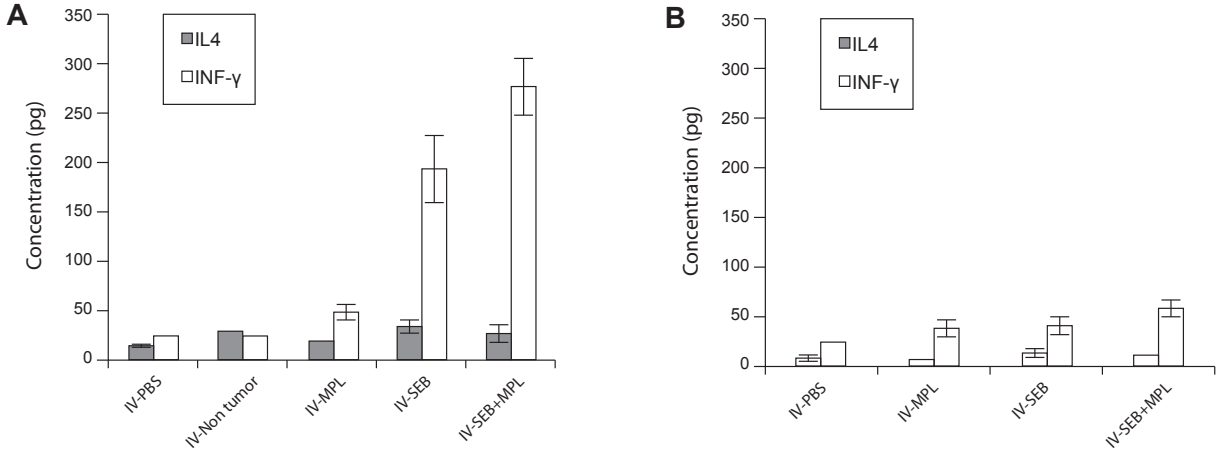

Figure 3. Production of IFN-γ and IL-4 by splenocytes in mice in the i.v.-SEB **(A)** and i.t.-SEB **(B)** groups. Splenocytes were cultured *in vitro* with SEB, MPL, SEB+MPL and PBS. The supernatant of each group was harvested after 72 h of culture, and released IFN-γ and IL-4 were measured by ELISA. These experiments were run in triplicate. i.v.-SEB+MPL group showed a significant very high level of IFN-γ producing ability ($p < 0.009$). On the other hand, no significant difference in the IL-4 producing ability was observed.

Tumor necrosis and apoptosis after SEB injection

To know the decreased tumor size in the SEB-i.v. group, we studied the incidence of necrosis and apoptosis. Tumors were collected, fixed in formalin and embedded in paraffin. Histological examinations were done using H&E stained sections, the summary of which is illustrated in Figures 4A and 5A, B. Significantly increased necrosis was evident in the i.v.-SEB+MPL group ($p < 0.001$). To elucidate the involvement of apoptosis, we further performed TUNEL assay. The summary is illustrated in Figures 4B and 5C, D. No significant difference was observed in apoptosis.

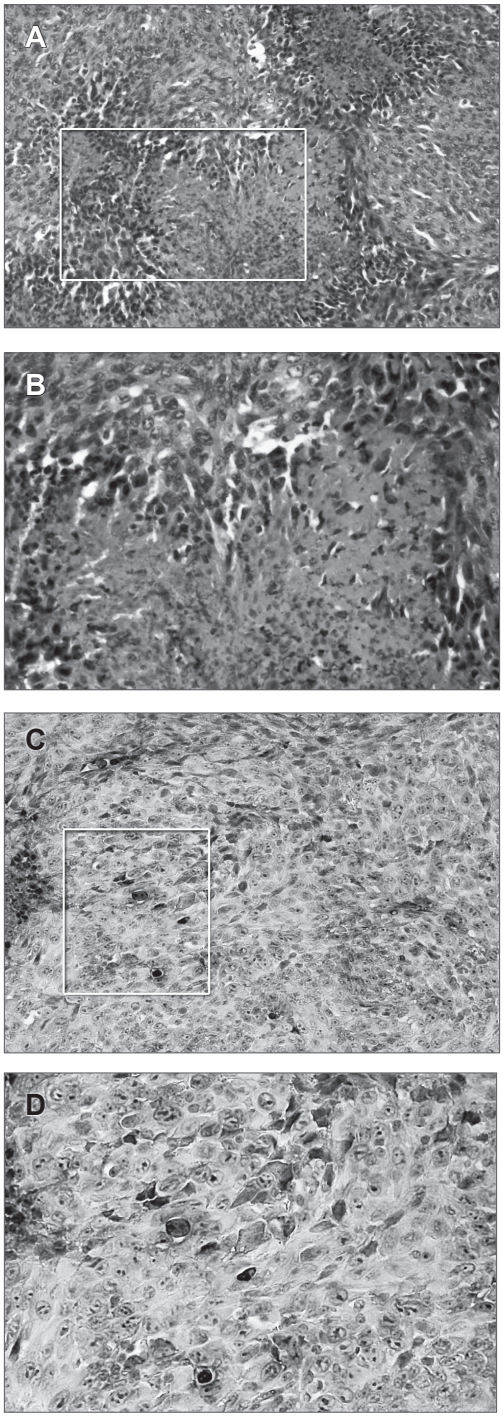

Figure 5. Results of the H&E staining in tumor tissues. **A:** 40× and **B:** 400× at the boxed region in A. **C, D:** Results of the TUNEL assay in tumor tissue; all slides are counterstained with hematoxylin. **C:** 40×, and **D** 400× at the boxed region in **C**.

Discussion

Researchers are constantly looking for innovating and better anticancer treatments. One of the most optimistic methods is the use of monoclonal antibodies. It was determined that activation of lymphocytes after treatment with monoclonal antibodies *in vivo* led to suppression of malignant tumors in mice [23]. Common treatments for cancers include surgery, chemotherapy and radiotherapy. In many cases the tumor recurs after a surgical operation due to either metastatic spread or incomplete operation. Furthermore, the above-mentioned approaches are unsafe and less efficient in preventing local relapse or metastasis. Results of many studies indicate the immune system is able to control cancer and prevent recurrence or metastasis under proper conditions [24].

Exploitation of the immune system against cancer cells is considered one of the convenient methods.

Usually, malignant tumor cells are resistant to immune system's antitumor activity, including apoptosis, necrosis etc. Therefore the use of factors such as SAgs can lead to enhancement of immune system's antitumor activity, leading to restriction of tumor growth and subsequently preventing metastasis [25].

One of the main objectives of tumor immunotherapy is the production of T-cells responsible to kill tumor cells. Tumor cells barely present their antigens to T-cells [8] and new approaches try to stimulate the antitumor response of T-cells. The SAgs, particularly bacterial and viral proteins, are able to stimulate large numbers of T-cells and monocytes with high antigenic specificity, leading to the production of considerable volume of different cytokines [26,27]. In current research, the synergistic effect of one of strongest bacterial Sags (SEB) with MPL was studied on mouse fibrosarcoma [2].

Since fibrosarcoma cells metastasize very rapidly, are more resistant to immune mechanisms and grow very rapidly compared with other types of cancer, they could be proper candidates for evaluation of the stimulation of the immune system by SEB+MPL [28].

For this reason, the current investigation studied the effect of SEB and MPL individually and cooperatively on mouse fibrosarcoma (WEHI-164) *in vivo,* administered i.t. and i.v. The obtained results from measuring the tumor size (Figure 1), measuring the cytokines IFN-γ and

IL4 (Figure 3A, 3B), counting $CD4^+$ and $CD8^+$ proliferated in tumor tissues (Figure 2) and the magnitude of tumor necrosis (Figure 4A) showed that i.t. injection in the experimental group compared with the negative control had no effect either to stop or reduce the growth of tumor. The lack of specific receptors for SEB and MPL in fibrosarcoma can be one of the main reasons for the ineffectiveness of SEB and MPL injected i.t. In view of the facts that: a) fibrosarcoma express low level of MHC-II molecules and b) normally cancer cells prevent the expression of MHC-II molecules, consequently low levels of SEB bind to MHC-II which are not sufficient for induction of antitumor activity by T-cells [9].

However, the obtained results from the i.v. groups were different from those of the i.t. groups. Tumor growth in the experimental group compared with the negative control displayed significant reduction ($p < 0.009$; Figure1). Measurement of IFN-γ and IL4 in this group showed that the amount of the produced IFN-γ compared with the produced IL4, in the i.t. and negative control groups was significantly different ($p < 0.009$; Figures 3A, B). The increase of IFN-γ compared with IL4 indicates that the activation of the immune system takes place via Th1.

Since MPL causes increased expression of MHC-II molecules on the surface of T-cells [29], it was expected that the i.v. injection of SEB+MPL compared with the individual injection of those substances would be more effective. Our measured immunologic parameters have verified this fact. The i.v. injection of SEB leads to binding them to their receptors on the surface of T-cells and causes their stimulation and proliferation; on the other hand, increased expression of these receptors leads to more intensified and sustained immune response. Tappan et al. obtained similar results by using the synergistic effect of staphylococcal enterotoxin type A (SEA) with protein A (SEA+PA) [21].

Two points for discussion emerge here**:** SEA individually, as well as SEB, is not able to stimulate the immune system and always has been used as combined factor with other components and/or antibodies [20,21]. Yet, in the current study the effects of SEB together with MPL were indicative of the fact that SEB individually and combined with MPL has better antitumoral effect than SEA+PA. Nevertheless, the tumor examined in this study is different from other investigations and so far the antitumor effect of SEB individually and combined with MPL, direct or indirect, has not been evaluated in mouse fibrosarcoma.

It has been determined that PA is effective in stimulating humoral immunity, but MPL, in addition to humoral immunity, is effective on cellular immunity too, therefore its antitumor activity is greater than PA. The ratio of the infiltrating $CD4^+$ and $CD8^+$ in tumor tissues of the i.v. group compared with the control group and the i.t. group injection was significantly increased ($p < 0.016$) (Figure 2). In addition, they were more effective in the i.v. group using SEB + MPL compared with any individual SEB and MPL. The results have shown increased level of both $CD4^+$ and $CD8^+$. The increased $CD8^+$ have an important antitumor role because they exert strong cytotoxic effect. But their effective and optimal activity is related to the activation of $CD4^+$ cells because $CD4^+$ play an important role in the conservation of memory cells and in antitumor activity functions as effective Th cells [30].

Shimizu et al. have shown that Vb of T-cells was specific receptor for SEB SAg and proliferation and antitumor activity of T-cells will be increased by binding of SEB to Vb [9]. In that study it was shown that SEB conjugate vaccine with mouse fibrosarcoma prevented the formation of fibrosarcoma [9]. In our research we used MPL and SEB alone or in combination to treat mouse fibrosarcoma and found that the combination gave better results. Although MPL has not been used for vaccine designing so far, it seems possible to use SEB+MPL conjugate vaccine in the future in order to prevent tumor development. Our results show that the magnitude of tumor necrosis in the i.v. injection group compared with the negative control group were significantly different ($p < 0.001$), and the magnitude of tumor necrosis caused by administering SEB + MPL in comparison with any individual SEB and MPL was higher; this finding goes together with the results indicating reduction of tumor size.

In the i.t. groups, despite the necrosis observed by the above-mentioned components compared with the negative control group, no significant difference was seen and necrosis could be attributed to trauma or inflammation due to the direct injection itself (Figures 4A, and 5A, B).

In 70% of cells containing Fas ligand, SEB causes apoptosis, while no apoptosis is seen in cells lacking this ligand. Probably, SEB has a slight effect in stimulating apoptosis in fibrosarcoma cells because of the following reasons: a) maybe the fibrosarcoma cells lack Fas ligand or Fas ligand concentration is not enough to induce apoptosis; and b) in addition to stimulation by SEB, Fas ligand needs a series of cellular mechanisms to express itself and these mechanisms possibly don't function well in fibrosarcoma or don't exist at all [32,33].

In this investigation it was seen that the induced apoptosis in the i.v. group was higher than the one in the i.t. group, but comparison with the negative control group showed no significant difference (Figures 4B and 5C, D). This result probably implies that fibro-

sarcoma cells possess low Fas ligand or other cellular mechanisms are involved in the expression of Fas ligand which prevent apoptosis in this tumor. On the other hand, SEB causes stimulation and proliferation of cytotoxic T-cells (CD4, CD8) that lead to production of cytotoxic cytokines including IL2, TNF-α, β, IFN-γ and etc, which play an important role in the lysis of tumor cells [34].

It seems that the level of production of these cytokines is such that causes necrosis of tumor cells rather than apoptosis and in our study it has been verified that the magnitude of necrosis in the i.v. group was greater than apoptosis and comparison with the negative control group showed significant difference. The above-mentioned results verified the reduction of growth rate and the size of tumor, the phenomenon of cellular lysis and the prevention of tumor growth by cytotoxic cytokines.

Of course it is possible that MPL and SEB may play a role in producing other necrotic factors, what requires more investigation. These experiments indicate that the use of Sags, both as i.v. injection and i.t. injection open a new perspective for the treatment of fibrosarcoma. Future researches will show whether using SEB+MPL by i.v. injection will be an effective approach in the treatment of fibrosarcoma in humans.

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