

The Effect of Intravenous Immunoglobulin (IVIG) on Fibrosarcoma Growth in CBA and C57BL/6 Mice

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Abstract

Background: The effect of IVIG in cancer treatment is likely to be mediated by immune cells that regulate tumor progression. Here we studied the effect of IVIG on tumor development in two immunologically contrasting mouse strains, CBA and C57BL/6. We compared the rate of tumor growth, the number of circulating neutrophils and oxidative characteristics of tumor tissue in IVIG-treated and control mice.

Results: The animals were inoculated with fibrosarcoma S37 cells, and 14 days after inoculation there was a significant difference between IVIG-treated and control mice: IVIG treatment inhibited tumor growth in CBA mice and stimulated it in C57BL/6. The changes in tumor growth rate were associated with biochemical alterations in tumor tissue. According to the data of biochemical tests, IVIG-treatment influenced activity of tissue myeloperoxidase (neutrophil azurophilic granule enzyme) in CBA and C57BL/6 and of some antioxidant enzymes (glutathione-S-transferase, catalase, glutathione peroxidase) in C57BL/6 mice. Neutrophils content was increased in IVIG-treated CBA mice while in C57BL/6 it remained unaltered and significantly lower than in CBA as was shown by direct count on smears.

Conclusions: These results suggest the tight link between the effects of IVIG upon neutrophils and tumor tissue redox balance as a part of regulatory mechanism of tumor growth.

Keywords: IVIG treatment; Fibrosarcoma S37; CBA and C57BL/6 mice; Tumor tissue myeloperoxidase; Glutathione-S-transferase; Glutathione peroxidase

Abbreviations: IVIG: Intravenous Immunoglobulin; OZ: Opsonized Zymosan; CL: Chemiluminescence; ROS: Reactive Oxygen Species; RBC: Red Blood Cells; GST: Glutathione-S-Transferase; CDNB: 1-chloro-2,4-dinitrobenzene; DTNB: 5,5'-dithio-bis(2-nitrobenzoic acid; GSH: Glutathione Reduced Form; ISOP: Isoprostane; GPx: Glutathione Peroxidase

Introduction

Multiple studies focus on the role of immune system in regulating of tumorigenesis [1]. Diagnostics of cancer and elaboration of efficient antitumor drugs require detailed characterization of blood cells [2,3].

Understanding of mechanisms of IVIG interaction with immune cells that regulate tumor progression determines the perspectives of IVIG application for cancer treatment [4-8].

The therapeutic effects of IVIG in cancer could result from a broad range of mechanisms among which are hindrance of nuclear factor κ B activation and I κ B degradation, suppression of tumor cell growth and of its angiogenesis, inhibition of matrix metalloproteinase-9 mRNA expression and others [6].

Among the experimental models in mice in which the anti-cancer and anti-metastatic effects of IVIG were demonstrated, there are melanoma, carcinoma, sarcoma, lymphoma [9-11].

The main aim of the present investigation was to study the IVIG action in the model of fibrosarcoma in different mouse strains.

Model systems that demonstrate intraspecies variability of immune reactions might contribute to elucidation of such mechanisms. The

impact of various response strategies on individual patient's response to treatment might be estimated with such models.

Previously we showed that neutrophils of two mouse strains (CBA and C57BL/6) developed different response to IVIG in vitro. The observed differences might result from the peculiarities of hemopoietic system, since the two strains varied in the amount of neutrophils, platelets and hemoglobin content [12].

The role of such peculiarities in tumor development and IVIG-response can be elucidated only experimentally. This work was aimed to estimate development of S37 fibrosarcoma in CBA and C57BL/6 control and IVIG-treated mice.

Materials and Methods

Reagents

K-phosphate salts, NaCl, Tris(hydroxymethyl)aminomethane,

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hydrogen peroxide, o-dianisidine, 1-chloro-2,4-dinitrobenzene (CDNB), 5,5'-dithio-bis(2-nitrobenzoic acid (DTNB), glutathione reduced form (GSH), t-butyl hydroperoxide, EDTA were purchased from Sigma-Aldrich Company, USA; NWLSSTM Isoprostane Assay kit was purchased from NWLSS (UK).

Tumor cells

The mouse fibrosarcoma S-37 tumor cell line was kindly provided by NIOPIK institute (Moscow, Russia). Cells were routinely maintained by injecting 0.5 ml of ascites fluid intraperitoneal into a male 20-22 g BALB/c (H-2d) white mice. After 7 days ascites was used as a source of cells for further injections and experimental procedures.

Experimental animal models

Male white BALB/c (H-2^d) mice, female CBA (H-2^k) and C57BL/6 (H-2^b) (18-22 g) used for this study were purchased from Russian State Medical University. The maintenance of animals and all experimental procedures were reviewed and approved by the Animal Care and Ethical Committee of Russian Medical University (Moscow, Russia).

To induce tumor growth 1×10^6 cells/0.1 ml were injected subcutaneously into the mouse thigh. The tumor volume (mm³) was calculated as $d_1 \cdot d_2 \cdot d_3 \cdot \pi / 6$, where d_1 , d_2 and d_3 - three diameters (mm) of tumor measured at the 7th and 14th day to evaluate the tumor growth parameters.

IVIg injection

Human immunoglobulins (IVIg) were obtained from Biotest (Germany).

Animals (CBA (n=36) and C57BL/6 (n=10) were divided into two groups. Both in the control and in the experimental groups the animals were injected with S-37 cells (day 0). Animals from IVIg-treated groups were injected with IVIg 24 hours after S-37 injection (day 1). IVIg solution was injected into the tail vein in the dosage of 10 mg IVIg/mouse (0.5 g/kg), and then repeatedly at the 3th and 6th days (three IVIg injections in total). The animals in the control groups were injected with sterile saline solution.

At day 7, animals were subjected to cervical dislocation; blood was taken from the right ventricle and mixed with EDTA. The tumor was dissected, weighed and measured in three dimensions. The samples were frozen at -20°C and stored at -120°C for biochemical analysis.

Blood sampling, preparation of whole blood smears, staining, total and differential WBC counting

Blood was thoroughly mixed with 3% EDTA (4:1). A 2 µL aliquot of blood was used for smear preparation. The whole blood smears were subjected to Romanowsky-Giemsa staining and analyzed with Motic 3B microscope (China).

Total WBC number was estimated with hemocytometer after 10-fold dilution with 5% acetic acid. WBC differential count was performed after analysis of 100-200 leukocytes.

Platelet counting

The number of individual platelets, aggregates and platelets in each aggregate was estimated in 15 microscopic fields in the central part of the blood smear at 40X magnification.

Biochemical analysis

Tumor tissue was incubated in 0.1 M K-phosphate buffer (pH7.4) and homogenized on ice using Potter grinder. The resulting suspension was centrifuged (900 g, 40 min, 4°C), and supernatant was taken for biochemical analysis.

Protein content was measured by Lowry method [13], activity of myeloperoxidase was estimated by o-dianisidine method [14], GST activity was measured in presence of CDNB [15], activity of catalase – by a direct reaction with hydrogen peroxide [16], activity of glutathione peroxidase was measured in presence of tert-butyl hydroperoxide [17]. The content of isoprostanes was measured with NWLSSTM Isoprostane Assay (UK).

Statistical analysis was performed using Statistica® 6.0.437.0 software (StatSoft).

Results and Discussion

The experiments were performed using two strains of mice. All animals were treated similarly, but the effect of IVIg on tumor progression was different.

At day 7 no difference between control CBA and C57BL/6 mice was observed. IVIg-treatment reduced tumor growth in CBA and increased tumor growth in C57BL/6 mice compared to control animals, and the difference between two strains was significant (Figure 1).

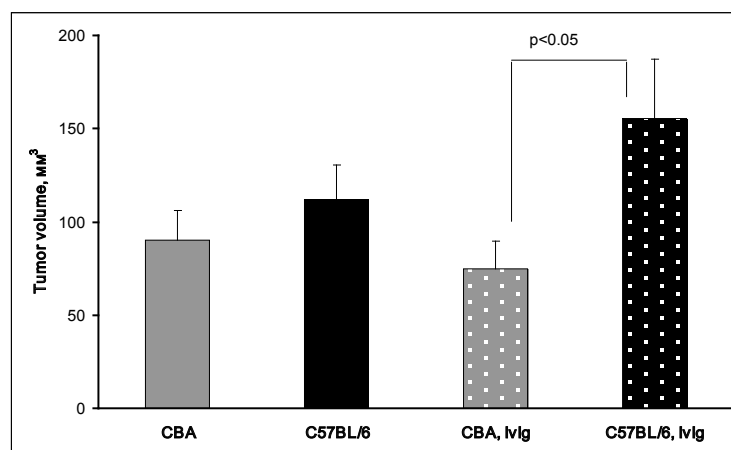


Figure 1: Tumor size (mm³) at day 7 after fibrosarcoma cells inoculation into mice of CBA (n=18/18) and C57BL/6 strains (n=10/10). Significance of differences was estimated by Mann-Whitney test. The results represent mean ± standard error of mean.

Previously we demonstrated that these two strains differed a lot in neutrophil and platelet content: CBA mice had almost 2-times more neutrophils and 1.5-times less platelets than C57BL/6.

We also noticed that morphology of platelets was different in two mice strains: platelets in CBA mice were of normal size, while C57BL/6 had enlarged platelets, and there were more aggregated platelets in C57BL/6.

Therefore, we compared leukocyte content in untreated CBA and C57BL/6 mice, as well as platelet count.

Here we compared blood cell content in IVIG-treated and control animals (Table 1). Analysis of monocytes at day 7 revealed reduction of their content in all animals compared to intact animals, no effect of IVIG and the absence of inter-strain differences.

Lymphocyte content was higher in intact mice C57BL/6 and the difference remained at day 7 (regardless IVIG treatment). Moreover, at day 7 the lymphocyte content was increased in C57BL/6 mice compared to intact animals. Overall, the correlation of lymphocyte content between groups was invert compared to neutrophils content, but less pronounced.

Dynamics of platelets was similar, although the initial content in CBA and C57BL/6 mice was different. Increased content of single platelets was observed in C57BL/6 mice compared to CBA at all experimental points. IVIG caused no significant effect.

The number of platelet aggregates was lower in intact CBA compared to C57BL/6. IVIG induced abrupt reduction of aggregates

in C57BL/6 (significant differences from intact and control animals (IVIG-untreated)).

Starting from 1865 when Trousseau’s syndrome was described, a lot of experimental and clinical data were obtained indicating an interplay between the platelets, the primary tumor and circulating tumor cells during metastasis [18]. More than 50% of patients with cancer and 90% of patients with metastases demonstrate coagulation disorders [19]

Since our results showed the decrease in the number of platelets in aggregates under IVIG treatment (in CBA and especially in C57BL/6 mice) one could suppose its inhibitory effect toward platelets aggregation in blood. Nevertheless, we can’t exclude the possible accumulation of aggregated of platelets in the area of tumor which could facilitate its growth.

According to the latest hypothesis, cancer-associated thrombosis can result from the release of extracellular traps by tumor-induced neutrophils [20]. G-CSF is supposed an important priming factor in this process [21].

In our study we observed that IVIG had different effect on neutrophil content in the two strains: neutrophil number was increased in IVIG-treated CBA mice, while in C57BL/6 it remained constant. Neither tumor growth nor IVIG treatment influenced the difference in neutrophil count between the strains.

Comparison of tumor volume with neutrophils content suggests correlation between these parameters. We noticed that inhibition of tumor growth was associated with the increased neutrophil number.

Parameters	Units	CBA mice			C57BL/6 mice		
		intact	day 7 after inoculation		intact	day 7 after inoculation	
			control	IVIG		control	IVIG
Monocytes	%	25 ± 3	16 ± 5 ^a	11 ± 3 ^a	27 ± 5	9 ± 2 ^a	13 ± 2 ^a
Lymphocytes	%	45 ± 3	53 ± 9	46 ± 3	56 ± 5 [*]	77 ± 6 ^{a*}	71 ± 4 ^{**}
Neutrophils	%	28 ± 1	31 ± 5	43 ± 5 [#]	15 ± 2 [*]	13 ± 3 [*]	16 ± 2 [*]
Single platelets	in 15 fields	167 ± 7	175 ± 46	140 ± 59	233 ± 8 [*]	269 ± 48 [*]	252 ± 64 [*]
Aggregated platelets	in 15 fields	64 ± 3	87 ± 29	45 ± 20	142 ± 6 [*]	92 ± 28	29 ± 5 ^{a#}

^a p<0.05 compared to intact animals
[#] p<0.05 compared to control animals of the same strain
^{*} p<0.05 compared to corresponding CBA

Table 1: Blood cell content of CBA andC57BL/6 mice before and at day 7 after S37 fibrosarcoma inoculation in control (untreated) and after IVIG-treatment.

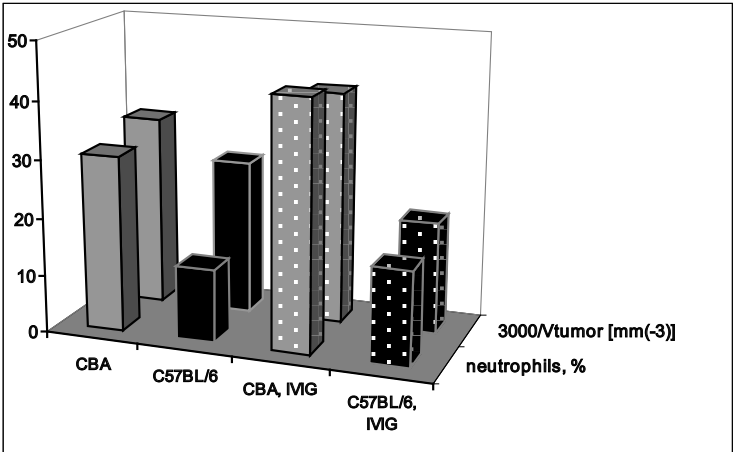


Figure 2: Comparison of neutrophil content (%) and reciprocal value of tumor volume (3000/V).

Figure 2 shows correlation of neutrophils content (%) reciprocal value of tumor volume.

The obtained data indicate a direct correlation between neutrophil content and tumor growth. Hence, one could suppose that neutrophils participate in tumor development and in response to IVIG.

Signal and regulatory role of neutrophils is associated with local release of cytokines, chloride species and generation of reactive oxygen via NADP-H oxidase activation. These compounds might activate or inhibit tumor development directly or are involved in release of neutrophil extracellular traps [22].

The quantity and effect of ROS in tissues depend on functioning antioxidant systems, which include antioxidant enzymes. To reveal the role of oxidative reactions in the observed differences, we analyzed activity of myeloperoxidase, antioxidant enzymes and isoprostane content in soluble fraction of tumor homogenates.

Activity of tissue myeloperoxidase reflects tissue infiltration by neutrophils. The ability of neutrophils to generate ROS locally might result in oxidative modification of tissue proteins and lipids. Isoprostanes, the products of peroxidation of polyunsaturated fatty acids, are considered as markers of lipid peroxidation [23].

IVIG-treated CBA mice showed increased MPO activity in tumor, while in C57BL/6 mice MPO activity was reduced. As a result, the differences between two strains became significant by day 7. Similar alterations (significant or trend-like) were observed for antioxidant enzymes (glutathione-S-transferase, catalase and glutathione

peroxidase (Table 2). Moreover, comparison of tumor weight and GST activity revealed an invert correlation (Figure 3).

Calculation of isoprostane amount per glutathione peroxidase activity unit (ISOP/GPx) shows that in CBA mice this parameter is unaltered after IVIG-treatment, while in C57BL/6 it is significantly increased (Figure 4).

The results indicate that the tumor is infiltrated by neutrophils and the balance of ROS generation and activity of antioxidant enzymes is changed.

Our data demonstrate that tumors of IVIG-treated C57BL/6 mice exhibit lower MPO activity compared to control and reduced activity of some antioxidant enzymes.

ROS play a dual role in tumor development: they can enhance tumor growth and damage adjacent regions of normal tissues, but at concentrations that exceed the capacity of tumor defense resources, they may be harmful for tumors [24-27].

Comparison of IVIG-treated CBA and C57BL/6 mice that demonstrated different rate of tumor growth at early stages of development revealed the elevation of ISOP/GPx ratio and reduction of GST and catalase activity in C57BL/6.

Current concept of regulation of lipid peroxidation considers the ISOP/GPx ratio as an indicator of the balance of pro- and antioxidant processes in tissues [28].

Parameter	Units	CBA		C57BL/6	
		Control	IVIG	Control	IVIG
Myeloperoxidase	uM/g tissue	7.3 ± 1.3	11.8 ± 0.6 [#]	9.8 ± 1.9	5.5 ± 0.5 ^{**}
Glutathione-S-transferase	uM/min/g tissue	0.26 ± 0.03	0.3 ± 0.03	0.26 ± 0.02	0.17 ± 0.02 ^{**}
Catalase	Un/mg tissue	0.25 ± 0.04	0.3 ± 0.04	0.24 ± 0.05	0.11 ± 0.02 ^{**}
Glutathione peroxidase	Un/g tissue	4.2 ± 0.5	5.3 ± 0.6	4.9 ± 0.9	3.3 ± 1.2
Isoprostanes	ng/g tissue	1.3 ± 0.2	1.2 ± 0.3	1.2 ± 0.5	1.2 ± 0.7

significant difference from control IVIG-untreated mice.
* significant difference from corresponding CBA mice
(p<0.05, Mann-Whitney test).

Table 2: Biochemical characteristics of tumor tissues (day 7 after inoculation) obtained from CBA and C57BL/6 control and IVIG-treated mice. The results represent mean ± standard deviation (n=5 for each group).

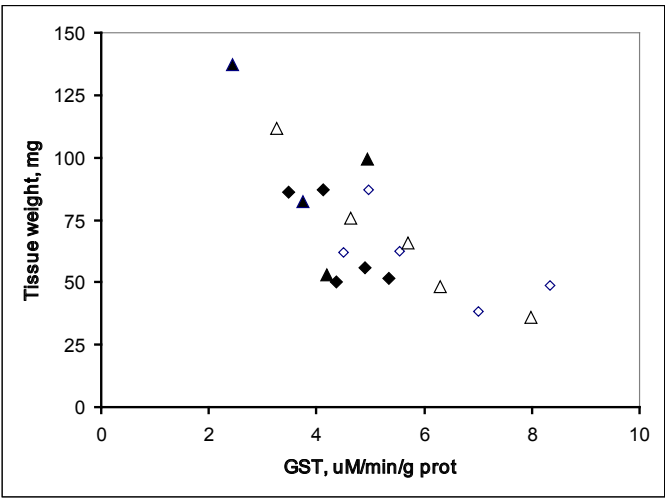


Figure 3: Correlation between tumor weight and GST activity in tumor tissues. White triangles – CBA mice, dark triangles – C57BL/6, rhombs – no IVIG, squares – IVIG (p<0.05).

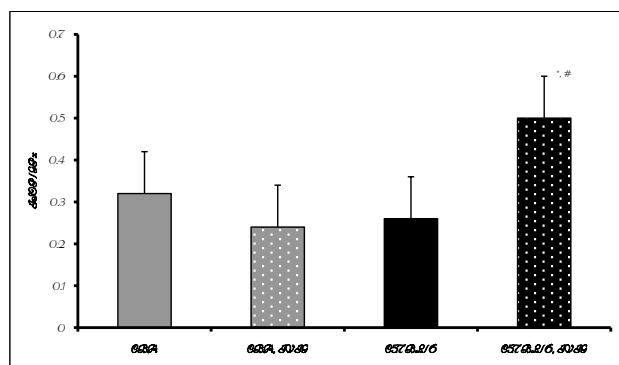


Figure 4: Significance of the IVIG effect (* $p < 0.05$) and of differences between groups (# $p < 0.05$) were estimated by Mann-Whitney test.

Prooxidant phenotype might enhance division rate of tumor cells at this time [19], which explains negative feedback between GST activity and tumor growth and elevation of ISOP/GPx ratio in C57BL/6 mice with increased tumor growth rate.

Measurement of MPO activity indicates reduced neutrophils infiltration of tumor in IVIG-treated C57BL/6 mice. However according to our previous results, neutrophils in IVIG-treated mice demonstrate an increased radical-generating activity soon after fibrosarcoma inoculation (day 1). Such an effect on circulating neutrophils might change the redox balance of cell's microenvironment and elevate the amount of oxidants due to local activation of primed cells. As a result, the effect of oxidants on neighboring cells is enhanced even after less active neutrophil infiltration. Under these conditions ROS-resistant tumor cells divide faster [24]. Another effect of tumor-induced neutrophil activation can be the release of extracellular traps and tumor-associated thrombosis facilitating tumor growth.

In CBA mice, MPO activity was significantly increased in tumor tissues, whereas activity of antioxidant enzymes remained unaltered. Moreover, previous experiments showed that IVIG reduced chemiluminescence of in vitro activated neutrophils. The later indicates that neutrophils might induce lower oxidative burden as a result of ROS generation. At the same time, these neutrophils might retain anti-tumor functions, but such an interpretation requires experimental confirmation.

Indeed, IVIG-treated C57BL/6 mice showed enhanced tumor growth rate at day 7, while CBA showed an opposite tendency of tumor reduction. To confirm this observation we examined tumor growth at day 14.

It turned out that at day 14 tumor growth rate was significantly lower in IVIG-treated CBA mice compared to control, and higher in IVIG-treated C57BL/6 (Figure 5).

Thus our data show that the properties of the innate immune system namely of neutrophils and platelets can influence the different effects of IVIG upon fibrosarcoma S-37 growth in CBA and C57BL/6 mice. These effects of IVIG could be mediated by regulating of neutrophil NADPH oxidase activation (presumably via Fc-receptors) followed by changes in reactive oxygen species generation and release of extracellular traps.

If the same mechanisms function in humans, understanding of IVIG effects at different stages of tumor development is required for optimization of treatment conditions regarding individual immune reaction of patients.

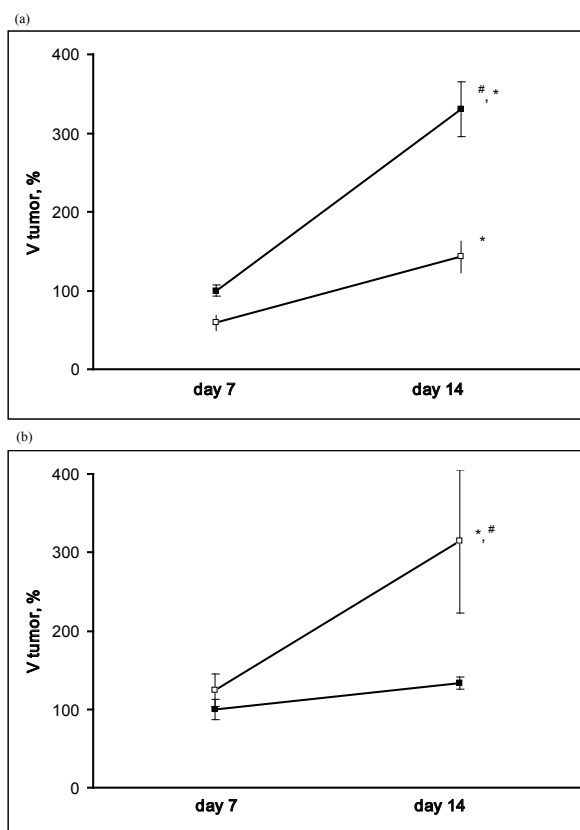


Figure 5: Tumor growth in CBA (a) and C57BL/6 (b) control (dark squares) and IVIG-treated (white squares) mice. Significance of the IVIG effect (* $p < 0.001$) and of differences between groups (# $p < 0.001$) were estimated by Mann-Whitney test. For each experiment the data are normalized to the mean tumor volume in control and represented as mean \pm standard error of mean.

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