

# The Effect of Intravenous Immunoglobulin (IVIG) on *In vitro* Activation of Circulating Neutrophils from CBA and C57BL/6 Mice with Fibrosarcoma S37

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## Abstract

**Background:** The application of IVIG for cancer treatment is associated with the problem of variable patient's response to IVIG, which might result from the peculiarities of neutrophil's activation. Individual variations in the response to IVIG treatment can be elucidated by the comparison of CBA and C57BL/6 mouse strains. These two strains are considered as contrasting ones regarding function of their immune system. Here we studied zymosan-induced activation of circulating neutrophils from CBA and C57BL/6 mice developing fibrosarcoma.

**Results:** The circulating neutrophils of CBA and C57BL/6 mice were studied at early stages of tumor growth. The WBC total and differential counting revealed the increase of neutrophil content in blood up to the day 7 after fibrosarcoma cells S37 inoculation. The neutrophils' activity was measured as opsonized-stimulated (OZ) chemiluminescent (CL) response of whole blood samples at various stages of tumor growth. At days 1 and 7 after inoculation the tumor-associated neutrophils priming was detected in both mouse strains. The effect was more prominent in C57BL/6 if measured in the presence of calcium. In addition to tumor associated priming, the IVIG-enhancing effect was registered at day 1 after inoculation of fibrosarcoma cells. As for normal neutrophils this effect was observed only in the presence of extracellular calcium. At day 7 after inoculation IVIG inhibited *in vitro* CL responses of blood samples stimulated with OZ (both of CBA and of C57BL/6).

**Conclusions:** The activity of circulating neutrophils changes with the tumor development via the mechanisms, which depend on the strain type. These mechanisms are sensitive to IVIG and the effects of IVIG vary also with the stage of tumor growth.

Developing fibrosarcoma induced priming of neutrophils in both mouse strains, mainly by calcium-dependent pathway. OZ-stimulated neutrophils from CBA were less active compared to C57BL/6, and implicated calcium-independent pathways even at day 7 of tumor development.

**Keywords:** IVIG; Neutrophils; Fibrosarcoma; Chemiluminescence; Zymosan; Calcium

**Abbreviations:** IVIG: Intravenous Immunoglobulin; OZ: Opsonized Zymosan; CL: Chemiluminescence; ROS: Reactive Oxygen Species; TAN: Tumor Associated Neutrophils; TNF $\alpha$ : Tumor Necrosis Factor alpha; WBC: White Blood Cells

## Introduction

The preparation of intravenous immunoglobulins G obtained from thousands of patients (IVIG) is used for treatment of numerous diseases associated with inflammation [1,2]. Along with it, IVIG are efficient in cancer treatment [1,3-7], yet the molecular mechanism of their action remains unknown. The therapeutic effect might result from the interaction of IVIG with cancer cells or with immune cells with anti-tumor activity. In humans most immune cells are represented by neutrophils. Their major function is antibacterial defense; however recent data indicate importance of tumor-associated neutrophils (TANs) in carcinogenesis. Neutrophils can play a dual role in tumorigenesis: some of them (N2) neutrophils promote tumor growth due to the secreted chemokines, reactive oxygen species and stimulation of angiogenesis, while others (N1) neutrophils exhibit proinflammatory and anti-cancer activity [8]. Patients with bronchoalveolar carcinoma [9], metastatic melanoma [10] and renal carcinoma [11] with increased number of circulating neutrophils have worse prognosis. High level of neutrophils infiltration in tumor is often associated with severe human

gliomas [12] and pancreatic tumors [13]. However in other cases (gastric cancer, [14]), the correlation is negative. The therapeutic effect of IVIG might result from their effect on TANs [15-17].

The application of IVIG for clinical practice is hindered by variable responsiveness of patients to IVIG. The nature of the observed variation is unknown, and experiments with immunologically contrasting mouse strains (CBA and C57BL/6) might shed light on the problem. Previously we found that these strains responded differently to IVIG

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**Received** September 03, 2013; **Accepted** October 11, 2013; **Published** October 15, 2013

**Citation:** Basyreva LY, Brodsky IB, Gusev AA, Zhapparova ON, Mikhailchik EV, et al. (2013) The Effect of Intravenous Immunoglobulin (IVIG) on *In vitro* Activation of Circulating Neutrophils from CBA and C57BL/6 Mice with Fibrosarcoma S37. Immunome Res 9: 065. doi: [10.4172/1745-7580.1000065](https://doi.org/10.4172/1745-7580.1000065)

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treatment of tumors, and IVIG had different effects on neutrophils activation *in vitro*. We suggested that IVIG might have different effects on circulating neutrophils in CBA and C57BL/6 mice with fibrosarcoma.

Activity of neutrophils is often assessed as production of reactive oxygen species (ROS) [15,17], expression of specific proteins [15] and by cell's degranulation [16]. Reactive oxygen species (ROS) are generated by NADPH oxidase. The multisubunit complex can assemble at plasma membrane (under soluble stimuli) or at phagosome membrane that surrounds engulfed particles [18-20]. The rate and duration of ROS synthesis depends on the stimulus, i.e. on the activated signaling pathway. Thus, soluble phorbol myristate acetate (PMA, a structural analog of diacylglycerol), interacts directly with protein kinase C, inducing phosphorylation of NADPH oxidase subunits independently of extracellular calcium. Corpuscular stimuli (zymosan, bacterial cells) can activate neutrophils via specific receptors that require calcium mobilization from both intra- and extracellular sources [21].

Here we studied zymosan-induced activation of circulating neutrophils from CBA and C57BL/6 mice developing fibrosarcoma(s).

## Materials and Methods

The following reagents were used: zymosan, luminol, Krebs-Ringer medium, EDTA (Sigma-Aldrich, USA, IVIG (Intraglobin, Boitest, Germany).

### 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 intraperitoneally into a male 20-22 g white mouse (BALB/c). 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<sup>d</sup>) mice, female CBA (H-2<sup>k</sup>) and C57BL/6 (H-2<sup>b</sup>) (18-22 g) used in this study were purchased from Russian State Medical University. Maintenance of animals and all the experimental procedures were reviewed and approved by the Animal Care and Ethical Committee of Russian Medical University (Moscow Russia).

To induce tumor growth S-37 (1×10<sup>6</sup> cells/0.1 ml) were injected subcutaneously into the mouse thigh.

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

At 24 h and at day 7 after S37 inoculation the animals were subjected to cervical dislocation, then 100 µL of blood were taken from the right ventricle after thorotomy. Blood was added to 3% EDTA (4:1) and mixed thoroughly. 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).

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

### 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 x 40 magnification.

### Chemiluminescent (CL) assay

The CL assay was performed using LKB Wallac luminometer in continuous measurement mode at 37°C. Final volume of the samples was 1 ml.

The cuvette was filled with Krebs-Ringer solution (Sigma) containing the following reagents (g/l): D-glucose 1.8; MgCl<sub>2</sub> 0.0468; KCl 0.34; NaCl 7.0; Na<sub>2</sub>HPO<sub>4</sub> 0.1; NaH<sub>2</sub>PO<sub>4</sub> 0.18; NaHCO<sub>3</sub> 1.26. CaCl<sub>2</sub> was added separately to final concentration of 0.144 g/l. Luminol (0.2 mM) was used as a CL-sensitizing (enhancing) agent. Spontaneous CL was recorded for 6 min after addition of 20 µL of whole blood into warm medium with luminol followed by 200 µL of IVIG (50 mg/ml) or 200 µL of NaCl. Then the cells were activated with zymosan (0.2 mg/ml) and the peak value was measured. CL response to zymosan was found as the difference between maximal and basement (spontaneous) CL values. The result was expressed as CL response (mV) per 10<sup>5</sup> neutrophils.

**Zymosan opsonization:** 10 mg of zymosan (Sigma) were thoroughly suspended in 1 ml of 0.9% NaCl and added to 1 ml of fresh human blood serum. The suspension was incubated for 30 min at 37°C and intermittent shaking, and then zymosan was pelleted by centrifugation and washed three times with 10-fold volume of NaCl. The obtained pellet was resuspended in 0.9% NaCl to final concentration of 4 mg/ml.

## Results

### Luminol-dependent chemiluminescence (CL) of neutrophils from CBA and C57BL/6 mice

Activity of circulating neutrophils was assessed as luminol-dependent CL response to opsonized zymosan (OZ) measured before and at day 1 and day 7 after S37 inoculation.

Blood samples from CBA and C57BL/6 mice were analyzed at each data point, and CL was measured under different conditions (in the presence and the absence of calcium ions and IVIG).

We showed previously that in calcium-free medium OZ-induced activation of neutrophils was more pronounced in case of CBA mice compared to C57BL/6. At the same time, no difference between strains was observed in the presence of calcium.

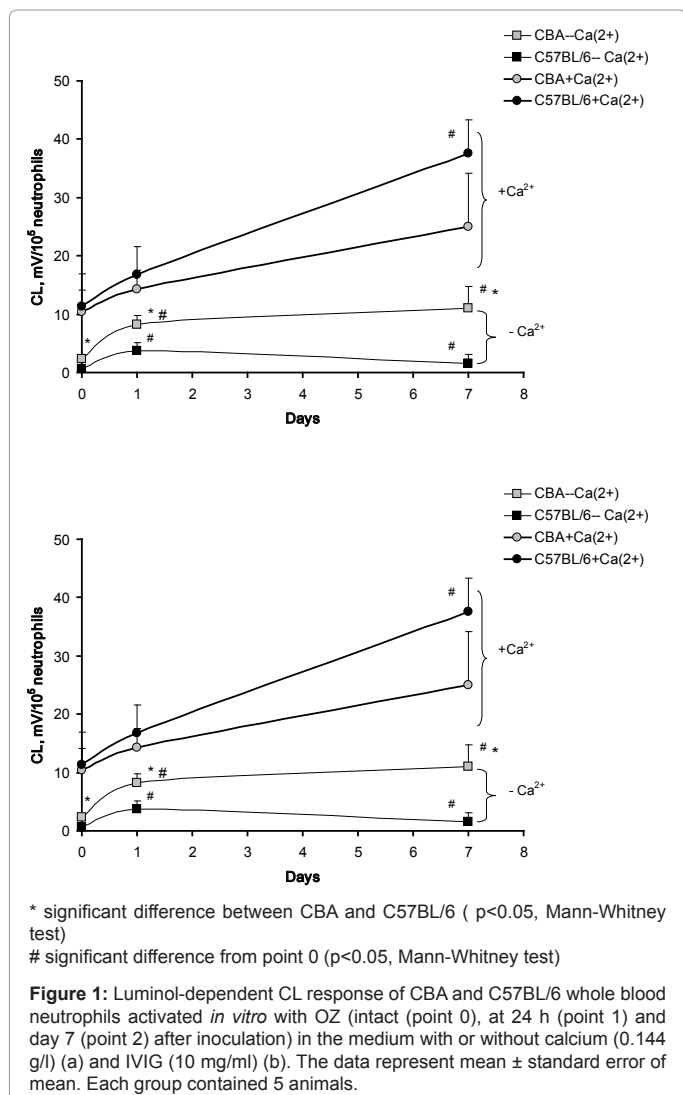
Time-course analysis of OZ-activated CL revealed the dependence of CL response on stage of the experiment (Figure 1).

At an early stage of tumor growth (24 h after inoculation, data point 1), the calcium-independent OZ-induced CL of whole blood neutrophils was significantly (7-fold) higher compared to intact animals (day 0) (Figure 1a). Such an effect was observed in both mouse strains, the difference between the strains still being significant (the calcium-independent CL was higher for CBA).

At day 7, the calcium-independent CL response of CBA neutrophils remained at the same level as at point 1 (1 day after inoculation), and was reliably higher compared to intact animals. In case of C57BL/6 neutrophils, this parameter was lower compared to point 1, but still higher than at point 0 (Figure 1a).

Apparently, starting from early stages, tumor growth is accompanied by neutrophils' priming, expressed as their enhanced OZ-induced calcium-independent activation.

In the presence of calcium, the reliable increase of neutrophils'



activity with tumor development was observed only in C57BL/6 mice at day 7 after inoculation (the value was 3.5-fold increased).

Neutrophils from CBA mice showed similar tendency, but the neutrophils' activity changed insignificantly, and no reliable difference from intact animals was observed.

The obtained result indicates that tumor-associated neutrophils' priming depended on extracellular calcium, and the effect was more prominent in C57BL/6 mice.

Previously we found that IVIG inhibited normal neutrophils' activation in calcium-free medium (CBA mice), and enhanced neutrophils' activation in the presence of calcium (C57BL/6 mice) (Figure 1 and Figure 2, point "0").

Therefore, we made an attempt to specify the impact of IVIG-sensitive activation pathways in the time-course of tumor growth.

Calcium-independent CL of neutrophils in the presence of IVIG increased at 24 h after inoculation compared to intact animals (CBA and C57BL/6), with no difference between CBA and C57BL/6 (Figure 1b).

At day 7, the CL decreased and became indistinguishable from point 0.

Thus, in calcium-free medium with IVIG, no tumor-associated neutrophils priming was observed at day 7 after inoculation.

In the presence of calcium and IVIG there was no increase in neutrophils' activity compared to intact animals.

As mentioned above, in the presence of calcium and IVIG neutrophils' responses in points 0 and 1 were more prominent in case of C57BL/6 mice compared to CBA.

By day 7, in the presence of calcium and IVIG zymosan-induced response of neutrophils from C57BL/6 mice was decreased to the level of intact animals (point 0) (Figure 1b).

For clarity, the effect of IVIG is represented as the ratio of luminol-dependent chemiluminescence of IVIG-treated sample to IVIG-untreated sample (Figure 2).

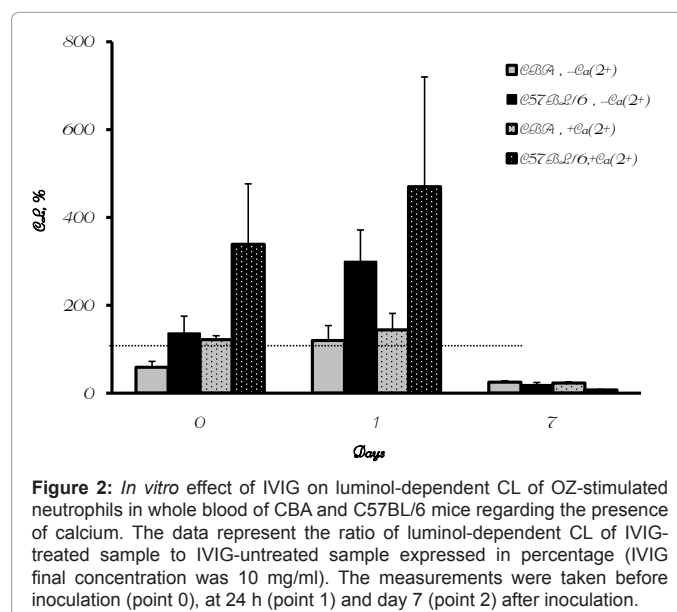
Figure 2 shows that all values are over 100% at 24 h after inoculation. The data suggest that IVIG do not influence OZ-induced activation of neutrophils (in CBA mice), or enhance the response. At day 7 the values are far below 50% indicating the inhibitory effect of IVIG on OZ-induced activation of neutrophils in both strains.

### Differential WBC counting

Analysis of WBC revealed that C57BL/6 mice had less percentage of neutrophils than CBA mice at day 0 and at day 1 after inoculation. At day 7 no difference was found (Table 1). In CBA mice, the number of lymphocytes was reduced at day 1 after inoculation, but by day 7, the number of lymphocytes returned to normal values. At day 7, the number of monocytes was less than in intact animals.

CBA mice showed no significant alteration of lymphocytes and monocytes count.

The percentage of eosinophils was similar in intact CBA and C57BL/6 mice [1,1(0,2) and 1,7(0,3), respectively]. However, development of tumor was associated with reduced percentage of eosinophils in CBA at 24 h after inoculation, which did not alter by day 7. In C57BL/6 the content of eosinophils was reduced only by day 7 (Table 1).



	Intact mice					
	Day 0		Day 1		Day 7	
	CBA	C57BL/6	CBA	C57BL/6	CBA	C57BL/6
neutrophils, %	29 ± 1	15 ± 2*	25 ± 2	18 ± 1*	28 ± 2	25 ± 4#
lymphocytes, %	45 ± 3	56 ± 5*	43 ± 3	46 ± 2#	49 ± 3	56 ± 6
monocytes, %	25 ± 3	27 ± 4	31 ± 3	34 ± 2	22 ± 3	18 ± 3#
eosinophils, %	1.1 ± 0.2	1.7 ± 0.3	0.3 ± 0.2#	1.6 ± 0.4*	0.1 ± 0.1#	0.4 ± 0.4#
total leukocytes, 10 <sup>6</sup> /ml	3.1 ± 0.2	3.2 ± 0.7	1.3 ± 0.2#	3.0 ± 0.6*	3.9 ± 0.6	6.3 ± 0.3##

\* - significant difference from CBA (p<0.05, Mann-Whitney test)  
# - significant difference from intact mice (p<0.05, Mann-Whitney test)

**Table 1:** Differential WBC count in CBA and C57BL/6 mice (%). The data are represented as X ± m, where X corresponds to mean, and m – to standard error of mean (SEM).

Tumor inoculation and development caused the most prominent effect on the number of circulating neutrophils.

In case of C57BL/6 reliable increase of circulating neutrophils was observed at day 7 after inoculation. In case of CBA, the number of neutrophils was reduced at 24 h after inoculation and reached normal valued by day 7.

### Estimation of total platelet content

The number of single platelets did not alter in the course of tumor development in both mouse strains. However, C57BL/6 mice had 50% more platelets. It should be noted that at points 0 and 1 C57BL/6 mice had more aggregates and platelets per aggregate compared to CBA.

Therefore, here we revealed that the number of neutrophils, eosinophils and platelets in blood of CBA and C57BL/6 varies in course of tumor development.

### Discussion

Previously we have shown that the differences in functioning of neutrophils from CBA and C57BL/6 mice might result from the peculiarities of their haemopoiesis and perhaps due to the strain MHC difference.

We used the model of early tumorigenesis to compare the changes in WBC content, in the quality and number of platelets and in activity of circulating neutrophils in CBA and C57BL/6 mouse strains.

Activity of neutrophils was analyzed by measurement of luminol-dependent chemiluminescence (CL) sensitive to ROS production. We compared *in vitro* response of neutrophils regarding the presence of calcium in incubation medium and estimated the impact of calcium-dependent and -independent pathways on the ROS generation.

We showed that inoculation of tumor cells induced priming of neutrophils in both mouse strains at day 1, via calcium-independent mechanisms, and the effect was detectable up to day 7. At day 7 in C57BL/6 mice the neutrophils priming partly depended on the extracellular calcium.

We specified the following differences between neutrophils from CBA and C57BL/6 mice:

1) Regardless of the stage of the experiment the calcium-independent chemiluminescence of neutrophils from C57BL/6 mice was reliably lower compared to CBA;

2) In the presence of calcium (conditions closer to physiological) the difference between CL responses was detected at day 7th (in the course of tumor development OZ-stimulated neutrophils from C57BL/6 mice were more active compared to CBA).

3) At day 7, CL of neutrophils from C57BL/6 depended on extracellular calcium and was higher compared to intact animals. The data indicate that tumor-associated priming of neutrophils requires mainly calcium-dependent activation of cells. In CBA mice neutrophils implicated calcium-independent pathways even at day 7 of tumor development.

Detailed analysis of mechanisms underlying OZ-induced activation of neutrophils during tumor development requires further research. Nevertheless, priming (raise in calcium-dependent CL response to OZ) of neutrophils in C57BL/6 mice suggests that they differ from CBA neutrophils in functional potency (ability to undergo polarization, DNA trap, secretion of MPO, cathepsin G, elastase, etc.) [8,22].

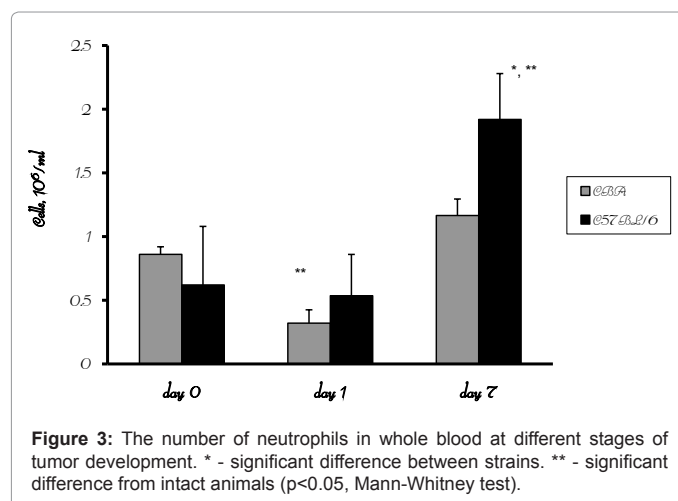
Activation of neutrophils by opsonized zymosan can be mediated by various membrane receptors (including FcR), which trigger phosphorylation of NADPH oxidase subunits. We studied the role of immunoglobulins on neutrophils' activation *in vitro*, adding IVIG to the cells before OZ-activation.

We did not observe IVIG-induced respiratory burst at any stage of the experiment. At the same time, other authors described IVIG-induced generation of ROS by isolated neutrophils and showed that pretreatment with TNF-α significantly enhanced the effect [17].

A dose-dependent effect of IVIG on neutrophils *in vitro* was demonstrated by Casualli et al. [15], low concentrations of IVIG induced activation of neutrophils, whereas high concentrations induced inhibition.

Our results suggest dependence between the effect of IVIG and the stage of tumor development on one hand, and a genetic susceptibility of response to IVIG treatment on the other hand.

In intact animals, IVIG had an inhibitory effect on calcium-independent CL of zymosan-induced neutrophils from CBA mice, but the effect disappeared at day 1 after inoculation. In C57BL/6 mice neutrophils' response at points 0 (in the presence of calcium) and 1 (with and without calcium) significantly increased after addition of



**Figure 3:** The number of neutrophils in whole blood at different stages of tumor development. \* - significant difference between strains. \*\* - significant difference from intact animals (p<0.05, Mann-Whitney test).



IVIg to the incubation medium. We considered this enhancement as IVIG-induced priming *in vitro*.

At day 7, the effect of IVIG on both mouse strains regardless the presence of exogenous calcium became unidirectional and reduced the CL responses.

It should be noted that significantly increased OZ-activated CL at day 0 and 1 is a specific feature of C57BL/6 mice. The effect of IVIG varied in the time-course of tumor growth, and addition of IVIG to the incubation medium completely eliminated priming of neutrophils *in vivo*.

Since tumor-promoting and anti-tumor roles of neutrophils are associated with their ability to generate ROS [23], the observed effects might be parts of the mechanism, which mediates the effect of IVIG on carcinogenesis.

At the same time, qualitative (functional), as well as quantitative, parameters of circulating leukocytes are important for the process.

At the present time, some markers of inflammation are tested as potential predictors of tumor development outcome. The list includes the number of circulating neutrophils, lymphocytes, platelets and their ratio [24-31].

Here we revealed that the number of neutrophils, eosinophils and platelets in blood of CBA and C57BL/6 mice vary during tumor development.

The blood neutrophil concentration and the number of platelets in aggregates (PA) changed in the same mode with tumor growth (Figure 4).

The typical feature was a decrease in neutrophil count (in CBA) and in PA (in CBA and in C57BL/6) at day 1. The measured parameters of platelet aggregation differed between the CBA and C57BL/6 mainly before and one day after inoculation of fibrosarcoma cells. At these time-points the different *in vitro* effects of IVIG upon the OZ-stimulated CL of blood neutrophils were shown. So in the blood of C57BL/6 there were more platelets in aggregates (before inoculation) and platelet aggregates in blood (before and one day after inoculation) and at the same time IVIG raised the level of OZ-stimulated neutrophil CL *in vitro*.

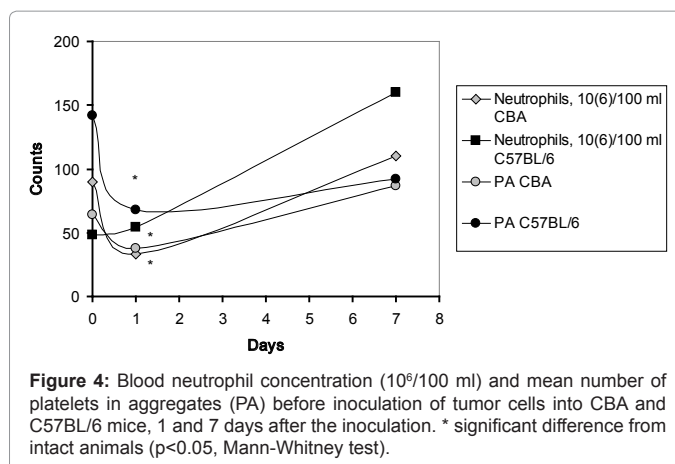
Platelet aggregation can depend upon the formation of neutrophil extracellular traps (NETosis) [32,33]. NETosis results from activation of certain signal pathways, like Raf-MEK-ERK [34] in cooperation with NADPH-oxidase activation [33] and superoxide production [35].

Cancer-associated microthrombosis is supposed to result from neutrophils activation by G-CSF, NETosis included [32]. The priming of neutrophil NADPH-oxidase by G-CSF was shown earlier [36].

	Intact mice					
	Day 0		Day 1		Day 7	
	CBA	C57BL/6	CBA	C57BL/6	CBA	C57BL/6
SP	167 ± 10	233 ± 11*	161 ± 23	234 ± 31	175 ± 29	245 ± 40
PA	64 ± 4	142 ± 9 *	38 ± 7 #	68 ± 7* #	87 ± 18	92 ± 17 #
NA	19 ± 1	31 ± 3 *	17 ± 3	31 ± 3*	28 ± 4	30 ± 4

SP – the number of single platelets in 15 microscopic fields  
 PA – the number of platelets in aggregates in 15 microscopic fields  
 NA – the number of aggregates in 15 microscopic fields  
 \* - significant difference from CBA (p<0.05, Mann-Whitney test)  
 # - significant difference from intact animals (p<0.05, Mann-Whitney test)

**Table 2:** The number and aggregation status of platelets from whole blood of CBA and C57BL/6 mice. The data are represented as X ± m, where X corresponds to mean, and m – to standard error of mean (SEM).



**Figure 4:** Blood neutrophil concentration (10<sup>6</sup>/100 ml) and mean number of platelets in aggregates (PA) before inoculation of tumor cells into CBA and C57BL/6 mice, 1 and 7 days after the inoculation. \* significant difference from intact animals (p<0.05, Mann-Whitney test).

So the decrease in neutrophil count and PA found one day after fibrosarcoma inoculation could be explained by the mechanism which includes neutrophils-NADPH oxidase –NETs- platelets and G-CSF. This result is consistent with cancer-associated thrombocytopenia and neutropenia in mice [32]. Taking in account the possible role of NADPH oxidase in NETosis and the enhancement of CL responses of C57BL/6 blood neutrophils in presence of IVIG (before and one day after inoculation), one could expect different effects of IVIG treatment in CBA and C57BL/6 mice.

Our data show that with tumor growth, the differences between CBA and C57BL/6 mice in mean platelets number per aggregate and in blood aggregates number disappears by the 7<sup>th</sup> day. At this time-point IVIG inhibited OZ-stimulated CL of blood neutrophils, probably via Fc-receptors. Such an inhibition could signal the positive role of IVIG in prevention of microthrombosis. The survival assay could confirm this hypothesis.

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