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The effect of granulocyte colony-stimulating factor (G-CSF) on the activity of granulocyte enzymes in children with cancer who developed neutropenia after chemotherapy

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ABSTRACT

Purpose: G-CSF is a cytokine that stimulates the proliferation and maturation of granulocyte precursor cells. The results of *in vitro* and *in vivo* investigations conducted on animal models revealed that this cytokine influences the functions of mature granulocytes increasing the activities of the granulocyte enzymes participating in phagocytosis.

Material and methods: The investigation was conducted on a group of 26 children (age: 1.5 – 17 years) with cancer who developed neutropenia after chemotherapy and were treated with G-CSF. The control group included 29 healthy children (age: 5 –17 years). The heparinized blood samples were taken before the injection of the stimulator (time 0) and after the 2nd and 5th injection of G-CSF (on day 3 and 6). Activities of granulocyte enzymes involved in the process of phagocytosis (myeloperoxidase, acid and alkaline phosphatase and esterase) in blood smears were evaluated.

Results: It has been found that G-CSF affects the activity of granulocyte enzymes by the normalization of decreased values of myeloperoxidase, acid phosphate and increasing the normal values of alkaline phosphate activity. The enzyme activities increased during the following days of treatment.

Conclusion: Based on the obtained results, we can conclude that G-CSF activates the formation of fully competent granulocytes in cytostatic-treated children with various neoplastic diseases.

Key words: granulocyte-colony stimulating factor, neutropenia, cancer

INTRODUCTION

It is known that G-CSF not only stimulates the granulopioesis but also regulates the functions of mature neutrophils. The influence of this stimulator on chemotaxis [1-6], adhesion [7], phagocyte and bactericidal [6, 8-11] abilities has been proved.

One of the phagocytosis stages is the enzymatic digestion of phagocyted material. Mature neutrophils are equipped with enzymes located in their granules.

Myeloperoxidase (MPO) is a typical enzyme for azurophilic granules that takes part in bactericidal activity dependent on oxygen. With the use of H_2O_2, myeloperoxidase oxidizes chloride, bromide, and iodide ions to the form of acids (hypochlorous, hypobromous and hypoiodous) that indicate strong bactericidal [12-13] activity.

Alkaline phosphatase (AP) is considered by some authors to be an indicator of granules specificity, which are more often connected with secretory vesicles that are believed to be type four of neutrophilic granules next to primary, secondary and tertiary granules. In the course of phagocytosis during the granules degranulation AP is the first to be set free. This is the beginning of the degradation of a foreign particle membrane. AP is also believed to be an indicator of granulocyte maturity.

Granulocyte esterase plays the small role in the process of killing. It is an enzyme of azurophilic granules, and takes part in detoxication and phagocytosis.

The neutrophil acid phosphatase (AcP) is recognized as a typical lysosomal marker located in the azurophilic granules and possesses an ability to digest the bacteria that had been killed.

Figure 1. **MPO activity in granulocytes of neutropenic children after G-CSF injection, data are means ± SD from 26-29 observations, significantly different from: control group ✶ (p≤ 0,05); 0 day ● (p≤ 0,05).**

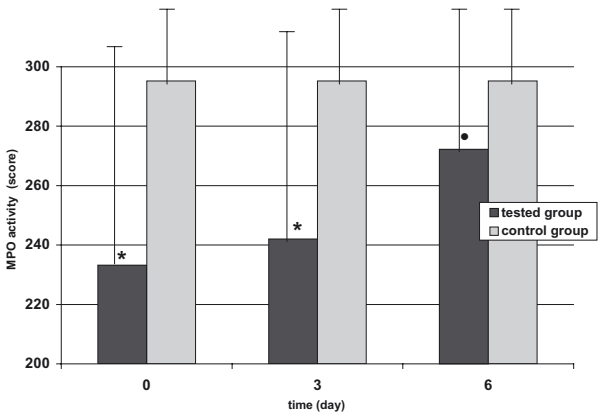
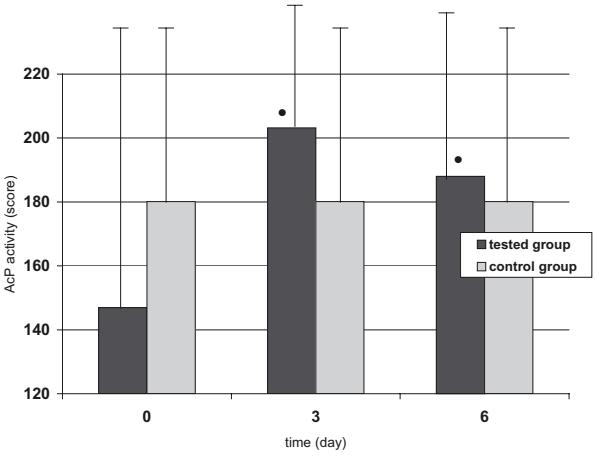


Figure 2. **AcP activity in granulocytes of neutropenic children after G-CSF injection, data are means ± SD from 26-29 observations, significantly different from 0 day ● (p≤ 0,05),**



The purpose of this work was to investigate two questions: (1) Does G-CSF administered to children with neutropenia after chemotherapy in the course of cancer, apart from hemotopoiesis regulation, influences the activity of granulocyte enzymes taking part in the process of phagocytosis? (2) Do the estimated parameters depend on the time of G-CSF injections? The increased activity of these enzymes would be a direct biochemical indicator of G-CSF – dependent phagocytosis activation *in vivo*.

MATERIALS AND METHODS

The investigation was conducted on a group of 26 children (13 boys and 13 girls) at the ages of 1.5 to 17 years (average age was 8.5 years) with solid tumors and systemic neoplasms (acute lymphoblastic leukaemia – 9, lymphomas – 7, solid tumors – 10). One week after chemotherapy, the children developed neutropenia (absolute number of granulocytes – 328.8/μl). In order to stimulate granulopoioesis, G-CSF (Neupogen) was given to children subcutaneously at a dose of 4.4 to 12.9 μg/kg/d (average 6.66 μg/kg/d) for the whole period of experiments, lasting from 5 to 7 days.

The control group consisted of 29 healthy non-treated children (15 boys and 14 girls) at the ages of 5 to 17 years (average age 12.5 years). Interviews were used to determine if the children qualified to be in the control group. They included a lack of immunological disorders and the absence of clinical symptoms of infection for the period of the preceding two months and during the tests.

Blood samples were collected after overnight fasting in the morning before the G-CSF injection (time 0) and after the 2nd and 5th stimulator injection (3rd and 6th day). Parallel fasting blood samples were taken and the tests were repeated 2-5 times in the control group.

The activity of enzymes was evaluated in the cells of peripheral blood smears observed in the immersional magnification of the light microscope and expressed by a 'score' coefficient being a sum of a products of individual degrees of reaction (0 – 4) multiplied by the number of cells with that degree.

Degrees of reaction:

- (0) – lack of granules in cytoplasma,
- (1) – 1-10 granules,
- (2) – 11-20 granules,
- (3) – 21-30 granules,
- (4) – above 31 granules.

AP, AcP and chloracetate esterase activities were assessed with standard mix provided by Sigma-Aldrich (Poznań, Poland).

Myeloperoxidase was assessed with Graham-Knoll's method [14]. Blood smears were submerged with assay medium containing ethylic alcohol 75% (25ml), H_2O_2 - 3% (0.1ml), and benzidine (0.125g), followed by staining with a Giemsa reagent.

The Kolmogorov-Smirnov test was used to check the fit of the data to the Gaussian distribution pattern (p≥0,1 was considered to be indicative for Gaussian distribution). Differences between the two experimental groups were tested by unpaired Student t-test. Results obtained after treatment in the same person were compared by the paired Student t-test. P ≤0,05 was considered as an indicator of significant difference. Standard deviations (SD) were also calculated and indicated.

The investigation was approved by the Bioethical Committee of the Medical University in Bialystok and written informed consent was obtained from the parents of each tested subject (R-I-003/84/2002).

RESULTS

Fig. 1 presents the influence of G-CSF on the activity of MPO. Before the G-CSF application, the activity of that granulocyte enzyme in the cancer group (233.2 score) was

Figure 3. **AP activity in granulocytes of neutropenic children after G-CSF injection, data are means ± SD from 26-29 observations, significantly different from: control group ✶ (p≤ 0,05); 0 day ● (p≤ 0,05).**

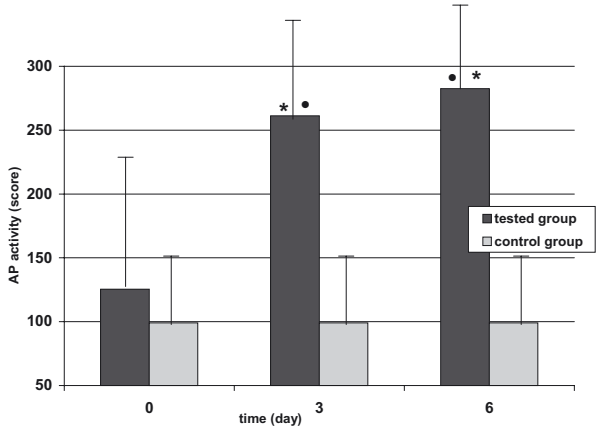
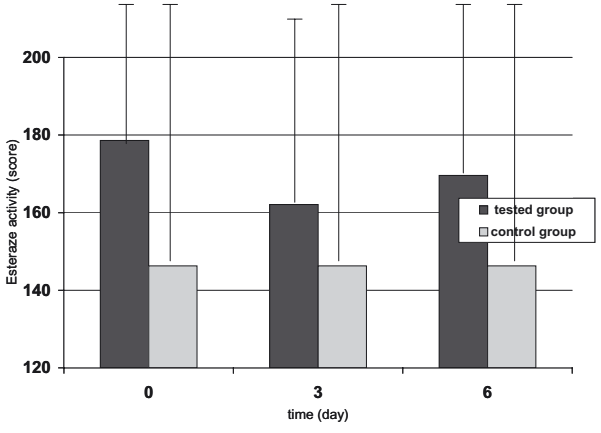


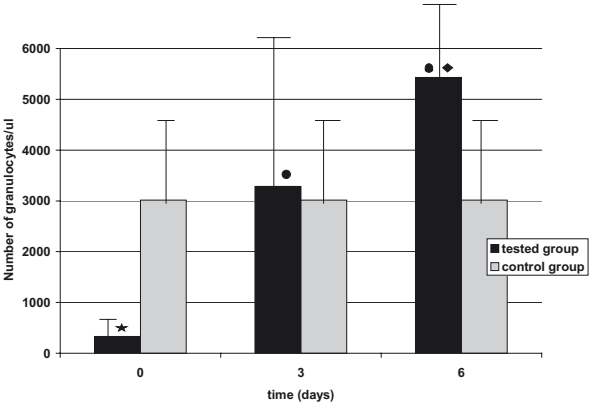
Figure 4. **Esterase activity in granulocytes of neutropenic children after G-CS injection, data are means ± SD from 26-29 observations.**



significantly lower than in the control group (295.2 score). After the second stimulator injections (3rd day), the MPO activity was still statistically lower than in the control group. After the 5th injection of G-CSF (6th day) a significant increase of MPO activity to a score of 272.2, which did not differ significantly from the enzyme activity of the control group. In the experimental group, the increased enzyme activity on day 6 in comparison to day 0 was statistically considered as significant.

Before the G-CSF injection (day 0), the activity AcP in granulocytes of the tested group (146.9) did not differ from that in the control group (180.1) (*Fig. 2*). On the 3rd day (after 2 injections) of treatment, a significant increase of AcP activity to a score of 203.2 was observed. These values were higher than in the control group. On day 6 (after 5 injections), enzyme activity (188,0score) did not change significantly in respect to day 3; however, it was still higher than enzyme activity on day 0 and the difference was statistically significant.

Figure 5. **The number of granulocytes of neutropenic children after G-CSF injection, data are means ± SD from 26-29 observations significantly different from: control group ✶ (p≤ 0,05); 0 day ● (p≤ 0,05); 3 rd day ♦ (p≤ 0,05).**



Before the G-CSF injection, the activity of AP in the patients was similar to that in the control group (*Fig. 3*). In the patients, the score was 125.5; whereas, in control group, the score was 99.0. After two injections (3rd day) of G-CSF, AP activity in the patient group increased to 261.2 (p< 0,05), which was 150% higher than in the control group. On the 6th day of experiment, further AP activity, up to 282.0, was observed.

No significant differences in esterase activity were found in the granulocytes of the control and patient groups (*Fig. 4*). G-CSF treatment did not change esterase activity in patient group.

Before the G-CSF injection, the number of granulocytes (328/μl) in the blood of the patients treated with cytostatics was significantly lower than in the control group (3014/μl) (*Fig. 5*). After the G-CSF treatment of patient group, the number of granulocytes significantly increased to 3284 /μl on the 3rd day and to 5430/μl on the 6th day. The results obtained on the 6th day were also significantly higher from those obtained on the 3rd day.

DISCUSSION

Neutropenia after chemotherapy is one of the most frequently used clinical indicators for the influence of G-CSF on cancer patients [9]. G-CSF, apart from the influence on the proliferation and maturation of the neutrophils, is also presumably responsible for the stimulation of mature granulocyte functions, especially phagocytosis.

In our investigation, the G-CSF influence on the phagocytic activity of mature neutrophils *in vivo* in children with cancer was tested. Neutropenic children after chemotherapy received Neupogen in order to stimulate granulopioesis. To confirm the influence of G-CSF on the functions of mature cells, the activities of granulocytes enzymes (MPO, AcP, AP and esterase) taking part in the process of phagocytosis were evaluated.

Other research papers are available about the influence of G-CSF on a MPO and AP activity *in vivo* in human granulocytes. Fukumasu et al. [15] have conducted investigations on patients with neutropenia treated with chemotherapy in the course of cancer in reproductive organs and receiving G-CSF. They have noticed the increased activity of AP in neutrophils during application of this factor. The results of our experiments agree with that report [15] (*Fig. 1, 3*). Leavey et al. [16] observed a similar G-CSF effect on the activity of granulocytes AP and the lack of influence on the MPO activity.

In vitro experiments on neutrophils from patients with noncompensated diabetes have shown that their incubation with G-CSF increased the initially low MPO activity [17]. Our experiment also indicated the beneficial effect of this factor on MPO activity (*Fig. 1*).

Szmitkowski et al. [18] evaluated the activity of enzymes: AcP, MPO, AP and esterase in mice granulocytes that received G-CSF in three different doses (1, 10, 100 µl/kg/d). The increase of activity for each tested enzyme was G-CSF-dose dependent. The increase of AcP activity in children's granulocytes after G-CSF injections in the tested group remains in agreement with aforementioned animal study (*Fig. 2*).

According to Saito's et al. [19] investigations with the immunocryomicroscopy method in human neutrophils, we can differentiate 5 types of primitive (azurophilic) granules. The problem of final neutrophil granules qualification still seems to be open. Probably the lack of response of esterase activity to G-CSF treatment might be caused by the different localization of that enzyme, e.g. in different types of azurophilic granules than AcP and MPO (*Fig. 4*). Differences in the reaction of enzyme activity obtained in our tests and on mice, indicate the need for further researches.

All of the obtained results indicate that G-CSF administered in order to stimulate granulopioesis in children with neutropenia after chemotherapy in the course of cancer normalizes and often increases the activity of granulocyte enzymes, thereby improving phagocytic functions of neutrophils.

CONCLUSIONS

1. G-CSF administered to children with neutropenia after chemotherapy in the course of cancer influences the activity of granulocytes enzymes, normalizing low values of MPO and AcP activity and increasing normal values of AP activity.
2. A time-dependent increase of enzyme activity in granulocytes was observed after G-CSF applications to cytostatics-treated children with cancer.
3. Obtained results prove that G-CSF, apart from granulopoiesis stimulation, also has to the ability to activate the non-specific granulocyte dependent immunity.

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