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Neutrophils with Toxic Granulation Show High Fluorescence with Bis(Zn^{2+}-dipicolylamine) Complex

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Abstract. Although blood neutrophils with toxic granulation provide an excellent means of evaluating acute bacterial infections, the methods are labor-intensive and their reproducibilities depend on the staining technique and the observer's judgment. We measured the flavin adenine dinucleotide (FAD) content of normal neutrophils and neutrophils with toxic granulation by flow cytometry after incubating them with bis(Zn^{2+}-dipicolylamine) complex. A total of 122 blood samples (78 with neutrophils with toxic granulation, 44 with normal neutrophils without toxic granulation) were analyzed. The mean autofluorescence levels of neutrophils in the toxic granulation (+) group and toxic granulation (-) group were both 1.9 × 10^3 MESF (mean equivalent soluble fluorochrome) values. However, after incubating neutrophils with bis(Zn^{2+}-dipicolylamine) complex for 15 min, the mean fluorescence intensities of neutrophils in the toxic granulation (+) and toxic granulation (-) groups were significantly enhanced by 71- and 19-fold to 138±78 × 10^3 MESF and 37±37 × 10^3 MESF, respectively ($p < 0.001$). An MESF cutoff value of 79 x 10^3 showed a sensitivity of 93.2% and a specificity of 81.8% for neutrophils with toxic granulation. Similarly, the MESF level ratio, defined as the ratio of the MESF value after incubation with bis(Zn^{2+}-dipicolylamine) complex to the autofluorescence MESF value, at a cutoff value of 41, showed a sensitivity of 92.3% and a specificity of 81.8% for the detection of neutrophils with toxic granulation. The MESF values of neutrophils after incubation with bis(Zn^{2+}-dipicolylamine) complex did not correlate with the leukocyte ($p > 0.05$) or neutrophil counts ($p > 0.05$). In conclusion, measurement of FAD fluorescence intensity in neutrophils by flow cytometry after incubation with bis(Zn^{2+}-dipicolylamine) complex is an easy, objective, and reliable method of detecting neutrophils with toxic granulation.

Keywords: neutrophil, toxic granulation, bis(Zn2+-dipicolylamine) complex, flavin adenine dinucleotide, flow cytometry

Introduction

Toxic granules are dark blue to purple intracytoplasmic granules in neutrophils, and are found during acute bacterial infections or other toxic conditions [1]. Because total leukocyte counts and absolute neutrophil counts vary in the presence of bacterial infections, the presence in neutrophils of toxic granulation and/or toxic vacuoles provides valuable diagnostic information [1-8]. However, the reproducibility of detection of neutrophils with toxic granulation is low, because it depends on staining quality and observer's skill [2,3,9]. Hence, an accurate and objective method is needed to detect neutrophils with toxic granulation.

Flavin adenine dinucleotide (FAD) is a cofactor for numerous redox enzymes, such as dehydrogenases, oxygenases, oxidases, and reductases, in human cells, including neutrophils, to generate hydrogen peroxide or superoxide ion that can damage ingested organisms [10,11]. FAD content of neutrophils is reduced in some patients with chronic granulomatous disease, in whom neutrophil antimicrobial activity is diminished [12].

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Neutrophils with toxic granulation have prominent azurophilic granules that contain a variety of antimicrobial enzymes [13]. Therefore, neutrophils with toxic granulation could contain more FAD than neutrophils without toxic granulation. However, the FAD content in individual neutrophils has not been estimated. The natural autofluorescence of neutrophils is due to FAD, but it is too weak to allow comparisons of intracellular FAD levels in neutrophils with toxic granulation versus neutrophils without toxic granulation [14].

Recently, we developed a new flow cytometric method to measure the FAD content of blood eosinophils semiquantitatively using bis(Zn^{2+}-dipicolylamine) complex [15]. The fluorescence intensity of FAD was enhanced selectively by more than 7-fold versus autofluorescence after incubation with this complex [15]. In the present study, we used the bis(Zn^{2+}-dipicolylamine) complex to compare the FAD contents of neutrophils with and without toxic granulation, and to probe its possible application as a diagnostic tool to detect neutrophils with toxic granulation.

Materials and Methods

Seventy-eight patients who had neutrophils with toxic granulation were studied (Table 1). Of these patients, 8 had received hematopoietic stem cell transplantation (HSCT), 23 had leukemia, and 24 had bacterial infections including sepsis. Ten of the HSCT and leukemia patients had recently received granulocyte colony stimulating factor (G-CSF). Fifty-one of the patients were male and 32 were female. Patients ages ranged from 6 to 91 yr (mean 48 yr). The control group of 44 subjects included 27 healthy volunteers and 17 patients with neutrophils without toxic granulation. Mean age of the control group was 45 yr (28 male and 16 female).

Table 1. Patient characteristics.

Diagnoses	No. of cases	Mean age (yr)	Sex (M/F)
Toxic granules (+)			
Bacterial infections	24	59	17/7
Neutrophilia, unknown origin	6	53	2/4
Post-operation	6	50	2/4
Leukemia	23	41	16/7
Cancer	5	67	3/2
Post-HSCT*	10	29	7/3
Other diseases	4	37	1/3
Toxic granules (-)			
Bacterial infections	5	61	5/0
Neutrophilia, unknown origin	2	64	2/0
Post-operation	2	44	0/2
Leukemia	1	46	0/1
Post-HSCT*	2	16	1/1
Other diseases	5	40	2/3
Healthy volunteers	27	43	18/9

*Post-HSCT, Post-hematopoietic stem cell transplantation.

EDTA-anticoagulated peripheral blood samples remaining after routine laboratory tests were used within 4 hr after collection. The samples were stored at room temperature before use. The study subjects all provided written consent for the use of their blood samples.

Leukocyte and neutrophil counts were determined using a hematologic analyzer (XE-2100, Sysmex, Kobe, Japan). The cutoff point used for neutrophilia in this study was ≥8.0 × 10^6/ml, as described in European and American studies of reference ranges [16,17]. Peripheral blood smears were stained with Wright's giemsa stain. Morphologic examinations of peripheral blood smears were performed by two specialists. Neutrophils with toxic granulation were defined by the presence of dark blue to purple cytoplasmic granules in the neutrophils (Fig. 1).

For flow cytometry, 100 µl samples of blood were used. The erythrocytes were lysed using lysing solution (Becton-Dickinson, San Diego, CA, USA). After washing with 2 ml of saline, the cells were fixed and permeabilized using a Cytofix/Cytoperm kit (BD Pharmingen, San Diego, CA, USA), according to the manufacturer's instructions. Then 100 µl of bis(Zn^{2+}-dipicolylamine) complex (3.33 mmol/L in saline) was added and cells were incubated at room temperature for 15 min. The cells were washed with the Perm/Wash solution (supplied with the kit) and they were analyzed using a flow cytometer (FACSCalibur, Becton-Dickinson, San Jose, CA, USA) and the CellQuest program (Becton-Dickinson). An argon-ion laser (488 nm) was used to induce fluorescence, which was measured at 530 (±10) nm. Neutrophils were gated using forward scattering (FSC) and side scattering (SSC) characteristics. Fluorescent microparticles, Quantum^{TM} FITC medium level (Bangs Laboratories, Fishers, IN, USA), were run together with the cell samples for comparative fluorescence analysis. Mean values of fluorescence intensities were converted to mean equivalent soluble fluorochrome (MESF) values (QuickCal software, v. 2.3, Bangs Labs).

For statistical analysis, the MESF values of neutrophils with toxic granulation (toxic granulation (+) group) and without toxic granulation (toxic granulation (-) group) were compared using the Student's t-test and the Mann-Whitney U test. Correlations between MESF values of the neutrophils to other clinical and laboratory findings were analyzed using Pearson's correlation test. Values of $p < 0.05$ were considered significant.

Results

The total leukocyte count in the toxic granulation (+) group was 16,160±13,807 x 10^3/µl, which was

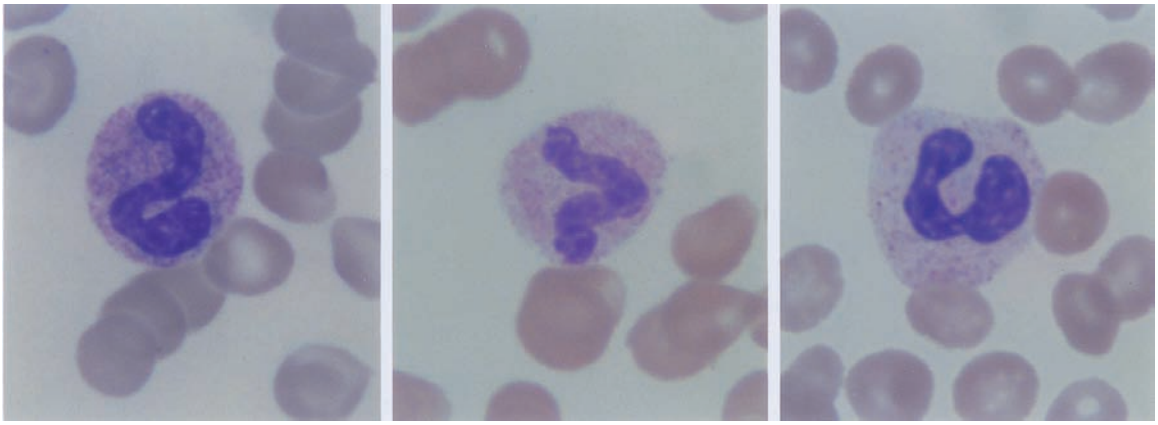

Fig. 1. A neutrophil with toxic granulation (left) found in a patient with sepsis. The cell revealed many dark colored granules after staining for 5 min. Another neutrophil from the same patient after staining for 4 min (middle) shows only pink granules without toxic granulation like the normal neutrophil shown on the right (x 1,000).

greater than in the toxic granulation (-) group (8,300±3,920 x 10^3/µl, p <0.001) (Table 2). The neutrophil count in the toxic granulation (+) group was 8,820±9,330 x 10^3/µl, which was also greater than in the toxic granulation (-) group (5,850±2,090 x10^3/µl, p <0. 01). However, only 38 of the 78 toxic granulation (+) samples showed neutrophilia (48.7%); the other 40 samples (51.3%) showed normal or reduced neutrophil counts. In the toxic granulation (-) group, 7 of 44 samples (15.9%) had neutrophilia.

Mean autofluorescence levels of neutrophils in the toxic granulation (+) and toxic granulation (-) groups were both 1.9 × 10^3 MESF. However, after neutrophils were incubated with bis(Zn^{2+}-dipicolylamine) complex for 15 min, the MESF values of neutrophils in the toxic granulation (+) and (-) groups were significantly enhanced by 71- and 19-fold, respectively (p <0.001). Furthermore, the MESF of neutrophils in the toxic granulation (+) group after incubation with bis(Zn^{2+}-dipicolylamine) complex was 138±78 x 10^3 MESF, which was significantly higher than in the toxic granulation (-) group (37±37 x 10^3 MESF) (p <0.001) (Fig. 2).

The MESF value of neutrophils of post-HSCT patients receiving G-CSF whose neutrophils had toxic granulation was 149±25 x 10^3 MESF and that of post-HSCT patients with neutrophils without toxic granulation and who had not received G-CSF was 38±25 x 10^3 MESF (p <0.01).

An MESF cutoff value of 79 x 10^3 showed a sensitivity of 93.2% and a specificity of 81.8% for the detection of neutrophils with toxic granulation. Similarly, the MESF level ratio, defined as the ratio of the MESF value after incubation with bis(Zn^{2+}-dipicolylamine) complex to the autofluorescence MESF value, at a cutoff value of 41, showed a sensitivity of 92.3% and a specificity of 81.8% for detection of neutrophils with toxic granulation.

The MESF values of neutrophils incubated with bis(Zn^{2+}-dipicolylamine) complex were not correlated with the total leukocyte count (p >0.05) or the neutrophil count (p >0.05).

Table 2. Fluorescence intensities of neutrophils in the two groups after incubation with bis(Zn^{2+}-dipicolylamine) complex.

Parameter	Toxic granules (+)	Toxic granules (-)	p
No. of cases	78	44	
Sex (M/F)	47/31	28/16	
Age (yr, mean ± SD)	48 ± 20	45 ± 16	>0.05
Total blood eukocyte count (×10^3/µl)	16,160 ± 13,807	8,299 ± 3,919	<0.001
Blood neutrophil count (×10^3/µl)	8,815 ± 9,331	5,853 ± 2,087	<0.01
Fluorescence intensity of neutrophils (MESF) (×10^3)*	138 ± 78	37 ± 37	<0.001

*Mean equivalent soluble fluorochrome (MESF) values.

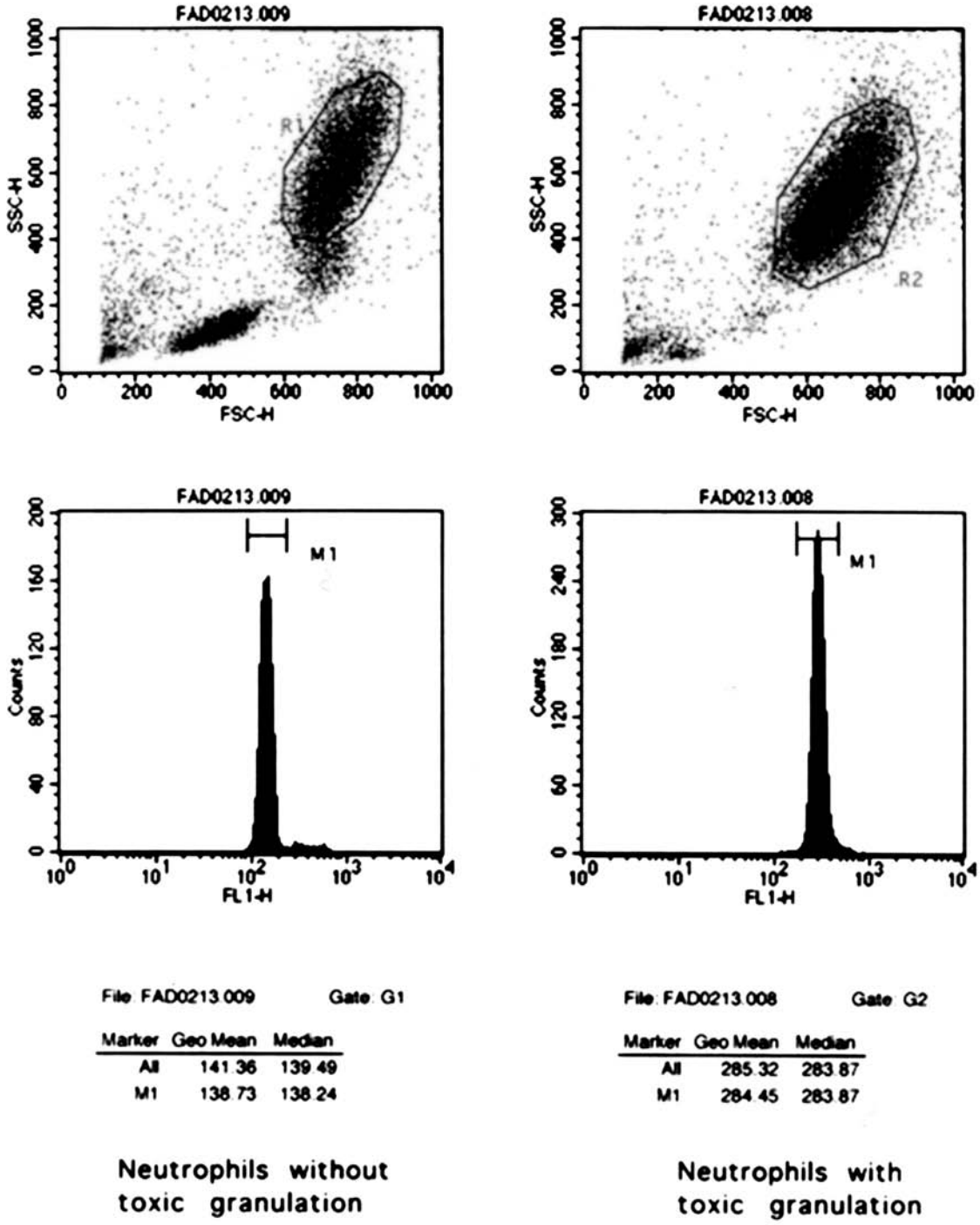

Fig. 2. After incubation with bis(Zn^{2+}-dipicolylamine) complex for 15 min, neutrophils with toxic granulation showed higher mean fluorescence intensities (right, file no. FAD0213.008) than neutrophils without toxic granulation (left, file no. FAD0213.009).

Discussion

The mean leukocyte and neutrophil counts in the toxic granulation (+) group were significantly higher than in the toxic granulation (-) group. However, more than half of the cases of the toxic granulation (+) group had normal or decreased neutrophil counts, and 15.9% of the toxic granulation (-) group had neutrophilia, which means that only a limited portion of patients whose neutrophils with toxic granulation have neutrophilia, and that many patients whose neutrophils show toxic granulation have normal neutrophil counts or are even neutropenic. Furthermore, some patients with neutrophilia had neutrophils without toxic granulation. These results show that the presence of toxic granulation is a separate marker of neutrophil abnormality.

The origin of toxic granules has been considered to be related to abnormal neutrophil maturation with persistence of azurophilic granules [18]. The azurophilic granules are formed during the promyelocytic stage and contain many anti-microbial compounds [13]. Normal bone marrow granulocyte maturation is associated with progressive decreases of azurophilic granule enzymes (myeloperoxidase, defensins, lysozyme, azurocidin, etc.), and acid mucosubstance. However, neutrophils with toxic granulation show abnormal maturation of azurophilic granules with persistence of the acid mucosubstance that causes intense staining with the Wright or Romanowsky stains [13,19]. Therefore, neutrophils with toxic granulation could contain more FAD than neutrophils without toxic granulation. The mean autofluorescence levels of neutrophils with and without toxic granulation were similar ($p > 0.05$), which means that FAD content differences between neutrophils with and without toxic granulation are undetectable without amplification. However, after neutrophils were incubated with bis(Zn^{2+}-dipicolylamine) complex (which enhances FAD fluorescence) for 15 min, fluorescence was significantly amplified in both groups, but more amplified in the toxic granulation (+) group. The MESF of neutrophils in the toxic granulation (+) and (-) groups were increased 71- and 19-fold, respectively ($p < 0.001$), which suggests that the neutrophils with toxic granulation have more FAD and that this is bound by bis(Zn^{2+}-dipicolylamine) complex to emit strong fluorescence. Using this amplification method, patients that have neutrophils with toxic granulation can be differentiated from those having neutrophils without toxic granulation with a sensitivity of >90% and a specificity of >80%. Both the absolute MESF values and the MESF ratios before and after incubation with bis(Zn^{2+}-dipicolylamine) complex appear to be very useful.

The MESF values of neutrophils of post-HSCT patients who had received G-CSF, without a bacterial infection, and whose neutrophils showed toxic granulation, were much higher than those of post-HSCT patients who had not received G-CSF, without a bacterial infection, and whose neutrophils did not have toxic granulation ($p < 0.01$). This finding suggests that G-CSF may induce toxic granules in neutrophils.

The MESF value of neutrophils after incubation with bis(Zn^{2+}-dipicolylamine) complex was not significantly correlated with the leukocyte or neutrophil counts ($p > 0.05$), which suggests that the fluorescence intensities of neutrophils after incubation with bis(Zn^{2+}-dipicolylamine) complex are a useful marker for the detection of neutrophils with toxic granulation.

In conclusion, measurement of FAD fluorescence intensity in neutrophils by flow cytometry after incubation with bis(Zn^{2+}-dipicolylamine) complex is proposed as an easy, objective, and reliable method of detecting neutrophils with toxic granulation in patients with infection and in patients being treated with G-CSF.

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