Ann Hematol (1999) 78:73–77

ORIGINAL ARTICLE

K. Hübel · K. Hegener · R. Schnell · G. Mansmann
F. Oberhäuser · P. Staib · V. Diehl · A. Engert

Suppressed neutrophil function as a risk factor for severe infection after cytotoxic chemotherapy in patients with acute nonlymphocytic leukemia

Received: July 3, 1998 / Accepted: September 9, 1998

Abstract Severe infections are a major problem in patients suffering from acute nonlymphocytic leukemia (ANLL) undergoing myeloablative chemotherapy. Possible factors leading to infectious complications in these patients are suppressed immune defense mechanisms existing prior to therapy, including those involving the neutrophil granulocyte department. In this study we investigated whether neutrophil function as measured by oxidative burst and phagocytosis before the start of treatment correlates with the severity of infection after therapy. Forty-four patients were included, 27 men and 17 women. Their median age was 46 years (range 20–70 years). According to the development of infectious complications the patients were assigned retrospectively to group 1 (no or only mild infections, $n=29$) or to group 2 (severe infection or death due to infection, $n=15$). The phagocytic activity was significantly reduced in group 2 as compared with group 1 [113.7±13.7 (SEM) vs 170.0±19.2, mean channel fluorescence; $p=0.04$]. In contrast, the oxidative burst as measured by FMLP stimulation was pronounced but not significantly enhanced in group 2 (24.8±6.1 vs 14.5±3.4, mean channel fluorescence). In conclusion, patients with severe infections after chemotherapy might already have preactivated neutrophils with suppressed function prior to treatment. Thus, evaluating function parameters could help to estimate the individual risk of infection for a patient with ANLL.

Key words Acute nonlymphocytic leukemia · Chemotherapy · Infection · Neutrophil function

K. Hübel · K. Hegener · R. Schnell · G. Mansmann
F. Oberhäuser · P. Staib · V. Diehl · A. Engert (✉)
Klinik I für Innere Medizin der Universitat zu Köln,
Joseph-Stelzmann-Strasse 9, D-50924 Köln, Germany
Tel.: 0049-221-478-5966, Fax: 0049-221-478-6733)

Introduction

Infections are a frequent cause of morbidity and mortality in patients with hematological malignancies undergoing chemotherapy. In acute leukemia, infections have been the major obstacle to treatment, since systemic therapies are required to effect a cure [9]. Many factoss predispose to infection in this patient population. Neutropenia is probably the most important [4], although not the only one. The risk of infection varies considerably depending on type and doses of drugs administered. Humoral and cellular immune mechanisms mediated by B and T lymphocytes, respectively, can be profoundly altered in patients with leukemia. Furthermore, the functional capabilities of phagocytes like neutrophils, eosinophils, and mononuclear cells, which play a fundamental role in the response to infection, may be intrinsically defective. The ability of polymorphnuclear neutrophils to engulf and digest pathogenic micro-organisms is essential in the early phase of infection-preventing mechanisms. Hence, in tests of neutrophil function, it is desirable to determine their phagocytic and killing activity [5, 14, 16].

The present study was intended to identify patients suffering from acute nonlymphocytic leukemia (ANLL) at risk for infection after chemotherapy. Therefore, we analyzed the phagocytic and bactericidal capacities of neutrophils before administration of cytotoxic agents and examined the correlation between depressed neutrophil function and infection after therapy.

Patients and methods

Patients

Eligible were patients aged 18–70 years who were undergoing myeloablative chemotherapy because of ANLL. The diagnosis had to be confirmed by bone marrow puncture. Patients were required to have an ECOG performance status of 0–2, no concur-

rent second malignancy, serum bilirubin and serum creatinine levels <3 times the upper limit of normal values and serum GPT, serum GOT, and serum AP <2.5 times the upper limit. Patients who received concurrent treatment with growth factors or other cytokines before evaluation of neutrophil function were excluded from the study.

Protocol design and objectives

This was an open-label, prospective, nonrandomized study. The objectives were to determine whether the neutrophil function parameters, oxidative burst and phagocytosis, measured before administration of chemotherapy correlated with the incidence and severity of post-treatment infectious complications. According to the extent of infections, patients were retrospectively assigned to group 1 or to group 2. Group 1 consisted of patients who developed no or only mild infections (low-risk patients). Patients with severe infections or those who died of infection were assigned to group 2 (high-risk patients). Detailed characteristics of each group are listed in Table 1.

All patients received oral prophylaxis consisting of trimethoprim-sulfamethoxazole and an amphotericin B suspension. In case of infection, full antibiotic treatment was given.

Chemotherapy

Patients with primary ANLL received induction therapy consisting of thioguanine (2×100 mg/m^2 per day per os, days 3–9), cytosine arabinoside (100 mg/m^2 per day i.v., days 1–8), and daunorubicin (60 mg/m^2 per day i.v., days 3–5), followed on day 21 by cytosine arabinoside (2×3 g/m^2 per day i.v., days 1–3) and mitoxantrone (10 mg/m^2 per day i.v., days 3–5). Patients with secondary or relapsed ANLL received a regimen consisting of fludarabine (25 mg/m^2 per day i.v., days 1–5), cytosine arabinoside (2×1 g/m^2 per day i.v., days 1–5), and idarubicin (8 mg/m^2 per day i.v., days 1, 3, 5). In this protocol, granulocyte colony-stimulating factor (G-CSF) was administered routinely from the beginning of chemotherapy until recovery of blood cells.

Table 1 Criterions of the two groups. Patients were retrospective assigned to one group according to their clinical course

group 1	"low risk patients" with
	no infection
	fever due to the leukemic process
	fever of unknown origin (FUO)
	fever <38.5 °C with proven infection
	local infections, e.g.
	viral upper respiratory infections
	pharyngitis
	tracheobronchitis
	otits media
	skin abscesses
	cellulitis
	urinary tract infections
group 2	"high risk patients" with
	fever >38.5 °C with proven infection
	organ infections, e.g.
	pneumonia
	pulmonary abscesses
	brain abscesses
	requiring ICU due to infection
	disseminated infection, septicemia
	patients who died because of infection

Experimental analyses

Evaluation of neutrophil function was performed immediately before the start of chemotherapy.

Measurement of the oxidative burst

Intracellular H_2O_2 release in neutrophils was measured by analyzing the oxidation of nonfluorescent 2,7,-dichlorofluorescein-diacetate (DCF-DA, Molecular Probes Europe, Leiden, Netherlands, 5 m*M* dissolved in ethanol) to the fluorescent 2,7,-dichlorofluorescein (DCF) using the methods of Bass et al. [3], with modifications. Briefly, 1 µl DCF-DA solution was added to 100 µl of freshly drawn citrated peripheral blood. Simultaneously, 10 µl FMLP (N-formyl-L-methionyl-L-leucyl-L-phenylalanine, Sigma Chemicals), dissolved in DMSO (10^{-2} *M*), PBS (10^{-4} *M*), and RPMI 1640 (Gibco Laboratories, Eggenstein, Germany, 10^{-5} *M*) was added (final concentration: 10^{-6} *M*). An aliquot sample without addition of FMLP was prepared as control and processed accordingly. The samples were incubated at 37 °C, 5% CO_2 for 30 min. Alter incubation, the tubes were placed in a Coulter Q-Prep Immunology Workstation (Coulter Inc., Krefeld, Germany). This station is a matched, three-reagent system. Reagent-A (formic acid 1.2 ml/l) lyses erythrocytes, reagent-B (sodium carbonate 6.0 g/l, sodium chloride 14.5 g/l, sodium sulfate 31.3 g/l) stabilizes leukocytes, and reagent-C (paraformaldehyde 10.0 g/l) is a cell membrane fixative. Finally, the samples were washed four times with PBS to remove untrapped DCF-DA and then analyzed by flow cytometry (FAC Scan, 488-nm argon laser, Becton Dickinson Immunocytometry Systems, Heidelberg, Germany). Data of neutrophils were acquired using selective gating based on light-scatter properties (forward light scatter and side scatter) and peak channel of log intensity of DCF fluorescence was measured by automated flow-cytometric cell analysis (Cell Quest, Becton Dickinson). The difference in the mean channel fluorescence of the stimulated probe, and the control probe was taken as a surrogate marker for the oxidative burst activity of neutrophils.

Measurement of phagocytic activity

For determination of the phagocytic activity, albumin-coated fluorescent latex particles were incubated with blood cells and the mean channel fluorescence of the neutrophils was analyzed by flow cytometry. The albumin-coated latex beads were prepared as follows: 0.5 ml of 2.5% green-fluorescent latex beads (Fluorescent Carboxylated Microsheres, nominal mean diameter; 1.0 µm, Polyscience Ltd., Eppelheim, Germany) were washed twice in 0.1 *M* carbonate buffer (pH 9.6), then resuspended and washed three times in 0.02 *M* sodium phosphate buffer (pH 4.5). Following the addition of freshly prepared 2% carbodiimide hydrochloride (Merck-Schuchardt, Hohenbrunn, Germany) dissolved in phosphate buffer, the beads were mixed for 3–4 h at room temperature using an end-to-end mixer (Heidolph Reax 2, Kelheim, Germany). The cells were then washed three times in phosphate buffer to remove unreacted carbodiimide and resuspended in 0.2 *M* borate buffer pH 8.5). For coupling, 400 µg albumin (BSA) was added and mixed gently overnight at room temperature with constant stirring. To block the unreacted sites on the microparticles, 0.25 *M* ethanolamine was added, mixed for 30 min, and removed by centrifligation. The pellet was resuspended in 10 mg/ml BSA solution dissolved in borate buffer and mixed for 30 min at room temperature. This step was repeated once, and the resuspended beads were stored in a buffer containing PBS with 10 mg/ml BSA, 5% glycerol, and 0.1% NaN_3 (pH 7.4).

Phagocytosis was measured according to Andoh et al. [1]. A 2.5-µl suspension of fluorescent latex particles was sonicated and added to 100 µl of citrated blood. Simultaneously with the phagocytic particles, 10 µl FMLP (preparation as described above) was added; a control tube without FMLP was also assayed to assess the internalization of latex beads, samples were incubated at

37 °C, 5% CO_2 for 30 min. Alter incubation, the tubes were processed in the Q-Prep as described above. Prior to analysis by flow cytometry, the samples were washed four times to remove noninternalized latex beads. The difference between the mean channel fluorescence of the stimulated probe and that of the control probe was determined by selective gating of the cells and calculated automatically by the flow-cytometric program (see above).

Statistical analyses

Data are displayed as mean plus standard error. Statistical significance of differences between means was evaluated using Student's *t*-test for independent samples.

Results

Patient characteristics

The characteristics of the 44 patients are shown in Table 2. Twenty-nine patients were assigned to group 1, 15 patients to group 2. The median age was 45 years in group 1 and 47 years in group 2. For one patient in group 1 the neutrophil assays were performed two times because ANLL relapsed after complete remission had been achieved. Overall, 31 patients had a first diagnosis of ANLL, five had relapsed disease, and eight were treated in complete remission. The majority of patients had M2 leukemia according to the French-American-British (FAB) Group, followed by M4 (eight patients) and MS (eight patients) leukemia. There was no M3 leukemia. Each group had three patients with secondary ANLL.

Table 2 Demographic and clinical characteristics of the patients in the two groups

	group 1	group 2
no. of patients	29	15
no. of tests performed	30	15
median age (years)	45	47
range	20–70	22–69
sex		
male	17	10
female	12	5
untreated ANLL	22	9
relapsed ANLL	3	2
ANLL-complete remission	4	4
primary ANLL	26	12
secondary ANLL	3	3
FAB M1	3	0
FAB M2	11	5
FAB M3	0	0
FAB M4	5	3
FAB M5	4	4
FAB M6	0	0
FAB M7	1	0

Hematological characteristics

Prior to the start of chemotherapy, there were no significant differences in leukocyte count or hemoglobin level between patients in group 1 and those in group 2. However, patients in group 1 had significantly higher thrombocyte values as compared with patients in group 2 (93.600/µl ± 15.000/41 vs 33.300/µ1 ± 6.400/µl; $p = 0.02$). More information is listed in Table 3.

Infections

In any case of fever or suspected infection, blood cultures were examined. In group 2 we found three patients with coagulase-negative *Staphylococcus* infections. One patient had an *Acinetobacter* infection, one patient was infected with *Streptococcus viridans.* We had one patient who died of pneumonia. In this patient, *Aspergillis* was detected by bronchoscopy. In total, five patients died of septicemia. In group 1, the main microorganisms detected by blood culture, bronchoscopy, or urine analyses were *Streptococcus salivarius; Pseudomonas aeruginosa, Escherichia coli, Enterococcus,* and *Candida.* For more details see Table 4.

In group 1, the mean duration of i.v. antibiotic treatment was 19.1 ± 2.0 days, whereas in group 2 it was 27.1 ± 3.4 days. This difference is statistically significant ($p = 0.04$).

Neutrophil function tests

Figures 1 and 2 summarize the results of the neutrophil function tests in group 1 and in group 2. There is a significant difference in neutrophil function as measured by phagocytosis when group 1 is compared with group 2 (170.0 ± 19.2 vs 113.7 ± 13.7, mean channel fluorescence; $p = 0.04$). In contrast, the FMLP-stimulated oxidative burst was more pronounced, though statistically not significantly enhanced, in group 2 (14.5 ± 3.4 vs 24.8 ± 6.1, mean channel flourescence). We concluded that this phenomenon might be attributable to latent infection prior to chemotherapy in patients of group 2.

Table 3 Hematological characteristics before chemotherapy. Data are expressed as mean ± standard error. The difference in thrombocytes between group 1 and group 2 is statistically significant ($p = 0.02$)

	group 1	group 2
Leukocytes (/µl)	18.200 ± 4.900	16.700 ± 8.800
Thrombocytes (/µl)	93.600 ± 15.000	33.300 ± 6.400
Hemoglobin (g/dl)	9.7 ± 0.6	8.8 ± 0.5

Table 4 Microorganisms causing infections in group 1-/group 2-patients and the site of detection

	group 1	group 2
blood culture		
Staphylococcus, coagulase negative		3
Acinetobacter		1
Streptococcus viridans		1
Streptococcus salivarius	1	
Pseudomonas aeruginosa	1	
E. coli	1	
bronchoscopy/lavage		
Aspergillus		1
Enterococcus	1	
Bacteroides	1	
Candida	2	
urine		
Enterococcus		2
Pseudomonas aeruginosa	1	
Candida	1	
central venous catheter		
Staphylococcus, coagulase negative	1	
Corynebacterium	1	

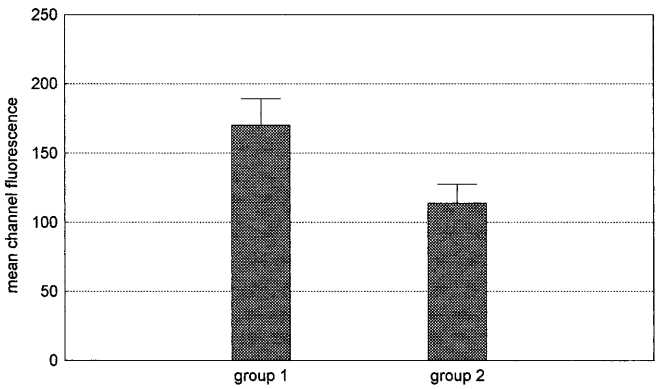

Fig. 1. Comparison of group 1 with group 2 concerning the average phagocytosis capacity of one neutrophil. Difference is statistically significant ($p = 0.04$). Data are expressed as mean plus standard error

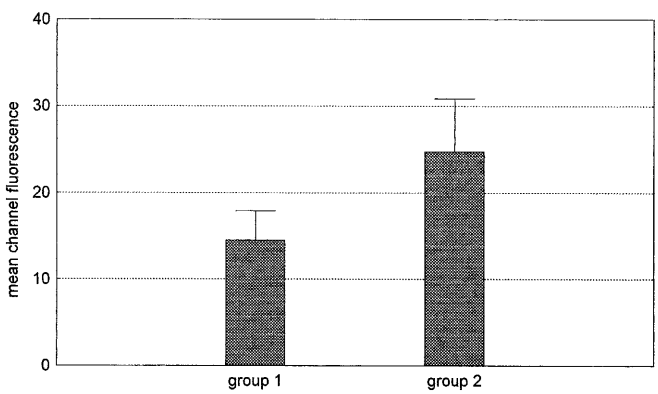

Fig. 2. Comparison of group 1 with group 2 concerning the FMLP-stimulated oxidative burst. Data are expressed as mean plus standard error

Discussion

The growing intensity of acute leukemia treatment has led to an increase in the incidence of severe infections, which currently play an important role in the morbidity and mortality of this patient group. Approximately two thirds of treatment-associated deaths are caused by infections [6]. The micro-organisms that are most frequent causes of infection after chemotherapy are fungi such as *Aspergillus* sp., aerobic gram-negative bacilli, especially *E. coli, Klebsiella* sp., *Enterobacter* sp., *Serratia* sp., *Proteus* sp., *P. aeroginosa;* and gram-positive bacteria, such as *Staphylococcus aureus, Staphylococcus epidermitis,* and *Streptococcus viridans* [11, 13]. These findings are comparable to the results in our cohort. However, the underlying micro-organism was identified in only 15 patients.

The high rate of infection has usually been ascribed to neutropenia. However, dysfunction of the remaining neutrophils can be an additional promoting factor, making the infection complicated and serious [16]. Neutrophils have two important roles in the immune response: to ingest and to kill invading organisms. Using flow cytometry, it is relatively easy to determine the phagocytic activity of the cell with fluorescent latex beads and to measure the oxidative burst by incorporation of DCF-DA. As to the gating of neutrophils on the cytogram, when peripheral white cells contain a large number of immature cells such as blasts, it is possible to gate mature cells separately from immature cells in terms of different dimensions of forward scatter (number of cells) and side scatter (granularity of cells) on flow cytometry. This differentiation of blasts and mature neutrophils by flow cytometry is also used by other investigators [12].

The phagocytic and bactericidal capacities of neutrophils in leukemia have been examined repeatedly by a large number of investigators. Tsurumi et al. analyzed the hydroperoxide production by peripheral neutrophils in various hematological diseases [12]. Sixteen patients had ANLL; nine of these patients had not received antileukemic agents prior to study inclusion, and the remaining seven were examined during complete remission induced by chemotherapy. Interestingly, in untreated leukemia, the H_2O_2 production was reduced, while in the complete remission stage of the disease the level was normal, suggesting recovery from normal clones. Kubo and colleagues examined phagocytic and bactericidal capacities and generation of oxygen radicals by neutrophils in patients with ANLL [8]. In untreated patients, the neutrophils showed a significant decrease of hydroxyl radical generation and bactericidal capacity, whereas the superoxide anion release did not differ from that of a control cohort. Yamamoto et al. investigated the phagocytic and bactericidal activities of the circulating neutrophils from 13 patients with acute myelogenous leukemia [16]. They found a significantly impaired bactericidal capacity of the neutrophils,

whereas the phagocytic activity did not differ from that in normal controls, indicating that the ingestion of bacteria had proceeded normally in the neutrophils from patients with acute myelogenous leukemia. Unfortunately, their values showed a wide deviation.

In our study, we found a significantly suppressed phagocytic capacity of the neutrophils before treatment in those patients who developed severe infectious complications (group 2) as compared with patients with no or only mild infections after chemotherapy (group 1). Furthermore, the FMLP-stimulated oxidative burst was enhanced in group 2. An increased burst as a response to a less potent stimulus like FMLP can be explained as preactivation of the neutrophils, which is often found in inflammatory diseases [10]. In conclusion, our data show a correlation between reduced neutrophil function and severe infection after therapy in patients with ANLL. We showed that the investigation of neutrophil function before the beginning of chemotherapy might identify patients with a high risk of infection. Consequently, there is a high interest in approaches to infection prophylaxis for these patients.

For several years, recombinant human granulocyte colony-stimulating factor (rHuG-CSF) has been used in clinical hematology, and it is now routinely employed to treat chemotherapy-induced neutropenia, since it can significantly shorten the duration of neutropenia [2]. However, despite the body of information available on the use of G-CSF in neutropenic patients, there are very few data on its prophylactic applications. Weiss et al. reported a small study using G-CSF prophylaxis in a heterogeneous group of high-risk intensive care patients [15]. The continuous infusion of 1 μg/kg per day G-CSF (filgrastim) for 4 days and 0.5 μg/kg per day for another 5 days resulted in a significant increase in the neutrophil oxygen radical production. In contrast to three of ten patients in an untreated control group, none of ten patients in the G-CSF-treated group developed sepsis. In our department, we investigated the effects of prophylactic administration of G-CSF on the function of neutrophils and the rate of infectious complications in patients with esophageal cancer undergoing esophagectomy [7]. Neutrophil function was significantly enhanced during the G-CSF treatment, whereas the infection rate after surgery was significantly reduced in the study patients compared with a control group. Despite these promising data, however, the prophylactic use of G-CSF in hematological disorders needs to be evaluated in clinical studies.

In conclusion, we have demonstrated that ANLL patients with suppressed neutrophil function prior to treatment are predisposed to developing severe infections after chemotherapy. Reflecting the growing intensity of cytotoxic protocols, e.g., the administration of high-dose chemotherapy prior to bone marrow or stem cell transplantation, our results could provide a chance to estimate the individual risk of infection for a patient.

References

1. Andoh A, Fujiyama Y, Kitoh K, Hodohara K, Bamba T, Hosoda 5 (1991) Flow cytometric assay for phagocytosis of human monocytes mediated via Fcg-receptors and complement receptor CR1 (CD 35). Cytometry 12:677–686
2. Bacigalupo A (1994) The clinical benefits of recombinant human granulocyte colony-stimulating factor in the treatment of cancer patients. Eur J Cancer 30A [Suppl 3]: S26–29
3. Bass DA, Parce JW, Dechatelet LR, Szejda P, Seeds MC, Thomas M (1983) Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. J Immunol 130:1910–1917
4. Bodey GP (1986) Infection in cancer patients. Am J Med 81 [Suppl lA]: 11–25
5. Clarkson B, Strife A (1993) Linkage of proliferative and maturational abnormalities in chronic myelogenous leukemia and relevance to treatment. Leukemia 7:1683–1721
6. Estey EH, Keating MJ, McCredie KB, Bodey GP, Freireich EJ (1982) Causes of initial remission induction failure in acute myelogenous leukemia. Blood 60:309–315
7. Hübel K, Schäfer H, Hölscher AH, Diehl V, Engert A (1997) Perioperative treatment with granulocyte-colony stimulating factor (G-CSF) in patients with esophageal cancer enhances neutrophil function and reduces infectious complications after esophagectomy. Proc Am Soc Clin Oncol 16:395
8. Kubo A, Sasada M, Yamamoto K, Nishiyama H, Nishimura T, Ando S, Nakamura T, Uchino H (1985) Neutrophil function in leukemia patients – the correlation between bactericidal capacity and generation of oxygen radicals, especially hydroxyl radical. Acta Haematol Jpn 48:1381–1389
9. Ohno R, Tomonaga M, Kobayashi T, Kanamaru A, Shirakawa S, Masaoka T, Omine M, Oh H, Nomura T, Sakai Y, Hirano M, Yokomaku S, Nakayama S, Yoshida Y, Miura AB, Morishima Y, Dohy H, Niho Y, Hamajima N, Takaku F (1990) Effect of granulocyte colony-stimulating factor after intensive induction therapy in relapsed or refractory acute leukemia. N Engl J Med 323:871–877
10. Rothe G, Kellermann W, Schaerer B, Valet G (1994) Measurement of phagocytic hydrogen peroxide production of granulocytes and monocytes with dihydrorhodamine 123. In: Schmitz G, Rothe G (eds) Flow cytometry in the clinical cell diagnosis. Schattauer, Stuttgart, pp 331–350
11. Talbot GH, Provencher M, Cassileth PA (1988) Persistent fever after recovery from granulocytopenia in acute leukemia. Arch Intern Med 14:129–135
12. Tsurumi H, Shimazaki M, Takahashi T, Moriwaki H, Muto Y (1993) Flow cytometric determination of active oxygen (hydroperoxide) produced by peripheral blood neutrophils in patients with hematological disorders. Int J Hematol 57:213–219
13. Verhoef J (1993) Prevention of infections in the neutropenic patient. Clin Infect Dis 17 [Suppl 2]: S359–367
14. Wandall JU (1988) Neutrophilic granulocyte function. Quantitative leukocyte mobilization and function of circulating and exudative neutrophils. Dan Med Bull 35:237–252
15. Weiss M, Gross-Weege W, Schneider M, Neidhardt H, Liebert S, Mirow N, Wernet P (1995) Enhancement of neutrophil function by in vivo filgrastim treatment for prophylaxis of sepsis in surgical intensive care patients. J Crit Care 10:21–26
16. Yamamoto K, Sasada M, Nishiyama H, Uchida M, Sawada H, Nakamura T, Maekawa N, Uchino H (1982) Ingestion and killing of bacteria by neutrophils from patients with leukemia – a new method for evaluating phagocyte function at short incubation periods. Acta Haematol Jpn 45:56–65