Haematologica 1999; 84:114-118 original paper

Fusarium infections in patients with severe aplastic anemia: review and implications for management

CORRADO GIRMENIA, ANNA PAOLA IORI, FEDERICA BOECKLIN, ANTONELLA TOROSANTUCCI,* PAOLA CHIANI,* PAOLO DE FABRITIIS, FABRIZIO TAGLIETTI, ANTONIO CASSONE,* PIETRO MARTINO
*Dipartimento di Biotecnologie Cellulari ed Ematologia, University "La Sapienza", and *Department of Bacteriology and Medical Mycology, Istituto Superiore di Sanità, Rome, Italy*

ABSTRACT

Background and Objective. The prognosis of severe fungal infections, such as fusarium infections, in patients with aplastic anemia is directly related to the recovery of bone marrow functions. In this study, *in vitro* anti-*Fusarium* activity of granulocytes was investigated, the case of disseminated infection in a child with very severe aplastic anemia is reported, and implications for management of such infective complications are discussed.

Design and Methods. The *in vitro* efficiency of PMNL from three untreated, normal blood donors and from two G-CSF-treated WBC donors in contrasting the growth of the *Fusarium sp* strain isolated from the patient we present was measured by a ^{3}H-glucose uptake inhibition assay and confirmed by microscopic examination.

Results. Basic growth inhibitory activity of unstimulated PMNL on *Fusarium* cells was significantly enhanced in the presence of GM-CSF in all three blood donors tested. In one of the two G-CSF-treated donors, *in vitro* efficiency of PMNL in contrasting the growth of the fungus increased notably after G-CSF treatment. We report the case of a 3-year-old girl with very severe aplastic anemia unresponsive to conventional immunosuppressant therapy who developed a disseminated fusarium infection. The child initially responded to liposomal amphotericin B and granulocyte transfusions from G-CSF stimulated donors. Subsequently she was given a cord blood stem cell transplantation but died of disseminated infection.

Interpretation and Conclusions. Including the present case, there are only ten reports of invasive infections caused by the genus Fusarium in aplastic anemia patients and only two of the patients survived. *In vitro* data seem to suggest that *in vivo* treatment with rh-G-CSF could have a stimulatory effect on the anti-Fusarium activity of neutrophils. Despite the efficacy of granulocyte transfusions by G-CSF-stimulated donors in the temporary control of fusarium infection, treatment of the underlying hematologic disease is required to cure the infection in patients with severe aplastic anemia. Granulocyte transfusions by G-CSF-stimulated donors while awaiting bone marrow recovery following the blood stem cell transplant should be considered.

Correspondence: Corrado Girmenia M.D., Dipartimento di Biotecnologie Cellulari ed Ematologia, University "La Sapienza", via Benevento 6, 00161 Rome, Italy.
Phone: international +39-06-857951 – Fax: international +39-06-44241984.

Key words: aplastic anemia, fusariosis, granulocyte transfusions, G-CSF, transplantation

Fusarium is a pathogen which is emerging as a cause of invasive infections in immunocompromized patients, particularly those with severe and prolonged neutropenia.[1,2] Amphotericin B therapy has no significant activity on the control of invasive fusarium infection while the patient is still neutropenic and recovery of bone marrow function is the most important factor predicting the outcome.[1,2]

In this report, we describe a case of disseminated fusariosis in a patient with very severe aplastic anemia (SAA) who initially responded to liposomal amphotericin B associated with transfusions of granulocytes obtained from volunteer donors pre-treated wíth recombinat human granulocyte colony stimulating factors (rhG-CSF) and subsequently underwent unsuccessful cord blood transplant.

We also measured *in vitro* efficiency of granulocytes, under different conditions, in contrasting the growth of the *Fusarium sp.* strain isolated from the patient.

We also give a review of the literature on invasive *Fusarium* infections in patients with aplastic anemia.

Design and Methods

Granulocyte collections

Fifteen normal adults (10 males, 5 females; nine were relatives of the patient) volunteered to be granulocyte donors following the administration of rh-G-CSF. Informed consent for rh-G-CSF administration and granulocyte donation was obtained using a protocol approved by our local institutional review board. The dose of rh-G-CSF for all donors was 300 µg/day, starting between 12 and 18 hours before the first leukapheresis until the last leukapheresis. Three

granulocyte collections (one every other day), were taken from twelve donors, a single collection was taken from the other three donors. Granulocyte collections were performed with an automated, continuous-flow blood cell separator (Cobe Spectra, Englewood, CO, USA), processing 6 to 9 L of blood during 150 to 220 minute periods. Each leukapheresis product was irradiated with 30 Gy prior to infusion. Granulocyte concentrates were infused within 6 hours of being collected.

Granulocyte functional studies

Preparations of purified polymorphonuclear leukocytes (PMNL) were obtained from heparinized venous blood samples from three untreated, normal blood donors and from two G-CSF-treated granulocyte donors. Two blood samples were taken from the G-CSF donors, one immediately before rh-G-CSF administration and one 24 hous after. PMNL were isolated by density gradient centrifugation in Lymphoprep solution (Nyegard, Oslo, Norway), as described elsewhere.[3] Microscopic examination of Giemsa-stained preparations showed that cells isolated by this procedure were > 99% PMNL as judged by morphologic criteria.

The strain of *Fusarium sp.* isolated from our patient was used as the target for PMNL functional *in vitro* testing. Fungal cultures were routinely maintained on Sabouraud agar plates at 28°C. *Fusarium* conidia were gently resuspended from the surface of 48h-old cultures, counted in an hemocytometer and diluted to the desired concentration (5×10^4/mL) in assay medium (see below).

The antifungal activity of PMNL was measured by a growth inhibition assay as described elsewhere.[4] Briefly, PMNL were suspended at different cell densities (5×10^6 to 10^5/mL, in order to obtain the final PMNL/fungal cells ratios of 100:1, 50:1 and 10:1) in complete assay medium [RPMI 1640 supplemented with 25 mM HEPES, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 5% heat-inactivated fetal calf serum (Gibco)] and placed (50 µL/well) into triplicate wells of a 96-well microplate. Fungal cell suspensions (50 µL) were added to each well, and co-cultures were incubated at 37°C in a 5% CO_2 atmosphere for 48 h. Control triplicate wells with Fusarium cells in the absence of PMNL were also included in the experiment. GM-CSF (R&D System,MN,USA) was added, when specified, at a final concentration of 10 U/mL for PMNL activation. At the end of the incubation period, fungal growth was monitored microscopically and PMNL were lysed by the addition of H_2O. The fungal cells were labeled with 5 µCi of D-(5,6) ^{3}H-glucose (specific activity 70 Ci/mmol; Du Pont-NEN, Boston, Mass., USA) and incubated for three additional hours at 37°C. Finally, the fungal cells were harvested from the plates and counted in a β-counter (Betaplate, LKB, Bromma, Sweden). Values of ^{3}H-glucose incorporation by fungal cells were taken as a measure of fungal growth and percent growth inhibition was calculated by comparing label incorporation by fungal cultures grown in the presence of PMNL against incorporation into control cultures without PMNL. Inhibition units (IU)/10^7 PMNL were calculated on the basis of the different E:T ratios used. One inhibition unit is defined as the number of PMNL causing 20% growth inhibition of the fungal cells.

Results

Granulocvte collections

Following the administration of rh-G-CSF there was a rapid increase in peripheral WBC count. The majority of cells were mature neutrophils, although most of the donors had some myelocytes by day 4 after the start of the rh-G-CSF administration. The median numbers of peripheral WBC reached on days 2, 4 and 6 of rh-G-CSF administration were 29,500/µL (range 16,000-37,500), 32,400/µL (range 25,400-49,100) and 36,000/µL (range 26,600-58,700), respectively. The increase in circulating WBC levels resulted in the collection of larger concentrations of nucleated cells (a median of 99,000/µL, range 56,000-157,000 on day 2; a median of 150,000/µL, range 57,500-174,500 on day 4; a median of 193,000/µL, range 65,000-261,000 on day 6) and in an increased yield of granulocytes (a median of 2.7×10^{10}, range 0.8-6.6 on day 2; a median of 4.6×10^{10}, range 1.6-6.7 on day 4; a median of 6.2×10^{10}, range 1.9-10 on day 6). The patient received a mean of 2.1×10^9 cells/kg/day.

In vitro anti-Fusarium activity by granulocytes from normal and rh-G-CSF-treated subjects

In preliminary experiments, we investigated the effect of GM-CSF stimulation on *in vitro* anti-*Fusarium* activity of PMNL from three normal subjects. Basic growth inhibitory activity on *Fusarium* cells of unstimulated PMNL was variable, depending on the subject (Figure 1). However, in all three donors tested, inhibition was significantly enhanced in the presence of GM-CSF, with inhibition unit values being 2-10 times higher than those observed for the unstimulated control PMNL.

We, therefore, attempted to verify whether *in vivo* G-CSF administration could similarly activate PMNL anti-*Fusarium* functions. To this aim, we compared the activity of PMNL isolated from volunteer granulocyte donors immediately before and 24 hours after G-CSF administration.

Two subjects were assessed for PMNL activity after G-CSF infusion (Figure 2): in one of them, the basal, unstimulated antifungal activity was not increased by G-CSF treatment, since PMNL isolated before and after G-CSF administration showed comparable anti-*Fusarium* activity. In contrast, in the second subject studied, anti-*Fusarium* activity increased notably after G-CSF treatment (100 IU/10^7 versus 880 IU/10^7 for PMNL cultures tested before and after G-CSF injection,

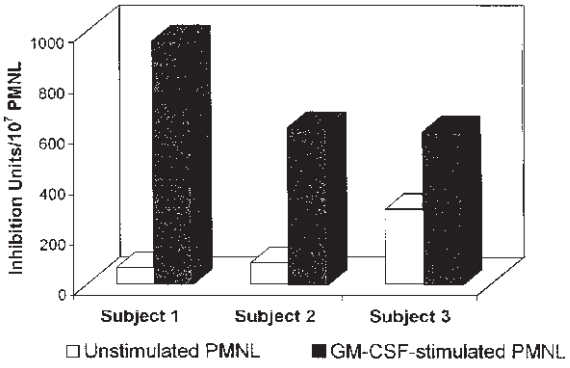

Figure 1. In vitro anti-Fusarium growth inhibitory activity by human granulocytes is increased by GM-CSF stimulation. PMNL were co-cultured (48 h, 37°C, 5% CO_2) with Fusarium conidial cells, at different effector:target ratios. Where specified, GM-CSF (10 U/mL) was added to the co-cultures for PMNL stimulation: Inhibition of fungal growth was evaluated by a ^{3}H-glucose uptake inhibition assay, as described in the Methods section, and confirmed by microscopic examination. Inhibition units were calculated by comparing label incorporation by Fusarium cultures grown in the presence of PMNL and control cultures without PMNL.

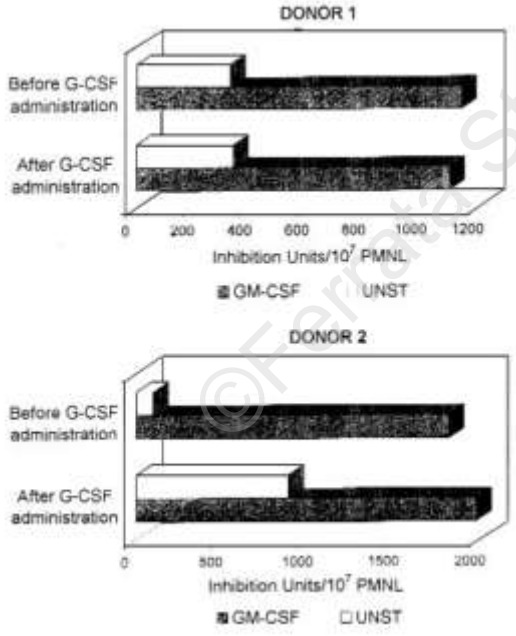

Figure 2. Effect of in vivo administration of G-CSF on the anti-Fusarium activity of granulocytes. Two voluntary granulocyte donations were assayed for their PMNL anti-Fusarium activity immediately before and 24 hours after G-CSF infusion: PMNL cultures from the two donations were tested either unstimulated (UNST) or stimulated in vitro by the addition of GM-CSF, 10 U/mL (G-CSF). Growth-inhibitory activity of PMNL was evaluated as described in the Design and Methods section and in the legend of Figure 1.

respectively). However, in both subjects, PMNL isolated either before or after *in vivo* G-CSF administration maintained a comparable responsiveness to a subsequent *in vitro* stimulation with GM-CSF itself, suggesting that in no case had *in vivo* G-CSF brought granulocytes to their maximal stimulation levels.

Case Report

A 3-year-old girl was diagnosed as having very severe SAA in May 1995. She was unsuccessfully treated with a 4 month regimen of cyclosporine, antilymphocyte serum, methylprednisolone and rh-G-CSF. In the absence of a histocompatible family member, an unrelated bone marrow and cord blood donor search was initiated and a class I serological and class II molecularly mismatched cord blood unit was identified in the New York Blood Center. In January 1996 the patient developed pyrexia and disseminated, nonpruriginous, painful, erythematous, maculopapular skin lesions. A skin biopsy was performed and a *Fusarium* infection was histologically and microbiologically documented. An *in vitro* antifungal susceptibility test to amphotericin B showed a minimal inhibítory concentration of 2 µg/mL. Amphotericin B therapy was started but patient continued to be febrile and developed new skin lesions and renal failure. After two weeks amphotericin B therapy was substituted with liposomal amphotericin B (AmBisome) at a dose of 6 mg/kg/day and granulocyte transfusions (mean 2.1 $\times 10^9$ cells/kg/day) from rh-G-CSF-stimulated donors were administered (a total of 39 transfusions over 52 days). The number of peripheral neutrophils rapidly increased, remaining within a range from 100 to 500 PMNL/mm^3 and the patient's clinical condition improved (drop in body temperature and decrease in the number and size of the skin lesions). On February 19th, she underwent a cord blood stem cell transplantation. The transplant conditioning regimen consisted of cyclophosphamide 50 mg/kg/day for 4 days, antilymphocyte serum (Lymphoglobulin, Merieux, France) from day –5 to day –1, and methylprednisolone (2 mg/kg/day). This regimen was chosen in view of its high immunosuppressant activity, with the aim of obtaining autologous bone marrow reconstitution in the event of failure of the cord blood stem cells to engraft. Prophylaxis for GVHD consisted of cyclosporine (3 mg/kg/day) by continuous intravenous infusion from day –1. Three weeks after the start of granulocyte transfusions, the patient developed a high fever, chill and severe hypertension during granulocyte transfusions and her peripheral neutrophil count decreased significantly. Persistent pancytopenia and bone marrow examination on days +16 and +25 after cord blood infusion demonstrated lack of engraftment. The patient continued to receive high dose liposomal amphotericin B and daily granulocyte transfusions. Nonetheless, she developed new skin and multiple pulmonary lesions and died on day +40 from cord blood transplantation.

Discussion

Of 79 evaluable cases of deep-seated *Fusarium* infections that we previously reviewed,[2] only 28% survived with apparent resolution of the infection, and the improvement in 86% was associated with recovery of bone marrow function. Seventy-six percent of patients who recovered from neutropenia survived, as opposed to only 6% of patients whose neutropenia persisted. Because of the high mortality rate of disseminated fusarium infection in the setting of persistent, profound neutropenia, granulocyte transfusions by rh-G-CSF-stimulated donors in association with amphotericin B treatment have been recommended in patients with hematologic malignancies in an attempt to control the infection and *buy time* until spontaneous recovery from myelosuppression occurs.[1]

In agreement with previous observations,[1,5] the clinical evidence presented here shows that the rh-G-CSF-stimulated granulocyte transfusions were effective in the initial control of disseminated fusarium infection. The high number of granulocytes collected from rh-G-CSF-stimulated donors seems to be of primary importance in the efficiency of this procedure. There is, however, some evidence that rh-G-CSF can also directly modulate the antimicrobial efficiency of PMNL *in vivo*.[4,6,7] We attempted to verify whether G-CSF administration *in vivo* could enhance PMNL anti-*Fusarium* functions and whether PMNL from rh-G-CSF-treated subjects might retain their possible preactivated status at the time of WBC donation. In agreement with recent data by Liles *et al*.[7] who observed increased activity of PMNL isolated from blood of 3 normal human volunteers after administration of rh-G-CSF *in vivo* against *Aspergillus fumigatus* and *Rhizopus arrhizus*, our preliminary findings suggest that *in vivo* treatment with rh-G-CSF could also have a stimulatory effect on the anti-*Fusarium* activity of PMNL. In fact, in one of the two subjects we studied, anti-*Fusarium* activity by PMNL increased notably after rh-G-CSF treatment, compared to that measured in the same subject before rh-G-CSF administration. Due to the very small number of subjects studied, the possibility that *in vivo* treatment with rh-G-CSF has a stimulatory effect on the anti-*Fusarium* activity of PMNL, though fully plausible, should be confirmed.

To our knowledge, including the present case, only ten invasive infections caused by the *Fusarium* genus have been reported in patients with SAA.[2,8-14] Table 1 summarizes the clinical features of these cases. All but two patients died due to disseminated infection. Of the two patients who survived, one patient with sinonasal and skin involvement received rh-G-CSF at the first evidence of *Fusarium* infection obtaining a lasting rise in the neutrophil count and cure of the mycosis;[2] in the second case a localized sinonasal fusariosis was cured by surgical excision.[14]

While duration of chemotherapy-induced neutropenia is limited in cancer diseases, SAA is characterized by prolonged neutropenia lasting for months or even years and this different course of neutropenia seems to be a determinant prognostic factor.[15] The development of alloimmunization in the absence of available HLA-matched donors is a major problem following multiple granulocyte transfusions and seems to contribute to the loss of activity of prolonged procedures.[16] Consequently, the provision of granulocytes by transfusion could lead to only limited control of the infection. In view of the above, the resolution of severe fungal infections, such as those caused by *Fusar-*

Table 1. Summary of published cases of invasive fusarium infections in patients with aplastic anemia.

Ref.	*Sex/age*	*Previous therapy for SAA*	*Documented sites of infection*	*Organism isolated*	*Treatment*	*Outcome*
2*	F/34	CSA	Lung, heart, liver, spleen, kidney,gut	*Fusarium sp*	AmB	Died
2*	M/29	CSA, PDN, ALG	Paranasal sinus, skin	*Fusarium sp*	AmB, G-CSF	Survived
8	F/66	Androgens, PDN	Esophagus, liver, spleen, cecum	*F. oxysporum*	no	Died
9	M/46	BMT	Skin, lungs, spleen, kidneys, testes, lymph nodes	*F. solani*	AmB, ketoconazole	Died
10	M/50	No	Skin, lung	*Fusarium sp*	AmB, miconazole	Died
11*	M/43	ALG	Nail, skin, blood	*F. solani*	AmB	Died
12	F/15	Unreported	Paranasal sinus, skin	*Fusarium sp*	no	Died
13	M/49	BMT	Skin, lungs, kidneys, esophagus, heart	*F. oxysporum*	AmB, itraconazole	Died
14	F/40	ALG	Sinonasal infection	*F. chlamydosporum*	AmB, itraconazole, surgical excision	Survived
Present case*	F/3	CSA, ALG, G-CSF, PDN	Skin, lungs	*Fusarium sp*	Liposomal AmB, WBC transfusions from G-CSF stimulated donors	Died

*ALG = antilymphocyte globulin, CSA = cyclosporin-A, PDN = prednisone, AmB = amphotericin B. * observed at the Dipartimento di Biotecnologie Cellulari ed Ematologia, University "La Sapienza", Rome, Italy.*

ium spp, in neutropenic patients depends on the recovery of peripheral neutrophil count , which, in patients with SAA, can only be obtained by curing the underlying disease.

The case of a patient with SAA and suspected fungal infection cured by receiving rh-G-CSF-stimulated granulocyte transfusions while awaiting bone marrow recovery following matched unrelated bone marrow transplantation was recently reported.[17] In this experience, the high risk transplantation procedure was the only chance of curing both the underlying hematologic disease and the infective complication. Unfortunately, mismatched cord blood transplantation was not able to cure our patient.

In conclusion, the prognosis of patients with SAA complicated by a fungal infection resistant to antifungal therapy, such as disseminated fusarium infection, is nearly always fatal. As shown by the clinical course of our patient and by *in vitro* evidence, PMNL transfusions from G-CSF-stimulated donors might be able to control the severe infection but the prognosis of the mycosis seems to be directly related to the rise of endogenous peripheral PMNL, i.e. to the cure of the underlying hematologic disease. In our opinion, in patients who do not respond to conventional immunosuppressant therapy and who have an invasive fungal infection resistant to antifungal therapy, such as Fusarium infection, bone marrow or other blood stem cell transplantation should be considered despite the limited chances of success.

Contributions and Acknowledgments

CG, AC and PM formulated the design of the study and wrote the paper. API and FB were responsible for clinical assessment and data handling. FT collected the clinical data. PD was responsible for the aphereses (collection/manipulation of peripheral white blood cells). AT, PC and AC were responsible for the in vitro studies.

Funding

This study was supported by grants from the Italian National Research Council (CNR), ACRO Project (Applicazioni Cliniche nella Ricerca Oncologica) (920221939) and National Research Council, Special Project FAT.MA. (Fattori di Malattia) (94.00683.41).

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received July 17, 1998; accepted October 26, 1998.

References

1. Boutati EI, Anaissie EJ. *Fusarium*, a significant emerging pathogen in patients with hematologic malignancy: ten years' experience at a cancer center and implications for management. Blood 1997; 90:999-1008.
2. Martino P, Gastaldi R, Raccah R, Girmenia C. Clinical patterns of *Fusarium* infections in immunocompromised patients. J Infect 1994; 28 (Suppl 1):7-15.
3. Palma C, Serbousek D, Torosantucci A, Cassone A, Djeu J. Identification of a mannoprotein fraction from *Candida albicans* that enhances human polymorphonuclear leukocytes (PMNL) functions and stimulates lactoferrin in PMNL inhibition of candidal growth. J Infec Dis 1992; 166:1103-12.
4. Cohen DM, Bhalla SC, Anaissie EJ, Hester JP, Savary CA, Rex JH. Effects of *in vitro* and *in vivo* cytokine treatment, leucapheresis and irradiation on the function of human neutrophils: implications for white blood cell transfusion therapy. Clin Lab Haematol 1997; 19:39-44.
5. Spielberger RT, Falleroni MJ, Coene AJ, Larson RA. Concomitant amphotericin B therapy, granulocyte transfusions, and GM-CSF administration for disseminated infection with *Fusarium* in a granulocytopenic patient. Clin Infect Dis 1993; 16:528-30.
6. Vecchiarelli A, Monari C, Baldelli F, et al. Beneficial effect of recombinant human granulocyte colony-stimulating factor on fungicidal activity of polymorphonuclear leukocytes from patients with AIDS. J Infect Dis 1995; 171:1448-54.
7. Liles WC, Huang JE, van BuriK JAH, Bowden RA, Dale DC. Granulocyte colony-stimulating factor administered in vivo augments neutrophil-mediated activity against opportunistic fungal pathogens. J Infect Dis 1997; 175:1012-5.
8. Gutmann L, Chou SM, Pore RS. Fusariosis, myasthenic syndrome, and aplastic anemia. Neurology 1975; 25:922-6.
9. El-Ani AS. Disseminated infection caused by *Fusarium solani* in a patient with aplastic anemia. N Y State J Med 1990; 90:609-10.
10. Rutten A, Hantschke D, Goos M. Kutane Manifestation einer Pilzsepsis. Hautarzt 1990; 41:392-3.
11. Girmenia C, Arcese W, Micozzi A, Martino P, Bianco P, Morace G. Onychomycosis as a possible origin of disseminated *Fusarium solani* infection in a patient with severe aplastic anemia. Clin Infect Dis 1992; 14:1167.
12. Nucci M, Spector N, Lucena S, et al. Three cases of infection with *Fusarium* species in neutropenic patients. Eur J Clin Microbiol Infect Dis 1992; 11: 1160-2.
13. Mohammedi I, Gachot B, Grossin M, Marche C, Wolff M, Vachon F. Overwhelming myocarditis due to *Fusarium oxysporum* following bone marrow transplantation. Scand J Infect Dis 1995; 27:643-4.
14. Segal BH, Walsh TJ, Liu JM, Wilson JD, Kwon-Chung KJ. Invasive infection with *Fusarium chlamydosporum* in a patients with aplastic anemia. J Clin Microbiol 1998; 36:1772-6.
15. Weinberger M, Elattar I, Marshall D, et al. Patterns of infection in patients with aplastic anemia and the emergence of aspergillosis as a major cause of death. Medicine 1992; 71:24-43.
16. Schiffer CA, Aisner J, Daly PA, Schimpff SC, Wiernik PH. Alloimmunization following prophylactic granulocyte transfusion. Blood 1979; 54:766.
17. Clarke K, Szer J, Shelton M, Coghlan D, Grigg A. Multiple granulocyte transfusions facilitating successful unrelated bone marrow transplantation in a patient with very severe aplastic anemia complicated by suspected fungal infection. Bone Marrow Transplant 1995; 16:723-6.