

Addition of granulocyte colony-stimulating factor to chemotherapy in patients with AIDS-related lymphoma: effects on neutrophil Fc γ receptor expression and soluble Fc γ RIII plasma levels

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Summary. AIDS-related neutropenia and neutrophil dysfunction can (partly) be reversed by granulocyte-colony stimulating factor (G-CSF). We studied the effect of G-CSF on neutrophil increment and levels of soluble Fc γ receptor type III in 15 patients with AIDS-related lymphoma (ARL) undergoing chemotherapy. In six of these patients we performed a detailed kinetic analysis of the membrane expression of the functionally important Fc γ -receptors type I, II and III. In all these patients G-CSF induced Fc γ RI positive neutrophils with a decreased expression of the Fc γ RIII receptor. These changes were similar to those seen both in healthy volunteers and in non-HIV-infected individuals

treated with chemotherapy. Interestingly, the mean neutrophil and sFc γ RIII increment were significantly lower and more patients had a nadir granulocyte count $<0.5 \times 10^9/l$ after the first cycle than after the second cycle of chemotherapy. This may be related to a therapy-associated decrease in HIV-1 viral load.

The conclusion is that patients treated with chemotherapy for ARL have a qualitatively normal response to G-CSF.

Keywords: rh-G-CSF, Fc γ R expression, sFc γ RIII levels, AIDS, non-Hodgkin's lymphoma.

Although CD4-lymphocyte depletion is the hallmark of HIV-1 infection, patients with AIDS often also have neutropenia, and several studies have demonstrated evidence of neutrophil dysfunction, such as defective chemotaxis, bacterial killing and phagocytosis (Ellis *et al.*, 1988; Murphy *et al.*, 1988; Gabrilovich *et al.*, 1994; Elbim *et al.*, 1994). HIV-1 infected persons have a higher incidence of bacterial infection (e.g. pneumonia) as compared with the general population (Polsky *et al.*, 1986; Moore *et al.*, 1995). This may partly be iatrogenic (myelosuppressive effect of anti(retro)viral drugs), but has also been attributed to HIV-infection itself or to HIV-1-induced dysregulation of cytokines (Ellis *et al.*, 1988; Busch *et al.*, 1986). Myelosuppressive chemotherapy renders these patients especially sensitive to bacterial infection, causing severe morbidity and mortality (Gill *et al.*, 1987; Sawka *et al.*, 1992; Kaplan *et al.*, 1989).

Granulocyte colony stimulating factor (G-CSF) is a lineage-specific growth factor that stimulates the production, differentiation and functional activation of neutrophilic

granulocytes (Lieschke & Burgess, 1992). Recombinant human (r-H) G-CSF shortens the period of neutropenia induced by high-dose chemotherapeutic regimens (Framp-ton *et al.*, 1994). Also in patients with AIDS, G-CSF can overcome neutropenia and neutrophil defects (Vecchiarelli *et al.*, 1995; Laursen *et al.*, 1995; Hermans *et al.*, 1996).

The high affinity IgG receptor Fc γ RI is usually absent from mature neutrophils in healthy individuals but can be up-regulated during infection (presumably via production of gamma-interferon, IFN γ) or upon treatment with G-CSF (Gericke *et al.*, 1995; Kerst *et al.*, 1993a). Antibody-dependent cellular cytotoxicity is mediated by Fc γ RI and has been shown to be defective in patients with AIDS (Kinne & Gupta, 1989). Fc γ RIII (CD16), a low-affinity receptor for IgG, is expressed by neutrophils, natural killer cells and macrophages. A soluble form of Fc γ RIII originating mainly from release by neutrophils can be detected in plasma. The plasma concentration of sFc γ RIII reflects total body mass of neutrophils, and is not influenced by shifts of neutrophils from one pool to another (Huizinga *et al.*, 1994). sFc γ RIII levels are therefore presumed to be a better measure of total body neutrophil mass and neutrophil defence than the peripheral blood neutrophil count and might be a better

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predictor of the risk of infection in neutropenic patients (unpublished observation). sFc γ RIII levels have been shown to be increased early in HIV-1 infection and to be severely depressed in patients with clinical AIDS (Khayat *et al.* 1990).

In view of the neutrophil defects found in patients with AIDS, we studied the effect of G-CSF on neutrophil increment, levels of sFc γ RIII and membrane expression of Fc γ RI, II and III in patients with AIDS-related lymphoma undergoing chemotherapy, and related this to the rate of infection and to endogenous G-CSF levels. These data were also compared with results previously obtained in healthy volunteers (Kerst *et al.* 1993a).

PATIENTS AND METHODS

Patients. We studied 15 patients with AIDS-related NHL who were treated at our hospital in a phase II trial of CNOP chemotherapy in combination with G-CSF. In the first six patients detailed kinetic studies of neutrophil Fc γ receptor expression and soluble Fc γ RIII were performed. Clinical characteristics of the patients will be described elsewhere (unpublished observation). Briefly, they were 15 homosexual HIV-1 seropositive males with biopsy-proven, previously untreated, intermediate or high grade NHL. Two patients had bone marrow infiltration. None of the patients had symptoms or signs of an (opportunistic) infection at the start of chemotherapeutic treatment. The median CD4 cell number was $0.06 \times 10^9/l$ (range 0.01 – $0.67 \times 10^9/l$).

Chemotherapy regimen. Chemotherapy consisted of cyclophosphamide, 750 mg/m^2 , mitoxantrone, 10 mg/m^2 and vincristine, 1.4 mg/m^2 i.v. on day 1, and oral prednisone 50 mg/m^2 days 1–5. This cycle was repeated every 3 weeks. In cases of granulocytopenia ($<1.0 \times 10^9/l$) and/or thrombocytopenia ($<100 \times 10^9/l$) chemotherapy was postponed for 1 week; in cases of persistent pancytopenia a 50% dose-adjustment for cyclophosphamide and mitoxantrone was made. G-CSF (Neupogen, Amgen, Thousand Oaks, Calif.) was self-administered on days 3–12 ($300 \mu\text{g}$ subcutaneously). Antiretroviral therapy was stopped during chemotherapy and resumed after stopping chemotherapy. All patients received *Pneumocystis carinii* pneumonia prophylaxis (either trimethoprim–sulphamethoxazole 480 mg daily or aerosolized pentamidine, 300 mg once every month).

Collection of blood samples. For the first six patients, during the first three cycles of chemotherapy venous blood samples (serum and EDTA anticoagulated blood) were obtained on day 1 (prior to start of chemotherapy), day 3 (prior to start of G-CSF), and days 7–8, 11–12, 15, 18 and 22 (prior to the next cycle of chemotherapy). For the other nine patients, blood was obtained at day 1 and prior to every cycle of chemotherapy. Total white blood cell (WBC) counts and differential counts were performed with the H1 system (Technicon Instruments, Tarrytown, N.Y.).

Fc γ receptor expression. Fc γ R expression was analysed by flowcytometry (FACScan, Becton Dickinson, San Jose, Calif.) essentially as described (Kerst *et al.* 1993a), using standard indirect immunofluorescence techniques. Monoclonal antibodies (MoAb) used were FcRgran/1 (5D2) for CD16, AT10 for CD32 (CLB, Amsterdam, The Netherlands) and 32.2 for

CD64 (Medarex Inc., West Lebanon). As controls, irrelevant murine antibodies of the appropriate Ig subclass were used. Binding of the antibodies was visualized with FITC labelled polyclonal goat-anti-mouse Ig F(Ab')₂ (DakoPatts, Denmark). Measurements were made after live-gating on neutrophils. The mean fluorescent intensity (MFI) was used as a relative measure of receptor expression, after subtraction of background fluorescence.

Soluble Fc γ RIII. Plasma levels of soluble Fc γ RIII were determined by sandwich ELISA as described (Koene *et al.* 1996), using the MoAb CLB FcRgran1 as the primary antibody and a polyclonal biotinylated antibody as the secondary antibody. A calibration curve was constructed by pooling plasma of 90 healthy donors and measuring serial dilutions of this pooled plasma in the ELISA. The concentration of sFc γ RIII in this pool was arbitrarily set at 100 units.

G-CSF. G-CSF levels were measured with a commercial sandwich ELISA (Quantikine, R&D Systems, Minneapolis, Minn.) according to the manufacturer's instructions. The sensitivity of this assay is 10.9 pg/ml ; 95% of levels in healthy volunteers are $<40 \text{ pg/ml}$.

Statistical analysis. Statistical analysis was performed using the Number Cruncher Statistical System (NCSS, Kaysville, Utah). A *P* value of <0.05 was considered to represent statistical significance. For normally distributed values a paired Student's *t*-test was used and for skewed data the nonparametric Wilcoxon test was used. Baseline values of G-CSF, sFc γ RIII and neutrophils were correlated with Spearman's rank-correlation coefficient.

RESULTS

Neutrophil counts

At baseline, the median WBC count was $3.5 \times 10^9/l$ (range 0.9 – $12.9 \times 10^9/l$). Two patients had neutropenia (absolute neutrophil count 0.4 and $0.8 \times 10^9/l$ respectively). These two patients did not have lymphomatous bone marrow infiltration or neutrophil antibodies, but were treated with zidovudine in one case and with zidovudine and ganciclovir in the other case. After the first cycle of chemotherapy in combination with G-CSF, neutrophils rose from a median of $3.5 \times 10^9/l$ (range 0.6 – 14.8) at baseline to $8.3 \times 10^9/l$ (range 2.5 – 37 ; $P=0.04$; mean increment $6.5 \times 10^9/l$). Interestingly, the neutrophil increment after the second cycle of chemotherapy was significantly more pronounced than after the first cycle (6.5 v $14.0 \times 10^9/l$; $P=0.009$). Of 15 patients, seven had a nadir granulocyte count $<0.5 \times 10^9/l$ after the first cycle; the median duration of neutropenia was 3 d (range 3–8 d). Only one patient had a nadir neutrophil count $<0.5 \times 10^9/l$ after the second cycle.

sFc γ RIII levels

Before chemotherapy, sFc γ RIII levels ranged from 47 to 294 arbitrary units (mean \pm SD: 129.3 ± 77.6 a.u.). Baseline levels of neutrophils did not correlate with sFc γ RIII levels ($P>0.1$). For the six subjects studied in detail, the kinetics of sFc γ RIII levels are shown in Fig 1C. Peak levels of sFc γ RIII were reached 13–16 d after the start of G-CSF: 214 ± 91 a.u. (mean \pm SD, $P=0.07$). After the second cycle, sFc γ RIII levels rose from 149 on day 22 to 328 a.u. on day 39

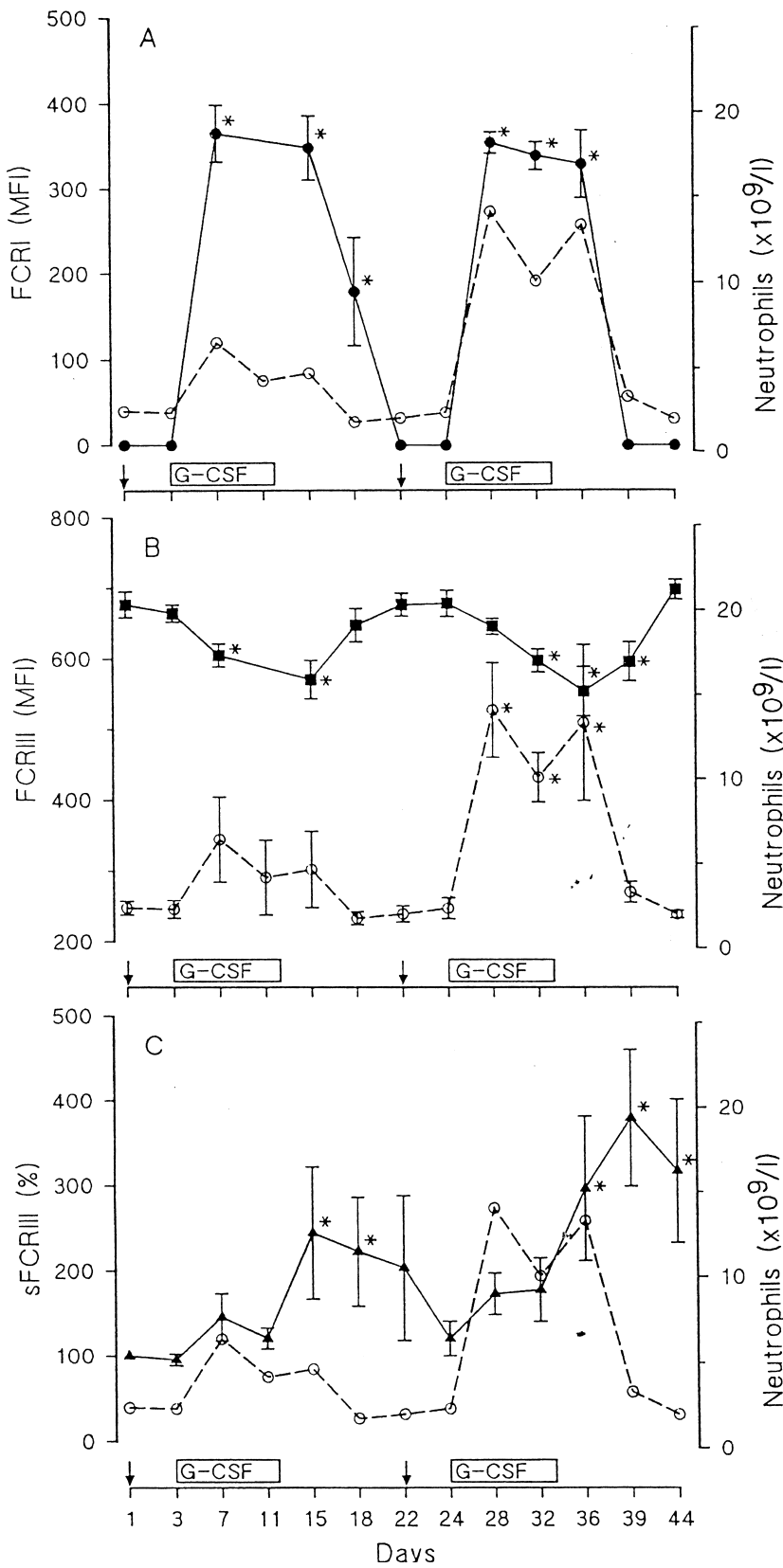


Fig 1. Membrane expression of Fc γ RI (A) and Fc γ RIII (B) on neutrophils as well as soluble Fc γ RIII levels (C) are shown during treatment with chemotherapy and G-CSF in patients with AIDS-related NHL. On the x-axis the time in days is shown. The arrow at days 1 and 22 indicates the start of chemotherapy; the box indicates the administration of G-CSF (days 3–12 and days 24–33). Data are shown for six individuals (mean \pm SEM). Asterisks indicate statistically significant increases or decreases ($P < 0.05$). (○) in panels A, B and C represents the mean number of circulating neutrophils ($\times 10^9/l$; right axis). Panel A: (●), membrane expression of Fc γ RI (mean fluorescent intensity (MFI; mean \pm SEM); left y -axis) as measured by flow cytometry using MoAb 32. Panel B: (■), membrane expression of Fc γ RIII (mean fluorescent intensity (MFI; mean \pm SEM); left y -axis) as measured by flow cytometry using MoAb CLB FcRgrn/1. Panel C: (▲), plasma levels of soluble Fc γ RIII as measured by sandwich ELISA. Data are expressed as percentage of baseline value of sFc γ RIII.

($P=0.04$). Thus the increment in sFc γ RIII was also higher after the second than after the first cycle of chemotherapy.

Neutrophil Fc γ receptor expression

In all six subjects studied for neutrophil Fc γ receptor expression, Fc γ RI expression was consistently absent at the start of chemotherapeutic treatment and at day 3 (before administration of G-CSF). After treatment with G-CSF, in all subjects Fc γ RI-positive neutrophils were found: at days 7–15 90–99% of all neutrophils showed expression of Fc γ RI. Up-regulation of Fc γ RI coincided with the neutrophil increment, indicating that most of the newly produced neutrophils were Fc γ RI positive. Fc γ RI expression was lost again at day 22, before the start of the next cycle. Similar induction of Fc γ RI-positive neutrophils was observed after the second cycle of chemotherapy (Fig 1A) and also after the third cycle in the three patients who received three cycles (data for third cycle not shown). As to Fc γ RIII, the mean MFI for the six patients at baseline was 676 (SD 44.7), whereas the minimum Fc γ RIII expression (reached at days 7–15) was 571 ± 55.4 ($P=0.009$, Fig 1B). Also after cycle 2 a statistically significant decrease in Fc γ RIII expression from 687 to 586 on day 11 and 538 on day 15 was observed ($P=0.002$).

No significant changes were seen in the expression of Fc γ RII or the granulocyte activation marker CD67 during treatment with G-CSF (data not shown).

G-CSF levels

Baseline endogenous G-CSF levels were between 16 and 421 $\mu\text{g/l}$ (mean \pm SD: $122 \pm 136.2 \mu\text{g/l}$), and were above 40 $\mu\text{g/l}$ (the 95% confidence limit of normal values) in 10/15 patients. No correlation could be demonstrated between endogenous G-CSF levels and either pretreatment neutrophil or sFc γ RIII levels ($P>0.1$). Also, no correlation was found between endogenous G-CSF levels and neutrophil increment or sFc γ RIII increment.

Infectious episodes

In these 15 patients, four infectious episodes were seen. One patient had CMV colitis after the fifth cycle of chemotherapy, and three patients had localized bacterial infection (all three perianal abscess). Two of the abscesses occurred after the third cycle and one after the first cycle. These patients did not have neutropenia at the time of infection. Pretreatment sFc γ RIII levels were low in two of the three patients with infection (30, 46 and 116 a.u.) but not statistically significantly different from levels seen in patients without infection.

DISCUSSION

This is the first study of the effect of G-CSF on neutrophil Fc γ R expression in patients with AIDS. Previously, we have demonstrated *in vitro* that under the influence of G-CSF committed myeloid progenitor cells give rise to Fc γ RI-positive neutrophils, which at the same time have a strongly decreased expression of the PI-linked Fc γ RIII receptor (Kerst *et al*, 1993b). The G-CSF-induced expression of high-affinity receptors for IgG is probably instrumental in improving effector cell functions such as antibody-dependent

cellular cytotoxicity (ADCC; van de Winkel & Andersen, 1991). Since patients with AIDS are at increased risk of bacterial infections, especially when treated with chemotherapy, and have been shown to have impaired ADCC, we were interested to find out whether treatment with G-CSF would induce up-regulation of Fc γ RI. Our results show that after G-CSF treatment virtually all circulating neutrophils are Fc γ RI positive. In addition, these neutrophils have decreased Fc γ RIII expression as compared with baseline. These changes were similar in magnitude when compared with both effects seen in healthy volunteers (Kerst *et al*, 1993a) and in non-HIV-infected individuals treated with chemotherapy and G-CSF (Spiekermann *et al*, 1994). In contrast to a previous report by Capsoni *et al* (1992), we could not demonstrate expression of Fc γ RI on neutrophils before start of treatment. This could be due to the fact that none of our patients had signs of active infection at that time. We did in fact find Fc γ RI expression in one patient no longer treated with G-CSF during an infectious episode, possibly caused by increased IFN γ production.

In nine of the 15 patients the neutrophil increment was significantly higher after the second than after the first cycle of chemotherapy. Since also the increase in sFc γ RIII levels was significantly higher after the second cycle, the difference in neutrophil increment is probably not merely the result of shifts from one pool to another but a reflection of a higher production of neutrophils in the bone marrow. Moreover, significantly more patients had a nadir neutrophil count $<0.5 \times 10^9/\text{l}$ after the first than after the second cycle (7/15 v 1/15; $P=0.02$). The difference in increment or nadir could not be explained by differences in doses of chemotherapy, by presence or absence of lymphomatous bone marrow infiltration, by the use of concurrent myelosuppressive medication, or by baseline neutrophil or sFc γ RIII levels. Although G-CSF levels were elevated in 10/15 patients, no correlation could be demonstrated between pretreatment endogenous G-CSF levels and neutrophil increment after exogenous G-CSF. The difference observed could be explained by priming of the bone marrow by G-CSF treatment. Another hypothesis could be that the decrease in HIV-1 viral load we have observed in some of these patients (unpublished observation) and subsequent diminished infection of myeloid precursors in the bone marrow might result in improved neutrophil production. In fact, patients with a difference in neutrophil increment after the first and second cycle were more often p24 antigenaemic (8/9) at the start of treatment than patients with a similar increment after both cycles (2/6; $P=0.05$ with Fisher's exact test). In all but one patient with HIV-1 p24 antigenaemia, p24 Ag levels decreased significantly after the first cycle.

We could not demonstrate a correlation between either neutrophil or sFc γ RIII levels and endogenous G-CSF levels, as has been shown to exist for patients with aplastic anaemia (Watari *et al*, 1989). The fact that endogenous G-CSF levels were increased in two-thirds of the patients probably reflects decreased neutrophil production in the bone marrow, which, in view of the relatively normal sFc γ RIII levels, appears to be compensated. The number of infections seen in this patient group was too low to evaluate the prognostic value of sFc γ RIII levels.

In conclusion, in patients receiving chemotherapy for AIDS-related NHL, G-CSF induced changes in neutrophil FC γ receptor expression similar to those observed in both healthy volunteers and non-HIV-infected persons undergoing chemotherapy. Marked differences were seen in neutrophil response after the first and second cycle of chemotherapy, which may be related to differences in HIV viral load.

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