# Reducing anti-DT IgG concentrations to improve the efficacy of a diphtheria fusion protein

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**Preformed antidiphtheria toxin (anti-DT) IgG limits the development of diphtheria fusion proteins because the anti-DT IgG binds and removes the diphtheria fusion protein from the circulation. In our phase I trial of DT–granulocyte macrophage colony stimulating factor (GMCSF), a truncated DT linked to human GMCSF, in relapsed or refractory acute myeloid leukemia, patients with high concentrations of preexisting anti-DT IgG (>2.5 μg/ml) had significantly lower DT-GMCSF concentrations. This study details the fate of anti-DT IgG during the patient's treatment with DT-GMCSF and describes how we could lower anti-DT IgG concentrations and increase the patient's exposure to DT-GMCSF. Using an enzyme immunoassay, we measured anti-DT IgG concentrations before the first cycle of treatment (baseline) and on day 2 (after one dose of DT-GMCSF) and on day 5 (after four doses of DT-GMCSF). Thirty-three patients with relapsed or refractory acute myeloid leukemia in the phase 1 trial received DT-GMCSF at doses from 1 to 5 μg/kg/day intravenously for 5 days. The mean anti-DT IgG concentration pretherapy was 1.3 μg/ml (range: undetectable to 7.8) and significantly decreased to a mean concentration of 0.7 μg/ml on day 2 ($P=0.007$) and to 0.5 μg/ml on day 5 ($P<0.0001$). In two individuals in whom we measured DT-GMCSF concentrations on day 1 and day 5, we observed that a decrease in anti-DT IgG concentrations was associated with an increase in DT-GMCSF concentrations. No relationship was observed between dose of DT-GMCSF and the absolute change in anti-DT IgG concentrations on day 2 ($r=-0.01$, $P=0.98$) or day 5 ($r=-0.12$, $P=0.53$). For patients with high baseline anti-DT IgG concentrations, a single dose of DT-GMCSF could be used to lower the anti-DT IgG concentrations and potentially result in a significant increase in DT-GMCSF concentrations and efficacy.***Anti-Cancer Drugs*** **19:1007–1011 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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## Introduction

Accounting for approximately 13 290 new cases each year in the United States, acute myeloid leukemia (AML) is the second most common type of leukemia [1]. Standard induction treatment for AML (excluding the promyelocytic subtype) includes a topoisomerase II inhibitor (e.g. daunorubicin, idarubicin, or mitoxantrone) and cytarabine, and this combination produces complete remission in 50–80% of patients. The majority of patients, however, experience relapses. Unfortunately, patients with relapsed or refractory AML have a poor prognosis, and life expectancy on average is less than 6 months. Most of the leukemia blasts found in these patients exhibit multiple mechanisms of resistance to chemotherapy agents that inhibit DNA synthesis or cell proliferation [2]. The development of agents with novel mechanisms of action is needed to overcome resistance and improve outcomes.

One innovative class of antileukemia agents is diphtheria toxin (DT) fusion proteins. These differ from standard therapy in regards to their mechanisms of killing blast cells by modifying elongation factor-2, which inhibits cellular protein synthesis leading to apoptosis. DT naturally consists of three binding domains: the receptor binding domain, the catalytic domain, and the transmembrane domain. The receptor-binding domain binds nonspecifically to cell surface carbohydrates. For fusion proteins, this nonspecific binding domain is replaced with a more specific ligand, such as granulocyte macrophage colony stimulating factor (GMCSF), to direct the fusion protein toward leukemia blasts [3]. GMCSF is a ligand that specifically targets cells expressing the GMCSF receptor. GMCSF receptors are minimally expressed on normal hematopoietic stem cells but extensively expressed on myeloid progenitors, myeloid leukemias, monocytes, granulocytes, and macrophages [4]. Therefore, most normal cells, aside from myeloid progenitors, will not be the target of DT-GMCSF. Once the ligand binds to the GMCSF receptor, the diphtheria (DT) fusion protein undergoes endocytosis. The acidic pH of the endosome cleaves the DT fusion protein, and the transmembrane domain facilitates the translocation of

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the catalytic domain into the cytosol. Once in the cytoplasm, the catalytic domain inhibits elongation factor-2, preventing protein synthesis from occurring; resulting in apoptosis. DTs are extremely potent; only one molecule can lead to cell death [3].

To establish safety data regarding DT-GMCSF, a phase I trial was recently completed in patients with relapsed or refractory AML. Patients were treated at one of six dose levels of DT-GMCSF (1–5 μg/kg/day) for up to 5 consecutive days. The maximum tolerated dose was 4 μg/kg/day for 5 days. Hepatotoxicity, manifested as grade III or grade IV, was the dose-limiting toxicity. Patients with high anti-DT IgG concentrations had lower peak DT-GMCSF concentrations ($r = -0.64$, $P = 0.001$); additionally, these patients with high anti-DT IgG concentrations had less hepatic toxicity ($r = -0.45$, $P = 0.011$) [5]. It is hypothesized that the antibody response against DT-GMCSF differentiates responders (those who have high DT-GMCSF concentrations) from nonresponders (those who have low DT-GMCSF concentrations).

In patients who have been previously vaccinated against DT, the anti-DT IgG immediately binds the DT-GMCSF and leads to the rapid removal of DT-GMCSF from the body. Therefore, the half-life of DT-GMCSF is shortened; in turn, a decreased exposure to DT-GMCSF is seen. In our phase I clinical trial of DT-GMCSF, anti-DT IgG concentrations greater than 2.5 μg/ml were associated with undetectable DT-GMCSF concentrations [5]. One method to lower preexisting anti-DT IgG concentrations would be to bind the anti-DT IgG with DT-GMCSF. Therefore, the objectives of this analysis were (i) to determine if during their 5-day treatment with DT-GMCSF if their anti-DT IgG concentrations decreased and (ii) to determine if the lowered anti-DT IgG concentrations lead to an increased exposure to DT-GMCSF.

## Methods

### Patients and treatment

Our clinical trials of DT-GMCSF involved 37 patients with relapsed or refractory AML. All patients provided written informed consent before entry into the study. Nine patients received a second course of DT-GMCSF [4,5]. This study is a retrospective analysis of anti-DT IgG concentration in these patients. For this analysis, only 20 patients had anti-DT IgG concentrations that could be analyzed at baseline and on days 2 (approximately 24 h after one dose DT-GMCSF) and 5 (approximately 24 h after the fourth dose of DT-GMCSF) whereas we had 33 patients who had baseline and day 5 anti-DT IgG samples. In this study, we also had five patients who received a second course of treatment. The characteristics of the 33 patients are outlined in Table 1.

**Table 1 Patient characteristics**

| | |
|---|---|
| Age | 12–84 years (median age = 55 years) |
| Sex | |
| Male | 15 (45.5%) |
| Female | 18 (54.5%) |
| Disease status | |
| Relapse | 14 (42.4%) |
| Refractory | 19 (57.6%) |
| Dose (μg/kg/day × 5 days) | |
| 1 | 3 Patients |
| 2 | 6 Patients |
| 3 | 4 Patients |
| 4 | 12 Patients |
| 4.5 | 5 Patients |
| 5 | 3 Patients |

The patients were treated with DT-GMCSF at one of six dose levels (1, 2, 3, 4, 4.5, or 5 μg/kg/day) for up to 5 consecutive days. The DT-GMCSF was infused over 15 min [4]. DT-GMCSF concentrations were drawn at baseline, 17 min, 30 min, 1, 2, 4, 8, and 24 h. The protocol specified for days 1 and 5 pharmacokinetics for only five patients, and we had only enough detectable DT-GMCSF concentrations to calculate an area under the serum concentration–time curve (AUC) in two of these individuals. Only the maximum concentration ($C_{max}$) was detectable in the other three individuals. We calculated a 24-h AUC using both the linear and log trapezoidal rule. Results are presented as linear trapezoidal rule calculations.

### Measurement of anti-DT IgG and diphtheria toxin–granulocyte macrophage colony stimulating factor concentrations

An enzyme immunoassay (EIA) was used in this study to determine anti-DT IgG concentrations. This is a direct antibody EIA with 100 μl of DT-GMCSF at a concentration of 0.5 μg/ml coated to the EIA plate (Immulon 1B plates; Thermo Labsystems, Franklin, Massachusetts, USA). After overnight incubation at 4°C, the plates were then washed three times with PBS plus Tween-20 0.1% and blocked with PBS plus 5% bovine serum albumin (BSA) for 30 min at 37°C. The controls, standard curve, and unknowns were then added for 2 h at 37°C. The standard curve consisted of purified anti-DT-GMCSF IgG a patient in the phase I trial using a previously described affinity chromatography protocol [6]. Unknowns were diluted from 1 : 5 to 1 : 120 in PBS plus 1% BSA. All samples were run in duplicate. After the 2-h incubation, the plates were again washed with PBS plus Tween-20 0.1%, and then a 1 : 10 000 dilution of an alkaline phosphatase-labeled goat antihuman IgG (Sigma, St Louis, Missouri, USA) was added to each well for 1 h at room temperature. After three washings, the amount of bound antibody was visualized with the addition of a 1 mg/ml *p*-nitrophenyl phosphate (Sigma) solution and read at 405 nm (Dynatech Laboratories, Chantilly, Virginia, USA). Two controls were run with each assay,

and the assay was rejected if the controls were not within 20% of the predicted value for the assay. The lower limit of detection for the assay is 0.02 μg/ml. The interassay coefficient of variation was less than 20%, and the intra-assay coefficient of variation was less than 10% [6,7]. A previously published bioassay was used to determine DT-GMCSF concentrations [8].

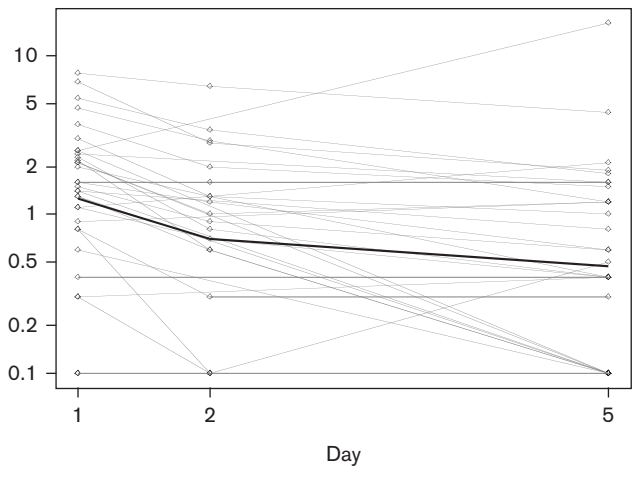
### Statistical analysis

Random effects linear regression was used to model the association between time and anti-DT IgG concentration where anti-DT IgG concentration was transformed to the log scale to adhere to the assumptions of the model. Random intercepts were used to account for correlation due to repeated measures on the same patients. Slopes were tested using Wald tests based on the linear regression results. Pearson correlations were used to describe associations with dose and assumption of linearity was explored using scatterplots. For anti-DT IgG concentrations below the limit of detection, we used 0.1 μg/ml. A $P$ value of less than 0.05 was considered significant.

## Results

Overall during the first course of treatment with DT-GMCSF, 17 of 20 patients (85%) of patients exhibited a decrease in their anti-DT IgG concentrations between baseline and day 2 (i.e. after one dose of DT-GMCSF) (Fig. 1). The estimated absolute change in anti-DT IgG concentrations between baseline and day 2 was −0.6 μg/ml with a range of 0 to −4 μg/ml ($P = 0.007$). Similarly, 25 of 33 patients (76%) treated with DT-GMCSF exhibited a decrease in their anti-DT IgG concentrations between baseline and day 5, after four doses of DT-GMCSF, with their first course of treatment. The estimated absolute change in anti-DT IgG concentrations between baseline and day 5 was −0.8 μg/ml with a range of −4.9 to +13 μg/ml (Fig. 1) ($P < 0.0001$). The change in anti-DT IgG concentrations between days 2 and 5 was not significantly different (Fig. 1) ($P = 0.06$).

**Fig. 1**




Anti-DT IgG concentrations significantly declined after 1 ($P = 0.007$) and 4 days ($P < 0.0001$) of treatment with diphtheria toxin-granulocyte macrophage colony stimulating factor. The bolded line represents the median data. Y-axis = anti-DT IgG concentration (μg/ml).

A limited number of patients were treated with a second course of DT-GMCSF. Only one patient had anti-DT IgG concentrations drawn at baseline, day 2, and day 5. This patient's anti-DT IgG slightly decreased from a cycle 2 pretherapy concentration of 27.4 μg/ml to 26.7 on day 2 to 25.8 μg/ml on day 5. Five patients who received a second course had anti-DT IgG concentrations drawn at baseline as well as day 5. Two patients exhibited an increase in their anti-DT IgG concentrations whereas three patients had decrease anti-DT IgG concentrations during the second course of treatment. The greatest decrease in anti-DT IgG concentrations occurred in one of these three patients from 23.1 to 6 μg/ml.

We measured DT-GMCSF concentrations on days 1 and 5 in five individuals, but we had only enough measurable DT-GMCSF concentration in two patients to calculate a 24-h AUC. Results from these two patients on days 1 and 5 of treatment demonstrated a decrease in anti-DT IgG concentrations resulted in an increased $C_{max}$ and AUC of DT-GMCSF (Table 2).

No correlation was observed between the dose of DT-GMCSF with absolute change in anti-DT IgG concentrations on either day 2 ($r = -0.01$, $P = 0.98$) or day 5 ($r = -0.12$, $P = 0.53$) (Fig. 2).

## Discussion

In our phase 1 trial of DT-GMCSF, we observed the median concentration of anti-DT IgG in patients with unmeasurable serum concentrations of DT-GMCSF was 2.5 μg/ml whereas the median anti-DT IgG concentration in patients with measurable DT-GMCSF concentrations was 0.6 μg/ml [5]. On the basis of these results, our current strategy for subsequent trials with diphtheria fusion proteins is to only enroll patients with anti-DT IgG concentrations less than 2.5 μg/ml to increase the likelihood of measurable DT-GMCSF concentrations. A strategy to expand the use of diphtheria fusion proteins in patients with anti-DT IgG concentrations above 2.5 μg/ml would be give a single low dose of the diphtheria fusion protein to bind up and lower the circulating anti-DT IgG concentration before starting the therapeutic regimen of diphtheria fusion protein.

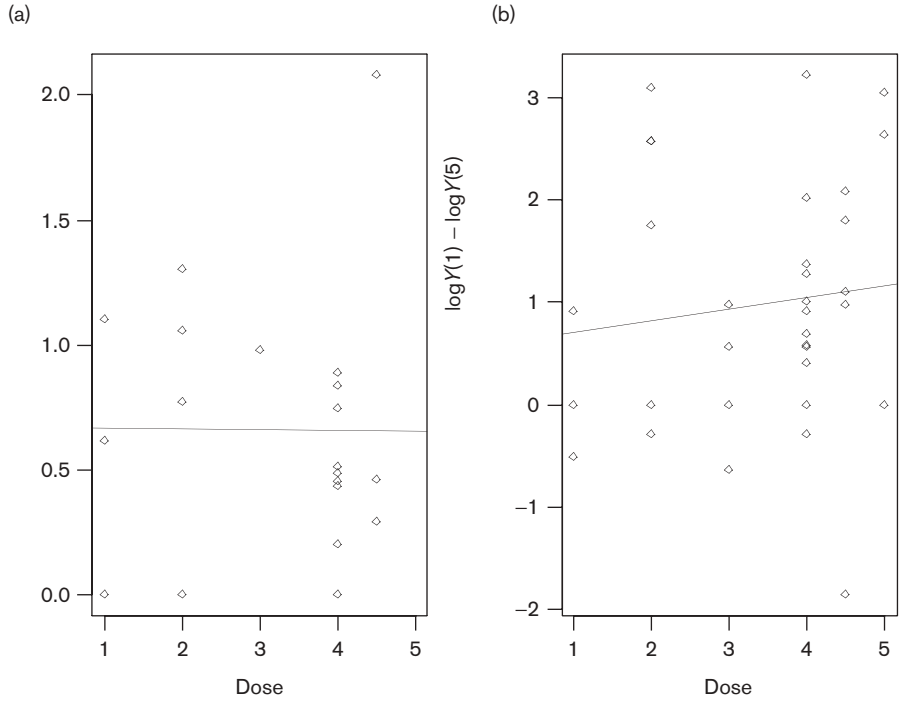
As shown in Fig. 1, the majority of the decrease in anti-DT IgG occurred after only one dose of DT-GMCSF. In addition, we found no relationship between the dose of

**Table 2 Relationship between decline in anti-DT IgG and DT-GMCSF pharmacokinetics**

| Patient | Day of treatment | Dose of DT-GMCSF | WBC $\times 10^3$ cells/μl | ANC $\times 10^3$ cells/μl | Anti-DT IgG (mg/ml) | $C_{max}$ (ng/ml) | AUC (ng-h/ml) |
|---|---|---|---|---|---|---|---|
| 21 | 1 | 4.5 | 0.9 | 0.243 | 0.8 | 52.8 | 40.4 |
| | 5 | 4.5 | 0.4 | 0.048 | BLD | 101.1 | 233 |
| 27 | 1 | 5 | 0.5 | 0.135 | 0.9 | BLD | BLD |
| | 5 | 5 | 0.4 | 0.108 | BLD | 336 | 822.2 |

ANC, absolute neutrophil count; AUC, area under the serum concentration-time curve; BLD, below the limit of detection; DT-GMCSF, diphtheria toxin–granulocyte macrophage colony stimulating factor; WBC, white blood cell.

**Fig. 2**




No relationship was observed between the dose of diphtheria toxin–granulocyte macrophage colony stimulating factor (DT-GMCSF) and the absolute decrease in anti-DT IgG concentrations (*Y*-axis) on day 2 (after 1 day of treatment with DT-GMSCF) (a) or on day 5 (after 4 days of treatment with DT-GMCSF) (b).

DT-GMCSF and the decline in anti-DT IgG (Fig. 2). This indicates that a single low dose could be used to bind up (i.e. serve as an antigen sink) the anti-DT IgG and lower the anti-DT IgG that should then increase the exposure to DT-GMCSF. Unfortunately, we did not measure DT-GMCSF concentrations on day 2 to address this hypothesis. We, however, did have DT-GMCSF concentrations in two patients on days 1 and 5. In Table 2, two patients' exposure as measured by $C_{max}$ and AUC increased between days 1 and 5 due to a significant decrease in anti-DT IgG concentrations. This increased exposure to DT-GMCSF was not due to a significant decrease in the white blood cell (WBC) nor absolute neutrophil count (ANC). Neutrophils and monocytes express the GMCSF receptor [9,10]. Therefore, these cells could bind the DT-GMCSF and prevent it from binding to AML blasts, but the WBC and ANC counts did not significantly change between days 1 and 5. Moreover, both the WBC and ANC counts were extremely low throughout treatment. Thus, it is doubtful that increased exposure of DT-GMCSF on day 5 compared with day 1 was due to a change in WBC and/or ANC, but the increased exposure was due to the drop in anti-DT IgG concentrations.

## Conclusion

Our results demonstrate that 1 and 4 days of treatment with DT-GMCSF significantly lowers anti-DT IgG concentrations in patients with relapsed or refractory AML. In two patients this reduction in anti-DT IgG resulted in an increase in a patient's DT-GMCSF exposure. These results indicate that a single dose of DT-GMCSF could significantly lower anti-DT IgG and potentially result in a major increase in DT-GMCSF

concentrations. Thus, potentially increasing the efficacy of DT-GMCSF in AML. These results may be applicable to other diphtheria fusion proteins.

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