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Cytokine Dysregulation in Chronic Graft Versus Host Disease

V. BARAK,[1] F. LEVI-SCHAFFER,[2] B. NISMAN[1] and A. NAGLER[3]

Departments of Oncology,[1] Pharmacology[2] and Bone Marrow Transplantation,[3] Hadassah University Hospital and Hebrew University, Jerusalem, Israel

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Cytokines play a key role in the pathogenesis of chronic Graft versus Host Disease (cGVHD) and various studies have shown abberant production of cytokines by immune cells from GVHD patients. Based on these findings and others showing that high TNF levels precede the development of GVHD, we evaluated inflammatory cytokine levels following BMT and during the development of cGVHD. In this study, patients undergoing bone marrow transplantation (BMT) who consequently developed chronic GVHD were analyzed as to their cytokine production during cGVHD and this was correlated with their clinical manifestations. A positive correlation was found between the severity as well as the number of major clinical complications and high levels of inflammatory cytokines (IL-1βIL-6 and TNFα) compared to control patients or to normal donors. Patients undergoing BMT who did not develop GVHD, did not produce high levels of IL-1βIL-6 or TNF. High levels of cytokines may be used as a tool for assessing novel therapeutic modalities and response to GVHD treatment.

KEY WORDS: cytokines chronic graft-versus-host disease (cGVHD) bone marrow transplantation (BMT)

INTRODUCTION

Chronic Graft versus Host Disease is a major obstacle to successful allogeneic bone marrow transplantation (BMT). GVHD following allogeneic BMT is caused by donor graft T lymphocytes that recognize antigenic disparities between donor and recipient.[1–3] Tissue damage associated with GVHD is thought to be caused by T cell mediated cytotoxicity[4,5] and it is possible that additional networks of inflammatory cytokines act as mediators of acute GVHD.[6,7] GVHD is viewed today as a three step process, consisting of upregulation of HLA and leukocyte adhesion molecules on host target cells; followed by activation of donor immunocompetent T cells by host histocompatibility antigens, which then proliferate. Finally, these activated T cells, then secrete cytokines, recruit additional cells, induce the expression of histocompatibility antigens, and focus their attack on recipient targets.

Cytokines play a key role in each of the above steps. [8–11] Many cytokines are produced in an aberrant fashion in patients with cGVHD[12–14] including Interleukin-2 (IL-2), soluble IL-2 Receptor (sIL-2R), Tumor Necrosis Factor—alpha (TNF-α) and beta (TNF-β), Interferon γ (IFNγ), Interleukin-1 (IL-1α and IL-1β), Interleukin-6 (IL-6) and Interleukin-4 (IL-4).

TNF-alpha has a major function in the first step of GVHD. In various studies TNF-α has been shown to be released from host tissues and to be elevated both in the tissues and the serum of mice and humans with chronic GVHD.[15] Moreover, Holler *et al.*[16] found a direct correlation between high TNF levels and transplant related complications or survival. Furthermore, the rise in TNF levels were shown to precede the development of GVHD.[16] The results of bioassays and immunoassays of

Address for correspondence: Dr. Arnon Nagler, Bone Marrow Transplantation Dept., Hadassah University Hospital, P.O. Box 12000, Jerusalem, Israel 91120.

TNF levels in patients do not always correlate with the clinical picture, suggesting the presence of cytokine inhibitors. An example for this would be IL-1 receptor antagonist (IL-1RA), which inhibits IL-1, and also prevents GVHD.[17]

In addition, high levels of IL-6 have been correlated with the onset of acute GVHD as well as the development of hepatorenal dysfunction (HRD), and bilateral lung infiltration (BLI).[18] High amounts of granulocyte macrophage-colony stimulating factor (GM-CSF) have also been detected in sera from patients with GVHD[19] and it has been suggested that IFN is an additional mediator of GVHD.[20]

This study was undertaken in order to assess whether the increase in the production of inflammatory cytokines could explain the consequent development of cGVHD in many patients post-BMT.

MATERIALS AND METHODS

Cytokine levels were examined in the sera of BMT patients who were diagnosed as having cGVHD according to the Seattle clinical and histopathological criteria.[21]

In most of the patients more than one evaluation was performed during the cGVHD period. Thirty normal individuals of compatible age and sex served as controls. In addition, we evaluated cytokine profiles 5–12 months post transplant in 11 acute lymphoblastic leukemia (ALL) patients, 8 acute myeloid leukemia (AML) patients and 6 chronic myeloid leukemia (CML) patients who underwent allogenlic BMT and did not develop cGVHD. Transplant conditioning regimens, allograft cell numbers and treatment were similar between the study groups and the controls.

Patients

Thirteen consecutive patients (10 males and 3 females) who developed cGVHD were enrolled in this study; mean age 16 (4–31) years. Six patients had ALL, four AML, one CML and two severe aplastic anemia (SAA). All patients underwent HLA matched bone marrow transplantation (BMT). Of the leukemic patients, four were in first complete remission (CR), four in second CR, one in third CR and one in relapse. The CML patient was transplanted in the first chronic phase.

Eight patients were conditioned with fractionated total body irradiation (TBI), (1200 cGy in 6 fractions) followed by administration of etoposide (1500 mg/m^2), cyclophosphamide (60 mg/kg) and melphalan (60 mg/m^2) and fractionated total lymphoid irradiation (TLI) (600 cGy in 4 fractions). Two patients were conditioned with Busulfan (16 mg/kg in 4 days), cyclophosphamide (200 mg/kg in 4 days) and TLI. The patient with CML was conditioned with fractionated TBI (1200 cGy) and cyclophosphamide (120 mg/kg in 2 days). The patients with SAA were conditioned with fractionated TLI (1600 cGy in 8 fractions) and cyclophosphamide (120 mg/kg in 2 days).

Bone marrow cells were purged with CAMPATH-1 (IgG2b), a monoclonal rat anti human lymphocyte antibody (anti-CDW52) (1 ug/10^6 nucleated cells) kindly supplied by Drs. G. Hale and H. Waldmann, Cambridge University (Cambridge, U.K.) in order to deplete T lymphocytes.[2] No other anti GVHD prophylaxis was given. The patients with SAA were treated with cyclosporine A in order to prevent graft rejection. The patient with CML was transplanted with non T depleted marrow and received a combination of cyclosporine A and methotrexate as GVHD prophylaxis.

All patients had cGVHD according to biopsy results which included a typical histopathologic picture.[1] Three patients had mild cGVHD, 5 moderate and 5 severe extensive cGVHD according to the Seattle grading criteria.[21] All cases showed cutaneous involvement, either generalized or localized, most had other organ involvement (oral mucosa, ocular, liver, gastrointestinal and musculoskeletal). Eight of the cGVHD patients developed acute GVHD in the early post BMT period. Three patients developed grade I, 2 grade II and 1 grade III. All were treated with cyclosporine A (6 mg/kg) and methylprednisolone (1–5 mg/kg) and 7 of them had no signs of active GVHD by day 100. Five patients had de novo cGVHD. Median onset of cGVHD was 120 days, range 100–166 days. They were treated with prednisone, cyclosporine A, azathioprine with modulation depending on clinical parameters. In addition, thalidomide (2 patients), colchicine (2 patients) and low dose TLI (2 patients) were also given without substantial clinical benefit.

The following cytokine levels were determined in patients' sera by means of radioimmunoassay (RIA) or enzyme immunoassay (EIA): IL-1β, IL-2, IL-6, TNFα, GM-CSF, γIFN. Cytokine determinations were performed within 5–27 months (mean 11) following the onset of cGVHD. Some of the patients sera was tested more than once.

Statistical analysis

For comparison of mean levels of cytokines in test patients, controls and normal donors, we used the Student T-test. The correlation between disease severity and mean cytokine levels was determined by Spearman correlation coefficients.

RESULTS

Cytokine production was evaluated in allogeneic BMT patients with cGVHD compared to BMT patients who did not develop cGVHD as well as in normal individuals.

The following cytokines were evaluated: IL-1β, IL-2, IL-6, TNFα, INFγ and GM-CSF. The level of TNF was found to be high in 10/13 patients or 19/24 evaluations. Only 3 patients (5 evaluations) had undetectable levels of TNF (Figure 1). The level of TNFα in most of the patients was very high (10–1000 pg/ml compared to normal levels <10 pg/ml) ($p < 0.001$). We observed that the three patients who had undetectable levels of TNF developed very mild cGVHD (n = 3) while patients who had high or extremely high levels of TNF had moderate to severe cGVHD (n = 10) (c.c = –0.496). There was no difference in TNFα levels between patients with moderate and severe cGVHD (3 of the patients with very high TNF had severe cGVHD while 2 had moderate cGVHD). The level of IL-6 was found to be very high (in comparison to normal levels $p < 0.001$) in 11/12 pts or 15/17 evaluations ranging between 1000–6000 pg/ml. Only 1 patient's serum (or 2 evaluations) contained relatively low amounts of IL-6—although much higher than the level found in normal human sera (< 100 pg/ml) (Figure 2). This particular patient had very mild cGVHD. All of the patients with moderate or severe cGVHD, had very high levels of IL-6. The remaining 2 patients who developed mild cGVHD, had high and very high levels of IL-6, respectively. IL-1β was found also in high levels in 11/13 pts or 25/31 evaluations (600–10000 pg/ml) as compared to normal donors ($p < 0.001$). 2/13 patients (or 6 evaluations) were in the normal range. The 2 patients with low levels of IL-1β had mild cGVHD. The third patient with mild cGVHD, had elevated levels of IL-1β. All of the patients with moderate and severe cGVHD had high or very high levels of IL-1 β (Figure 3).

GM-CSF was found to be elevated in 10/12 patients (or 13/17 evaluations)—although it was considered to be high only in 5/12 patients. Two patients had undetectable levels and the 2 patients with low GM-CSF had mild cGVHD (Figure 4).

Most (8/12) of the patients or 10/14 evaluations had undetectable or low levels of IFN γ. Only 4/12 patients (or 4/14 evaluations) had high levels (Figure 5). Out of the 4 pts with high levels of IFN γ, 2 had moderate and 2 had severe cGVHD.

IL-2 levels were undetectable in all of the patients (Data not shown). As an additional control, we analyzed sera from patients diagnosed with ALL, AML and CML who underwent allogeneic BMT and were matched in age and sex to the test group. These patients did not produce high

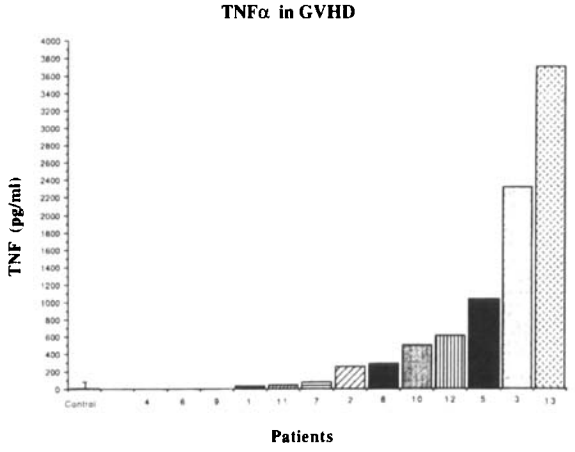

Figure 1 TNFα levels in GVHD patients and control normal donors.

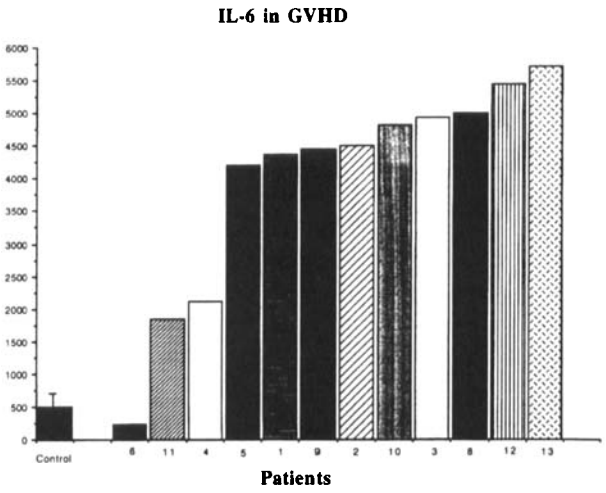

Figure 2 IL-6 levels in GVHD patients and control normal donors.

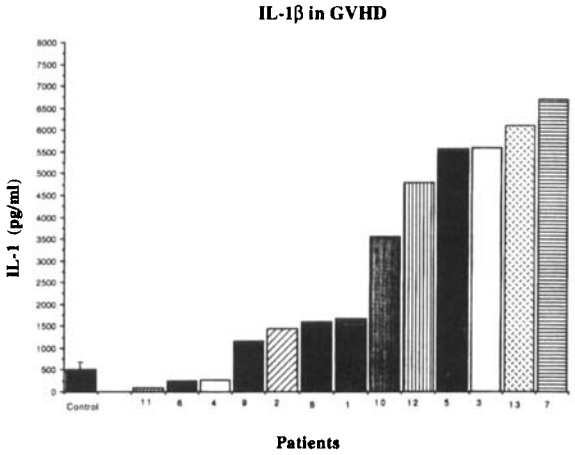

Figure 3 IL-1β levels in GVHD patients and control normal donors.

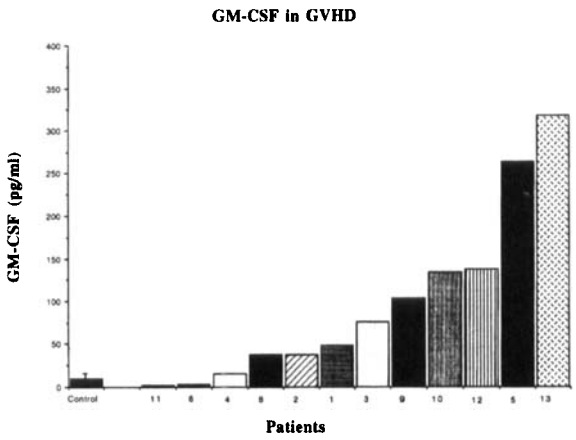

Figure 4 GM-CSF levels in GVHD patients and control normal donors.

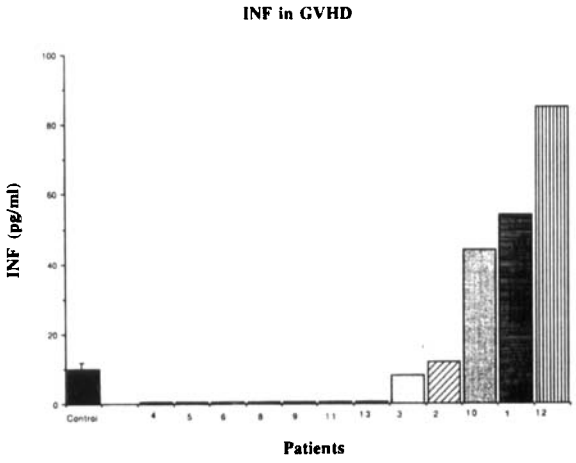

Figure 5 Interferon IFN levels in GVHD patients and control normal donors.

levels of IL-1β, IL-6 or TNFα in contrast to the patients who developed GVHD (Figure 6).

In conclusion, a majority of the patients who developed cGVHD had high levels of inflammatory cytokines (IL-1β, TNFα and IL-6) in their sera (Figure 6) compared to sera from normal healthy donors or controls.

DISCUSSION

Measurement of cytokines has become a valuable tool not only in elucidating pathophysiologic mechanisms but more importantly in diagnosis and prognosis of a variety of diseases. Previous studies carried out in patients with acute or chronic GVHD revealed a cytokine cascade that includes production of IL-1β, IL-6, TNFα and IFNγ.[6–9,11–14]

In this study we have shown that patients who underwent BMT and subsequently developed cGVHD produce high or even extremely high levels of inflammatory cytokines as IL-1β, IL-6, TNFα. Moreover, we found a good correlation between the severity of GVHD and the elevated cytokine levels.

In contrast, patients who underwent BMT and did not develop cGVHD, produced these cytokines within a normal range. The level of other cytokines such as GM-CSF or IFNγ was relatively low in most of these patients.

The level of TNF observed is in accordance with results obtained by Holler *et al.*[16] and in discrepancy with other studies which did not find any correlation between cytokine levels and the severity of GVHD.[13,18,20] The cause for these discrepancies may be dependent on the exact time of cytokine evaluation or on the different evaluation methods with different sensitivities.

Holler *et al.*,[16] however, showed that high levels of TNF generally precede GVHD. As we did not measure cytokine levels of these patients before the onset of GVHD, we cannot verify this observation. In a recent study by Tong *et al.*,[13] although TNF levels were shown to precede major complications and the development of cGVHD in transplant patients, this cytokine increased significantly within seven days post GVHD diagnosis, but did not correlate with disease activity. Additional studies did not find a correlation between IFNγ, IL-2 and GM-CSF, and the development of cGVHD.

Monitoring the level of serum TNF should help the evaluation of severity of chronic GVHD and allow a greater degree of differential treatment in the future.

Future anti-GVHD therapy could perhaps be directed towards the blocking of the production of and/or the effects of cytokines, whereas most of the established therapies today are directed towards immunosuppression of the

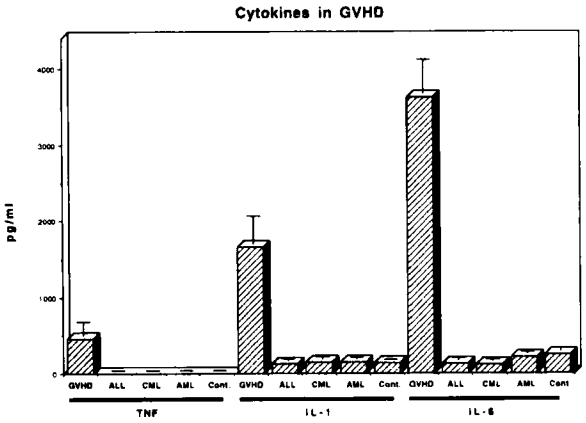

Figure 6 Cytokines (IL-1, IL-6, TNF) production in controls, GVHD and control ALL, CML and AML patients.

cytotoxic T lymphocyte (CTL) using cyclosporin A, FK506, Rifamicin, steroids, methotrexate, or monoclonal antibodies. One method attempted to avoid GVHD was T cell depletion[1] while cytokine antagonists (IL-4, IL-10) or combinations of antagonists such as IL-IRA and sTNF-R could be used as an alternative approach. Recently, anti TNF compounds such as pentoxifylline and ciprofloxacin and anti TNF monoclonal antibodies, have been introduced as a means of prevention and treatment of transplant related toxicity and GVHD.[22–24] It is important to note that cytokine release (TNF-α, IL-1β) from host macrophages and endothelial tissues in response to injury due to conditioning regimens and/or endotoxins, is independent of HLA-restriction. This may explain the occurrence of GVHD despite genotypic HLA identity between the donor and the recipient.

IL-1RA has been shown to prevent GVHD in a murine model without impeding the immunologic and hematopoietic reconstitution,[17] and recently, clinical phase I/II trials using IL-IRA to treat steroid resistant GVHD have begun.

In summary, we have shown increased levels of inflammatory cytokines (IL-1β, IL-6, TNFα) in cGVHD patients. Moreover, a statistically significant correlation was found between the severity of cGVHD and the levels of these cytokines. We believe that this correlation may be due to the role of inflammatory cytokines in the pathophysiology of cGVHD. Furthermore, levels of these cytokines may be used as a tool for assessing new therapeutic modalities as well as efficacy and response in GVHD.

REFERENCES

1. O'Reilly, R. J. (1982) Allogeneic bone marrow transplantation: current status and future directions. *Blood*, **62**, 941–964.
2. Slavin, S., Ed. (1984) Bone marrow and organ transplantation: Achievements and Goals. Amsterdam; Elsevier.
3. Slavin, S. and Nagler, A. (1991) New developments in bone marrow transplantation. *Current Opinion in Oncology*, **33**, 254–271.
4. Ferrara, J. L. M. and Deeg, H. J. D. (1991) Graft versus host disease. *New Engl. J. Med.*, **324**, 667–674.
5. DeGast, G. C., Gratama, J. W., Ringden, O. and Gluckman, E. (1987) The multifactorial etiology of graft versus host disease. *Immunol. Today*, **8**, 209–212.
6. Jadus, M. R. and Wepsic, H. J. (1992) The role of cytokines in graft-versus-host reactions and disease. *Bone Marrow Transplantation*, **10**, 1–14.
7. Weinberg, K., Lawrence, K., Barrantos, B., Lenarsky, C. and Parkman, R. (1992) The role of cytokines in the pathogenesis of human graft versus host disease. *Exper. Hematol.*, **20**, 717 (Abstract).
8. Smith, S. R., Terminelli, C., Kenworthy-Bott, L. and Phillips, D. L. (1991) A study of cytokine production in acute GVHD. *Cell Immunol.*, **134**, 336–348.
9. Knulst, A. C. (1992) Cytokines in lethal GVHD. *Transplant Int.*, **5**, 5679–5683.
10. Ferrara, J. L. M. (1992) Advances in GVHD: Novel lymphocyte subsets and cytokine dysregulation. *BM Transplant.*, **10 (Suppl. 1)**, 10–14.
11. Amtin, J. H. and Ferrara, J. L. M. (1992) Cytokine dysregulation and acute graft versus-host disease. *Blood*, **80**, 2964–2968.
12. Parkman, R., Lenarsky, C., Santos, R., Barrantes, B. and Weinberg, K. (1991) Cytokines during acute graft versus host disease (GVHD) following matched unrelated donor (MUD) transplantation. *Blood*, **78**, 286a (Abstr.)
13. Tong, J., Viale, M., Bacigalupoe, A., Podesta, M., Sogno, G., Tedone, E. and Marmont, A. M. (1992) Cytokine serum levels and acute graft versus host disease after allogeneic bone marrow transplantation. *Hematologica*, **77**, 365–366.
14. Jadus, M. R. and Wepsic, H. T. (1992) The role of cytokines in graft versus host reactions and disease. *Bone Marrow Transplantation*, **10**, 1–14.
15. Fuji, Y., Koku, K. and Kaneko, T. (1992) TNF is associated with chronic GVHD in BMT recipients. *Bone Marrow Transplantation*, **10**, 194–195.
16. Holler, E., Kolb, H. J., Moller, A., Kempeni, J. *et al.* (1990) Increased serum levels of tumor necrosis factor precede major complications of bone marrow transplantation. *Blood*, **75**, 1011–1016.
17. McCarthy, P. L., Abhyanhan, S., Neben, S. *et al.* (1991) Inhibition of Interleukin-1 by an Interleukin-1 receptor antagonist prevents Graft-Versus Host Disease. *Blood*, **78**, 1915–1918.
18. Symington, F. W., Symington, B. E., Liu, P. Y., Viguet, H., Santhanam, U. and Sengal, P. B. (1992) The relationship of serum IL-6 levels to acute graft versus host disease and hepatorenal disease after human bone marrow transplantation. *Transplantation*, **54**, 4557–4562.
19. Kanamara, A. and Hara, H. (1987) Hematopoietic factors in graft versus host reaction. *Internatl. J. Cell Cloning*, **5**, 450–462.
20. Reyes, V. E. and Klimpel, G. R. (1987) Interferon alpha/beta synthesis during acute graft versus host disease. *Transplantation*, **43**, 412–416.
21. Barrett, A. J. (1989) Graft versus host disease—clinical features and biology. *Bone Marrow Transplantation*, **4**, 18–22.
22. Bianco, J. A., Appelbaum, F. R., Nemunaitis, J. *et al.* (1991) Phase I-II trial of pentoxifylline for the prevention of transplanted-related toxicities following bone marrow transplantation. *Blood*, **79**, 1205–1211.
23. Herve, P., Flesch, M., Tiberghien, P. *et al.* (1992) Phase I-II trial of a monoclonal anti TNF alpha antibody (B-C7) in the treatment of refractory severe acute GVHD. *Blood*, **79**, 3362–3368.
24. Keetter, Y., Singer, A., Nagler, A., Slavin, S. and Fabian, I. (1994) Ciprofloxacin enhances hematopoiesis and the antibacterial activity of peritoneal polymorphonuclears in lethally irradiated bone marrow transplanted mice. *Exper. Hematol.*, **22**, 360–365.