Increased serum concentrations of tumour necrosis factor in β thalassaemia: Effect of bone marrow transplantation

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Abstract

Aims: **Serum concentrations of tumour necrosis factor-α (TNF) were determined in β thalassemic patients before and after bone marrow transplantation (BMT) to evaluate whether changes in TNF concentrations after BMT were related to immune mediated complications.**

Methods: **Serum TNF concentrations were determined by enzyme linked immunoassay (EIA) in paired samples from 71 patients with β thalassemia before and after BMT. Serial samples from 13 patients were also studied for up to six months after BMT. Forty one normal healthy children matched for sex and age were studied as controls.**

Results: **β thalassemic patients had high serum TNF concentrations before transplantation compared with controls. These were not related to sex, age, duration of disease, number of blood transfusions, transferrin concentrations or splenectomy. DQw1 positive patients showed significantly lower TNF concentrations than non-DQw1 cases. Patients with severe liver fibrosis had significantly higher TNF concentrations. No correlation was found between TNF values and BMT outcome before transplantation but TNF α values fell significantly after BMT. The decrease persisted only in patients with successful engraftment. In serial samples studied for up to six months after BMT, TNF values decreased but in four out of five patients with graft rejection and in all five with acute graft versus host disease (GVHD) sharp increases occurred at the time of clinical symptoms. No correlation was found between the degree of GVHD and serum TNF-α concentrations nor between TNF-α concentrations after BMT and the presence of bacterial, viral, and fungal infections.**

Conclusions: **About 50% of β thalassemic patients have increased serum TNF, and the changes after BMT are related to the occurrence of immune mediate complications. The persistence of low TNF concentrations after successful engraftment may be due to the preparative regimen and the lack of adverse immune reactions.**

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Tumour necrosis factor α/cachectin (TNF) is a 17 kilodalton cytokine that is synthesised by monocytes/macrophages, natural killer cells/large granular lymphocytes, and T lymphocytes subsets.[1] Its role in inflammation is important, emphasised by the variety of infectious and immunological stimuli which can induce TNF synthesis and by the variety of proinflammatory actions exerted by this cytokine which include activation and degranulation of neutrophils, upregulation of surface cell adhesion molecules expressed on neutrophils and endothelial cells, induction of procoagulant activity, synthesis of prostaglandin E2 and IL-1 and fibroblast proliferation.[2] High affinity membrane receptors for TNF can be found in normal tissue including liver, kidney, intestine and lung.[3,4] All these properties make TNF a plausible candidate as a mediator of the major adverse immunological responses which can occur after bone marrow transplantation (BMT)—namely, graft *versus* host disease (GvHD) and graft rejection. Antibodies to TNF have already been shown to mitigate the effect of acute skin and bowel lesions in the acute phase of GvHD[5] when administered prophylactically to mice. Furthermore, in addition to endotoxaemia and severe infectious purpura,[6] raised circulating TNF concentrations were found during acute rejection episodes after kidney transplantation.[7]

Some immunological abnormalities have been documented in thalassaemia major,[8] including decreased natural killer cell and macrophage activity, reduced mixed lymphocyte reaction, and increased numbers of CD8 positive cells. Some of these changes can be related to the number of transfusions the patients received and others may be attributed to the immunological effects of iron overload.[9]

Methods

From the series of 222 patients reported elsewhere,[10–13] we randomly selected 71, classifying them according to outcome of bone marrow transplantation.[14] Nineteen had acute graft *versus* host disease (GvHD), 11 chronic GvHD, 23 had rejected the transplant and 18 were free of disease.

Twenty four had received up to 100 transfusions and 47 more than 100 transfusions. The

spleens had been removed from 16 patients before undergoing bone marrow transplantation. Patients were human leucocyte antigen (HLA) typed for A, B, C, DR and 37 were typed for DQw using standard techniques.[15 16]

Liver biopsy was performed in 60 patients as part of liver disease assessment before transplantation. The presence of siderosis and fibrosis was evaluated by semiquantitative scoring: absent, mild, moderate, severe. Fifteen patients presented with chronic active hepatitis and 13 with chronic persistent hepatitis; none had cirrhosis.

Serum samples were obtained from all patients (mean 31 (SEM 6) days) before transplantation and subsequently seven to 380 days after transplantation. In patients with GvHD or graft rejection the sample taken after transplantation was obtained at the onset of transplant complications. Serial serum samples were obtained from 13 patients (three with successful engraftment, five with graft rejection, and five with acute GvHD) (three to seven samples from each patient) during the first two to three months after transplantation. Blood samples were obtained from each patient between 0700 and 0900 hours and left to clot at room temperature. Serum samples were stored at −70°C until analysis.

The preparative regimen for bone marrow transplantation has been described previously.[11]

EVALUATION AFTER TRANSPLANTATION

Infection was diagnosed on the basis of clinical evaluation and blood cultures which were drawn whenever the patients had a temperature of 38°C or more. Oral cultures for herpes simplex virus and Candida were taken weekly. Documented sepsis was defined as a positive blood culture; a documented fungal infection was defined by positive histological results or positive cultures with appropriate clinical findings. Viral infection was defined by a positive blood culture or by a clinically important increase in IgM class specific antibodies.

Acute and chronic GvHD was diagnosed and graded according to established criteria.[17]

Engraftment was evaluated according to β globin chain synthesis or chromosomal analysis, or both.

Serum samples were obtained from 41 normal healthy children matched for sex and age attending the outpatient clinic at Pesaro hospital (seven cases) and at King's College Hospital, London (34 cases) for routine checks. No significant difference has ever been found in TNF serum concentrations between Italian and British children.

TNF ASSAY

Circulating TNF was determined by an enzyme immunoassay (EIA) with two monoclonal antibodies to TNF (T Cell Sciences, Cambridge, Massachusetts). The first monoclonal antibody was adsorbed on to polystyrene microtitre wells after 18 hours of incubation at 4°C. After washing, serum samples were added undiluted and incubated for two hours at 37°C. After washing, peroxidase conjugated anti-TNF monoclonal antibody was added and incubated for two hours at 37°C. Finally, OPD substrate was added and the reaction stopped. The plates were read in an EIA reader (Bio Rad), interfaced with an IBM personal computer XT. Mean absorbance (at 492 nm) values from patient samples were plotted on a standard curve obtained from reference standards of 0, 40, 150, 500, 1000 pg/ml of recombinant TNF. Standard curve coefficients of correlation always exceeded 0·95: 1 mg of TNF used as a standard is reported to be equivalent to 2×10^7 units of activity, as defined by actinomycin treated L 929 cytotoxicity assay. The test is reported to have a mean interassay coefficient of variation of less than 8%. The optical density units were converted into pg/ml by the Bio Rad ELISA data analysis software.

Because of the skewed distribution of TNF values, non-parametric tests were used to analyse data: Wilcoxon's test for paired observations, the Mann Whitney U test for independent values. Kendall's coefficient of correlation was used to compare TNF concentrations and the results of liver histology.

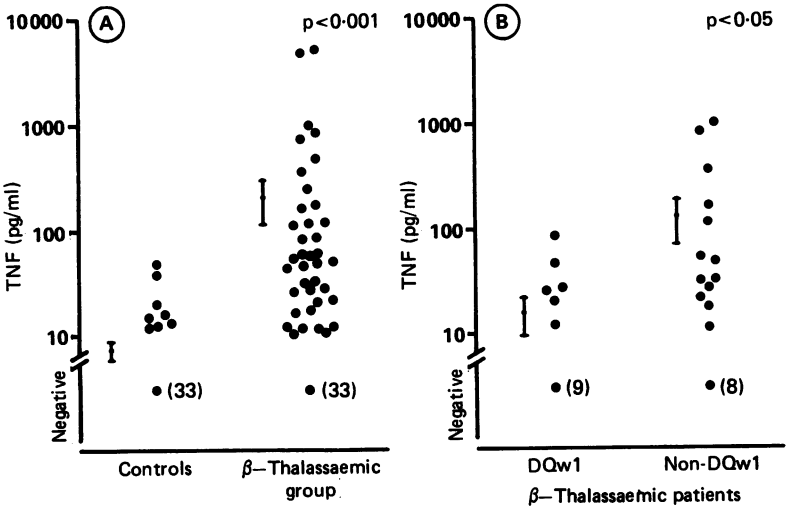

Figure 1 Serum TNF concentrations in normal controls, in thalassaemic patients (A) and DQw1 positive and negative thalassaemic patients (B). Mean standard (SEM) values are presented for each group. Numbers of negative cases in parentheses.

Results

BEFORE TRANSPLANTATION

No significant difference in cytokine concentrations among the pre transplant samples from the four bone marrow transplantation outcome groups was found. We therefore also considered the whole group of β-thalassaemic patients and compared them with the controls.

Beta thalassaemic patients had higher serum TNF concentrations than controls (fig 1A). TNF concentrations in β thalassaemic patients were not related to sex, age, disease duration, number of blood transfusions, splenectomy, transferrin concentrations or drug treatment for engraftment.

Patients carrying the DQw1 antigen showed significantly lower TNF concentrations compared with those without (fig 1B). No associa-

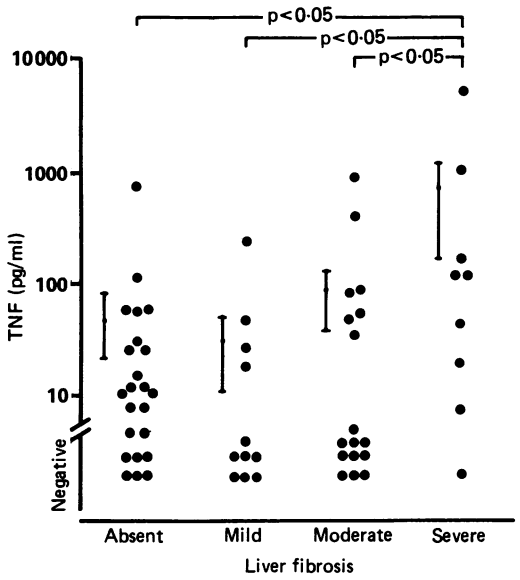

Figure 2 Serum TNF concentrations in β thalassaemic patients with different degrees of liver fibrosis. Mean (SEM) values are presented for each group.

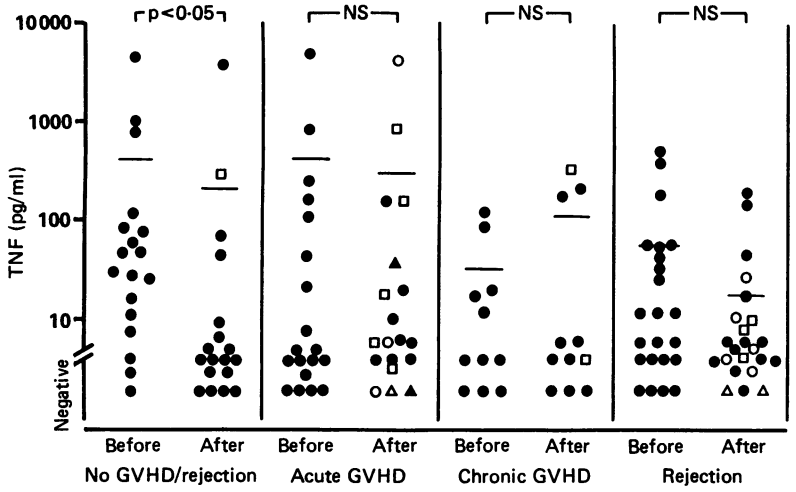

Figure 3 Serum TNF concentrations in thalassaemic patients before and after bone marrow transplantation. (□) Bacterial infection; (○) fungal infection; (▲) viral infection; (△) bacterial and fungal infection. Mean TNFG concentations are presented for each group.

tion was found between TNF concentration and DR2, DR3, DR4, DR5, DRw6, and DR7 antigens. Patients were severe fibrosis had significantly the higher TNF concentrations compared with those with no, mild, or moderate liver fibrosis (fig 2).

No relation was found between TNF concentrations and the presence of chronic persistent or active heptatitis, and the degree of siderosis (data not shown).

AFTER TRANSPLANTATION (FIG 3)

In patients who had a successful engraftment TNF fell significantly. In contrast, in patients with GvHD or rejection TNF concentrations were no different from the concentration before bone marrow transplantation.

No relation was found between TNF concentration and the presence of bacterial, viral, and fungal infections after transplantation (fig 3). TNF concentrations were > 10 pg/ml in only eight of 24 patients with infections.

Discussion

The main results of this study are: (1) an

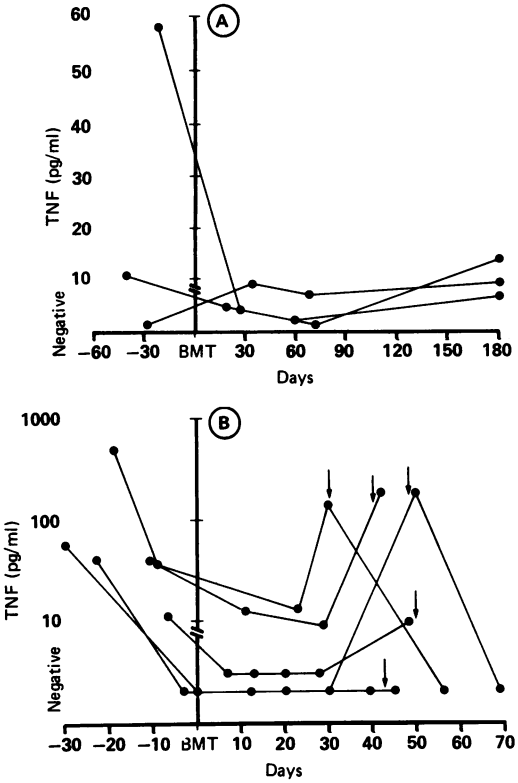

Figure 4 (A) Sequential serum TNF concentrations in three thalassaemic patients with successful engraftment. (B) Sequential serum TNF concentrations in five thalassaemic patients with graft rejection. Arrows indicate the day rejection was diagnosed.

increase in TNF in thalassaemia; (2) a corresponding decrease after bone marrow transplant; (3) an increase during immunological complications. The broad distribution of TNF concentrations accords with the significant differences among individuals in the levels of TNF synthesis by stimulated mononuclear cells observed by Jacob and coworkers[18] in healthy subjects.

We found that substantial increases in TNF occur in about 50% of β thalassaemic patients. It is noteworthy that the patients from our series with the highest TNF concentrations (> 100 pg/ml) did not present with symptoms or signs related to TNF associated syndromes (endotoxic shock or cachexia). Indeed, the immunoassay we used may detect TNF molecules without biological activity. Other investigators have found high TNF concentrations in healthy subjects.[19,20] We suggest that the lack of systemic harmful effects may be related to regulatory factors, such as autoantibodies[21] or soluble cytokine receptors[22,23] which may modulate the systemic biological activity of the cytokine. TNF is probably increased as a consequence of repeated immune stimulation. Thalassaemic patients are generally prone to several antigenic stimulations such as repeated blood transfusions and infections. In our study we used a crude evaluation of the transfusion received by the patients. The immunogenicity of blood transfusion also varies in different patients according to their blood groups; thus the lack of a relation between TNF concentrations and the number of transfusions is not surprising.

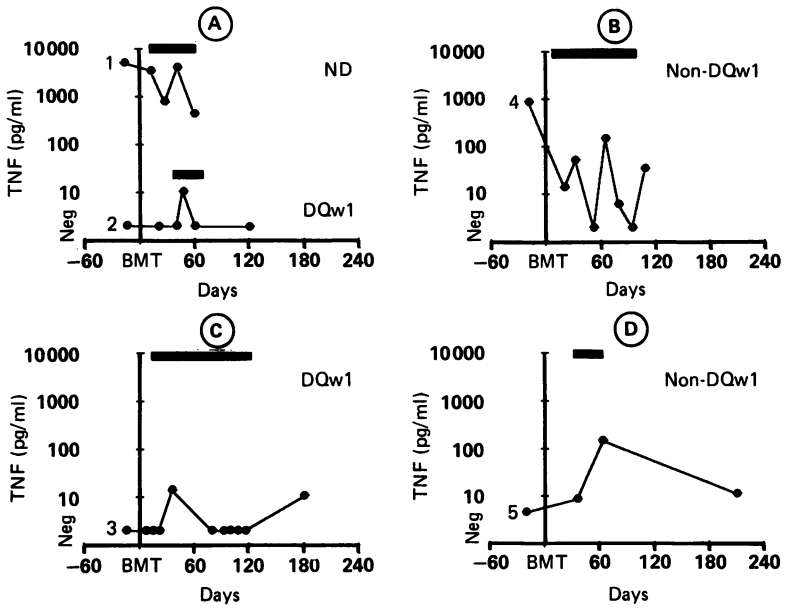

Figure 5 Sequential serum TNF concentrations in five thalassaemic patients with GVHD. Solid bars indicate clinical manifestation of acute GVHD. Four out of five patients were typed for DQw antigen. ND = DQw typing not done.

We observed that DQw1 positive patients had significantly less circulating TNF before bone marrow transplantation than DQw1 negative patients. The follow up study of patients with GvHD also confirms the relation between TNF synthetic capacity and DQw1 positivity. Our finding is in keeping with the previous report by Jacob and coworkers,[18] who found that DQw1 positivity was associated with a low TNF production in mitogen stimulated mononuclear cells from normal subjects. We did not find any association between TNF concentration and other HLA class II antigens in contrast to Jacob *et al*,[18] who found increased TNF production by DR3 and DR4 positive subjects and, conversely, decreased TNF production by DR2 positive subjects.

In our study high TNF concentrations before bone marrow transplantation were also associated with the presence of severe liver fibrosis. This is of particular interest as one of the best characterised properties of TNF is its growth promoting activity on fibroblast cell lines.[24 25] Furthermore, Piguet and coworkers[26] showed that TNF has a central role in the pathogenesis of experimental lung fibrosis. Our results may suggest that TNF is also involved in the pathogensis of thalassaemia associated liver fibrosis.

After bone marrow transplantation, patients with successful engraftment showed a significant decrease in TNF concentrations, probably as a consequence of the preparative drug regimen for GvHD prophylaxis and the lack of immunological complications. A temporary fall in TNF concentrations was also observed during the immediate period after transplantation in patients with acute GvHD or rejection, but sharp increases in TNF concentrations were shown when these complications arose. Because of our sampling procedure we were unable to show an early TNF increase before the clinical manifestation of GvHD or rejection. An early TNF increase before these episodes was documented by Holler *et al*,[27] who suggested a prognostic value for TNF. TNF also seems to be relevant in the pathogenesis of bone marrow transplantation complications.

Evidence in support of this hypothesis has been produced by Piguet *et al*,[5] who showed the effect of anti-TNF in skin and gut lesions of acute GvHD in mice. Surprisingly, these authors did not detect any TNF activity in the sera of transplanted mice. This discrepancy could be due to the different sensitivities of the assays used or, more probably, to the presence of TNF inhibitors interfering in the bioassay.[28]

In conclusion, our study documents a further immunological abnormality in thalassaemia major and suggests that there is a role for TNF in the hepatic fibrogenic process which occurs in this disorder. After bone marrow transplantation, in absence of the vigorous immune stimulations of GvHD and rejection, TNF falls to low levels. TNF perturbations in GvHD and rejection probably reflect the activation of monocytes/macrophages and lymphocyte subsets which occur during the immunological complications of bone marrow transplantation.[29]

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1 Le J, Vilcek J. Biology of disease. Tumor necrosis factor and interleukin 1: cytokines with multiple overlapping biological activities. *Lab Invest* 1987;56:234–48.
2 Dayer JM, Zavadil-Grobb C, Ucla C, Mach B. Induction of human interleukin 1 mRNA measured by collagenase- and prostaglandin E2-stimulating activity in rheumatoid synovial cells. *Eur J Immunol* 1984;14:898–901.
3 Aggarawal BB, Eessalu TE, Hass PE. Characterization of receptors for human tumor necrosis factor and their regulation by gamma-interferon. *Nature* 1985;318:665–8.
4 Tsujimoto M, Yip YK, Vilcek J. Tumor necosis factor: specific binding and internalization in sensitive and resistant cells. *Proc Natl Acad Sci USA* 1985;82:7626–31.
5 Piguet PF, Grau GE, Allet B, Vassalli P. Tumor necrosis factor/chachectin is an effector of skin and gut lesions of the acute phase of Graft-Vs-Host disease. *J Exp Med* 1987;166:1280–9.
6 Girardin E, Grau GE, Dayer JM, Roux-Lombard P. The J5 Study Group, Lambert PH. Tumor Necrosis Factor and Interleukin-1 in the serum of children with severe infectionous purpura. *N Engl J Med* 1988;319:397–400.
7 Maury CPJ, Teppo AM. Raised serum levels of cachectin/tumor necrosis factor in renal allograft rejection. *J Exp Med* 1987;166:1132–7.
8 Neri A, Callea V, D'Ascola DG, Martino B. Immune status in thalassaemia major. *Haematologica* 1986;71:511–6.
9 De Sousa M. Immune cell functions in iron overload. *Clin Exp Immunol* 1989;75:1–6.
10 Lucarelli G, Polchi P, Galimberti M, *et al*. Marrow transplantation for thalassemia following busulphan and cyclophosphamide. *Lancet* 1985;i(8442):1355–7.
11 Lucarelli G, Galimberti M, Polchi P, *et al*. Marrow transplantation in patients with advanced thalassemia. *N Engl J Med* 1987;316:1050–5.
12 Andreani M, Centis F, Lucarelli G, *et al*. Immunologic recovery in thalassemic marrow graft recipients following high dose busulfan and cyclophosphamide. *Transplantation* 1988;46:394–8.
13 Lucarelli G, Galimberti M, Polchi P, *et al*. Bone marrow transplantation in patients with thalassemia. *N Engl J Med* 1990;322:417–21.
14 Pocock SJ. *Clinical trials*. Chichester: John Wiley & Sons 1983:80–7.
15 Mittal KK, Mickey MR, Singal DP, Terasaki PI. Serotyping for homo-transplantation. XVIII. Refinement of microdroplet lymphocyte cytotoxicity test. *Transplantation* 1968;6:913–27.
16 Sengar DPS, Terasaki PI. A semimicro mixed leukocyte

culture test. *Transplantation* 1971;11:260–7.
17 Thomas ED, Storb R, Clift RA, *et al.* Bone-marrow transplantation (second of two parts). *N Engl J Med* 1975;292:895–902.
18 Jacob CO, Fronek Z, Lewis GD, Koo M, Hansen JA, McDevitt HO. Heritable major histocompatibility complex class II-associated differences in production of tumor necrosis factor: Relevance to genetic predisposition to systemic lupus erythematosus. *Proc Natl Acad Sci USA* 1990;87:1233–7.
19 Scuderi P, Sterling KE, Lam KS, *et al.* Raised serum levels of tumour necrosis factor in parasitic infections. *Lancet* 1986;ii:1364–5.
20 McLaughlin PJ, Davies HM, Aikawa A. Tumour-necrosis factor in normal plasma. *Lancet* 1990;336:1014–5.
21 Fomsgaard A, Svenson M, Bendtzen K. Auto-antibodies to tumour nerosis factor α in healthy humans and patients with inflammatory diseases and gram-negative bacterial infections. *Scand J Immunol* 1989;30:219–23.
22 Lantz M, Gullberg U, Nilsson E, Olsson I. Characterization in vitro of a human tumor necrosis factor-binding protein. A soluble form of a tumor necrosis factor receptor. *J Clin Invest* 1990;86:1396–402.
23 Porteu F, Nathan C. Shedding of tumor necrosis factor receptors by activated human neutrophils. *J Exp Med* 1990;172:599–607.
24 Sugarmann BJ, Aggarawal BB, Figari IS, Palladino MA. Recombinant human tumor necrosis factor: effects of proliferation of normal and transformed cells in vitro. *Science* 1985;230:943–7.
25 Tracey KJ, Vlassara H, Cerami A. Cachectin/tumor necrosis factor. *Lancet* 1989;i:1122–6.
26 Piguet PF, Collart MA, Grau GE, Kapanci Y, Vassalli P. Tumor necrosis factor/cachectin plays a key role in bleomycin-induced pneumopathy and fibrosis. *J Exp Med* 1989;170:655–63.
27 Holler E, Kolb HJ, Moller A, *et al.* Increased serum levels of tumor necrosis factor precede major complications of bone marrow transplantation. *Blood* 1990;75:1011–6.
28 Mosmann TR, Fong TAT. Specific assays for cytokine production by T cells. *J Immunol Methods* 1989;116:151–8.
29 Ferrara JLM, Deeg HJ. Graft-versus-host disease. *N Engl J Med* 1991;324:667–74.