Immunology 1994 **82** 211–214

Differential cytokine production associated with distinct phases of murine graft-versus-host reaction

P. GARSIDE, S. REID,* M. STEEL & A. McI. MOWAT *Department of Immunology, University of Glasgow, Western Infirmary, Glasgow*

SUMMARY

Previous studies which demonstrated that cytokines such as interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) are essential for the development of graft-versus-host reaction (GvHR) did not establish whether the individual cytokines were responsible for distinct features of the disease. In this report, we show that IFN-γ production is associated with the early proliferative phase of the disease, whereas the late, destructive phase correlates with production of TNF-α. These studies may assist in the development of specific immunotherapies aimed at individual aspects of immunologically mediated disease.

INTRODUCTION

Increased production of a wide range of cytokines has been demonstrated in murine models of graft-versus-host reaction (GvHR)[1–4] and *in vivo* depletion studies have shown that interferon-γ (IFN-γ) and tumour necrosis-factor-α (TNF-α) are essential for GvHR-associated damage to the epithelial surfaces of the skin and the gut.[5,6] We and others have shown that administration of TNF-α and IFN-γ to normal rodents can cause epithelial pathology similar to that found in GvHR[7,8] and both cytokines have direct effects on epithelial cells *in vitro*.[7] Although these studies suggest an important role for TNF-α and IFN-γ in GvHR, it is not known how each cytokine might contribute to individual aspects of the damage.

Here we show that the production of both TNF-α and IFN-γ is increased in mice with GvHR, but that the levels of the individual mediators correlate with distinct phases of the disease. Whereas increased production of IFN-γ is associated with the early proliferative stage of GvHR, elevated levels of TNF-α are found only in association with the later, destructive consequences of GvHR. These findings may assist the development of therapies aimed at individual components of immunologically mediated diseases.

MATERIALS AND METHODS

Mice

Specified pathogen-free (C57B16 × DBA/2)F_1(BDF_1)(H-2^{bxd}) and C57B1/6 (B6) mice were obtained from Harlan Olac (Bicester, U.K.) and first used at 6–8 weeks of age.

Received 29 November 1993; revised 5 February 1994; accepted 6 February 1994.

*Present address: Professorial Surgical Unit, St Bartholomew's Hospital, London EC1A 7BE, U.K.

Correspondence: Dr P. Garside, Dept. of Immunology, University of Glasgow, Western Infirmary, Glasgow G11 6NT, U.K.

Induction of GvHR

The GvHR was induced by intravenous injection of 10^8 viable C57B1/6 spleen cells into BDF_1 hosts. Control mice received 0·2 ml medium only. The intensity of the systemic GvHR was assessed by measurements of splenomegaly and body weights. Splenomegaly was assessed as the Spleen Index and an index of $> 1·3$ taken as evidence of significant GvHR.[5]

Production of cytokines in vitro

Single-cell suspensions were prepared in RPMI-1640 (Gibco BRL, Paisley, U.K.) by rubbing spleens through a stainless steel mesh and passing the resulting suspension through Nitex mesh (Cadisch & Sons, London, U.K.). After three washes in medium, the cells were resuspended at a final concentration of 10^6 cells/ml and cultured in 1-ml aliquots in 24-well tissue culture plates (Costar, Nucleopore, High Wycombe, U.K.) in RPMI-1640 containing 10% fetal calf serum (FCS), 100 U/ml penicillin/100 mg/ml streptomycin, 25 mM HEPES, 0·05 M 2-mercaptoethanol (all Gibco BRL) either alone or with 10 μg/ml concanavalin A (Con A) (Sigma, Poole, U.K.), 10 μg/ml LPS (Sigma) or 1 μg/ml lipopolysaccharide (LPS) + 200 U/ml recombinant murine (rm) IFN-γ (courtesy of Dr G. Adolf, BASF, Vienna, Austria). Preliminary studies indicated that peak cytokine production occurred after 24 hr of culture and, at this time, supernatants were harvested and stored at −70° until assayed.

Cytokine assays

IFN-γ and TNF-α production was quantified using sandwich ELISA techniques. Briefly, for measurement of IFN-γ, flexible 96-well microelisa plates (Falcon, ICN Flow, High Wycombe, U.K.) were coated overnight at 4° with 100 μl of monoclonal anti-murine IFN-γ (R46A2; courtesy of Dr T. Mosmann, DNAX, Palo Alto, CA) at 10 μg/ml in phosphate-buffered saline (PBS) (pH 7·2). Plates were then washed twice with PBS containing 0·05% Tween-20, after which non-specific protein

binding sites were blocked by incubation with 200 μl of PBS containing 10% FCS for 1 hr at 37°. Following blocking, the plates were washed × 3 as above and samples and standards, diluted in culture medium, were added to individual wells in a volume of 100 μl and incubated at room temperature for 2 hr. The plates were then washed × 3 and 100 μl/well of biotinylated anti-murine IFN-γ (XMG1.2; courtesy of Dr T. Mosmann) added at a concentration of 10 μg/ml. After incubation for 1 hr at room temperature, the plates were washed × 3 and 100 μl/well extravidin–peroxidase (Sigma) added at 2 μg/ml. Following a final incubation for 1 hr at room temperature, the plates were washed × 6 before 100 μl azino-bis (ABTS) substrate (Sigma) was added to each well and the absorbance read at 570 nm on an automatic plate reader. A similar protocol was followed for the measurement of TNF-α using a monoclonal anti-murine TNF-α (TN3; courtesy of Dr A. Morgan, Celltech, Slough, U.K.) as the capture antibody, followed by a polyclonal rabbit anti-TNF-α (courtesy of Professor F. Y. Liew, Glasgow, U.K.) and a goat anti-rabbit IgG conjugate (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) for detection. Serial dilutions of recombinant cytokines were included as standards on each plate assayed and the concentration in experimental samples calculated from the resulting standard curves.

Statistical analysis

Results are represented as the mean ± 1 SEM where indicated and were analysed using Student's *t*-test.

RESULTS

Weight loss and mortality

As we have shown previously,[9] BDF_1 mice with GvHR showed significant weight loss after day 16 and this continued until the end of the experiment on day 30 (Fig. 1a). In parallel, three of the BDF_1 mice with GvHR died on day 21 and one on day 30 (Fig. 1a).

Splenomegaly

BDF_1 mice with GvHR also developed splenomegaly which was significant by day 9, peaked at day 16 and returned towards control values after this point (Fig. 1b).

Production of IFN-γ

We next examined IFN-γ production in the spleen. Relatively low levels of IFN-γ were produced by control cells at any time-point, even after stimulation with Con A. In contrast, IFN-γ production could be detected using Con A-stimulated GvHR cells by day 2. This peaked on day 9 and returned to control values by day 16 (Fig. 2). Unstimulated GvHR cells also produced small amounts of IFN-γ at these time-points (data not shown). Thus, a peak of IFN-γ production was observed at the peak of the proliferative phase of the disease as indicated by the splenomegaly.

Production of TNF-α

Little or no TNF-α was produced by spleen cells from control mice at any time-point, even after stimulation with LPS and/or IFN-γ (Fig. 3). Spleen cells from BDF_1 mice with GvHR produced significant amounts of TNF-α when stimulated *in vitro* with LPS (data not shown) and this was even more marked when exogenous IFN-γ was added to the cultures (Fig. 3). However, this did not occur until day 16 of the GvHR and continued to rise thereafter. As the production of TNF-α correlates with the clinical evidence of destructive GvHR, including weight loss and mortality (Fig. 1a), we believe our immunoassay is measuring predominantly bioactive cytokine. However, direct proof of this will require parallel assays of TNF-α immuno- and bioactivity.

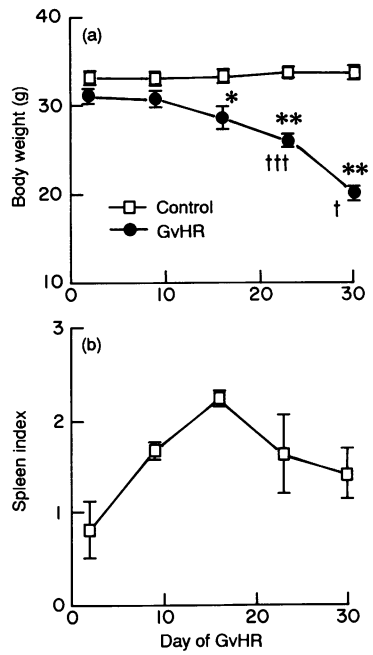

Figure 1. Systemic disease in BDF_1 mice with GvHR. (a) Mortality and body weights in GvHR and in control mice. (b) Splenomegaly in GvHR mice. Results shown are mean ± 1 SEM for four to five mice per group at the indicated times after inducing GvHR: $*P < 0{\cdot}01$; $**P < 0{\cdot}001$ versus control weight; †death.

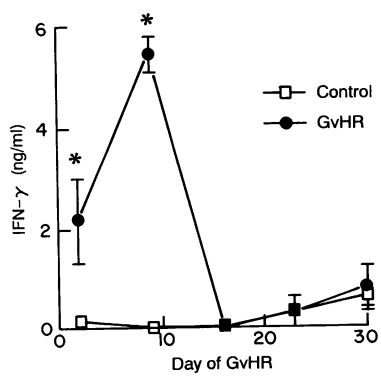

Figure 2. Kinetics of IFN-γ production in BDF_1 mice with GvHR. IFN-γ production by splenocytes pooled from GvHR and control mice in response to Con A. The results shown are the means ± 1 SEM of four replicates for each sample: $*P < 0{\cdot}05$ versus control. Similar results were obtained in two replicate experiments.

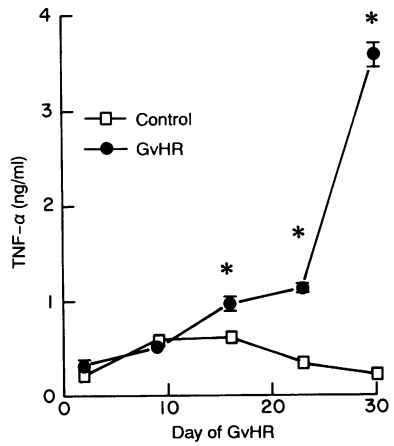

Figure 3. Kinetics of TNF-α production during BDF_1 GvHR. TNF-α production by splenocytes pooled from GvHR and control mice in response to LPS + IFN-γ. The results shown are means ± 1 SEM of four replicates for each sample: $*P < 0{\cdot}05$ versus control. Similar results were obtained in two replicate experiments.

DISCUSSION

We have shown that the production of two inflammatory cytokines correlates with individual aspects of immunopathology in murine GvHR. The model of GvHR used was chosen to provide a biphasic disease which begins with an early period of intense proliferation characterized by enhanced natural killer (NK) activity, splenomegaly and lymphoid hyperplasia.[9] This is superseded by a severe, destructive disease with cachexia, anaemia, immunosuppression, epithelial damage and eventually death.[9] We show here that an increased capacity to produce IFN-γ is associated with the proliferative phase of GvHR, while increased production of TNF-α only occurred when destructive disease developed.

Earlier reports which have implicated both TNF-α and IFN-γ in the pathogenesis of GvHR by showing increased production or by inhibiting epithelial GvHR by depleting the cytokines did not associate individual aspects of the disease with each mediator.[1–6] However, our current findings are consistent with other recent work showing that administration of IFN-γ to normal mice produces only mild proliferative pathology in the small intestine.[7] Although this association between IFN-γ production and proliferative pathology in GvHR appears to contrast with the generally cytostatic effects of IFN-γ *in vitro*,[10] IFN-γ induces hyperplasia of keratinocytes when given *in vivo*.[11] Thus, IFN-γ may play an indirect role in tissue-specific immunopathology *in vivo*, possibly by interacting with the other cytokines and cells which have been implicated in conditions such as GvHR.[12,13]

Although the production of IFN-γ in GvHR is transient, it appears to be essential for the full progression of the disease, as depletion of IFN-γ prevents all aspects of GvHR in severe models.[5] Our current findings suggest that one effect of IFN-γ may be to stimulate the release of TNF-α. In our experiments, TNF-α was only produced by lymphoid cells during the destructive phase of GvHR, confirming an earlier report in which TNF-α could only be found in the serum of mice with severe GvHR.[14] In both cases, the generation of TNF-α required additional stimulation *in vivo* or *in vitro* with LPS, suggesting that the early production of IFN-γ in GvHR primes macrophages (Mφ) to release TNF-α upon secondary stimulation with LPS. In severe progressive GvHR, this may occur spontaneously due to the increased amounts of LPS which appear in circulation.[14] Many of the manifestations of destructive GvHR such as cachexia and cellular apoptosis are characteristic of TNF-α[15] and we suggest that this also causes the villus atrophy found in the intestine late in GvHR.[9] This is supported by the ability of TNF-α to produce a destructive enteropathy in normal animals[7] and by its toxicity for enterocytes.[7] In addition to priming the production of TNF-α, IFN-γ may also potentiate the immunopathological effects of TNF-α in GvHR by up-regulating the expression of TNF-α receptors[16] or by direct synergy with TNF-α.[7,17] Finally, IFN-γ may be at least partly responsible for the increased amounts of gut-derived LPS that occur in GvHR,[14] via its ability to alter the permeability of the epithelial barrier.[18]

The sources of IFN-γ and TNF-α in GvHR remain to be established. Alloreactive $CD4^+$ and $CD8^+$ T cells can both produce IFN-γ when isolated from mice with GvHR,[2] while NK cells, which are activated during GvHR,[9] are also a potent source of this cytokine.[19] Although we believe that IFN-γ-activated Mφ may be the major source of TNF-α in GvHR, other cells which are activated in GvHR also have the capacity to produce TNF-α, including T cells,[15] NK cells[20] and mucosal mast cells.[21]

Together, our results show that IFN-γ and TNF-α are released during GvHR and indicate that each cytokine may be associated with distinct aspects of the disease. This may ultimately assist the design of specific therapies targeted at particular aspects of these conditions.

ACKNOWLEDGMENT

This work was supported by MRC grant G8823030.

REFERENCES

1. Smith S.R., Terminelli C., Kenworthy-Bott L. & Phillips D.L. (1991) A study of cyokine production in acute graft-vs-host disease. *Cell. Immunol.* **134,** 336.
2. Kelso A. (1990) Frequency analysis of lymphokine-secreting $CD4^+$ and $CD8^+$ T cells activated in a graft-versus-host reaction. *J. Immunol.* **145,** 2167.
3. Troutt A.B. & Kelso A. (1993) Lymphokine synthesis *in vivo* in an acute murine graft-versus-host reaction: mRNA and protein measurements *in vivo* and *in vitro* reveal marked differences between actual and potential lymphokine production levels. *Int. Immunol.* **5,** 399.
4. Allen R.D., Staley T.A. & Sidman C.L. (1993) Differential cytokine expression in acute and chronic murine graft-versus-host disease. *Eur. J. Immunol.* **23,** 333.
5. Mowat A.McI. (1989) Antibodies to γ interferon prevent immunologically mediated intestinal damage in murine graft-versus-host reaction. *Immunology*, **68,** 18.
6. Piguet P.-F., Grau G.E., Allet B. & Vassalli P. (1987) Tumor necrosis factor/cachectin is an effector of skin and gut lesions of the acute phase of graft-vs-host disease. *J. exp. Med.* **166,** 1280.
7. Garside P., Tomlinson R.C., Nichols B.L. & Mowat A.McI. (1993) Analysis of enteropathy induced by tumour necrosis factor α. *Cytokine*, **5,** 24.
8. Sun X-M. & Hsueh W. (1988) Bowel necrosis mediated by tumour necrosis factor is mediated by platelet activating factor. *J. clin. Invest.* **81,** 1328.

9. Mowat A.McI. & Felstein M.V. (1990) Experimental studies of immunologically mediated enteropathy. V. Destructive enteropathy during an acute graft-versus-host reaction in adult BDF_1 mice. *Clin. exp. Immunol.* **79,** 279.
10. Symington F.W. (1989) Lymphotoxin, tumor necrosis factor, and gamma interferon are cytostatic for normal human keratinocytes. *J. invest. Dermatol.* **92,** 798.
11. Nathan C.F., Kaplan G., Lewis W.R., Nusrat A., Witmer M.D., Sherwin S.A., Job C.K., Horowitz C.R., Steinman R.A. & Cohn Z.A. (1986) Local and systemic effects of intradermal recombinant interferon-γ in patients with lepromatous leprosy. *N. Engl. J. Med.* **315,** 6.
12. Mowat A.McI., Hutton A.K., Garside P. & Steel M. (1993) A role for interleukin-1α in immunologically mediated intestinal pathology. *Immunology*, **80,** 110.
13. Garside P., Hutton A.K., Severn A., Liew F.Y. & Mowat A.McI. (1992) Nitric oxide mediates intestinal pathology in graft-vs-host disease. *Eur. J. Immunol.* **22,** 2141.
14. Nestel F.P., Price K.S., Seemayer T.A. & Lapp W.S. (1992) Macrophage priming and lipopolysaccharide-triggered release of tumour necrosis factor α during graft-versus-host disease. *J. exp. Med.* **175,** 405.
15. Le J. & Vilcek J. (1987) Tumour necrosis factor and interleukin 1: cytokines with multiple overlapping activities. *Lab. Invest.* **56,** 234.
16. Tsujimoto M. & Vilcek J. (1986) Tumor necrosis factor receptors in HeLa cells and their regulation by interferon-gamma. *J. biol. Chem.* **261,**5384.
17. Kuale D., Brandtzaeg P. & Lvhaug D. (1988) Up-regulation of the expression of secretory component and HLA molecules in a human colonic cell line by tumour necrosis factor-alpha and gamma interferon. *Scand. J. Immunol.* **28,** 351.
18. Holmgren J., Fryklund J. & Larsson H. (1989) Gamma-interferon-mediated down-regulation of electrolyte secretion by intestinal epithelial cells: a local immune mechanism? *Scand. J. Immunol.* **30,** 499.
19. Brunswick M. & Lake P. (1985) Obligatory role of gamma interferon in T cell-replacing factor-dependent, antigen-specific murine B cell responses. *J. exp. Med.* **161,** 953.
20. Ostensen M.E., Thiele D.L. & Lipsky P.E. (1987) Tumor necrosis factor-α enhances cytolytic activity of human natural killer cells. *J. Immunol.* **138,** 4185.
21. Young J.D.E., Liu C.C., Butler G., Cohn Z.A. & Galli S.C. (1987) Identification, purification and characterisation of a mast cell-associated cytolytic factor related to tumor necrosis factor. *Proc. natl. Acad. Sci. U.S.A.* **84,** 9175.