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Interferon-γ production by peripheral blood mononuclear cells from patients with Behçet's syndrome

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SUMMARY

Interferon-γ (IFN-γ) production by peripheral blood mononuclear cells (MNC) from patients with Behçet's syndrome was compared with production by MNC from control donors. There was no evidence of significant spontaneous production of either IFN-γ or interferon-α (IFN-α) by unstimulated MNC from patients or controls but phytohaemagglutinin treatment of MNC induced IFN-γ in both patient and control groups. Significantly higher titres of IFN-γ were produced by MNC from patients with Behçet's syndrome than MNC from patients with other inflammatory diseases ($P=0{\cdot}005$), hospital controls ($P=0{\cdot}036$) or healthy controls ($P=0{\cdot}009$). No IFN was detected in plasma from patients or controls.

Keywords Behçet's syndrome interferon-γ autoimmune disease

INTRODUCTION

Behçet's syndrome is an inflammatory condition of unknown aetiology, features of which include buccal and genital ulcers, skin lesions, uveitis and arthritis (Mason & Barnes, 1969). From a study of the proliferative responses of peripheral blood mononuclear cells (MNC) stimulated with the mitogen, phytohaemagglutinin (PHA), it was concluded that there are more PHA responsive cells in the peripheral circulation of patients with Behçet's syndrome than matched controls (de Vere-Tyndall *et al.*, 1982). On the basis of this result the authors suggested that immune function may be altered in these patients. Interferon-γ (IFN-γ) is a lymphokine which may participate in the regulation of the immune response (reviewed by Moore, 1983). It may also be involved in the pathogenesis of Behçet's syndrome as it has previously been shown that lymphocytes from affected patients spontaneously produce IFN-γ *in vitro* (Fujii *et al.*, 1983).

Considerable attention has been given to the observation that sera from some patients with autoimmune disease contain IFN (Hooks *et al.*, 1979, 1982b, Preble *et al.*, 1982; Ytterberg & Schnitzer, 1982) and these reports indicate that an unusual, acid-labile, species of interferon-α (IFN-α) is the major subtype present. However, in a study of patients with Behçet's syndrome in Japan, Ohno *et al.* (1982) detected IFN-γ in serum and observed that the titre correlated inversely with disease activity. In order to confirm and extend these findings we measured IFN-γ produced by peripheral blood MNC cultured *in vitro* with and without PHA, and screened plasma from the same patients for IFN by two different bioassays. Our results indicate that MNC from patients with Behçet's syndrome produce more IFN-γ in response to PHA than MNC from control donors, but

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contrary to a previous report there was no spontaneous production of IFN-γ. In addition, plasma from these patients did not contain detectable levels of IFN.

MATERIALS AND METHODS

Patients and controls. Peripheral blood was obtained from 17 patients with Behçet's syndrome (age range 14–66 years) who fulfilled diagnostic criteria proposed by Mason & Barnes (1969) and 10 patients with other inflammatory diseases (age range 20–73 years); four systemic lupus erythematosus (SLE), three polyarteritis, two rheumatoid arthritis and one ankylosing spondylitis. The control groups consisted of seven healthy adults (age range 22–40 years) and six hospital controls with a variety of non-inflammatory disorders (age range 18–59 years). Disease activity in patients with Behçet's syndrome was assessed as previously described (Denman *et al.*, 1979). The main clinical features are summarized in Table 1. Five patients with Behçet's syndrome were receiving prednisolone, two warfarin, and one patient was receiving tolmetin. Three patients with other inflammatory disease were taking prednisolone and one of these was also treated with azathioprine. This study was performed with the approval of Northwick Park Hospital Ethical Committee.

Table 1. Behçet's syndrome—disease features in 17 patients studied

Feature	No.
Orogenital ulcers	17
Inflammatory arthritis	12
Thrombophlebitis	7
Skin lesions	7
Posterior uveitis	5
Iridocyclitis	2

Mononuclear cell separation. Heparinised peripheral blood specimens were layered onto Ficoll-Paque (Pharmacia) and centrifuged at 400*g* for 20 min. Plasma was carefully removed from the gradient and stored at −70°C for subsequent IFN assay. MNC were washed twice in L 15 (Flow Laboratories) and resuspended at 1×10^6 MNC per ml in RPMI 1640 (bicarbonate buffer; Flow Laboratories) containing 10% FCS, glutamine (2 mM), penicillin (100 u/ml) and streptomycin (100 μg/ml).

Production of IFN-γ. Five million MNC were cultured in 25 cm^2 plastic tissue culture flasks (Nunc). IFN-γ was induced with 5 μg/ml PHA (purified PHA; Wellcome) and parallel cultures without PHA were established. Incubation was at 37°C in a humidified incubator maintained with 5% CO_2 and 95% air, and supernatants were harvested after 4 days and stored at −70°C.

IFN assay. HEp 2 cells (Flow Laboratories) were seeded into Microtitre plates (Sterilin), 4×10^4 cells/0·1 ml per well in growth medium (GM; Eagle's MEM + 10% FCS). One-tenth of a millilitre volumes of serial dilutions of test samples in GM were added in duplicate and cultures were incubated overnight at 37°C. Medium was removed, cells were challenged with Semliki Forest virus (SFV, 5 pfu/cell) in maintenance medium (MM; Eagle's MEM + 2% FCS) and the assay was fixed when cytopathic effect (cpe) in virus control monolayers reached 100%. Monolayers were stained with crystal violet and dilutions of test samples protecting 50% of the cells from SFV infection were estimated using an inverted light microscope. A laboratory IFN-γ standard was included in all assays and IFN-γ titres are quoted in laboratory units. The standard consisted of the 4 day supernatant from PHA stimulated (5 μg/ml) MNC. All MNC culture supernatants were screened for IFN by this method. HEp 2 cells detect both IFN-γ and IFN-α although other cell lines such as EBTr are more sensitive to IFN-α (Bacon *et al.*, 1983).

Plasma specimens were diluted from a starting dilution of 1/5 in all cpe inhibition assays and were also screened for IFN-α activity in an assay measuring the inhibition of SFV RNA synthesis in

EBTr cells as previously described (Bacon *et al.*, 1983). The only modification to the method was that EBTr cells were grown in Microtitre plates and after fixation solubilised in NaOH. A laboratory IFN–α standard (Namalwa IFN, Wellcome) was used in all assays on EBTr cells and was expressed in terms of international units/ml (iu/ml) by reference to the international IFN-α standard MRC 69/19. Thus, all IFN-α titres are expressed in terms of iu/ml.

Preliminary characterization of the anti-viral factor in plasma. Neutralization tests were performed with two antisera against human IFN-α (HuIFN-α). Dilutions of sheep anti-HuIFN-α (received from Dr M. de Ley, Rega Instituut, Leuven) and calf anti-HuIFN-α (received from Dr G. M. Scott, University College Hospital, London) sufficient to neutralise 100 iu of IFN-α were mixed with aliquots of plasma and incubated for 1 h at 20°C. The samples were then titrated for residual anti-viral activity on EBTr cells as described previously. Acid stability of the anti-viral factor was assessed by titrating aliquots of plasma to pH 2 with 1 M HCl and incubating for 24 h at 4°C. An equal volume of 1 M NaOH was added after preparing a 1/10 dilution of the plasma in MM, and the sample was then assayed on EBTr cells.

Statistical analysis. The arithmetic mean and standard deviation of $\log_{10}$ transformed IFN titres were calculated for each group of results. Data were compared by the analysis of variance technique.

RESULTS

Production of IFN-γ in vitro

Detectable titres of IFN were obtained from unstimulated cultures of MNC from only two donors, one healthy control and one patient with Behçet's syndrome. However, in both instances IFN was barely detectable and therefore the significance, if any, remains uncertain. The spontaneous IFN may have been IFN-α (6–9 iu/ml) or IFN-γ (2–3 u/ml).

MNC from all donors responded to PHA as assessed by the production of IFN-γ. When the results were divided into the four disease categories (Fig. 1) and analysed it was found that MNC from patients with Behçet's syndrome produced significantly higher titres of IFN-γ than MNC from patients with other inflammatory diseases ($P=0{\cdot}005$), hospital controls ($P=0{\cdot}036$), or healthy controls ($P=0{\cdot}009$). Conversely, there was no association between IFN-γ response and age of the donor and no relationship for patients with Behçet's syndrome between IFN-γ response and disease activity, drug therapy, or pattern of disease.

Anti-viral activity in plasma

All plasma specimens were screened intitially by a cpe inhibition assay on HEp 2 cells. Although the majority of specimens were negative for IFN, there were some samples which, when titrated, did not give a clear end point. Because of the subjective nature of this assay all titrations were repeated in an assay which measured the inhibition of SFV RNA synthesis in EBTr cells. The sensitivity of this cell line to IFN-α is approximately 1 iu/ml. To verify that this system is capable of detecting serum IFN

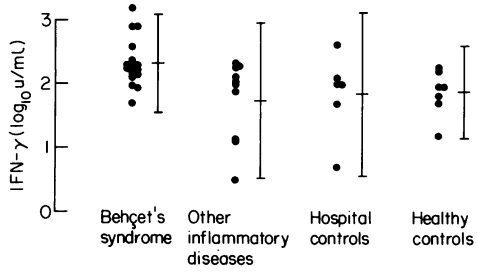

Fig. 1. IFN-γ production by MNC stimulated *in vitro* with PHA. The results are grouped according to disease. The bars represent the mean response ±2 s.d. for each group.

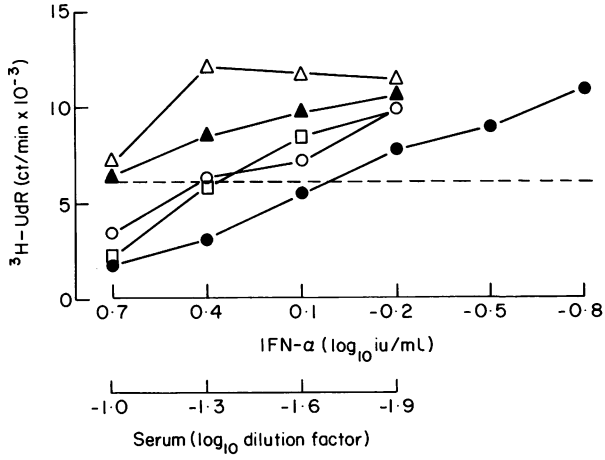

Fig. 2. Titration of IFN-α in a series of serum samples taken from a volunteer at various times after an intramuscular injection of IFN-α; O h = △; 1 h = ○; 2 h = □ and 4 h = ▲. The laboratory IFN-α standard = ●, was included in the assay for comparison. Fifty per cent inhibition of viral RNA synthesis is marked by a broken line.

we titrated a series of serum samples taken from a healthy volunteer at various times after intramuscular injection of 3×10^6 iu of NK 2-purified leucocyte IFN (sera were provided by Dr G. M. Scott, University College Hospital, London; NK2 IFN was provided by Drs Cantell and Secher) and the results are shown in Fig. 2. The gradients of the test samples are similar to that of the IFN-α standard, as assessed by eye, and the results clearly indicate that increasing levels of IFN-α were present in the serum of the volunteer reaching a maximun of about 24 iu/ml 2 h after injection. Before testing the plasma specimens it was confirmed that heparin at the concentration used in the original blood sample (25 u/ml) did not influence SFV RNA synthesis in EBTr cells.

All plasma specimens which, when diluted, inhibited SFV RNA synthesis by 50% or more, were re-tested in parallel with experiments to determine whether the anti-viral factor was resistant to pH 2 treatment and could be neutralised by anti-HuIFN-α sera. In preliminary experiments it was demonstrated that the laboratory IFN-α standard was resistant to pH 2 treatment, but conversely,

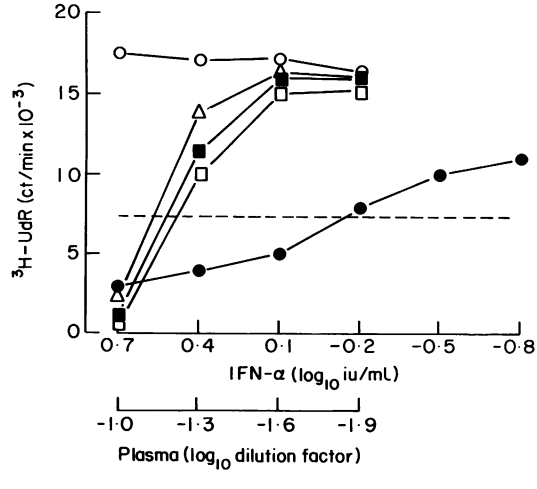

Fig. 3. Inhibition of viral RNA synthesis by plasma obtained from a patient with Behçet's syndrome. Untreated plasma = △; plasma after pH 2 treatment = ○; plasma incubated with calf anti-HuIFN-α = □; plasma incubated with sheep anti-HuIFN-α = ■. Laboratory IFN-α standard = ●. Fifty per cent inhibition of viral RNA synthesis is marked by a broken line.

the laboratory IFN-γ standard was almost completely inactivated. It was also verified that the two antisera neutralized 100 iu of IFN-α. Anti-viral activity in all test samples was eliminated at pH 2 but was not affected by the antisera. These results indicate that the anti-viral factor is active on a bovine cell line, labile at pH 2, but cannot be neutralised by antisera against HuIFN-α. Although a higher proportion of plasma specimens from individuals with Behçet's syndrome inhibited SFV replication when compared with other groups (seven of 17 patients with Behçet's syndrome; two of nine patients with other inflammatory diseases; and one of six hospital controls), no relationship was apparent in the Behçet's group between the presence of anti-viral activity in plasma and either drug therapy or disease activity. The results obtained when plasma from one patient with Behçet's syndrome was tested as previously described are shown in Fig. 3. Plasma treated at pH 2 at dilutions less than 1/40 markedly enhanced SFV RNA synthesis above that of the virus control. Finally, in contrast to the results plotted in Fig. 2 it should be noted that the gradient of the IFN-α standard differs markedly from the gradients of untreated plasma mixed with either antisera against HuIFN-α.

DISCUSSION

Peripheral blood MNC was from only one patient with Behçet's syndrome and one healthy control produced IFN spontaneously and in both cases the amount produced was barely detectable. However, Fujii *et al.* (1983) reported that lymphocytes from patients with Behçet's syndrome in convalescent phase produced significant titres of IFN-γ in the absence of an inducer. Possible explanations for these opposing results could relate to differences in experimental technique, or perhaps the patients with Behçet's syndrome in the Japanese study have a form of the syndrome which differs aetiologically or pathogenetically from that observed in the present study. Spontaneous IFN-γ production by lymphocytes from one patient with chronic lymphatic leukaemia has been described (Hooks *et al.*, 1982a) and Sugamura *et al.* (1983) recently reported that some cell lines established from normal human T cells after transformation by human T cell leukaemia virus constitutively produce IFN-γ. Furthermore, MNC from healthy donors cultured at high cell density spontaneously produce IFN-γ and acid labile IFN-α (Fischer & Rubinstein, 1983). Apart from these exceptions, IFN is generally induced in culture, for example, lymphocytes and fibroblasts produce IFN-α and IFN-β in response to viruses or polynucleotides, and IFN-γ is induced in lymphocytes by mitogens or specific antigens (Stewart *et al.*, 1980).

MNC from all donors produced IFN-γ in response to PHA, and significantly higher titres were produced by MNC from patients with Behçet's syndrome that MNC from controls, a finding which is consistent with the suggestion that there are more PHA responsive cells in the circulation of these patients (de Vere-Tyndall *et al.*, 1982). In MNC cultures treated with *S. aureus* protein A, IFN-γ is produced by T lymphocytes in co-operation with macrophages (Ratliff *et al.*, 1982). A suppressor T lymphocyte population may regulate the IFN-γ response (Johnson, 1981), therefore an alternative suggestion could be that there are fewer suppressor T lymphocytes in the peripheral circulation of patients with Behçet's syndrome. If correspondingly high levels of IFN-γ are produced *in vivo* after appropriate stimulation this could contribute to the pathogenesis of the disease, although at least in mice, IFN-γ titres produced *in vitro* and *in vivo* do not always correlate (Huygen & Palfliet, 1983). IFN-γ is considered to be a potent immunoregulatory molecule (Moore, 1983). This view is strengthened by recent experimental work demonstrating that IFN-γ can increase the expression of major histocompatibility antigens (Wallach, Fellows & Revel, 1982; Wong *et al.*, 1983) and that T lymphocytes respond to IFN-γ by producing receptors for interleukin-2 (Johnson & Farrar, 1983).

It has been noted elsewhere that MNC from patients with SLE respond poorly, if at all, to PHA as assessed by IFN-γ production (Preble *et al.*, 1983). Four patients with SLE were included in the present study and MNC from all donors produced IFN-γ (geometric mean for SLE group = 146 u/ml; healthy control group = 75 u/ml). Discrepancies such as this between studies suggest that IFN-γ responses should be measured over a range of culture conditions rather than under one set of conditions.

Plasma from some individuals which had been diluted 1/10–1/20 inhibited SFV RNA synthesis

in EBTr cells. The inhibitory 'factor' was inactivated at pH 2 but could not be neutralised by antisera against HuIFN-α. Dose–response curves of these plasma specimens and the IFN-α standard were distinct. Classical IFN-α can be detected on bovine cells and is pH 2 stable, but there is a subspecies which is inactivated at low pH (Preble *et al.*, 1982; Balkwill *et al.*, 1983). The present results suggest that there is a factor in plasma from some subjects which mimics IFN by inhibiting SFV RNA synthesis at concentrations $\geqslant 1/10$ but which is unrelated to IFN-α. It has previously been shown that high concentrations ($< 1/10$) of serum from healthy donors inhibit SFV RNA synthesis (Bacon *et al.*, 1983); perhaps the factor(s) responsible for this effect are present in varying amounts among the population. No IFN was detected in plasma on HEp 2 cells using a cpe inhibition assay and therefore it was concluded that the plasma from our patients did not contain detectable titres of either IFN-α or IFN-γ. Similarly, no serum IFN was detected in patients with systemic juvenile chronic arthritis (Bacon *et al.*, 1983). These findings contrast with other reports describing IFN-α (Hooks *et al.*, 1982b) and IFN-γ (Ohno *et al.*,1982) in the sera of patients with autoimmune diseases. We consider that the RNA inhibition assay is an objective assay for IFN and that there is some advantage in measuring the inhibition of a biochemical process before completion of the virus growth cycle rather than quantitating morphological changes in a virus infected cell monolayer. As an independent method of evaluating whether endogenous IFN is produced during the course of autoimmune diseases levels of the enzyme (2′–5′) oligoadenylate synthetase in peripheral blood MNC could be measured. Cells respond to IFN by producing (2′–5′) oligoadenylate synthetase (McMahon & Kerr, 1983) and it has been reported that the assay of this enzyme is more sensitive and reliable that the assay of serum IFN (Schattner *et al.*, 1981).

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