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Detection of Gamma Interferon in the Sera of Patients with Behçet's Disease

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The serum levels of interferon (IFN) in 58 patients with Behçet's disease were significantly higher than those in 79 normal controls ($P < 0.0001$). The average IFN titer was 51.4 ± 9.3 IU/ml in the patients and 5.9 ± 1.1 IU/ml in the controls. The patients were then divided into two groups according to the stage of ocular disease. Forty-six patients in the ocular convalescent stage had higher IFN levels (61.3 ± 11.2 IU/ml) than did 12 patients in the exacerbation stage (13.2 ± 3.0 IU/ml). Moreover, the kinetics of the IFN level in the circulation of seven patients showed a significant decrease of IFN in the exacerbation stage. IFN activity in sera from patients was destroyed by acid treatment and heating at 56°C for 30 min and was not neutralized with anti-human IFN-α and -β sera. In addition, it was demonstrated that vesicular stomatitis virus inhibitors detected in sera from both the patients and the controls had no effect on our IFN assay. Therefore, antiviral activity detected in sera of patients seems to be due to gamma interferon (IFN-γ). IFN-γ may play a significant role in the pathophysiology of Behçet's disease.

Behçet's disease occurs most frequently among the Japanese and Mediterranean populations (3); the disease is seen infrequently in the United States and the United Kingdom. The visual prognosis is poor, and there is no effective treatment confirmed yet (30). Although Behçet claimed that viral etiology would account for the disease (2), further evidence has not confirmed the hypothesis (27), and the exact cause remains unknown (26). Immunopathological investigations of this disease have shown a change from a type IV delayed hypersensitivity reaction in the early phase to a type III Arthus reaction in the later phase of ulceration (17). It was also reported that a variety of immune complexes were found in the sera of patients, and the relationship between immunoglobulin A (IgA) and IgG complexes was particularly revealing in that exacerbation of the disease was associated with an increase in IgG (and IgM) complexes but a decrease in IgA complexes (17). It is probable that some type of immunological reaction plays an important role in the manifestation of the disease process, depending on the immunogenetic basis associated with antigen HLA-B5 (22) or HLA-BW51 (21).

Recent studies on the interferon (IFN) system have revealed that IFN is classified into three groups, α, β, and γ (5). Viruses usually induce IFN-α in in vivo and in vitro cultures of mouse spleen cells and human peripheral blood mononuclear leukocytes. IFN-γ is also induced in vivo and in vitro, but the cellular source of IFN-γ might be different from that of IFN-α. IFN-γ is detected in the sera of *Mycobacterium bovis* BCG-sensitized mice by desensitization with either purified protein derivative or BCG cell wall (33) and is induced in mouse spleen cells or human peripheral blood mononuclear leukocytes by T-cell mitogens (7, 8) or some other agents (6, 16), as well as in sensitized lymphocytes by specific antigens (32; J. A. Green and S. Kibrick, Fed. Proc. 27:561, 1968). The cellular source for induction of IFN-γ might be T lymphocytes, whereas that of IFN-α might be non-T cells. Therefore, it is suggested that T-cell function may be closely associated with the production of IFN-γ.

Recently, Hooks et al. (13) reported that IFN-γ is detected in the circulation of patients with autoimmune diseases, such as systemic lupus erythematosus, and the level of IFN-γ in sera is closely correlated with disease activity.

In the present communication, we investigate the level and type of serum IFN in patients with Behçet's disease, after excluding serum vesicular stomatitis virus (VSV) inhibitors, and demonstrate the presence of IFN-γ, especially in the convalescent stage of ocular lesions.

MATERIALS AND METHODS

Patient population. Three hundred forty-six patients with Behçet's disease have been examined in the Uveitis Survey Clinic of Hokkaido University Hospi-

tal during the past 14 years. Among them, 58 patients with four characteristic major symptoms, such as aphthous stomatitis, ocular lesions, skin lesions, and genital ulcers, were studied for serum IFN titers. Thirty-eight of these patients were males and 20 were females. Their ages ranged from 17 to 56 years (mean, 39.2). The diagnostic criteria for inclusion in this study were those proposed by the Japanese Research Committee on Behçet's Disease (24). Thirty-seven of the patients were diagnosed as belonging to the complete type, with all four major symptoms mentioned above, and 21 belonged to the incomplete type, with three of four major symptoms. Seventy-nine healthy individuals of similar age and sex distribution served as controls.

Laboratory methods. Serum samples were collected at each visit and were stored at −70°C until use. The IFN assay was performed by the semimicro, dye-binding assay method (1). A continuous line of human amnion cells (FL) was suspended in RPMI 1640 medium containing 5% fetal calf serum, and 4×10^4 cells per 0.1 ml were inoculated into each well of a microplate (Nunc). After cultivation of FL cells for 24 h at 37°C in a CO_2 incubator, the culture fluid of the microplate was discarded; then, 0.1 ml of a serially twofold diluted serum sample was inoculated into the well and further incubated for 16 h at 37°C. Thereafter, the cells in each well were challenged with 0.1 ml of VSV (Indiana strain; 2×10^4 PFU/ml). When cytopathic effect induced by VSV was obvious, the cells were washed three times with a 0.85% NaCl solution, stained with gentian violet solution for 2 to 3 min, washed thoroughly with water, and dried at room temperature. The dye binding to cells was dissolved with methyl cellosolve for 2 to 3 h, and its optical density was measured at a 550-nm wavelength. A laboratory standard which had been calculated from a National Institutes of Health standard (G-023-901-527) was inoculated into each microplate. The IFN titer of samples was calibrated with the standard and expressed as international units per ml.

Thirty-six serum samples obtained from patients who had been randomly selected were dialyzed against 0.2 M KCl-HCl buffer (pH 2.0) for 48 h at 4°C and further dialyzed against RPMI 1640 medium to adjust the samples to pH 7.4. The antiviral activity was then measured by the same method. Six samples were also treated at 56°C for 30 min. To test the species specificity, monkey kidney cells (Vero) and mouse fibroblasts (L-929) were also used as targets.

Neutralization of IFN in six serum samples from patients was performed by anti-IFN-α and anti-IFN-β antisera. Anti-IFN-α and anti-IFN-β rabbit sera were kindly provided by S. Yonehara and S. Kobayashi of the Tokyo Metropolitan Institute of Medical Science. Anti-IFN-α serum had been prepared by injection into

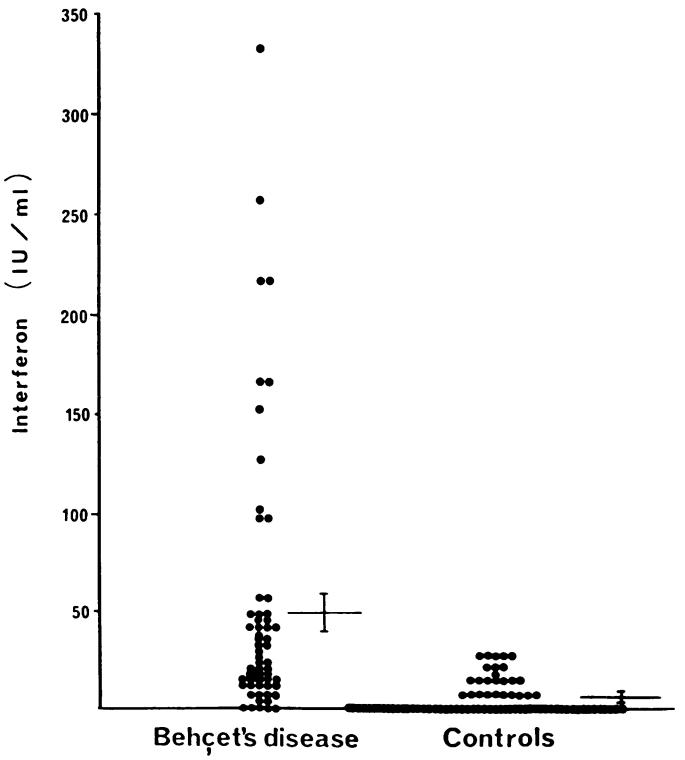

FIG. 1. Titers of IFN in sera of patients with Behçet's disease and in normal controls. The average titer was 51.4 ± 9.3 IU (mean ± standard error) per ml in 58 patients and 5.9 ± 1.1 IU/ml in 79 controls. The difference was statistically highly significant ($P < 0.0001$). Only the first measurement on each patient and control is illustrated.

rabbits of highly purified (>10^8 IU/mg of protein) human leukocyte IFN induced in Namalva lymphoblastoid cells. Anti-IFN-β serum had been prepared by injection into rabbits of highly purified (>10^7 IU/mg of protein) human fibroblast IFN induced in human foreskin fibroblasts by polyinosinic acid-polycytidylic acid. These sera were diluted to neutralize 100 IU of standard IFN-α and IFN-β per ml, obtained from the Blood Center of Hokkaido, Sapporo, and Torey Basic Research Institute, Kamakura, Japan, respectively. Serum samples were diluted serially twofold, and each dilution was mixed with an equal volume of antiserum. After incubation at 37°C for 30 min, the residual IFN activities were assayed.

VSV inhibitors in sera were measured in 15 samples from controls and 5 samples from patients. The mixture of 0.1 ml of a serially twofold diluted serum sample and 0.1 ml of VSV was incubated for 30 min at 37°C and then inoculated into wells and further incubated for 24 h at 37°C. The titer of the inhibitors was measured spectrophotometrically in a manner similar to that for the IFN assay.

RESULTS

Serum IFN in patients with Behçet's disease. Serum IFN titers of the controls were generally low; the average titer was 5.9 ± 1.1 IU/ml (mean ± standard error) in this group. The patients, on the other hand, showed increased IFN titers (Fig. 1). The mean titer was 51.4 ± 9.3 IU/ml on the first measurement on each of the 58 patients, and the difference was statistically highly significant ($P < 0.0001$).

Correlation between IFN titers and stage of ocular disease. Since the recurrent attack of ocular exacerbations can directly be observed with slit lamp microscopy and ophthalmoscopy, it is possible to detect the exact beginning of ocular exacerbations. Correlation between IFN titers and the stage of ocular disease was therefore investigated on the first measurement on each patient (Fig. 2). The mean IFN titer was 61.3 ± 11.2 IU/ml in 46 patients in the convalescent stage and 13.2 ± 3.0 IU/ml in 12 patients in the exacerbation stage. The convalescent stage showed significantly increased IFN titers as compared with both the exacerbation stage ($P < 0.0002$) and the controls ($P < 0.0001$).

Serial observations on seven patients at various stages of ocular disease showed that the exacerbation stage had decreased IFN activity compared with the convalescent stage (Fig. 3). The IFN titer was again increased in the post-exacerbation stage. This result was compatible with change in average titers of the patients.

VSV inhibitors in sera. VSV inhibitors were observed in sera from both healthy donors and patients (Tables 1 and 2). Activity of inhibitors ranged from 32 to 128 in both groups, and there was no significant difference between controls and patients. About 12.5 to 25% of activity of the inhibitors was found in microplates before the washing procedure, whereas no activity was observed after washing. On the other hand, it was also shown that these inhibitors were completely inactivated, or, at most, 3% were detected, after further incubation for 24 h even without the washing procedure. It was therefore shown that there are no unknown serum factors affecting the assay system in our method. Serum IFN activity was not depleted with the washing procedure, compared with that without it, although slight decrease of IFN activity was observed after washing. This decrease of IFN activity was considered to be due not to VSV inhibitors, but to the manipulation of sera.

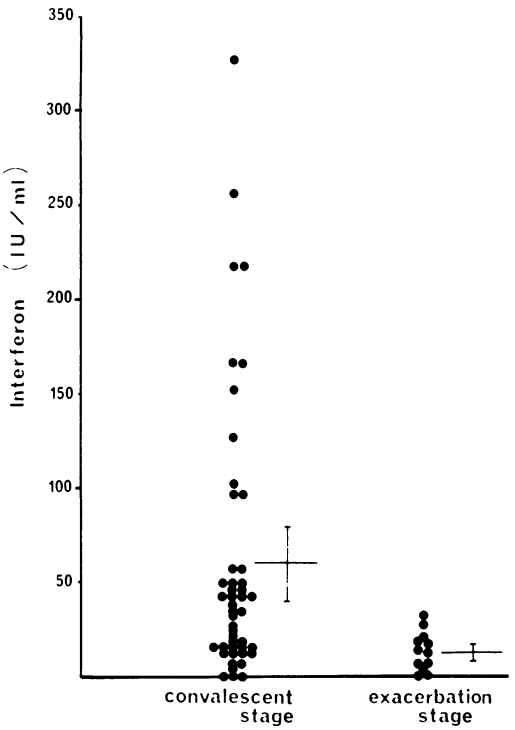

FIG. 2. Correlation between IFN titers and stage of ocular disease on the first measurement on 58 patients with Behçet's disease. The average titer was 61.3 ± 11.2 IU/ml in 46 patients in the convalescent stage and 13.2 ± 3.0 IU/ml in 12 patients in the exacerbation stage. The convalescent stage showed significantly increased IFN titers compared with both exacerbation stage ($P < 0.0002$) and controls ($P < 0.0001$).

Characterization of IFN in sera of patients with Behçet's disease. Characterization of IFN was performed by testing sera from six patients for antiviral activity of human amnion cells (FL), monkey kidney cells (Vero), and mouse fibroblasts (L-929). These sera were also treated at 56°C for 30 min. Dialysis at pH 2.0 in 0.2 M KCl-

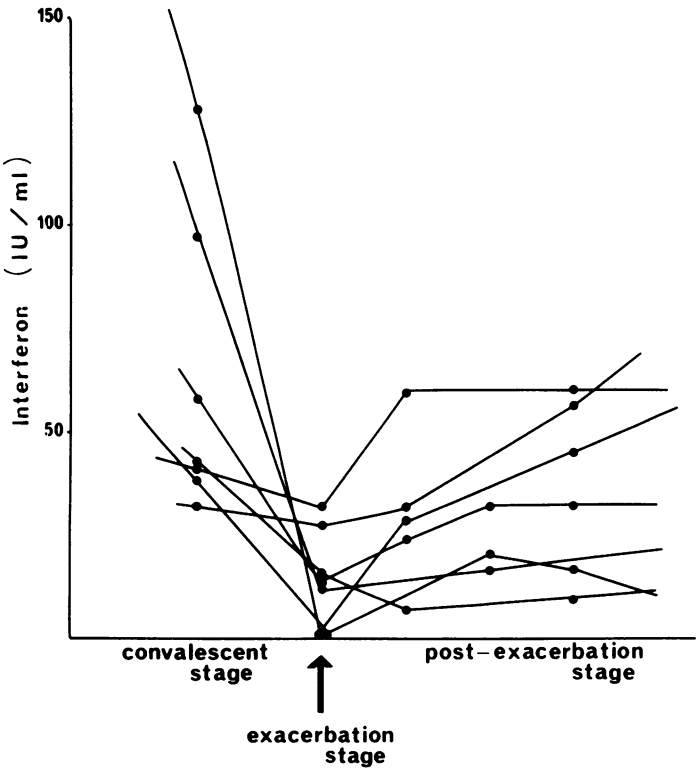

FIG. 3. Serial observations of serum IFN titers in seven patients (each represented by a set of points) with Behçet's disease. The arrow indicates the ocular exacerbation stage, with severe acute inflammation such as hypopyon iritis, uveoretinitis, or retinal vasculitis. The serum samples in the exacerbation stage were drawn within 1 week after the onset of exacerbation, whereas those in the convalescent or post-exacerbation stage were collected within 4 to 8 weeks before and after exacerbation, respectively.

TABLE 1. Detection of VSV inhibitors and IFN in sera of healthy donors

Donor	Activity (titer) of serum VSV inhibitors	Antiviral activity (titer) of serum in microplates after incubation for:			
		30 min		24 h	
		Without washing	With washing	Without washing	With washing
1	64	4	<2	<2	<2
2	58	2	<2	<2	<2
3	32	8	<2	<2	<2
4	32	4	<2	<2	<2
5	64	8	<2	<2	<2
6	64	4	<2	<2	<2
7	64	4	<2	<2	<2
8	64	2	<2	8	4
9	64	4	<2	4	4
10	48	2	<2	4	8
11	128	16	<2	8	8
12	64	2	<2	2	<2
13	64	4	<2	2	<2
14	64	4	<2	16	16
15	32	4	<2	4	4

TABLE 2. Detection of VSV inhibitors and IFN in sera of patients with Behçet's disease

Patient	Activity (titer) of serum VSV inhibitors	Antiviral activity (titer) of serum in microplates after incubation for:			
		30 min		24 h	
		Without washing	With washing	Without washing	With washing
1	64	8	<4	35	30
2	64	8	<4	24	21
3	64	8	<4	14	8
4	98	8	<4	11	10
5	64	16	<4	16	12

HCl buffer was performed on 36 serum samples. Table 3 summarizes the results of several treatments. IFN activity was detected only when targets were FL cells. All 36 serum samples were acid labile, and dialysis at pH 2.0 completely destroyed the antiviral activity, as was the case with six samples treated at 56°C for 30 min. IFN activity showed no change in six samples with treatment of either anti-IFN-α or anti-IFN-β antisera. It was therefore concluded that the detected IFN in Behçet's disease was primarily IFN-γ.

DISCUSSION

In this study we detected IFN-like activity in sera of patients with Behçet's disease, especially in patients in the convalescent stage of ocular disease. This IFN-like activity seems to be IFN-γ, since it was unstable to acid treatment, was not neutralized with anti-IFN-α and anti-IFN-β sera, and was shown to be species specific (Table 3). In addition, this activity was apparently not due to the VSV inhibitors detected in sera from both patients and the controls (Tables 1 and 2).

The cellular origins and mechanism of IFN-γ production in Behçet's disease are obscure. Our preliminary studies have shown that peripheral blood mononuclear leukocytes of the patients produced IFN spontaneously in vitro without any stimulation, but none was found in cultures from normal controls (unpublished data). Peak production occurred between days 3 and 7, and the average IFN titers in cultures were significantly higher in the convalescent stage than in the ocular exacerbation stage. It is therefore suggested that the circulating IFN-γ in sera of patients is also produced by peripheral blood mononuclear leukocytes, especially by T lymphocytes.

TABLE 3. Characterization of IFN in patients with Behçet's disease

Patient	IFN titer				
	FL cells			Vero cells	L-929 cells
	No serum treatment	56°C, 30 min	pH 2.0		
1	35	<4	<4	<4	<4
6	16	<4	<5	<4	<4
7	42	<4	<10	<4	<4
8	14	<4	<5	<4	<4
5	64	<4	<5	<4	<4
9	54	<4	<5	<4	<4

It was reported that IgG, IgA, and C1q binding immune complexes (18), as well as cold-precipitable immune complexes (17), were found in the sera of patients and that patients with neurological and ocular types of the disease had a higher incidence of these immune complexes (18). Since immune complexes may stimulate the host's peripheral blood mononuclear leukocytes to produce IFN (9), immune complexes may be one of the causative IFN inducers in Behçet's disease. Another possibility is that the number of IFN-producing T cells is increased in the patients, since T_γ lymphocytes have been reported to increase in number in this disease (10, 20).

It is worth noting that the titer of serum IFN-γ showed a significant negative correlation with disease activity. Myeloperoxidase activity in neutrophils, a lysosomal enzyme of leukocytes, has also been reported to decrease during the ocular exacerbation and then increase gradually with recovery of ocular inflammation in Behçet's disease (19). The serum lysosomal enzymes, on the other hand, show a higher level of activity in the patients, especially in the exacerbation stage (11). Ocular exacerbations are also associated with a corresponding decrease in IgA immune complexes and an increase in concentration of IgM and IgG immune complexes (17, 18). Complement components are not usually depressed in active Behçet's disease (15), but C3, C4, and C2 can be reduced significantly before an attack of uveitis (25). Complement activaition occurs by the classical pathway (25) and probably by an alternative pathway (18) in this disease.

Recent studies on the IFN system have revealed that IFN-γ has non-antiviral functions, such as immunodulation, and a close association with the immune response of the host (29). IgM

and IgG antibody synthesis, for example, is depressed by injection of IFN into mice (4), whereas the activity of natural killer cells (31), cytotoxic T lymphocytes (12), and macrophages (23) is enhanced by IFN treatment. Since it was reported that IFN-γ has a higher potential for immunomodulation than IFN-α or -β (14, 28), immunomodulation in Behçet's disease as described above seems to be strongly associated with IFN-γ detected in sera of patients.

However, it seems difficult at the present time to understand the exact biological role of IFN-γ in Behçet's disease because it is unknown whether the increased IFN-γ is the cause or the result of developing this disease. It may be possible that IFN-γ activates the cytotoxic T lymphocytes or natural killer cells and causes the tissue damage, such as aphthous stomatitis, uveoretinitis, skin lesions, and genital ulcers, frequently seen in this disease.

Further studies are needed to elucidate the mechanism of production and the function of IFN-γ in Behçet's disease.

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