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CD4 and CD8 cell responses to herpes simplex virus in Behcet's disease

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SUMMARY

The finding of part of the Herpes simplex virus type 1 (HSV1) genome in peripheral blood leucocytes of some patients with Behcet's disease (BD) led to investigations of T cell responses to HSV1 in this disease. A significantly impaired uptake of ^{3}H-thymidine by CD4 cells was found in BD, as compared with healthy HSV1 sero-positive subjects. The impaired cellular response appeared to be specific to HSV1, as neither cytomegalovirus nor varicella-zoster virus showed depressed CD4 cell responses in BD. A similar impairment of CD4 cell responses to HSV1 was found in patients with recurrent herpetic infections, known to be caused by latent HSV1 infections. However, in rheumatoid arthritis which was selected as an unrelated autoimmune disease. ^{3}H-thymidine uptake by CD4 cells stimulated with HSV1 was enhanced. CD8 cells showed generally rather a low uptake of ^{3}H-thymidine, nevertheless, the values in BD and recurrent herpetic infection were again lower than those in sero-positive controls or rheumatoid arthritis. The results are consistent with the hypothesis that HSV1 might be involved in the immunopathogenesis of BD.

Keywords CD4 cells Herpes simplex Behcet's disease

INTRODUCTION

Behcet's disease (BD) is a multisystem inflammatory disease most commonly found in Japan and the Eastern Mediterranean countries. It is characterized predominantly by oral and genital ulcers and a variety of cutaneous, ocular, arthritic, vascular and neurological manifestations. Although no micro-organism has been consistently isolated from patients with BD, there is some evidence for the involvement of herpes simplex virus type 1 (HSV1). Eglin, Lehner & Subak-Sharpe (1982) have shown by *in situ* DNA–RNA hybridization that at least part of the HSV1 genome is transcribed in mononuclear cells of some patients with BD. This has been confirmed by Bonass *et al.* (1986) who detected HSV1 DNA in patients with BD by dot blot DNA–DNA hybridization.

These findings supported the viral hypothesis comprehensively investigated by Denman *et al.* (1980), in that phytohaemagglutinin (PHA) transformed lymphocytes from patients with BD were unable to support the growth of HSV1, unlike those from healthy subjects. They also reported that peripheral blood mononuclear cells (PBMC) from patients with BD produce a significantly higher concentration of interferon-γ after PHA stimulation than cells from healthy controls or patients with other inflammatory diseases (Bacon *et al.*, 1984). Furthermore, the concentration of the interferon-induced enzyme, 2′-5′-oligoadenylate synthetase, is increased in PBMC from patients with BD (Hylton *et al.*, 1986). Chromosomal damage characteristic of a viral infection was also found in lymphocytes from these patients (Denman *et al.*, 1980). We have found significant HSV1 specific immune complexes *in sera* from patients with BD, compared with those from controls (Hussain *et al.*, 1986).

Changes in the proportions of T cell subsets have been reported. There is agreement that the number of CD4 cells is slightly, though significantly decreased (Kotani & Sakane, 1982; Lehner, 1982; Victorino *et al.*, 1982) but there is some uncertainty about any changes in the proportion of CD8 cells. However, some impairment in the T cell suppressor function has been found during the prodromal phase of the disease (Sakane *et al.*, 1982). In a preliminary investigation we have reported an impaired proliferative response of T cells stimulated by HSV1 (Pugh & Lehner, 1986). The objectives of this study were to investigate the lymphoproliferative responses of CD4 and CD8 cells to HSV1 and to other viruses in BD, in sero-negative and sero-positive subjects, in recurrent herpetic infections of the lips and face which is known to be caused by HSV1 and in rheumatoid arthritis which is an autoimmune disease. Depressed responses were found with both CD4 and CD8 cells from patients with BD when stimulated with HSV1.

MATERIALS AND METHODS

Patients and controls

A series of 50 patients and controls were investigated. Ten patients with BD consisted of five patients with the ocular type

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Table 1. Clinical manifestation and HLA typing of 10 patients with Behcet's disease

Patient	Age	Sex	Oral ulcers	Genital ulcers	Skin lesion	Arthralgia and/or joint swelling	Eye lesion	Other manifestations	Treatment (daily dose)	HLA A	HLA B
1	24	F	MjAu	Vaginal	Pustules	+	—	—	—	ND	
2	40	M	MjAu	Scrotal	Folliculitis	+	—	—	5 mg prednisolone	1,2	15
3	45	F	MiAu	Vulval	—	+	—	—	Local mysteclin	24,32	44,62
4	35	M	MjAu	Penile	Pustules	+	—	Thrombophlebitis	50 mg azathioprine	1,2	51,35
5	53	F	MiAu	Vulval	Perineal ulcers	+	—	—	Local mysteclin	9	7,22
6	43	F	MjAu	Vaginal	—	+	Uveitis	Thrombophlebitis	5 mg prednisolone	1,2	8,12
7	36	M	MiAu	Scrotal	Pustules	−	Uveitis	Thrombophlebitis epididymo-orchitis	20 mg prednisolone	ND	
8	33	M	MiAu	Scrotal Penile	Erythema nodosa	+	Uveitis	Muscle pain	—	9,11	14,35
9	20	F	MiAu	Vulval	Pustules	−	Uveitis	—	Local betamethasone	2,3	8,34
10	46	F	MiAu	Vaginal	Perineal ulcers	−	Iritis	—	5 mg prednisolone	2,44	51

and five patients with the arthritic type of BD as defined by Lehner & Batchelor (1979). Four patients were male and six were female and their ages ranged from 20 to 53 years. Four patients were treated with prednisolone (5–20 mg per day), one received azathioprine (50 mg per day), three received topical oral mysteclin or betamethasone and two were untreated at the time of investigation (Table 1).

We investigated four control populations. Normal healthy adults with no history of recurrent herpetic infection were separated into (1) a sero-negative group (eight subjects) and (2) sero-positive group (12 subjects), according to the presence and absence of serum antibodies to HSV1 antigen. (3) We selected 10 subjects who suffer from recurrent herpetic infection (RHI) of the lips or face. None of them had active lesions at the time of taking a blood sample, but each of them had been observed in the past by one of us to have one or more clinical herpetic lesions. (4) A group of 10 patients had rheumatoid arthritis (RA) as defined by the American Rheumatological Association criteria. The four control groups were matched for sex and age with BD patients.

Viral antigens

Herpes simplex virus type 1 (MacIntyre strain), grown in Vero cells, was kindly donated by Dr B. Thornton at CAMR, Porton Down, UK. Uninfected Vero cells were used as controls. The protein concentration of each antigen was measured (Lowry *et al.*, 1951) and adjusted to 1 mg protein per ml. The optimum antigen concentration was first determined (20 μg per ml), but usually several concentrations of HSV1 were used. Other viral antigens and their controls included cytomegalovirus (CMV) and varicella zoster virus (VZV) and these were obtained from the Public Health Laboratory Service (Colindale, London). The pre-determined optimum concentrations of 20 μg/ml of each of the viral antigens was used.

Radioassay for serum antibodies

Blood samples were taken from all subjects and the sera were tested for antibodies to the HSV1 antigen, using a solid phase radioimmunoassay (Smith & Lehner, 1981). The presence of specific antibodies was detected by the binding of ^{125}I-labelled goat anti-human IgG antibody (Tago, Burlington, CA) to serum IgG which was bound to the appropriate antigen coated plastic wells. Results were expressed as the percentage of bound radioactivity. Values greater than the mean plus 2 s.d. of the minimal binding found in sero-negative controls were taken to be sero-positive. On this basis there were eight sero-negative and 12 sero-positive control subjects. All but one of the patients with BD were sero-positive and this did not affect the results of the cellular studies.

Cell separation

Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood samples by centrifugation over a Ficoll-Isopaque (Pharmacia) density gradient for 30 min at 900 *g* (Böyum, 1968). Plastic adherent cells, hereafter referred to as monocyte enriched cells (MEC), were removed from the PBMC by panning on plastic dishes (Falcon 3002), for 1 h at 37°C, in a humidified atmosphere with 5% CO_2. The MEC were recovered from the dishes after overnight incubation at 37°C by vigorous washing with cold medium. T lymphocytes were prepared from the non-adherent cell population using a rosetting technique (Pellegrino, Ferrone & Theofilopoulos, 1976). The cells were mixed with a solution of AET (aminoethylisothiouronium bromide, Sigma Chemical Co., Poole) coated sheep red blood cells (SRBC, Gibco Europe Ltd) and the rosetted T lymphocytes were obtained after centrifugation over a Ficoll-Isopaque density gradient and lysing the SRBC with a hypotonic solution.

Complement dependent cell lysis was used to separate CD4 and CD8 cell subsets (Young & Lehner, 1987). Briefly, to separate CD4 cells the T cells were first incubated with 200 μl per 10^6 cells of anti-T8 monoclonal antibody supernatant (ATCC, Rockville, Maryland, USA, ref CRL8002), for 20 min at 37°C. The cells were centrifuged at 300 *g* for 10 min, the supernatant was discarded and the cells were resuspended in 100 μl per 10^6 cells of a 10% rabbit complement solution (Buxted Rabbit Co. Ltd, Sussex) for 1 h at 37°C. The procedure was repeated and the resulting CD4 cell population was washed four times with medium. To separate CD8 cells the CD4 cells were lysed, using 200 μl of anti-T4 monoclonal antibody supernatant per 10^6 cells, and rabbit complement as described above. Using this technique the CD4 population contained $91{\cdot}6 \pm 1{\cdot}9\%$ $CD4^+$ cells and $4{\cdot}2 \pm 1{\cdot}5\%$ $CD8^+$ cells, whilst the CD8 population contained

Table 2. Stimulation of T, CD4 and CD8 cells from five groups of subjects with HSV1; all cultures were reconstituted with 10% monocyte enriched cells (MEC)

Group	*n*	Stimulation index (mean (±s.e.m.))			Disintegration per minute (mean (±s.e.m.))					
					T+MEC		CD4+MEC		CD8+MEC	
		T+MEC	CD4+MEC	CD8+MEC	With antigen	Without antigen	With antigen	Without antigen	With antigen	Without antigen
Sero-negative*	8	1·3(0·4)	2·1(0·6)	1·0(0·1)	1442 (647)	1705(639)	1774 (646)	899(253)	264 (54)	321 (79)
Sero-positive*	12	21·9(7·8)	11·1(1·6)	4·2(2·2)	9028(2843)	930(407)	6316(2040)	573(220)	825 (431)	235 (34)
Recurrent herpetic infection	10	12·3(3·6)	7·7(2·1)	2·8(0·9)	8840(2655)	1039(222)	3964 (709)	1033(375)	754 (210)	488(123)
Rheumatoid arthritis	10	5·6(1·8)†	10·9(3·0)	5·9(1·3)	8053(3667)†	1356(490)	8643(3364)	1062(324)	5780(2449)	985(235)
Behcet's disease	10	7·7(3·6)	4·5(1·0)	1·7(0·5)	3483(1584)	603(221)	4653(2185)	755(227)	1130 (556)	561(271)

* To HSV1.
† $n=7$.

87·9±3·0% $CD8^+$ cells and 8·7±1·7% $CD4^+$ cells. Both subsets contained less than 1·0% surface Ig positive cells (detected by fluorescein conjugated goat anti-human $F(ab)_2$, Nordic Immunology, Tilbury, The Netherlands) and less than 1·5% non-specific esterase positive cells.

Cell culture

The cell subsets were cultured in RPMI 1640 medium (Gibco Europe), supplemented with 15 mM HEPES, 10% heat inactivated pooled human AB serum, antibiotics and the appropriate antigen. The cells were cultured in U-bottomed microtitre plates (TC3799, Costar), at 10^5 cells per well in a total volume of 200 μl, initially for 3–7 days. Previous studies of culture conditions have shown that 10% MEC yields optimimum stimulation of T cells from both controls and BD patients. The optimal duration of culture was 6 days. During the final 4 h of culture the cells were pulsed with 1 μCi per well of ^{3}H-thymidine (Amersham International). The cultures were then harvested onto glass-fibre filters, using a multiple automated sample harvester (Dynatech). The filters were dried and assayed by liquid scintillation counting. The results were expressed as the mean disintegrations per minute (d/min) of triplicate cultures, as well as stimulation index (SI) which was calculated as the ratio of dpm of HSV1 stimulated and the control cultures.

RESULTS

Uptake of ^{3}H-thymidine by T cell subsets stimulated with HSV1 antigen

There was little or no uptake of ^{3}H-Thy by T cells, CD4 or CD8 cells, with 10% MEC, from sero-negative subjects stimulated with HSV1 (Table 2). In contrast T cells from sero-positive subjects responded to stimulation with HSV1 to yield high SI. Whereas T cells and CD4 cells yielded high SI and d/min, CD8 cells showed much lower SI and d/min (Table 2).

T cells from patients with RHI showed lower SI and d/min than those from the sero-positive controls. There was, however, an increase in the SI and d/min of CD8 cells and d/min of CD4 cells from RA patients compared with any of the other four groups. However, the SI of unseparated T cells was lower than those of the three disease groups, though the d/min was comparable except for BD (Table 2).

In BD, CD4 cells showed a significant decrease in SI, as compared with cells from the sero-positive group ($t=3·253$, d.f. 20, $P<0·01$) or rheumatoid arthritis ($t=3·134$, d.f. 18, $P<0·01$), but not with those from RHI or sero-negative subjects. T cells showed similar changes to CD4 cells, except that the SI (but not d/min) appeared higher than that in RA because of the low count of the unstimulated cultures (Table 2). CD8 cells from BD also showed the lowest SI, though with the exception of RA the d/min were rather low. The possibility that $CD4^+$ cells from BD are more vulnerable to the isolation procedure or that the monocytes have a suppressive effect on the $CD4^+$ cells is unlikely, as the ^{3}H-Thy uptake of $CD4^+$ cells reconstituted with monocytes was not impaired when stimulated with cytomegalovirus or varicella-zoster virus (Table 3). Furthermore, a comparison of $CD4^+$ cells with unseparated T cell suggests that the ^{3}H-thymidine uptake was relatively higher in $CD4^+$ cells than T cells from BD, as compared with the sero-positive controls (Table 2).

Specificity of the cellular responses

The specificity of the HSV1 response in BD was tested with cytomegalovirus (CMV) and varicella-zoster virus (VZV; Table 3). CD4 cells from patients with BD showed the largest increase in SI with CMV (5·4±3·5) but not in d/min, unlike the decrease of both SI and d/min with HSV1. There was considerable variation from one patient to another, as indicated by the very high standard error of the mean and the results failed to reach statistical significance. VZV failed to yield significant increase or decrease in stimulating CD4 cells, as compared with other groups. CD8 cells yielded lower SI and d/min with both CMV and VZV than those from the sero-positive subjects but not necessarily those from the other groups, unlike the results with HSV1. Hence, both CD4 and CD8 cells from patients with BD responded differently to stimulation with HSV1 than to the other viruses and differed from the responses of cells from RA patients. However, a comparison with RHI which is known to be caused by HSV1 reveals similar decreases in CD4 and CD8 responses to those found in BD.

Table 3. Stimulation of CD4 and CD8 cells with the control cytomegalovirus or varicella-zoster virus

Group	*n*	Cytomegalovirus No antigen (d/min)	Cytomegalovirus (d/min)	Cytomegalovirus SI	*n*	Varicella-zoster virus No antigen (d/min)	Varicella-zoster virus (d/min)	Varicella-zoster virus SI
CD4+MEC								
Sero-negative*	5	3318(1312)	3271 (924)	1·4(0·3)	5	2238(1200)	10802(3205)	6·3(2·6)
Sero-positive*	6	4610(1953)	7092(2894)	2·2(1·4)	6	5944(1954)	9900(2314)	2·0(0·6)
Recurrent herpetic infection	3	3775(1901)	3515(1123)	1·0(0·4)	3	3775(1901)	3775(1902)	3·1(1·6)
Rheumatoid arthritis	7	1449 (475)	1834 (871)	1·2(0·2)	6	1130 (417)	5296(2038)	6·6(3·2)
Behcet's Disease	4	1338 (253)	4658(3000)	5·4(3·5)	3	1345 (250)	7420(5606)	4·5(2·3)
CD8+MEC								
Sero-Negative*	5	1041 (371)	1232 (497)	1·2(0·1)	5	1041 (371)	2483(1562)	2·0(0·6)
Sero-Positive*	6	3816(2365)	4712(2438)	3·2(1·8)	6	3816(2365)	3816(2365)	2·4(0·8)
Recurrent herpetic infection	3	675 (308)	483 (225)	1·0(0·5)	3	675(308)	1775(1064)	3·8(2·4)
Rheumatoid arthritis	7	1128 (233)	1333 (316)	1·2(0·2)	6	1255 (296)	4681(2623)	3·1(1·2)
Behcet's Disease	6	1407 (362)	2162 (707)	1·8(0·6)	5	1284 (417)	1573 (688)	1·0(0·3)

* To HSV1.

DISCUSSION

The finding of a portion of HSV1 genome in peripheral blood leucocytes of some patients with BD (Eglin *et al.*, 1982; Bonass *et al.*, 1986) prompted us to investigate the cellular responses to HSV1 and other viruses. However, the cells involved or the proportion of cells in which the HSV1 genome resides is as yet not known. We have separated healthy control subjects into a sero-negative group (those sera that do not have significant anti-HSV1 antibodies) and a sero-positive group (those that show anti-HSV1 antibodies), as only T cells from sero-positive subjects respond to HSV1 (Wilton, Ivanyi & Lehner, 1972; Rasmussen *et al.*, 1974). CD4 cells from patients with BD showed a higher uptake of ^{3}H-Thy than cells from sero-negative subjects, but a lower uptake of ^{3}H-Thy than sero-positive subjects, suggesting that although their cells were sensitized to HSV1 the responses were impaired. These results appeared to be specific for HSV1, as neither CMV nor VZV induced similar depression of CD4 cell responses in BD.

CD8 cells from BD also showed low responses to HSV1, but with the exception of RA the d/min of stimulated CD8 cells were altogether rather low. The results with CD8 cells failed to support the preliminary findings on a limited number of patients and controls that there is an increased response of CD8 cells to HSV1 (Pugh & Lehner, 1986).

T cells were also examined from patients with RHI in whom latent HSV1 is harboured in the trigeminal ganglion (Baringer & Swoveland, 1973; Stevens & Cook, 1974). A slight depression of T cell responses to HSV1 has been recorded in patients with RHI, though this is more evident before recrudescence of infection (Shillitoe, Wilton & Lehner, 1977; 1978; O'Reilly *et al.*, 1977). CD4 cells showed a similar decrease in d/min and to a lesser extent SI as those from BD and this is consistent with the hypothesis that HSV1 might be involved in the pathogenesis of BD. Impaired lymphokine production by T cells has been consistently demonstrated with RHI (Wilton *et al.*, 1972; Rasmussen *et al.*, 1974; Shillitoe *et al.*, 1977) and impaired production of interferon gamma is of particular significance (Rasmussen *et al.*, 1974). However, although lymphocytes from patients with BD do not produce interferon gamma spontaneously, there is no defect in synthesis of interferon gamma by phytohaemaglutinin stimulated lymphocytes (Bacon *et al.*, 1984).

The cellular studies were also carried out with T cells from patients with RA which is an unrelated autoimmune disease. The CD4 and CD8 cells showed maximum stimulation to HSV1, as compared with the other groups. This was rather surprising as a similar increase was not found with CMV or VZV. However, an impaired CD4 response to HSV1 was not found in RA but only in RHI, known to be caused by HSV1 and in BD in which part of the HSV1 genome has been detected. As the serum anti-HSV1 antibody titre in BD is not impaired (Hussain *et al.*, 1986), it is unlikely that the specific CD4 helper or suppressor inducer function for B cell antibodies is affected. The impaired CD4 cell activity affects production of lymphokines and may be associated with the finding of non-specific immune complexes (Williams & Lehner, 1977; Gupta *et al.*, 1978; Burton-Kee, Lehner & Mowbray, 1979) as well as HSV1-specific immune complexes (Hussain *et al.*, 1986). Until more information is gained concerning the subset of cells in which the HSV1 genome resides and the proportions of cells affected, any hypothesis attempting to interpret the results must be tentative. However, we have proposed (Lehner, 1986) that the HSV1 genome might affect immunoregulation by T cells in which lymphokine production, especially interferon might be impaired.

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