Detection of HSV-1 DNA in patients with Behçet's syndrome and in patients with recurrent oral ulcers by the polymerase chain reaction

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Summary. The polymerase chain reaction was used to detect HSV-1 DNA in genomic DNA extracted from peripheral blood leucocytes, in patients with Behçet's syndrome (BS), patients with recurrent oral ulcers and normal healthy controls. A 211-bp HSV-1 DNA fragment was found in a significant number of patients with BS ($p < 0.02$). Serum anti-HSV-1 antibodies were also found in a higher proportion of patients with BS ($p < 0.02$) than in healthy controls. However, virus-specific DNA was not detected in biopsy samples taken from oral ulcers in patients with BS.

Introduction

Behçet's syndrome (BS) is a multi-system inflammatory disease, characterised by oral and genital ulceration and various cutaneous, arthritic, ocular, vascular and neurological manifestations.¹ A viral aetiology has long been postulated for the disease.² Inclusion bodies have been found in scrapings from ulcers³ and in biopsies,^{4,5} and an unidentified virus has been isolated from patients with BS by some workers,^{6,8} but not by others.^{9–11}

However, there is indirect and direct evidence for the involvement of herpes simplex virus type 1 (HSV-1) in BS. Thus, phytohaemagglutinin (PHA)-stimulated peripheral blood lymphocytes from patients with BS, unlike those from normal subjects, are unable to support the growth of HSV-1^{12,13} and produce higher than normal amounts of interferon (IFN) γ .¹⁴ CD4 and CD8 cells from BS patients have been reported to have an impaired proliferative response to HSV-1,¹⁵ and increased levels of HSV-1 specific immune complexes have been found in serum.¹⁶

Examination of circulating lymphocytes, by in-situ hybridisation, has also revealed HSV-homologous RNA in patients with BS.¹⁷ This was confirmed by the demonstration of HSV-1-homologous DNA by the dot blot technique.¹⁸ We report here application of the polymerase chain reaction (PCR) to detect HSV-1 DNA sequences in DNA extracted from peripheral blood mononuclear cells from patients with BS and from control subjects, and in DNA extracted from biopsy samples from oral ulcers. The results are analysed by comparison with the serum anti-HSV-1 antibody level.

Subjects and methods

Patients and controls

Ninety-six patients and controls were investigated. Thirty-two patients with BS were diagnosed clinically¹ as mucocutaneous type (2), arthritic type (12), ocular type (12) and neurological type (6) (table I). Three age- and sex-matched control groups included normal healthy subjects without a history of recurrent herpetic infection (22), patients with recurrent herpes labialis (12), and patients with recurrent oral ulcers (30). The latter were diagnosed¹⁹ as minor aphthous ulcers (11), major aphthous ulcers (10), and herpetiform ulcers (9).

Peripheral blood mononuclear cells were separated from heparinised blood samples by centrifugation on Ficoll/Isopaque, washed in phosphate-buffered saline (PBS) and stored as cell pellets at -20°C until required for DNA extraction. Serum or plasma samples were stored at -20°C until assayed for antibodies to HSV-1 by solid phase radioimmunoassay. Biopsy samples of oral lesions were taken from 21 patients—11 from patients with recurrent oral ulcers, three from BS patients and seven from patients with miscellaneous oral diseases. The biopsy samples were stored at -70°C in Optimal Cutting Temperature (OCT) embedding compound (Miles Laboratories, Slough) and then examined by PCR.

Preparation of DNA samples

Cell pellets and finely chopped biopsy samples were thawed out into 5–10 ml of a buffer solution consisting of 10 mM Tris, pH 8.0, 10 mM EDTA, pH 8.0, 10 mM NaCl and SDS 0.5%, and incubated with proteinase K 100 $\mu\text{g/ml}$ (Boehringer Corp. London, Ltd) (BCL)

Table I. Clinical Manifestations of 32 patients with BS

Clinical type of BS	Number of patients	Sex	Age (y)	Number of patients with						
				oral ulcers	genital ulcers	skin lesions	arthralgia or joint swelling	eye lesions	CNS lesions	vascular lesions
Mucocutaneous	2	F	41–60	2	2	1	0	0	0	0
Arthritic	12	9F, 3M	29–55	12	12	8	12	0	0	2
Ocular	12	5F, 7M	24–50	12	9	6	5	12	0	2
Neurological	6	1F, 5M	30–52	6	5	5	1	2	6	3

for several hours at 37°C. After phenol-chloroform extraction, the DNA solution was reincubated at 37°C for 15 min with RNAase 50 µg/ml (BCL) and then for a further 30 min with proteinase K 100 µg/ml and SDS 1%. The solution was re-extracted with phenol-chloroform, and dialysed against a buffer solution of 200 mM Tris, pH 8.0, 10 mM EDTA, pH 8.0, and 10 mM NaCl, until the OD₂₇₀ of the dialysis buffer was <0.05. The DNA was precipitated by the addition of 0.1 volumes of 5 M ammonium acetate and one volume of ice-cold isopropanol and overnight incubation at –20°C. The precipitate was redissolved in a solution of 10 mM Tris, pH 7.5, and 1 mM EDTA, and the concentration and purity were determined by measuring the OD₂₆₀ and OD₂₈₀.

Polymerase chain reaction

Oligonucleotide primers specific to sequences bordering a 211-bp region of the *EcoRI* g fragment of HSV-1 DNA,²⁰ including two restriction sites for *HpaII* and a single site for *TaqI*, were purchased from Oswel DNA Service, Edinburgh University. The reactions were performed by a modification of the method of Saiki *et al.*,²¹ in 100 µl of a reaction buffer consisting of 67 mM Tris, pH 8.8 at 25°C, 16.6 mM ammonium sulphate, 10 mM 2-mercaptoethanol, 3.2 mM magnesium chloride, bovine serum albumin (BSA) 170 µg/ml, 200 µM of each dNTP, 1 µM of each primer, 1 µg of template DNA previously denatured by boiling and cooling on ice, and 2.5 units of AmpliTaq® (Perkin Elmer Cetus).

In some experiments, all the reactants except the template DNA were pre-incubated (37°C for 1 h, and then 75°C for 15 min) with the restriction endonuclease *HpaII* to remove any contaminating template which could lead to false positive results. Reaction mixtures were subjected to 20, 30 or 40 cycles of amplification on a Bio-excellence thermal cycler (Anglian Biotech). Each cycle consisted of 2 min at 94°C, 1 min at 60°C and 2 or 5 min at 72°C; 2.5 units of AmpliTaq were added to each reaction before cycling, and again after 20 cycles; 10-µl samples of amplified product were removed for analysis after 20, 30 and 40 cycles.

Analysis of PCR products

The 10-µl samples of amplified product were separated by electrophoresis on agarose 2% gels, containing ethidium bromide 1 µg/ml, and visualised under UV light. Gels were alkali blotted (0.4 M NaOH) on to nylon membranes (Biodyne B, Pall Europe), rinsed in 2 × SSC (20 × SSC is a solution of 3 M sodium chloride, and 0.3 M sodium citrate) and air dried. Pre-hybridisation was performed at 68°C for several hours or overnight in a solution consisting of 5 × SSC, 5 × Denhardt's (100 × Denhardt's is polyvinyl pyrrolidone 2%, Ficoll 2% and BSA 2%, SDS 0.1% w/v and heat-denatured salmon sperm DNA 100 µg/ml. Hybridisation with ³²P dCTP-labelled, oligonucleotide-primed *EcoRI* g fragment²² of HSV-1 was performed overnight at 68°C in fresh pre-hybridisation buffer. Blots were washed in a solution of 0.1 × SSC and SDS 0.1% w/v for 1 h at 68°C, and rinsed several times in the same solution pre-heated to 68°C before autoradiography.

Restriction digestion of amplified DNA was performed by adding 10 units of either *HpaII* or *TaqI* to 10-µl samples of PCR product and incubating for 3–4 h at 37°C (*HpaII*) or 65°C (*TaqI*). The digested DNA was electrophoresed on agarose 2% gels, containing ethidium bromide 1 µg/ml, and visualised under UV light.

Radioimmunoassay

The wells of microtitration plates (Dynatech, Billingshurst, Sussex) were coated with 50 µl of HSV-1 (MacIntyre strain) antigen (reconstituted from lyophilised HSV-1 infected Vero cells (PHLS, 61 Colindale Avenue, London) and diluted 1 in 20 in PBS, or control, non-infected Vero-cell antigen, and incubated overnight at room temperature. Unbound antigen was decanted and the wells were washed five times with PBS, and then incubated with 100 µl of BSA 0.5% in PBS (PBS/BSA) for 1 h at 37°C to prevent further non-specific binding.

The blocking buffer was decanted, and 50 µl of serum or plasma sample, diluted in PBS/BSA containing polyoxyethylene-sorbiton monolaurate (Tween

20), 0.05%, was added to wells in triplicate and incubated for 1 h at 37°C. After five washes with PBS the wells were incubated for a further hour at 37°C with 50 µl (> 50 000 cpm) of ¹²⁵I-radiolabelled goat anti-human IgG (Tago, Burlingame, CA 94010, USA) diluted in PBS/BSA/Tween 20. The wells were washed five times with PBS and air-dried, and the amount of bound ¹²⁵I-radiolabel was counted on a gamma counter.

The results were expressed as the percentage of bound radioactivity. Values greater than the mean plus two standard deviations of the radioactivity bound to the control Vero-cell antigen were taken to be seropositive.

Results

Polymerase chain reaction studies

The amplified DNA yielded a 211-bp band visible on ethidium bromide-stained gels under UV light (fig. 1), as well as on Southern blots after hybridisation with a labelled *Eco*R1 g probe. All the 211-bp bands, analysed by restriction endonuclease digestion yielded the expected 126-bp and 85-bp fragments when digested with *Taq*I, and 93-bp, 65-bp and 56-bp fragments when digested with *Hpa*II (fig. 2).

From the patients with BS, 16 (50%) out of 32 DNA preparations from the circulating mononuclear cells showed the 211-bp band (table II). This compared with only three (13.6%) out of 22 preparations from healthy controls ($\chi^2 = 6.05$, $p < 0.02$) and five (16.7%) out of 30 preparations from patients with recurrent oral ulcers ($\chi^2 = 6.26$, $p < 0.02$). However, preparations from five (41.7%) of the 12 patients with recurrent herpes labialis showed a positive PCR result, a proportion not significantly different from that of the patients with BS, or of the healthy controls ($\chi^2 = 2.01$). Of the PCR-positive BS patients, 17.6% were also known to suffer from recurrent herpes labialis, a percentage not significantly different from the 20% of PCR-negative BS patients with a history of recurrent herpes labialis. Analysis of the four clinical types of

Fig. 1. Electrophoresis of amplified DNA showing the 211-bp amplified band run on agarose 2% gel, stained with ethidium bromide and viewed under UV light. Lanes 1–3, positive controls; 4, negative control; 5–14, patient samples (positive samples in lanes 7, 8, 9, 10, 14).

BS showed little difference in their PCR results (45.4–66.6%) but there were relatively few muco-cutaneous and neurological patients in the study (table II). Of the five patients with recurrent oral ulcers who showed positive PCR results, three had major aphthous ulcers and two had herpetiform ulcers (table II).

There was no significant correlation of the PCR results with histocompatibility types HLA B51 or DR7. Two (18.2%) out of 11 PCR-positive BS patients were positive for HLA B51, and three (37.5%) out of eight for DR7, compared with two (22.2%) out of nine patients and two (33.3%) out of six patients, respectively, who were PCR-negative.

Examination of DNA extracted from the biopsy material showed only three positive results out of 21 samples examined by PCR—two out of 11 patients

Fig. 2. Electrophoresis of restriction endonuclease digested DNA showing cut and uncut samples run on agarose 2% gel, stained with ethidium bromide and viewed under UV light. Lanes 1, 4, 7 and 10, uncut 211-bp samples; 2, 5, 8 and 11, samples cut with *Hpa*II; 3, 6, 9 and 12, samples cut with *Taq*I.

Table II. Proportion of patients with HSV-1 211-bp bands detected by PCR amplification

Diagnosis	Number of patients	Number (%) positive	Significance	
			χ^2*	p value
BS	32	16 (50.0)	6.05	<0.02
mucocutaneous type	2	1 (50.0)	0.11	NS
arthritic type	12	5 (41.7)	2.01	NS
ocular type	12	7 (58.3)	5.47	<0.02
neurological type	6	3 (50.0)	1.86	NS
Recurrent oral ulcers	30	5 (16.7)	0.01	NS
minor aphthous ulcers	11	0 (0)	0.37	NS
major aphthous ulcers	10	3 (30.0)	0.37	NS
herpetiform ulcers	9	2 (22.2)	2.01	NS
Recurrent herpes labialis	12	5 (41.7)	2.01	NS
Healthy controls	22	3 (13.6)		

*The χ^2 analysis (with Yates's correction) was calculated against the healthy controls. NS = not significant.

with recurrent oral ulcers, and one other patient with severe artefactual ulceration of the lower lip which persisted for over a year. The three patients with BS who were examined gave negative results.

Presence of serum IgG anti-HSV-1 antibodies

IgG anti-HSV-1 antibodies (table III) were detected in only seven (31.8%) out of 22 healthy controls as compared with 19 (67.8%) out of 27 patients with BS ($\chi^2 = 5.77$, $p < 0.02$). Anti-HSV-1 antibodies were demonstrated in a high proportion of patients with recurrent oral ulcers—19 (65.5%) out of 29 ($\chi^2 = 4.42$, $p < 0.05$)—and, as expected, all 11 patients with recurrent herpes labialis possessed antibodies. The frequency of antibodies did not differ significantly among the four clinical types of BS patients. However, while all but one of the patients with minor aphthous or herpetiform ulcers had antibodies, only a third of the patients with major aphthous ulcers gave positive results (table III).

Table III. Proportion of patients with anti-HSV-1 antibodies

Diagnosis	Number of patients	Number (%) positive	Significance	
			χ^2*	p value
BS	27	19 (67.8)	5.77	<0.02
arthritic type	11	7 (63.6)	1.88	NS
ocular type	11	8 (72.7)	3.49	NS
neurological type	5	4 (80.0)	0.65	NS
Recurrent oral ulcers	29	19 (65.5)	4.42	<0.05
minor aphthous ulcers	10	9 (90.0)	7.13	<0.01
major aphthous ulcers	12	4 (33.3)	0.01	NS
herpetiform ulcers	7	6 (85.7)	4.25	<0.05
Recurrent herpes labialis	11	11 (100)	11.14	<0.001
Healthy controls	22	7 (31.8)		

*The χ^2 analysis (with Yates's correction) was calculated against the healthy controls. NS = not significant.

Discussion

The polymerase chain reaction detected the presence of 211 bp of the *EcoRI* g fragment of HSV-1 DNA in peripheral blood lymphocytes from patients with BS. The 211-bp fragment hybridised specifically to a labelled *EcoRI* g probe, and yielded the expected fragments when digested with *TaqI* or *HpaII* restriction endonucleases. Among the BS patients, 53% gave positive results for HSV-1 DNA and this is consistent with the findings of Eglin *et al.*¹⁷ (50% of BS patients positive) and Bonass *et al.*¹⁸ (41% positive). The former detected viral DNA in 80% of ocular and arthritic type patients, but in only 20% of mucocutaneous and neurological type patients. In the present study, there were no significant differences among the four groups (45–64%), although the numbers of mucocutaneous and neurological type patients were small. In both investigations, a part of the HSV-1 genome was detected in circulating lymphocytes, but there were differences in the proportions of those with the HSV-1 genome in the major and minor aphthous ulcer groups.

Despite the evidence for an association of HSV-1 with BS,^{12–18} the virus has not been isolated from patients.^{9–11} This could be due to the viral DNA being present in small selected fragments, which may or may not be integrated into the host genome, rather than as the intact viral genome found in latently infected ganglia.^{23–25} This could explain why we were unable to detect consistently HSV-1 DNA on Southern blots, despite dot blots being positive when hybridised with a *BamHI* n probe (unpublished data).

Small fragments of viral DNA in the nuclei of circulating peripheral blood lymphocytes could interfere with the transcription of genomic DNA, resulting in the absence of host cell transcripts, or the production of modified transcripts. It is unlikely that the presence of these fragments could lead to the production of specific viral mRNAs or immediate early DNA-binding proteins, such as infected cell polypeptide 4 (ICP4), which are found in latently infected ganglia^{26–29} and which are expressed as part of a co-ordinated and well-regulated cascade.³⁰ The presence in peripheral blood lymphocytes of HSV-1 DNA could affect T cell immunoregulation.³¹ Although the frequency of anti-HSV-1 antibodies (table III) and HSV-1 immune complexes¹⁶ are significantly increased in BS, the T-cell proliferative responses are impaired.¹⁵ These results argue in favour of an impairment of T-cell immunoregulation, associated with the HSV-1 genome, that does not affect T helper function. However, preliminary experiments have demonstrated the presence of viral DNA in B cells, and both CD4 and CD8 subsets of peripheral blood lymphocytes, as well as in macrophages.

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