

# Human Herpesvirus 6 and Multiple Sclerosis: A Study of T Cell Cross-Reactivity to Viral and Myelin Basic Protein Antigens

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Several reports have suggested an association of human herpesvirus 6 (HHV-6) with multiple sclerosis. Autoreactive T lymphocytes directed against myelin components seem to contribute to the pathogenesis of the disease. It has been suggested that molecular mimicry between viral and self-antigens might be one of the mechanisms that determine the onset of several autoimmune diseases. Following this hypothesis, the purpose of the present study was to evaluate if HHV-6 could play a role in activating T cells capable of cross-reaction with an important myelin component, the myelin basic protein. T cell lines were established from 22 multiple sclerosis patients and from 16 healthy controls, and their capability to react to both virus and myelin basic protein antigens was compared. The analysis of T cell cross-reactivity in patients and controls did not show significant differences in the HHV-6 ability to activate myelin basic protein-reactive T cells. Similarly, the evaluation of the humoral immune response to HHV-6 in patients and controls did not mirror any abnormality in the HHV-6 status in multiple sclerosis patients. Therefore, although the findings of activity in vitro of T cell lines with dual specificity are consistent with the hypothesis of molecular mimicry, the lack of differences in cross-reactivity between patients and controls do not support molecular mimicry as an important mechanism in the physiopathology of this disease. **J. Med. Virol. 68:268–272, 2002.** © 2002 Wiley-Liss, Inc.

**KEY WORDS:** molecular mimicry; T lymphocyte; herpesvirus; demyelinating disease

## INTRODUCTION

Multiple sclerosis (MS) is a common chronic demyelinating disease of unknown etiology, which leads to the

development of irreversible disability [Noseworthy et al., 2000]. Genetic, immunological, and environmental factors, such as viruses, have been considered for many decades as possible etiologic agents of MS. Many laboratories have searched, over the years, for a unique virus responsible for MS. More recently, this search has shifted toward ubiquitous viruses that might trigger the onset of the disease in individuals predisposed genetically. Human herpesvirus 6 (HHV-6) is a T cell lymphotropic herpesvirus with high seroprevalence in healthy adults, and it has been reported to infect also cells from the nervous system [Clark, 2000]. More importantly, the presence of HHV-6 was demonstrated recently in the plaques of MS tissue [Challoner et al., 1995]. After this initial observation, several other reports have been published both in favor or against the association between HHV-6 and multiple sclerosis [Soldan et al., 1997, 2000; Ablashi et al., 1998; Coates and Bell, 1998; Fillet et al., 1998; Jacobson et al., 1998; Mayne et al., 1998; Enbom et al., 1999; Akhyani et al., 2000; Knox et al., 2000].

Autoreactive T cells directed against myelin components, such as myelin basic protein (MBP) and Proteolipid Protein, seem to contribute to the pathogenesis of MS. It has been suggested that molecular mimicry between microbial and self-antigens could be an underlying triggering event that leads to the activation of autoreactive T cells in MS as in other autoimmune diseases [Wucherpfennig and Strominger, 1995]. This

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hypothesis has been proposed in the case of infection by human coronavirus, which has been shown to activate T cells cross-reacting with myelin basic protein in MS patients [Talbot et al., 1996]. Similarly, following infection with another ubiquitous virus, HHV-6, myelin basic protein-reactive T cells could be generated, based on epitope similarity between cellular and viral antigens [Steinman and Oldstone, 1997; Gautam et al., 1998]. This point has yet to be addressed experimentally. Therefore, virus-myelin cross-reactive T cells were sought in HHV-6 seropositive MS patients, and whether these cells were expressed preferentially compared to healthy seropositive controls.

## MATERIALS AND METHODS

### Subjects

Twenty-two multiple sclerosis patients (15 females, 7 males, ratio 2:1), not undergoing immunomodulatory therapy, with a median age of 37.2 years (age range 16–57) and diagnosed with relapsing remitting (RR; n = 17) and chronic progressive (CP; n = 5) MS were enrolled for this study. Within the RR group, 9 were in acute relapse at time of blood collection (Table I). Heparinized blood control samples were also obtained from 16 age- and sex-matched healthy individuals as controls. Informed consent was obtained from all patients. CSF samples were available from 9 of the patients, which were collected at enrollment for diagnostic purposes.

### Antigens

HHV-6 variant A (GS) was propagated in the human T lymphoid cell line HSB-2. Cells were maintained at

approximately  $5 \times 10^5$  cells/ml and when cytopathic effect was observed, fresh uninfected cells were added at a ratio of 1:2 (infected/uninfected). The cell extract supernatants were obtained from rapidly freezing and thawing infected (when approximately 70–80% of the cells appeared antigen positive by an immunofluorescence assay, IFA) and uninfected HSB-2 cells. To inactivate the virus, the supernatant was exposed to ultraviolet light (230 mW/sq cm for 5 min), and residual infectivity was checked on HSB-2 cells.

Bovine Myelin Basic Protein (MBP) was purchased from Sigma and used at a concentration of 10  $\mu$ g/well.

### HHV-6 DNA PCR

This was essentially as described previously [Cuomo et al., 2001]. Briefly, 10  $\mu$ l of acellular cerebrospinal fluid, after freezing and thawing several times, were submitted to nested PCR where a 287-bp outer fragment and a 163-bp inner fragment of HHV-6 DNA were amplified. The resulting product contained a Bam I and a Hind III digestion sites present selectively in "A" and in "B" type of HHV-6, respectively. Hind III digestion yielded two fragments of 66 and 97 bp, Bam I digestion produced a 90- and a 73-bp fragment. These enzymes were used to identify the type of HHV-6 amplified. Primer sequences and PCR conditions were as described [Lyll and Cubie, 1995]. The products of digestion were run in 3% agarose gel containing ethidium bromide.

### HHV-6 Serology

Sera were diluted serially and tested for the presence of antibodies against HHV-6 by an indirect immuno-

TABLE I. Clinical Characteristics and Status of HHV-6 Infection of Multiple Sclerosis Patients\*

Patient	Sex	Age	Type of disease	Disease duration (years)	Antibody titer <sup>a</sup>	Viral DNA in CSF
1	F	31	RRex	7	100	–
2	M	23	RRex	1	100	+
3	M	34	RRex	1st episode	200	–
4	M	23	RRex	1st episode	50	+
5	F	16	RRex	1st episode	200	+
6	F	39	RRex	1st episode	200	nd
7	F	24	RRex	1st episode	100	nd
8	F	39	RRex	2	100	nd
9	F	35	RRex	3	50	nd
10	F	29	RRrem	2	50	nd
11	F	33	RRrem	3	50	nd
12	F	32	RRrem	5	50	+
13	M	49	RRrem	4	50	nd
14	F	40	RRrem	1	100	nd
15	M	56	RRrem	9	50	+
16	M	45	RRrem	4	200	+
17	F	37	RRrem	10	50	nd
18	F	33	CP	2	100	nd
19	F	55	CP	2	200	nd
20	M	46	CP	1	200	+
21	F	43	CP	4	100	nd
22	F	57	CP	13	100	nd

\*RRex, relapsing-remitting, exacerbation phase; RRrem, relapsing-remitting in remission; CP, chronic progressive; nd, not done.

<sup>a</sup>Reciprocal values.

fluorescence assay on HSB-2 cells infected with HHV-6 (GS strain). Mock infected cells were used to evaluate nonspecific background fluorescence. Antibody titers were expressed as the highest serum dilution yielding detectable immunofluorescence on an appreciable number of antigen positive cells.

### Generation of Virus and Myelin Basic Protein Reactive T Cell Lines

Peripheral blood lymphocytes were separated by Ficoll-Hypaque (Pharmacia) density gradient centrifugation and resuspended at  $1 \times 10^6$  cells/ml in complete RPMI 1640 medium (EuroClone) containing 2 mM L-glutamine, 100 U/ml of penicillin, 100  $\mu$ g/ml of streptomycin, 10 mM HEPES, 50 mM 2-mercaptoethanol, and 10% (vol/vol) heat inactivated pooled human AB serum (EuroClone). Cells were seeded at  $2 \times 10^5$  cells per well (in 0.2 ml) into 96 well plates (round-bottom, Costar) together with viral antigen at a final dilution of 1:50 or with MBP (10  $\mu$ g/well). After 3–4 days of incubation, 0.1 ml of culture medium per well supplemented with 40 units/ml of human recombinant interleukin-2 (Immunex, Seattle, WA) was added, and this procedure was repeated every 3 to 4 days for a total of 14 days. Primary T cell lines were screened for antigen-dependent proliferation against viral antigen or myelin basic protein.

### Antigen-Specific Lymphoproliferation Assay

The T cell lines in microtiter plates were washed with complete medium to remove free IL-2, resuspended in 150  $\mu$ l/well of complete medium, and split into three new 96 well plates (50  $\mu$ l/well) to which autologous irradiated PBLs ( $1 \times 10^5$ ), as antigen presenting cells, and antigens (GS virus, Myelin Basic Protein or supernatant of mock-infected HSB-2 cells) were added. The cells were incubated for a total of 72 hr and 3H-thymidine (1  $\mu$ Ci/well, Amersham) was added for the last 8 to 15 hr. Cells were harvested onto glass microfiber filters (Packard) and counted using a Canberra Packard scintillation counter. A stimulation index was calculated as the ratio of radioactivity (cpm) incorporated in the presence of specific antigen over its control, and a value above 2, with at least 1,000 cpm incorporated considered significant.

## RESULTS AND DISCUSSION

A group of 22 multiple sclerosis patients and 16 healthy controls were examined for an HHV-6 specific serological response. All had specific antibodies of IgG class directed against viral cycle antigens, thus demonstrating past infection with HHV-6 infection. Antibody titers ranged from 1:50 to 1:200 (Table I). There were no significant differences between the antibody geometric mean titers found in the MS patients subgrouped according to the type of disease (RRex, 1:108; RRrem, 1: 64,8; CP, 1: 131,9) and the control group (1: 82, not shown). IgM specific antibodies were not found in either

patients or controls. Nine patients with MS were also investigated for the presence of HHV-6 specific Ig and of viral DNA in the cerebrospinal fluid, by indirect immunofluorescence and by nested-PCR, respectively. Consistent with the findings of Ablashi et al. [1998], seven patients had HHV-6 specific IgG. Viral DNA sequences were also detected in the same 7 patients, and, following restriction enzyme analysis, the type of virus present was determined as variant A in all cases. This finding is not unexpected, in view of the reported high neurotropism of HHV-6 variant A [Akhyani et al., 2000].

In order to obtain antigen specific T cell lines from both patients and controls, peripheral blood lymphocytes were stimulated with either HHV-6 type A cell extract or Myelin Basic Protein. Following HHV-6 stimulation, patients and controls gave rise to T cell lines at the same rate (68%, as calculated from data in Table II). Following Myelin Basic Protein stimulation, a higher, but again similar percentage of patients and controls responded to the stimulus (81 and 80%). To compare the degree of cross-reactivity to viral antigens and myelin in MS patients and healthy controls, all T cell lines established following stimulation with either antigen were challenged against the cross-reacting antigen. The results shown in Table II can be summarized as follows: (1) cross-reacting T cell lines were generated more frequently from MS patients (45 out of 124, 36.2%) than from controls (27 out of 104, 25.9%). Parallel conclusions were drawn in an earlier report from experiments using human Coronavirus 229E [Talbot et al., 1996]. The preferential occurrence of cross-reacting T cell lines in MS may be explained partially by the sustained T-cell mediated responsiveness found in the disease [Noseworthy et al., 2000]. (2) Within the MS group, cross-reacting T cell lines were generated more easily following Myelin Basic Protein stimulation (40 vs. 33%), whereas the viral extract was a better stimulus in controls (29 vs. 21%). (3) MS patients and controls differ significantly in giving rise to cross-reacting cell lines following Myelin Basic Protein stimulation (40 vs. 21%,  $P < 0.05$ ).

The finding that cross-reacting T cell lines are recruited more easily following stimulation with myelin basic protein in MS than in controls, can be explained by the specific relevance that this antigen plays in the disease. The recent observation on the encephalitogenic activity of other myelin proteins such as the myelin-associated glycoprotein and the oligodendrocyte-specific glycoprotein [Morris-Downes et al., 2002] supports further the relevance of these proteins and suggests additional studies. Moreover, the observation that T cells cross-reacting with HHV-6 and Myelin Basic Protein can be found in both MS patients and healthy controls is in agreement with the accepted notion that cross-reactivity between microbial and self antigens is a frequent event [Van Noort et al., 2000]. The difference in cross-reactivity between patients and controls is not as high as that found by other researchers for human coronavirus and Myelin Basic Protein

TABLE II. Patterns of Antigen Reactivity of Virus or Myelin Basic Protein Reactive T-Cell Lines\*

Donor group	Sel. Ag	No. of T-cell lines obtained per donor																				Total cell lines		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		21	22
MS	Virus	17	3	1	0	1	7	0	0	8	4	4	0	1	1	0	0	5	1	2	0	4	13	72
	Total lines	1	2	1	0	1	0	0	0	3	0	2	0	1	1	0	0	1	1	2	0	4	24 (33%)	
	Cross-reacting lines	8	2	2	nd	4	0	nd	2	nd	6	0	2	4	1	0	2	nd	1	nd	nd	14	52	
	MBP	7	1	1	nd	1	0	nd	0	nd	2	0	0	1	1	0	2	nd	0	nd	nd	1	21 (40%)	
Control	Virus	4	0	0	11	1	6	2	2	2	0	7	15	4	0	4	0	0	0	0	0	0	58	
	Total lines	1	0	0	1	1	1	1	0	2	0	4	3	3	0	0	0	0	0	0	0	0	17 (29%)	
	Cross-reacting lines	2	nd	nd	3	13	nd	5	0	0	6	15	1	1	nd	nd	nd	nd	nd	nd	nd	46		
	MBP	1	nd	nd	0	0	nd	5	0	0	0	2	1	1	nd	nd	nd	nd	nd	nd	nd	10 (21%)		

\*Virus or MBP-reactive T-cell lines from 22 MS patients and 16 healthy controls. MBP, myelin basic protein; nd, not done.

[Talbot et al., 1996], implying the need to study more patients and controls, but it could still indicate that a second ubiquitous human virus may contribute to the activation of autoreactive T cells directed against myelin components in MS. However, the question of whether there is a causative relationship between microbial infections and autoimmune responses to myelin antigens has yet to be solved. Molecular mimicry is one of the most attractive mechanistic hypothesis and our data, although consistent with this hypothesis, showing no differences in the percentage of cross-reactivity between patients and controls, do not support molecular mimicry as being the major mechanism responsible for the onset of MS.

Other experimental approaches are needed either to uncover other microbial antigens more effective than HHV-6 in eliciting cross-reacting T cell responses in MS patients, and/or to identify additional factors, such as cellular defects or genetic predisposition, which may contribute to the onset of an autoimmune demyelinating disease.

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