Detection of Antibodies Directed against Human Herpesvirus 6 U94/REP in Sera of Patients Affected by Multiple Sclerosis

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The association between human herpesvirus 6 (HHV-6) and multiple sclerosis (MS) is controversial. In fact, it is difficult to establish a causative role of HHV-6, due to the high prevalence of latent infected individuals in the healthy population. Therefore, the presence of virus sequences in tissue biopsy does not support a viral role, and serological assays do not show significant differences between MS patients and control populations. The only viral gene expressed during latency is U94/rep. Therefore, we have developed a serological assay for the detection of antibodies specifically directed against U94/REP protein. Different populations were analyzed by enzyme-linked immunosorbent assay, including healthy controls, MS patients, and subjects with diseases unrelated to HHV-6 infection, including other neurological diseases. The results show statistically significant differences (P > 0.01) between MS patients and control groups, both in antibody prevalence (87 and 43.9%, respectively) and in geometric mean titer (1:515 and 1:190, respectively). The detection of antibodies specific for HHV-6 U94/REP shows that the immune system is exposed to this antigen during natural infection. The higher prevalence and higher titers of antibodies to U94/REP suggest that MS patients and control groups might experience different exposures to HHV-6.

Human herpesvirus 6 (HHV-6) is a betaherpesvirus with a preferential tropism for CD4+ T lymphocytes. It was recently discovered that the cellular receptor of the virus is CD46, a ubiquitous protein expressed on the surfaces of different types of human cells (45). Consequently, the virus can infect a broad range of cells of different origin (13), including cells of the central nervous system (CNS) (15), generally supporting low levels of replication.

The primary infection with HHV-6 is associated with exanthema subitum, a benign pediatric disease (55). Following primary infection, HHV-6 establishes a latent infection persisting in monocytes/macrophages and in circulating mononuclear cells in the healthy population (42). Viral reactivation is induced by immunosuppression and can result in the development of severe diseases (10, 18, 35). HHV-6 has been associated with several pathological conditions, such as complications following solid organ and bone marrow transplantation (including pneumonia and bone marrow suppression [9] and thrombotic microangiopathy [36]), meningo-encephalitis (25), infectious mononucleosis (6, 48), persistent lymphadenopathy (38), fulminant hepatitis (5), autoimmune disorders (29), chronic fatigue syndrome (7), Kikuchi syndrome (22), and Rosai-Dorfman disease (30).

Several studies have documented the neurotropism of HHV-6, suggesting that viral infection of the CNS can play a role in disseminated demyelination (26), infarction of the basal ganglia (53), seizures and fatal encephalitis in children (21, 27), and AIDS dementia (28). Furthermore, several reports have associated virus infection of the CNS with multiple sclerosis (MS). In fact, high levels of HHV-6 DNA have been detected in the CNS and cerebrospinal fluid of MS patients (14, 46, 54), as well as in their sera (47). MS patients have increased titers of serum antibodies reactive with HHV-6 (3, 46), and 50 to 70% of them are positive for HHV-6-specific immunoglobulin M (IgM) antibodies (2, 3, 47). Nevertheless the evidence is still controversial. Due to the high prevalence of latent infected individuals in the healthy population, it is difficult to establish a causative role of HHV-6 in this disease. The majority of healthy subjects are seropositive for the virus, and studies based on the use of classical diagnostic methods failed to detect differences between MS patients and control populations (19, 39). Moreover, the mere presence of the virus is not supportive of a causal association due to the persistence of latent DNA in healthy tissues. Therefore, to establish a correlation, it is necessary to discriminate between latent and productive infections. Recently, it was shown that peripheral blood mononuclear cells from MS patients harbor HHV-6 DNA in a latent, nonproductive form, similar to the case for the control population (43).

HHV-6 is classified into two variants, HHV-6A and HHV-6B, which are different in regard to cell tropism and pathological implications (1). Both variants contain a linear double-stranded DNA genome of approximately 161 kbp with 112 open reading frames (ORFs), which include the ORF U94/rep, a spliced gene encoding a 490-amino-acid protein homologous to Rep78/68, a nonstructural protein from the human parvovirus adeno-associated virus type 2 (AAV-2) (20, 50). The gene is highly conserved between variants, since the difference is
limited to only 10 amino acid residues (40), and interestingly, it is unique to HHV-6 and not present in other herpesviruses. Transcriptional analysis of infected cells has shown that U94/rep represents a useful marker of latent infection, since it is the only immediate-early gene expressed during the latent phase of infection, in the absence of other transcripts (42). The AAV-2 rep gene product (REP) is known to possess several biological activities involved in the regulation of AAV-2 gene expression, including DNA-binding, site- and strand-specific endonuclease, helicase, and ATPase activities (23, 24). AAV-2 REP is necessary for the integration of the proviral DNA within the cellular genome (31, 32), inhibits transcription from the human immunodeficiency virus type 1 (HIV-1) long terminal repeat promoter in fibroblasts and T-cell lines (51), and represses the expression of cellular oncogenes (16, 49).

The HHV-6 U94/rep gene product shares 24% identity with AAV-2 REP at the amino acid level, suggesting that HHV-6 U94/REP may possess similar functions, as confirmed also by the observation that it can complement replication of a rep-deficient AAV-2 genome (51). Also, HHV-6 is able to integrate into the human genome (33, 34, 52), and it was recently shown that HHV-6 U94/REP can bind the human transcriptional factor TATA-binding protein (37). Furthermore, HHV-6 U94/REP suppresses the transformation by H-ras, inhibits transcription from the HIV-1 long terminal repeat in a T-cell line (4), and suppresses the lytic replication of the retrovirus in vitro (our unpublished data), and cell lines containing the HHV-6B U94/rep gene are refractory to productive infection by HHV-6A (42).

Thus, the role of U94/rep in the HHV-6 life cycle is particularly interesting, especially in regard to the pathological implications of the virus. For these reasons, in the present study we focused attention upon this nonstructural protein, setting up an enzyme-linked immunosorbent assay (ELISA) for the specific detection of antibodies to U94/REP and investigating the presence of antibody reactivity in sera from MS patients.

The presence of antibodies against nonstructural viral proteins in the sera of patients has been documented (41), and it could be relevant in the natural history of the disease (8); however, it is presently unknown whether the gene product of HHV-6 nonstructural ORF U94/rep elicits specific immune responses. To address this question, we developed a serological assay for the detection of antibodies specifically directed against U94/REP. Different populations were analyzed, including MS patients, healthy controls, and subjects with diseases unrelated to HHV-6 infection.

A recombinant U94/REP protein, produced in bacteria and purified, was used in ELISA and Western blot assay. The tests were validated by analysis of sera collected from children before and after seroconversion for HHV-6. The results showed that the majority of MS patients are positive for the presence of serum immunoglobulins specifically reacting against U94/REP. A statistically significant quantitative difference in the antibody prevalence and titer was observed between MS patients and control groups, including healthy controls and patients with other neurological disorders.

MATERIALS AND METHODS

Expression of the U94/rep gene in Escherichia coli and production of anti-REP antisera. U94/rep from the HHV-6B variant was cloned in the pSRS2P vector, giving the recombinant plasmid pSRS2P2H (42), and subsequently it was subcloned in the pQE30 vector (Qiagen) in frame with a stretch of six histidine residues (His6) at the amino terminus under the control of a T7 promoter-lac operator for protein production. The pQE-rep recombinant plasmid was used to transform Escherichia coli K-12. Upon addition of IPTG (isopropyl-β-thiogalactoside), the T7 promoter-K-12 is activated, and the fusion protein. The U94/REP protein was purified under denaturing conditions by ion-exchange chromatography, using a hydroxyapatite column and a sodium gradient in lysis buffer; purity of about 99% was obtained, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

To obtain a specific anti-REP serum, rabbits were inoculated according to a DNA vaccination protocol by direct injection of plasmid pSRS2P2H. DNA was prepared as described previously (11, 12), and the following protocol was used to immunize rabbits: two intramuscular injection of 300 μg of pSRS2P2H at 2-week intervals, followed by three boosts with the same amount of DNA injected twice intradermally and once subcutaneously. Animals were then bled, and samples were used for subsequent immunological analysis. Polyclonal rabbit antisera against HHV-6 U94/REP and preimmune serum were used as a positive and negative controls, respectively, in Western blot assay and ELISA.

Clinical samples. Sera were collected at the Section of Neurology, University of Ferrara, with the exception of sera from three children affected by exanthem subitum, collected before and after seroconversion, which were obtained at the Section of Virology, University of Modena. The seroconverter sera were used to assess the specificity and sensitivity of the ELISA.

Control blood samples, which were seronegative for HIV-1, HIV-2, hepatitis B virus, and hepatitis C virus, were obtained from 82 healthy blood donors. Sera from subjects with a clinical diagnosis of MS were obtained from 54 patients (42 females and 12 males). This group represents a cross-sectional population of patients; 20 patients were affected by relapsing-remitting MS and had received beta interferon therapy, whereas 34 were enrolled at an earlier stage of the disease and had not received any treatment. The mean age of MS patients was 40 years old (age range, 14 to 68).

In addition, sera from 20 patients with other neurological disorders of inflammatory (10 cases) or noninflammatory (10 cases) origin were also analyzed, together with sera from 15 patients affected by cervical dysplasia (CIN) of degree I to III. The group with other neurological disorders of inflammatory origin included patients with a mean age of 59 years (range, 43 to 73), with 80% male and 20% female patients. The group with other neurological disorders of noninflammatory was 60% female with a mean age of 51 years (range, 27 to 72). Patients of the CIN group were female with a mean age of 47 years (range, 29 to 68). All of the samples were aliquoted and frozen at −80°C until needed in order to avoid repeated freezing-thawing cycles.

ELISA. The presence and titer of serum antibodies directed against HHV-6 U94/REP were determined by ELISA, using twofold serial dilutions of serum and the recombinant U94/REP as the capture antigen. Control antigen, represented by mock bacterial extract treated in the same manner as the REP-containing bacteria, was also used. Briefly, Immunoplates (Nunc) were coated overnight at 4°C with purified recombinant U94/REP or mock lysate (obtained from E. coli K-12 cells transformed with the pP3UO30 vector alone) at a concentration of 5 μg/ml in 0.05 M sodium-bicarbonate buffer (pH 9.6). Excess antigen was eliminated by three washings with phosphate-buffered saline (PBS) (137 mM NaCl, 3 mM KCl, 80 mM Na2HPO4, 1 mM NaH2PO4, pH 7.4) containing 0.05% Tween 20 (PBS-T). A saturation step was performed by incubating plates for 90 min at 37°C with 200 μl of a PBS solution containing 10 mM CaCl2 and 5 mM MgCl2 (PBS-C) and 3% bovine serum albumin (BSA) (Sigma) per well. After three washings with PBS-T, 100 μl of serum diluted in saturation buffer (PBS-C plus 3% BSA) was added and tested in duplicate. The rabbit polyclonal anti-REP serum diluted 1:200 was used as a positive control, whereas negative controls were represented by preimmune sera from three children who subsequently seroconverted. Incubation was performed for 90 min at 37°C. The plates were further washed three times with PBS-T, and then 100 μl of a horseradish peroxidase (HRP)-labeled goat anti-human or anti-rabbit IgG (Roche Molecular Biochemicals) diluted 1:10,000 or 1:3,000, respectively, in PBS containing 0.1% Tween 20 and 1% BSA was added per well to reveal specific anti-U94/REP antibodies. Incubation was performed for 90 min at room temperature. Following three additional washings, 100 μl of ABTS (2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (Roche Molecular Biochemicals) per well was added and left for 45 min at room temperature. The optical density at 405 nm (OD405) was measured. Values higher than the mean control value plus three standard deviations were considered positive.

Western blot analysis. The specificities of anti-U94/REP antibodies of human and rabbit sera were checked by Western blot analysis. Briefly, 5 μg of U94/REP protein or mock lysate was separated by SDS-PAGE and then electrically transferred onto nitrocellulose paper by using a transfer buffer consisting of 25 mM...
protein was recovered in the insoluble fraction of the total extract (arrow). Following a purification step by ion-exchange chromatography, a highly purified U94/REP was obtained, as shown in the right panel. Sizes of molecular mass markers (MW) are shown.

FIG. 1. Production and purification of recombinant HHV-6 U94/REP. E. coli K-12 was lysed before induction (n.i.) or after induction by IPTG for 1, 3, and 5 h. Aliquots of bacterial extract were separated by SDS-PAGE and stained with Coomassie blue. The recombinant by IPTG for 1, 3, and 5 h. Aliquots of bacterial extract were separated by SDS-PAGE and stained with Coomassie blue. The recombinant protein was recovered in the insoluble fraction of the total extract (arrow). Following a purification step by ion-exchange chromatography, a highly purified U94/REP was obtained, as shown in the right panel. Sizes of molecular mass markers (MW) are shown.

Tris, 192 mM glycine, and 20% methanol. Blots were incubated for 1.5 h in saturation buffer, consisting of 5% dehydrated nonfat milk in 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl (TBS). After three washings for 10 min each with TBS containing 0.5% Tween 20 (TBS-T), nitrocellulose filters were incubated for 1 h in fresh TBS-T containing 5% dehydrated milk, and the appropriate dilution (1:500) of human serum was then added. Rabbit polyclonal antiserum diluted 1:200 was used as a positive control, whereas negative human sera were utilized as negative controls. After three additional washings in TBS-T, blots were incubated with HRP-labeled goat anti-human or anti-rabbit IgG (Roche Molecular Biochemicals) in TBS-T plus 5% dehydrated milk for 2 h. The blots were then further washed three times with TBS-T and developed with the addition of a chemiluminescent HRP substrate (SuperSignal West Pico chemiluminescent substrate; Pierce) according to the manufacturer’s protocol.

Statistical analyses. The Student t test was performed to assess the statistical significance of differences between the different sets of data from the various populations tested.

RESULTS

Detection of serum antibodies against HHV-6 U94/REP. To detect human immunoglobulins directed specifically against HHV-6 U94/REP, an ELISA was developed, using the recombinant U94/REP protein obtained in E. coli. The protein was extracted (Fig. 1) and subsequently purified by ion-exchange chromatography, with a purity of about 99% as judged by SDS-PAGE analysis. Crude bacterial extracts derived from bacterial cells harboring the vector alone were extracted, treated exactly as was the U94/REP-containing lysate, and used as a control. Rabbit anti-U94/REP polyclonal antiserum obtained by DNA immunization was used as a positive control.

Acute- and convalescent-phase sera from three children who had developed HHV-6 seroconversion were used to standardize the ELISA protocol. HHV-6 seroconversion was assessed by immunofluorescence assays with structural proteins of HHV-6 as the antigen. The results (Table 1) show that two children (children 1 and 3) were seronegative during the acute phase of the disease and that child 2 had a detectable titer already in the first serum, possibly due to the fact that sampling took place later during the acute phase of the disease. Nevertheless, all three children had an eightfold increase in their immunofluorescence titers to HHV-6. Acute- and convalescent-phase sera were tested at different dilutions against both REP-coated and mock-coated plates in order to check the sensitivity and specificity of the assay. As shown in Table 1, ELISA results correlated with the immunofluorescence titers. In particular, the acute-phase sera that were seronegative for HHV-6 were below the ELISA cutoff value, and the corresponding convalescent-phase sera showed a significant positivity for specific IgG by ELISA. The ELISA analysis confirmed the presence of antibodies to HHV-6 in the first serum from child 2 and the subsequent increase in the sample from the convalescent phase. No reactivity was observed when mock lysate was used as the coating antigen, suggesting that the observed reactivity was specific for U94/REP. Therefore, this ELISA is specific for HHV-6.

Analysis of anti-REP antibodies in different populations by ELISA. To determine the presence and titer of circulating anti-U94/REP IgG in different groups of subjects, five cohorts of human sera were analyzed, consisting of sera from 82 healthy donors, 54 MS patients, 20 patients with neurological disorders with causes other than viral infections (including 10 with inflammatory and 10 with noninflammatory origin), and 15 patients affected by CIN of different degrees (CIN I to CIN III). CIN patients were women 29 to 68 years old, and patients with neurological disorders with causes other than viral infections were 40% females and 60% males and of 27 to 73 years of age. All sera were serially diluted and tested in duplicate in at least three different ELISAs, using the conditions standardized with the seroconvertor sera from children. Negative controls were represented by sera from children before seroconversion, whereas the positive control was polyclonal rabbit anti-REP antiserum. Cutoff values were calculated as the mean of negative control values plus three standard deviations.

The results are shown in Fig. 2. In the control group of healthy donors, 36 out of 82 samples were positive for the presence of specific anti-U94/REP antibodies, with a seroprevalence of 43.9%. No serum reacted against the mock lysate, confirming the specificity of the ELISA. The mean titer of positive samples was 1:130 (range, 1:50 to 240; median titer, 1:166). Immunofluorescence analysis of these control sera showed a seroprevalence of 95% (mean titer, 1:190; range, 1:40 to 1,280), confirming the high prevalence of infection in the healthy population (data not shown).

Interestingly, 47 of 54 sera from MS patients (87%) showed a marked IgG response to U94/REP, with titers ranging from 1:200 to 1:1,200 (mean titer of positive samples, 1:515; median titer, 1:730). The difference in the fraction of positive samples...
between healthy controls and MS patients was statistically significant ($P < 0.01$).

Analysis of sera from patients affected by neurological disorders of different origins revealed a prevalence similar to that observed in the healthy population, with 7 out of 20 samples (35%) positive for anti-REP IgG, with a mean titer of 1:160 (range, 1:50 to 300; median, 1:190). Similar results were obtained for CIN patients, with 6 of 15 sera (40%) positive for anti-REP antibodies. The mean titer of positive sera was 1:175 (range, 1:60 to 260; median, 1:200).

**Analysis of anti-REP antibodies by Western blotting.** To further analyze the specificity of binding between HHV-6 U94/REP and the serum antibodies revealed by ELISA, 20 sera from different groups of patients were also analyzed by Western blotting. The analysis was performed with both positive and negative sera (as determined by ELISA). Instance, the positive control was represented by rabbit polyclonal anti-REP antiserum.

Figure 3 shows the results for three human sera representative of the conditions observed in negative and positive samples. Sera that were negative by ELISA did not react in Western blotting, whereas a strong immunoreactive band was present in all ELISA-positive sera. The intensity of the band was variable in the different sera and correlated with the IgG titer observed in the ELISA. No bands were detected in the lanes for mock lysate, confirming the specificity of the IgG detected with the ELISA method developed.

As expected, the positive control, represented by the rabbit polyclonal antiserum obtained by DNA vaccination, reacted strongly against the recombinant U94/REP protein, which resulted in the development of a clear specific band of the expected molecular mass (Fig. 3).
produced a recombinant U94/REP protein and developed both an ELISA and a Western blot assay to test sera from different populations.

The results show that HHV-6-seropositive individuals can develop antibodies specifically directed against U94/REP. The specificity of the assay is supported by the observation that seronegative children developed reactive antibodies to U94/REP only after a clinically diagnosed, laboratory-confirmed primary infection with HHV-6. This observation, and the lack of reactivity against the mock bacterial lysate, confirmed that the ELISA was specific. The specificity of the antigen-antibody reaction was further confirmed by Western blot assay, showing that positive sera reacted, strongly and directly, with the 56-kDa band of U94/REP.

Interestingly, the analysis by ELISA showed that MS patients had increased prevalence and higher titers of anti-REP immunoglobulins than control groups, represented by healthy blood donors, patients affected by CIN (a neoplastic pathology unrelated to HHV-6), and patients with noninflammatory or inflammatory neurological disease.

These results show for the first time that HHV-6 circulating immunoglobulins directed against HHV-6 U94/REP are developed during natural infection. The functions of U94/REP in HHV-6 biology are still unclear. The gene is highly conserved in all sequenced strains, but the transcript is synthesized with low abundance during lytic replication (40). U94/REP has been associated with viral latency, being expressed in healthy adults in the absence of other lytic genes (as detected by reverse transcription-PCR (42)). Furthermore, cells expressing U94 survive HHV-6 infection, whereas control cell lines are killed by cell lysis. These observations suggest that U94/REP might have enzymatic functions, playing an important role in regulation of viral DNA replication. However, the detection of U94-specific antibodies in human sera suggests that this antigen is exposed to the human immune system during natural infection and that seroconversion to HHV-6 implies specific reactivity also to U94.

The higher prevalence and titers of anti-U94/REP antibodies in MS patients than in healthy donors or in patients with other pathologies suggests that differences in exposure may take place. However, HHV-6 is latent in the peripheral blood of these MS patients, and the viral load is similar to that in controls (43, 44). Therefore, increased titers to U94/REP, the product of a potential latency-associated gene, suggest that these patients might experience variations in U94 production, or frequent switches between latency and active replication, leading to an increased sensitization to this viral antigen.

Presently, it is not possible to establish a positive association between antibodies to U94/REP and MS, and further study is necessary. In particular, the increased titer of specific antibodies at early stages of MS disease could hint at potential applications of the U94/REP ELISA for diagnostic purposes.

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