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Investigating the presence of human herpesvirus 7 and 8 in multiple sclerosis and normal control brain tissue

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Abstract

Multiple sclerosis (MS) is an important demyelinating disease of the central nervous system, the aetiology of which is thought to have a possible viral component. In this study we investigated the possible involvement in MS of two herpes viruses: the neurotropic human herpesvirus 7 (HHV-7) and the related human herpesvirus 8 (HHV-8). Utilising fluorescent in situ hybridisation (FISH) techniques, we examined human post mortem tissues for the presence of immediate early and late viral gene or protein expression in MS patient normal appearing white matter (NAWM), lesional tissue and normal control brain samples. HHV-7 and/or HHV-8 mRNA or protein was detected in some individuals in all three sample categories and was restricted to oligodendrocytes, as determined by double mRNA FISH analysis or immuno fluorescence (IF). No samples showed evidence of viral mRNA when subjected to RT-PCR on extracted ribonucleic acid. We therefore conclude that there is little evidence in our particular sample cohort to suggest involvement of either HHV-7 or HHV-8 in MS.

Keywords: Multiple sclerosis; Human herpes virus 7 and 8; Oligodendrocyte; mRNA FISH

1. Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the CNS. Autoimmune and genetic factors are unlikely to wholly explain the aetiology of this disease, and an environmental component has long been postulated to be involved in MS pathogenesis [1–3]. Pathogens such as bacteria and viruses are the predominant candidates for the environmental factor in MS. Of these two biological candidate groups, viruses have been and remain the leading contenders and MS relapsing episodes are frequently preceded by common viral infections [4]. The infection theory of MS is strengthened by the observation that the CSF of MS patients contain temporally stable oligoclonal IgGs that could be the result of an antigen-driven chronic activation of possibly poly-specific B-cells [4–7]. It is possible that this reaction is triggered by a combination of viruses, the order in which these infections takes place influencing the subsequent immunological reaction [8] With an ever increasing list of viral candidates, including the more recent additions of endogenous retroviruses, coronaviruses and several recently discovered herpesviruses [9–12] there are many ‘contenders’ to chose from and little evidence to favour one above the other.

We are currently investigating some herpesviruses for a possible role in MS aetiology since they have as a family several properties that make them credible candidates. Among these properties are the ability to initiate and maintain a latent infection in ganglionic neuronal cells with subsequent reactivation (prime examples being cold sores and shingles, caused by herpes simplex virus (HSV) and varicella zoster virus (VZV), respectively) and neurotropism [13,14]. Herpesviruses which can cause demyelinating pathology and infection by other viruses are capable of triggering reactivation of latent herpesviruses [15]. Earlier
investigations by others and ourselves have provided evidence to implicate a possible role of HHV-6 in MS pathogenesis [9,16–22]. We have recently shown that Human Herpesvirus 6 (HHV-6) activity is increased in MS lesion tissue compared to both normal appearing white matter (NAWM) and normal control tissue [22]. As a logical development of this work we decided to investigate whether the closely related human herpesvirus 7 (HHV-7) and human herpesvirus 8 (HHV-8) were also detectable in MS brains.

Human herpesvirus 7 (HHV-7) is a member of the β-herpesvirus family, closely related to HHV-6. HHV-7 was isolated in 1990 [23] and comprises a 145 kb genome encoding over 70 proteins, many of which share close homologies with HHV-6, Eppstein Barr virus (EBV) and cytomegalovirus (CMV) proteins. Infection generally occurs early in childhood, with most individuals seropositive for HHV-7 antibodies by the age of 3 [24,25], with 80% of adults testing positive for HHV-7 DNA in PBMCs [26]. Some report that HHV-7 may not undergo a latent stage, but remains persistent at low levels throughout the lifespan of an individual [27,28], others that the virus produces no mRNA in the latent state [29]. Primary infection has been associated with some cases of exanthem subitum [30,31] and encephalopathy [32,33], although more have been attributed to HHV-6 infection. Another condition associated with both HHV-7 and HHV-6 is pityriasis rosea, an inflammatory skin condition which occurs in adults in their 20’s and 30’s, which is attributed to reactivation of the two herpesviruses [34]. Although HHV-7 reactivation has been seen frequently in patients with demyelinating diseases of the peripheral nervous system [35], HHV-7 has also been found in the CNS at varying levels. A review by Dewhurst [36] concludes that HHV-7 is found in 5% of brain tissue samples. This result is consistent with that of Chan et al. [37], but whilst only 5% of the brain samples were positive, multiple samples were taken from patients so that total patient positivity was 37%. It has also been shown that HHV-7 can reactivate HHV-6 from latency in peripheral blood mononuclear cells [38], which may explain the co-occurrence of these two viruses in pityriasis rosea.

Human herpesvirus 8 is a member of the γ-herpesvirus family, of the genus Rhadinovirus (known to infect lymphocytes and associated with immortalisation and transformation). Probably better known as Kaposi’s sarcoma associated virus, HHV-8 comprises of a 170 kb genome bearing close resemblance to EBV and herpesvirus saimiri, although it more closely resembles HSV in its regulation. Although largely known for its role in Kaposi’s sarcoma and several other cancers, it has also been shown to be able to invade the CNS with increased incidence of HHV-8 DNA seen in brain samples with increased patient age [39,40]. HHV-8 has been associated with encephalitis in some immunocompromised individuals, and there is a suggestion that it may play an indirect role in certain primary CNS lymphomas [30]. The incidences of HHV-8 infection and Kaposi’s sarcoma have risen and are a frequent complication in HIV/AIDS patients. The mode of transmission of HHV-8 is debated and although the main route is believed to be through sexual contact, the finding of HHV-8 DNA in brain tissue from stillborn babies indicates that transmission can occur from mother to child in the womb [40].

Aspects of the virus that decrease the probability of its involvement in MS aetiology include the lack of evidence for an increased risk of MS in the spouses/partners of MS patients [41,42]; nor is there evidence for an MS epidemic amongst the homosexual population (the highest prevalence of HHV-8 being found in homosexual males in the areas where MS risk is greatest) [43]. The demographics of HHV-8 and MS then do not tally, thus making any link between the two weak, but nevertheless the possibility cannot be discounted without examination.

Although HHV-7 looked the more promising candidate virus, we decided to investigate both viruses simultaneously. By examining immediate early and late gene transcription (HHV-7) or protein products (HHV-8), we aimed to differentiate between latent/persistent and active viral infection. By comparing normal control tissue with MS normal appearing white matter (NAWM) and MS lesional tissues, we investigated whether there were any differences in viral activity in these three tissue types.

2. Materials and methods

2.1. Samples

Tissue samples were kindly provided by the UK Multiple Sclerosis tissue bank and consist of normal control white matter (3 samples), MS NAWM (6 fixed samples, 5 unfixed) and MS lesional (8 fixed samples, 4 unfixed, all chronic lesions) white matter (see Table 1). Tissues were either fixed frozen or snap frozen, and were used for FISH and making gDNA and cDNA for PCR, respectively.

One mouse brain was kindly provided by Peter Humphreys, University of Glasgow Department of Neurology, Division of Clinical Neurosciences.

| Sample ID | Age and sex | MS type and duration | PM interval | Days in fixation |
|-----------|-------------|----------------------|-------------|-----------------|
| MS 53     | M 66        | 2° PMS 34 yrs        | 26 h        | 6               |
| MS 61     | F 56        | Prog. relapsing MS 34 yrs | 6 h | 8               |
| MS 77     | F 57        | MS 31 yrs            | 28 h        | 8               |
| MS 80     | F 71        | 2° PMS 35 yrs        | 24 h        | 12              |
| MS 88     | F 54        | Chronic MS 20 yrs    | 22 h        | 10              |
| MS 97     | M 55        | MS details NA        | 31 h        | 14              |
| Control 14| F 64        | COD: Cardiac failure | 18 h        | 22              |
| Control 15| M 82        | COD: Schizophrenia   | 21 h        | 18              |
| Control 16| M 92        | COD: Cardiac failure, old age | 13 h | 10              |
Wax embedded samples of Kaposi’s sarcoma tissue was kindly provided by David Blackbourn, Institute of Virology, University of Glasgow.

2.2. Extraction of RNA and DNA from snap frozen tissues

RNA was extracted using the RNA Lipid Tissue Mini Kit from Qiagen (cat 74804). DNA was extracted using the QIAamp DNA Mini Kit from Qiagen (cat 51304).

2.3. Primers and probes

Labelled probes were made by MWG; PCR primers were made by Sigma Genosys. Claudin-11 (BC013577): FISH oligo cocktail = 241–270, 301–330, 361–390, 421–450, 481–510.

HHV-7 (J strain, X83413): Immediate early (U42) FISH oligo cocktail = 63132–63161, 63351–63380, 63459–63488, 63767–63796, 64013–64042. Outer PCR primers: F’ = 63821–63844, R’ = 63131–63154. Inner PCR primers: F’ = 63557–63580. Late (U11) FISH oligo cocktail = 16015–16044, 16102–16131, 16193–16222, 16287–16316, 16844–16873.

HHV-8 PCR primers taken from ORF K9-3 [44]. FISH oligo cocktail probe (fluorescein conjugated) against T1.1 mRNA from Novocastra Laboratories (NCL-HHV-8). Visualisation by Universal ISH Detection Kit (Novocastra Laboratories, NCL-ISH-D).

2.4. PCR, RT-PCR

RT reactions were performed on the RNA samples extracted from the snap frozen tissues using either specific (HHV-7 outer nested primers) or oligo dT/random hexamer primers in conjunction with Durascript RT-enzyme/Durascript RT-PCR kit from SIGMA (cat. A4464/H SRT-20). PCR reactions were performed using RedTaq Ready Mix PCR reaction mix from SIGMA, 45 cycles at 55 °C. As a positive control, HHV-7 and -8 genomic DNA (Autogen Laboratories) was used. The HHV-7 gDNA stock was 50 ng/μl, with good PCR results achieved at concentrations down to 10 x 10^{-3} ng. The HHV-8 gDNA stock was 1.3 x 10^{4} copies/μl, with PCR detection of 50–100 copies.

2.5. Fluorescent in situ hybridisation (FISH)

Tissue preparation, sectioning, prehybridisation and hybridisation are as described in [22]. Probes were cocktails of five antisense or sense 30-mer oligonucleotides (40 ng/ml) based on cDNA sequences labelled with either rhodamine red (clau-11, 3’only) or digoxigenin (DiG) (HHV-7, S’ and 3’) by suppliers (MWG-BIOTECH AG, Germany). Secondary detection was with Sheep anti-DiG conjugated to either rhodamine or fluorescein (Roche) followed by either rat anti-FITC antibody conjugated to FITC (Serotec Ltd, UK), or a combination of mouse anti-rhodamine followed by goat anti-mouse IgG conjugated to rhodamine (both AbCam Ltd). Sections were blocked in incubation buffer (no antibody) for 15 min (in 100 mM Tris–HCl pH 7.4, 15 mM NaCl, 1% serum). Antibody incubation (1/250 dilution for anti-DiG, 1/100 dilution for anti-Fluorescein, 1/300 for mouse anti-rhodamine and 1/500 for goat anti-mouse IgG–rhodamine) was for 30 min at room temperature followed by 3 x 5 min washes in buffer minus serum. Sections were mounted using Vectashield Mounting medium with antifading agent (+/- DAPI). Images were captured using a Zeiss Axioplan microscope fitted with a cooled CCD camera (Pro-Series High Performance) and analysed with Image-Pro Plus software version 4.1 (Media Cybernetics).

HHV-8 mRNA ISH was performed using a commercial oligo cocktail probe (HHV-8, fluorescein conjugated against T1.1 mRNA, Novocastra Laboratories NCL-HHV-8), following manufacturer’s instructions, although experimentation demonstrated that incubation overnight gave equally good results and allowed for parallel processing with HHV-7 FISH slides. This did not provide a signal, so amplification of the probe signal was achieved using the Universal ISH Detection Kit recommended for use with the commercial probes (NCL-ISH-D, Novocastra Laboratories).

2.6. Immunofluorescence (IF)

Immunofluorescence was performed on hybridised sections using a goat primary antibody directed against MOBP (myelin-associated oligodendrocytic basic protein, Autogen-bioclear UK) with AMCA-conjugated (7-amino-4-methyl-coumarin-3-acetic acid) secondary rabbit anti-goat IgG (Vector Laboratories). Dilutions were 1/250 and 1/100, respectively. Mouse monoclonal antibodies raised against HHV-7 pp85 and HHV-8 virion envelope glycoprotein ORF K8.1A/B (both diluted 1/250, Autogen-bioclear UK) were used, with goat anti-mouse IgG conjugated to rhodamine (1/500 dilution, AbCam Ltd) as the secondary. Investigation of latent HHV-8 infection was performed using an antibody against HHV-8 latent nuclear antigen and the accompanying protocol (1/20 dilution, Novocastra Laboratories).

3. Results

3.1. PCR and RT-PCR

Using the unfixed tissue, DNA and RNA was extracted and subjected to PCR/RT-PCR analysis for the presence of HHV-7 (IE) and HHV-8 DNA and RNA. Using nested PCR with forty-five rounds of amplification for each step, two samples tested positive for HHV-7 genome (see Table 2). Both samples were from MS NAWM, neither the normal control samples nor the lesional MS samples tested positive for HHV-7 genomic DNA. Two of the genomic samples tested positive for HHV-8 (see Table 2), albeit for very low
levels of the viral genome. Using either random primed or specific (HHV-7 IE outer primers) primed cDNA, the PCR reactions were repeated. Neither HHV-7 (IE) nor HHV-8 mRNA was detected in our samples, although the endogenous housekeeping gene β2-microglobulin was seen in abundance. These results suggested that if either of the two viruses were present and active in our tissues, the level of RNA expression was likely to be low.

3.2. IF

Immunofluorescence was performed on fixed frozen sections of brain from normal and MS tissue (lesional and NAWM). IF against HHV-7 pp85 did not produce a positive signal in normal control tissue. Out of 6 MS patient samples with good tissue morphology and both NAWM and lesional tissue available, only two exhibited convincing positive signal (see Table 1) with a further two very weakly positive. In all cases the positive HHV-7 protein signal co-located with that for the oligodendrocyte specific protein MOBP (see Fig. 1A). The protocol for antigen unmasking for the latent HHV-8 antibody proved to be detrimental to tissue morphology and could not be used. The antibody against HHV-8 virion envelope glycoprotein ORF K8.1A/B was successful. The two sample sets exhibiting a strong positive signal for HHV-7 protein were also positive for HHV-8 envelope protein, again locating to oligodendrocytes (see Fig. 1B). The control Kaposi’s sarcoma tissue was also found to exhibit cytoplasmic positive staining for the envelope protein (see Fig. 1D). No signal was seen in the 3 normal control brains. Although these results failed to provide any substantial evidence for a link between HHV-7 or -8 viral activity and MS, it was possible that poor tissue preservation meant that viral protein could have been

Fig. 1. HHV-7 protein and mRNA signal seen in cytoplasm of oligodendrocytes (A and C). Total numbers of positive cells are low and tend to be seen in clusters rather than evenly distributed throughout positive tissue samples. HHV-8 protein signal seen in cytoplasm (B and D), in brain tissue limited to oligodendrocytes. HHV-8 mRNA probe is against nuclearly transcribed T1.1. As with HHV-7, positive staining cells are rare and tend to appear in clusters. HHV-8 protein signal seen in cytoplasm (B and D), in brain tissue limited to oligodendrocytes. HHV-8 mRNA probe is against nuclearly transcribed T1.1. As with HHV-7, positive staining cells are rare and tend to appear in clusters.

Table 2
List of all tissue samples and their positivity for HHV-7 and 8 DNA, RNA and protein

| Tissue          | HHV-7 pp85 | HHV-7 gDNAa | HHV-7 mRNA FISH | HHV-8 K8.1A/B protein | HHV-8 gDNAa | HHV-8 mRNA FISH |
|-----------------|------------|-------------|-----------------|------------------------|-------------|-----------------|
| Control 14      | X          | X           | X               | X                      | X           | X               |
| Control 15      | X          | X           | X               | X                      | X           | A few           |
| Control 16      | X          | X           | X               | X                      | X           | X               |
| MS 53 NAWM      | X          | A few       | X               | X                      | X           | A few           |
| MS 53 Lesion    | X          | X           | X               | X                      | X           | X               |
| MS 61 NAWM      | X          | X           | [+/- Faint]     | X                      | X           | X               |
| MS 61 Lesion    | [+/- Faint]| X           | X               | X                      | X           | X               |
| MS 77 NAWM      | X          | X           | [+/- Faint]     | X                      | X           | X               |
| MS 77 Lesion 1  | X          | –c          | X               | X                      | X           | –c             |
| MS 77 Lesion 2  | X          | –c          | X               | –c                     | –c          | –c             |
| MS 77 Lesion 3  | X          | –c          | X               | –c                     | –c          | –c             |
| MS 80 NAWM      | X          | –c          | X               | X                      | X           | X               |
| MS 80 Lesion    | [+/- Faint]| X           | X               | [+/- Faint]             | –c          | X               |
| MS 88 NAWM      | –c         | X           | X               | –c                     | X           | –c             |
| MS 88 Lesion    | –c         | X           | X               | –c                     | X           | –c             |
| MS 97 NAWM      | X          | X           | [+/- Faint]     | X                      | X           | X               |
| MS 97 Lesion    | X          | X           | X               | X                      | X           | X               |
| Kaposi’s sarcoma| –c         | –c          | –c              | –c                     | –c          | –c             |

Claudin-11 mRNA was found to be present in all samples by mRNA FISH, the one exception being Kaposi’s sarcoma.

a gDNA = genomic DNA examined using PCR. RT-PCR failed to provide evidence for either HHV-7 or HHV-8 mRNA in frozen tissue samples.
b X = not found.
c –c = not done.
degraded and thus antigen positivity may have been underestimated.

3.3. Double mRNA FISH

The fluorescent in situ hybridisation for HHV-7 mRNA was performed on fixed frozen sections of brain tissue from normal control patients and MS samples (both lesional and normal appearing white matter). As an internal control and identifier of oligodendrocytes (for validation see [22]), the myelinating cells of the CNS, a cocktail of 10 30 base rhodamine labelled oligonucleotide probes against the oligodendrocyte specific gene claudin-11 was used in conjunction with the DiG-labelled probe cocktail against HHV-7 IE and Late genes (U42 and U11, respectively). The DiG labelled probes (5 for each gene, labelled 5' and 3') were visualised using an antibody raised against DiG with a fluorescein hapten attached (Sheep anti-DiG-FITC, Roche).

The claudin-11 mRNA signal is known to require 3 rounds of amplification in order to obtain a strong positive signal (mouse anti-rhodamine followed by anti-mouse IgG conjugated to rhodamine, both AbCam) [22]. Unsurprisingly, therefore, the initial visualisation step failed to provide a positive signal for HHV-7 mRNA. The signal was amplified using rat anti-FITC conjugated to FITC (Serotec), with 12 rounds needed before any positive signal
was seen. The mRNA FISH results were similar to the protein IF results for HHV-7, with no evidence of viral infection in normal control tissue and a strong viral mRNA signal only seen in two samples (see Fig. 1C) with a further two individuals exhibiting samples with a weak viral mRNA signal. As with the protein results, viral expression appeared to be limited to oligodendrocytes as the signal co-localised with that for claudin-11 (see Fig. 1C and Table 2).

Using the HHV-8 fluorescein commercial probe against the nuclear T1.1 mRNA, no direct fluorescent signal was observed in any of the tissues, including the KS control. The ISH fluorescein-conjugated probe detection kit (NCL-ISH-D, Novocastra) was used to amplify the signal with a rabbit anti-FITC/AP antibody and BCIP/NBT. Strong nuclear staining was seen in the KS positive control tissue (see Fig. 1D), although positive cells were visible in clusters with large areas of tissue seen without any positive signal (see Fig. 1F). No signal was seen in the mouse brain negative control tissue (see Fig. 1F). One of the normal control brain samples exhibited a small number of positive staining nuclei (see Table 1). Definite nuclear staining was seen in 5 MS tissue samples (see Fig. 1E), 2 from NAWM and 3 in lesional tissue encompassing a total of 3 individual patients (see Table 2).

4. Discussion

We have shown that low levels of active HHV-7 and HHV-8 infection can be found in MS NAWM and lesional brain tissue, with one example of HHV-8 infection also seen in normal control brain. Viral activity appears to be confined to oligodendrocytes and is seen in only a proportion of the samples examined. Within positive tissue, the infected cells appear to be randomly distributed in clusters with no more than one in 20 views exhibiting positively labelled cells (or an estimated upper level of oligodendrocytes infected with either virus of 5%). As the mRNA FISH signal needed 12 rounds of amplification, we conclude that levels of viral expression in infected cells must be low.

There is the possibility that HHV-7 or HHV-8 may be involved in initial MS lesion development, followed by clearance of the virus. As all our samples are chronic lesions from patients where MS duration is in excess of 20 years (see Table 1) such an involvement would be impossible to detect in our study. Future studies will also need to examine acute MS lesional tissues to answer this point. It is also recognised that the sample size was relatively small due to the paucity of suitable autopsy MS tissues that are not degraded. Notwithstanding this limitation the data obtained were internally consistent with clear negative results.

The low level of HHV-7 mRNA expression observed in the positive samples may be consistent with the possibility that HHV-7 can remain in a persistent state with a small amount of transcription rather than undergoing a latent stage [27,28]. It is also possible that HHV-7 is present latently without any viral mRNA transcription whatsoever [29] and we detected normal low-level reactivation of HHV-7 mainly as a consequence of the inflammatory responses in MS.

Our finding of convincing HHV-7 protein, genomic DNA and mRNA in 2/6 (protein and gDNA) and 2–4/6 (mRNA FISH) individuals examined (or 2/9 and 2–4/9 for the combined normal and MS samples) is comparable to the results reported for prevalence of HHV-7 DNA in the brain of a normal population [37]. The latter authors also found an uneven distribution of viral nucleic acid within the positive tissues, which is mirrored by, and consistent with, our protein and mRNA results. This uneven distribution of positive cells within the tissue may help account for the poor PCR and RT-PCR results, and suggests that multiple sampling must take place for a confident PCR result.

Although HHV-7 can reactivate HHV-6 from latency [38], its low abundance compared to HHV-6 reported in the same set of tissues [22] suggests that this is not occurring here. With reactivation of HHV-7 reported as being common in patients with PNS demyelinating disease [35] it was reasonable to suggest that this may be the case in CNS demyelination as well, but our findings suggest otherwise. Overall our results indicate that the low level of HHV-7 expression seen in our MS tissues does not support a role for this virus in MS.

The T1.1 mRNA and HHV-8 protein results confirmed that HHV-8 infection is present at a very low level in 2–3/6 MS sample sets and in only 1/3 normal control brains. As with HHV-7 results, we could only see a very small number of positively staining cells per section (in most cases a total of 2 views containing no more than a dozen positive cells in all out of almost one hundred views in total). Our results compare favourably to those reported by Chan et al. [39] on HHV-8 DNA prevalence in post mortem brain tissue. In their study, 10 samples from each of 30 individuals were examined and a total of 14% of these samples gave positive results for HHV-8 DNA. When this is tallied with the number of individuals that gave at least one positive sample, 63.3% of the individuals were positive for HHV-8 DNA in the brain. It is therefore probable that if we had access to multiple samples from all individuals in our study, a higher proportion would have scored positively for HHV-8 in both our PCR and in situ studies, including the normal control samples.

As with the HHV-7 results, the low level of protein and mRNA expression and the small number of positive individuals in our sample population does not in our opinion support a role for this virus in MS.

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