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Molecular and diagnostic aspects of the protein p41 of HHV-6 and silencing of the CD46 receptor by RNA interference

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To Xiaoping and My Parents

ABSTRACT

Multiple sclerosis (MS) is one of the most common diseases of the central nervous system (CNS), leading to sensory and motor disability in young adults. There is no efficient treatment of the disease. Its etiology is unknown. Identification of an infectious cause of MS will be of importance to establish an efficient strategy in treating or preventing the disease. HHV-6 is a lymphotropic herpesvirus and one cause of exanthema subitum (ES). All HHV-6 isolates can be classified into two variants, HHV-6A and HHV-6B. CD46 has been found to be a cellular receptor for HHV-6 entry. A link between human herpesvirus 6 (HHV-6) and the pathogenesis of MS has been suggested on the basis of some laboratory findings. Presence of HHV-6 early protein (p41) in MS brains and an elevated serum IgM response to p41 in MS patients are the main evidence.

In order to further examine a possible role of HHV-6 in MS, p41 genes (U27) from HHV-6A strain GS and HHV-6B strain Z29 were cloned and expressed in HEK-293 cells and as glutathione-S-transferase fusion proteins (GST-p41) in *E. coli*. The transfected cells and recombinant p41 proteins were detected with two monoclonal antibodies (mAb to p41/38 and mAb to p41). The mAb to p41/38 recognized the HHV-6A but not the HHV-6B. The epitope recognized by the antibody and the critical amino acid of the epitope were identified by peptide ELISA. Serum IgM and IgG responses to p41 were measured in MS patients and controls by ELISA using the recombinant GST-p41 proteins as the antigen. No significant difference was observed between the examined groups. Our data do not indicate a link between HHV-6 and MS, and suggest that p41 is not an ideal antigen for HHV-6 serology.

RNA interference (RNAi) is a gene silencing generated by double-stranded RNAs. Small interfering RNAs (siRNA), composed of double-stranded RNAs of about 21-nucleotides, can induce specific RNAi and don't activate a nonspecific protein kinase mediated activity as long double-stranded RNAs do. RNAi is a promising method for probing gene functions and for treatment of human diseases. Some data suggests that siRNAs act by the antisense strands. It is unknown if there is an association between the antisense accessible sites and the siRNA accessible sites in the mRNAs. We tested 12 pairs of antisense DNAs and siRNAs against reporter luciferase genes in cultured mammalian cells. Both the antisense DNA and siRNA in one pair were directed towards the same site. Five siRNAs and two antisense DNAs turned out effective, but no overlapping was found among the sites targeted by those effective antisense DNAs or siRNAs. These results suggest that effective antisense DNAs and siRNAs have different target sites in the mRNA. Targeting the exogenous luciferase and endogenous CD46, the duplex siRNAs were more potent than the antisense-stranded siRNAs as RNAi triggers. Antisense-stranded siRNAs had a shorter duration in the transfected cells than the duplex siRNAs, but it doesn't seem to be a main reason for the fact that antisense-stranded siRNAs have significantly lower efficiencies than duplex siRNAs in mammalian cells. Double-stranded siRNAs and antisense-stranded siRNAs may act in cells by different mechanisms. CD46 expression was efficiently blocked by the specific duplex siRNAs in human cells. This CD46-specific RNAi model could be used in study of the biological roles of CD46 in human diseases and the interaction between cells and microorganisms using the CD46 receptor.

LIST OF PUBLICATIONS

I. **Xu Y,** Linde A, Dahl H and Winberg G. 2001. Definition of a divergent epitope that allows differential detection of human herpesvirus 6 variant A and B early protein p41. J Clin Microbiol 39; 4: 1449-1455

II. **Xu Y,** Linde A, Fredrikson S, Dahl H and Winberg G. 2002. HHV-6 A- or B-specific p41 antigens do not reveal virus variant-specific IgG or IgM responses in human serum. J Med Virol 66; 394-399

III. **Xu Y,** Zhang H-Y, Thormeyer D, Lasson O, Du Q, Wahlestedt C and Liang Z. 2003. Effective small interfering RNAs and phosphorothioate antisense DNAs have different preferences for target sites in the luciferase mRNAs. (Manuscript)

IV. **Xu Y,** Linde A, Thormeyer D, Elmen J, Lasson O, Steir S, Wahlestedt C and Liang Z. Comparison of double- and single-stranded siRNAs in RNA interference against luciferase and CD46 in cultured mammalian cells. (Manuscript)

CONTENTS

Paper I: Xu, Y et al. 2001. J Clin Microbiol 39:1449-1455

Paper II: Xu, Y. et al. 2002. J Med Virol 66:394-399

Paper III: Xu, Y et al. 2003 (manuscript)

Paper IV: Xu, Y et al. 2003 (manuscript)

LIST OF ABBREVIATIONS

asRNA	Antisense-stranded ribonucleic acid
AIDS	Acquired immunodeficiency syndrome
C. elegans	*Caenorhabditis elegans*
C. pneumonia	*Chlamydia pneumonia*
CSF	Cerebrospinal fluid
CNS	Central nervous system
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
ES	Exanthema subitum
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate
GST	Glutathione-S-transferase
HEK-293	Human embryonic kidney cells
HeLa-ATCC	Human cervical carcinoma cells
HHV-6	Human herpesvirus 6
HHV-6A	HHV-6 variant A
HHV-6B	HHV-6 variant B
HIV	Human immunodeficiency virus
IF	Immunofluorescence
kb	Kilo-basepairs
kD	Kilo-dalton
mAb	Monoclonal antibody
MCP	Membrane cofactor protein
MBP	Myelin basic protein
MS	Multiple sclerosis
NIH/3T3	Mouse fibroblast cells
nt	Nucleotide
OD	Optical density
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
RdRP	RNA dependent RNA polymerase
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
siRNA	Small interfering RNA
SK-N-MC	Human neuroblastoma cells
ssRNA	Sense-stranded ribonucleic acid

Aminno acid single letter codes

A	Alanine
C	Cysteine
D	Aspartic acid

E	Glutamic acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine

1 INTRODUCTION

1.1 HUMAN HERPESVIRUS 6 (HHV-6)

Human herpesvirus 6 (HHV-6) was first isolated from the peripheral blood leukocytes of six patients with a variety of lymphoproliferative disorders and AIDS in 1986 [1]. The novel herpesvirus was initially called human B-lymphotropic virus (HBLV) based on a suggested tropism for B cells [1]. Further studies showed that the virus has a wide host-cell range with a predominant CD4+ T-lymphocyte tropism, and it was subsequently renamed HHV-6. All HHV-6 isolates are classed into two major variants: HHV-6A and HHV-6B based on difference in genetic, immunologic and biological properties [2]. HHV-6 is a cause of exanthema subitum (ES) [3]. CD46 has been found to be a cellular receptor for HHV-6 entry [4].

1.1.1 Structural and molecular biology

As for all herpesviruses, HHV-6 virons have four main structural elements: an electron-dense core, a capsid with icosahedral symmetry, a tegument and an outer envelope (Figure 1). Mature virons are approximately 200 nm in diameter. The complete nucleotide sequences of HHV-6A (strain U1102) and HHV-6B (strain Z29 and strain HST) have been determined [5-7]. The genomes are 160-162 kb in size including a unique region of 143-144 kb bounded by terminal direct repeats of 8-9 kb. HHV-6B is predicted to contain 119 open reading frames, 9 of which are absent in HHV-6A (U1102). The HHV-6A and HHV-6B genomes are collinear. The conserved genes in HHV-6 variants A and B show an identity of greater than 94%.
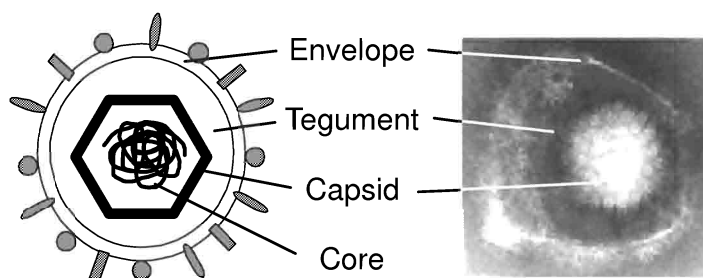

Figure 1. Schematic structure and electron micrograph of HHV-6

Seven genes are conserved in the HHV-6 variants and necessary for virus replication: U27, the polymerase processivity factor; U38, a DNA polymerase; U41, a single-stranded DNA binding protein; U41/U74/U77, the components of the helicase/primase complex; U73, the origin binding protein. The U27 gene product, a DNA polymerase accessory protein, designated p41, can complex with the U38 gene product and greatly increase DNA synthesis activity [8]. It is believed that herpesviruses can avoid host immunity by viral chemokines and/or chemokine receptors. Similar to US28, a protein encoded by human cytomegalovirus (HCMV) [9, 10], the U12, U51, U83 products of HHV-6 have been showed to function as chemokine receptors [11-13].

1.1.2 Cellular tropism and latency

HHV-6 is predominantly regarded as a T-cell tropic virus, although it can also replicate with varying efficiency in a wide array of host cell types. These cells include epithelial cells, endothelial cells, fibroblasts, natural killer cells and a number of neural cells (including adult oligodendrocytes). Activated CD4+ T-cells appear to be the preferential target, but CD4 is not the receptor for HHV-6 [14]. CD46 has been found to be a cellular receptor for HHV-6 entry [4]. CD46, also designated as membrane cofactor protein (MCP), is a glycoprotein expressed on the surface of all human nucleated cells [15]. The wide expression of CD46 can explain why HHV-6 has a broad spectrum of host cells. Primary infection of HHV-6 leads to a life-long latency in the host. Monocytes/macrophages [16] and early bone marrow progenitor cells [17] might be host cells of latency, but the exact site has to be determined. HHV-6 virons and genomes are often detected in saliva [18, 19].

Integration of complete HHV-6 genome into the short arm of chromosome 17 of human peripheral blood mononuclear cells (PBMC) has been reported in one MS case and in other patients [20]. This is possibly a biologically important phenomenon. In single cases, integration has been mapped to chromosome 17p13.3 [21], chromosome 22q13 [22] and chromosome 1q44 [23]. A woman who had Burkitt's lymphoma carried HHV-6 DNA integrated at chromosome 22q13, and her asymptomatic husband also carried HHV-6 DNA integrated at chromosome 1q44. Their daughter had HHV-6 DNA on both chromosomes 22q13 and 1q44, identical to the site of viral integration of her mother and father, respectively, suggesting a possible inheritance of chromosomally integrated HHV-6 [23]. At present, the underlying molecular mechanisms that control integration of HHV-6 to chromosomal sites and the clinical relevance of this type of

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latency are unknown, although it is remarkable that HHV-6 has captured a homologue of the AAV2 rep protein (HHV-6, U94) which catalyses site specific integration of AAV2 in chromosome 19 [24].

1.1.3 Epidemiology

Seroepidemiological investigations show that HHV-6 is ubiquitous in all populations in the world, with a prevalence of at least 90% in adults. The virus is acquired by more than 95% of children within the first two years of life. There is no reliable serological technique available to distinguish between antibodies to HHV-6A and HHV-6B. Prenatal or congenital infection seems to occur in about 1% [25]. A mother and her infant usually have the same strain of virus, but it is possible to acquire more than one HHV-6 variant [26, 27]. HHV-6B accounts for the majority (97%) of symptomatic HHV-6 infections in infants in USA. Salivary contact is likely to be a pathway of the spread of HHV-6B. The prevalence of HHV-6A is unclear, and probably underestimated by mainly testing blood and saliva. HHV-6A, compared with HHV-6B, is more frequently detected in healthy skin biopsies and cerebrospinal fluid (CSF) [28, 29].

1.1.4 Clinical manifestations

Primary HHV-6 infection is frequently associated with exanthema subitum (ES), also known as roseola infantum [3], and other febrile illnesses in infancy [30]. Most (60-74%) clinically diagnosed ES cases are caused by HHV-6 infection. ES is characterized by 3-5 days of fever, often to 39-40 °C, and a maculopapular rash on the infant's trunk and neck, which appears as the fever subsides. The characteristic rash of roseola occurs in a minority of infants. HHV-6 infection is the single most common cause (10-40%) of hospital visits in young infants with fever [30, 31]. HHV-6 is estimated to account for one third of all febrile seizure in children younger than 2 years of age [30]. Other complications associated with HHV-6 infection include diarrhea, respiratory tract symptoms and CNS infection. In most cases, the prognosis of HHV-6 infection in young children is excellent.

Reactivation of HHV-6 occurs frequently in immunocompromised hosts, including transplant-recipients and persons with HIV infection. HHV-6 reactivation usually occurs in the first month after transplantation, causing encephalitis, pneumonitis, and possibly early and late graft failure, bone marrow suppression and delayed engraftment

[32-37]. HHV-6 infection is frequently accompanied by human cytomegalovirus infection in liver or renal transplant patients and may cause severe diseases [38-40]. It is debated whether HHV-6 is a cofactor for **HIV infection**. Some clinical data suggests that HHV-6 infection accelerated the progression of HIV disease [41, 42]. Such clinical findings are supported by experimental data, e. g. HHV-6 transactivates the HIV long terminal repeats [43] and renders human CD8+ T-cells or NK cells susceptible to HIV-1 infection [44, 45]. In contrast to those observations, a reduced HHV-6 prevalence in PBMC was observed in patients with HIV infection [46, 47] and HHV-6 was found to be able to suppress HIV-1 replication or transcription in vitro experiments [48, 49]. Those discrepancies might be due to differences in virus strains, patients and methods in individual clinical investigations and experimental studies.

Neurological complications occur in patients with HHV-6 infections. HHV-6 may play a causative role in febrile seizures in childhood. The incidence of febrile seizure in primary HHV-6 infection was reported to be 8% to 31% in different populations [30, 50, 51]. HHV-6 can invade the central nervous system (CNS) of patients with primary HHV-6 infection [52, 53]. A greater neurotropism has been found for HHV-6A, compared with HHV-6B [29]. Most of the HHV-6 associated neurological complications are found in immunocompromised adults, as mentioned above. HHV-6 associated encephalitis or encephalopathy has also been found in children [53, 54]. The potential association between HHV-6 and MS will be described later.

1.1.5 Laboratory diagnosis of HHV-6 infections

Diagnosis of primary or reactivated HHV-6 infections may be achieved by one of the following procedures [55]:

a) Demonstration of an HHV-6-specific IgG antibody seroconversion (primary infection) or a significant change in IgG antibody titres between acute and convalescence sera (primary or reactivated infection, cross-reactive IgG antibody response can be seen in Epstein-Barr virus or human cytomegalovirus infection [56]).

b) Demonstration of HHV-6 specific IgM in serum (mostly primary infection).

c) Presence of low avidity HHV-6-specific IgG in serum (primary infection) [57-59].

d) Positive PCR from serum or plasma (primary or reactivated infection).

e) Positive reverse transcription PCR from PBMC targeting a gene activated in the lytic cycle (primary or reactivated infection).

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f) A combination of lack of HHV-6 IgG and serum/plasma/whole blood PCR positive (primary infection).

1.2 MULTIPLE SCLEROSIS (MS)

MS is a demyelinating disease of the CNS in humans. It represents the most common disease leading to sensory and motor disability in young adults. MS typically begins in early adulthood and has a variable prognosis. The pathologic features of MS are white matter plaques characterized by primary demyelination and necrosis of oligodendrocytes within the center of the lesions. The etiology of MS remains a mystery despite study for decades. There is no efficient treatment of the disease.

1.2.1 Epidemiology

The prevalence of MS varies considerably around the world [60]. The prevalence is highest in northern Europe, southern Australia, and the middle part of North America (>30 cases/100,000 persons), while in many developing countries in Africa and Asia the prevalence is low (<5 cases/100,000 persons). The reasons for this geographic difference are unclear. Environmental and genetic factors may contribute singly or in combination to these variations in susceptibility. Epidemiologic data indicated that MS is a familial. Almost one quarter of patients have an affected relative.

1.2.2 Clinical features

Chronic neurological disability is the main clinical feature of MS. No treatment can halt the progress of disability. Different types of MS exist. **Relapsing-remitting MS**, presenting in 80 percent of patients, is characterized by episodes of acute or subacute neurologic dysfunction, associated with new or expanding areas of demyelination, followed by periods of improvement and stabilization with remyelination. This MS type typically begins in the second or third decade of life. **Primary progressive MS** has a slow progression of signs and symptoms. **Secondary progressive MS** has a relapsing-remitting course, causing slow accumulation of neurologic signs and symptoms.

1.2.3 Treatment

Current therapies have no impact on existing neurological deficits in MS patients. The management of MS should be a comprehensive strategy consisting of education and

support, disease-modifying therapies, management of acute relapses and symptomatic treatments and rehabilitation [61]. Interferon beta has been found to be effective in decreasing the number of relapses and delaying the progression of disability in the patients with relapsing-remitting MS [62-64]. The mechanism of such therapy is unclear. Relevant biological effects may include antagonism of the effects of interferon gamma, inhibition of T cell proliferation and improvement of suppressor cell function. Glatiramer acetate (a mixture of polypeptides) [65] and intravenous immunoglobulin [66] have also been tried clinically. A randomized, double-blind, placebo-controlled clinical trial demonstrated that acyclovir-treated patients with relapsing-remitting MS had a significant reduction of exacerbations, although neurological parameters were essentially unaffected in those patients [67]. It suggests that acyclovir-susceptible viruses might be involved in the pathogenesis of MS. Antigen specific therapy has the potential to suppress undesirable autoimmunity, while leaving the rest of the immune system intact. This aim could be achieved by induction of an antigen specific Th1-to-Th2 shift [68].

1.2.4 Etiology

Genetic factors play an important role in the pathogenesis of MS, but genetic influence alone is insufficient for the development of MS. In most twin pairs only one twin develops the disease, yet the identical twin rate of MS is about 26% [69]. Results from genomic screens stress the complexity of MS genetics. No single locus has been found to contribute significantly to familial risk. Becker et al. made a comparison of the linkage results from 23 genome-wide scans of autoimmune or inflammatory diseases. Human MS and murine EAE were included in this study. Overlapping of susceptibility loci were found between MS and other human immune diseases. Similar phenomenon was found in conserved regions in EAE and other experimental autoimmune/immune disease models (Figure 2) [70].

The involvement of **environmental factors** in the pathogenesis of MS is suggested by the distinct geographic distribution of the disease in the world. There is a north-south declining gradient in the Northern hemisphere, the opposite in the Southern one, and a higher incidence in temperate climates than in the tropics. The risk of MS has been linked to high rates of cow milk protein consumption. Abnormal T cell immunity to such proteins has been found in MS patients. One peptide of the protein, BSA_{193} (EDKGACLLPKIE), has structural homology with MBP exon 2

(GLCHMYK). An immune response to the peptide was found in MS patients. The observation of BSA_{193} inducing EAE in mice indicated a potential pathogenic role of this protein in MS [71].
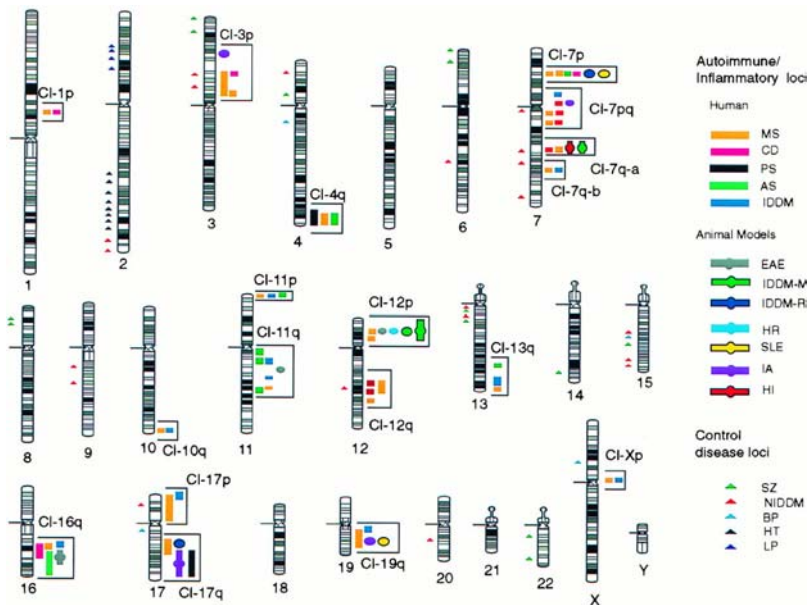
Figure 2. Clustering of autoimmune candidate loci. MS, multiple sclerosis; CD, Crohn disease; EAE, experimental autoimmune encephalomyelitis; IA, rat inflammatory arthritis; PS, familial psoriasis; AS, asthma; IDDM, insulin-dependent diabetes (type I) (IDDM-H, human; IDDM-M, mouse; IDDM-R, rat); HR, B. *pertussis*-induced histamine sensitization; SLE, murine lupus; HI, humoral immunity; SZ, schizophrenia; NIDDM, non-insulin-dependent diabetes (type II); BP, bipolar disorder; LP, leptin-associated obesity; and HT, hypertension [70] (Becker, K.G., et al. Proc Natl Acad Sci USA, 1998;95(17):9979-9984, reproduced with permission from National Academy of Sciences/Proceedings).

Abnormal immune reactivities are frequently found in MS patients. They are presented by increased T- or B-cell reactivity to autoantigens, especially to myelin basic protein, proteolipid protein [72], myelin-associated glycoprotein [73] and

myelin/oligodendroglia glycoprotein [74, 75]. An immunologic background of the disease is also suggested by some observations in clinical trials, such as the benefit for MS patients of recombinant interferon [62-64]. Many candidate autoantigens have been supposed to be involved in MS [76].

The involvement of **Infectious agents** in MS was primarily suggested by the familial and nonfamilial clusters of MS outbreaks [77]. The risk of developing MS may be associated with acquirement of certain infectious childhood diseases at a later age in comparison to normal controls [78]. Many viruses have been postulated as possible causes of MS (Table 1). However, none of them has withstood the test of time. HHV-6 [79-82] and *C. Pneumoniae* [83, 84] turned up at the center of the stage in the past a couple of years. It is noticed that two of the human viruses (measles virus and HHV-6) listed in the Table 1 use CD46 as cellular receptor [4, 85-87]. CD46 plays an important role in protecting host cells from homologous complement attack [15, 88].

Table 1. Human viruses assumed to be involved in the pathogenesis of multiple sclerosis (MS)

Virus	Reference
Rabies	[89]
Measles	[90]
Parainfluenza virus 1	[91]
Human T-lymphotropic retrovirus	[92]
Coronavirus	[93]
Tick-bone encephalitis virus	[94]
Herpes simplex virus 1	[95]
Herpes simplex virus 2	[96]
Epstein-Barr virus	[97]
Varicella-zoster virus	[98]
Human herpesvirus 6	[80]

1.2.5 Hypothesis of virus involving in MS

Virus infections can cause damage directly on the myelin-forming cells [99], but it is not likely to be a major mechanism in the pathogenesis of MS. A more likely mechanism is one of the following:

- **Molecular mimicry** (see Figure 3). A viral antigen or peptide sharing homology with myelin proteins can activate myelin protein-specific T cells or B cells. This hypothesis was supported by induction of EAE with hepatitis B peptides [100] or Theiler's murine encephalomyelitits virus (TMEV) in animals [101, 102]. Many human viral proteins share short stretches of amino acids with similarity to human myelin proteins [103]. Only four native MBP residues are required to stimulate MBP-specific T cells. A six-residue peptide can sensitize MBP-specific T cells to cause EAE [104]. Certain homologues found between human viruses and MBP are shown in Table 2.

- **Epitope spreading** (see Figure 3). A viral infection of the CNS can cause upregulation of human major histocompatibility complex class I and class II molecules on the cell surfaces, T cell infiltration, local tissue degradation or accessibility of normally hidden autoantigens. These pathologic or immunologic effects will lead to the activation of normally quiescent autoreative T cells. This mechanism was indicated by induction of a chronic demyelinating disease with Theiler's murine encephalomyelitis virus in animals [105].
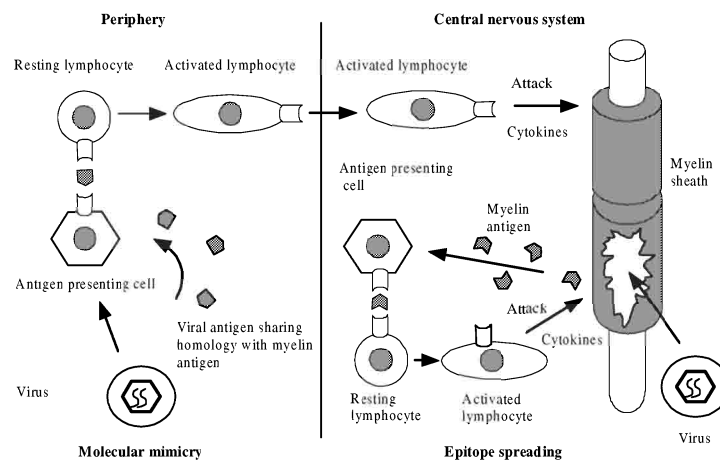

Figure 3. Schematic representation of molecular mimicry and epitope spreading

- **Bystander activation.** A chronic CNS autoimmune disease induced by lymphocytic choriomeningitis virus infection can be enhanced by a second infection with an unrelated virus [106].

1.2.6 HHV-6 and MS

HHV-6 is assumed to be one of the infectious agents that are involved in MS for several reasons: (1) HHV-6 establishes life-long latent and lytic infections, and may dysregulate the immune system. (2) HHV-6 has a wide range of host cells, including astrocytes, oligodendrocytes and other CNS cells, and HHV-6A has a great neurotropism in vivo. (3) One of the fundamental properties of herpesviruses is their tendency to reactivate. The same factors that often lead to herpesvirus reactivation, such as stress and infection, have also been associated with MS exacerbation.

The first evidence suggesting the involvement of HHV-6 in MS was the observation of significantly higher anti-HHV-6 IgG titres in MS patient [107]. A potential pathogenic role HHV-6 in MS was supported by several recent reports. First, HHV-6 protein p41 and p101 expression was observed in MS brains but not in the controls [80]. Second, an increased IgM response to p41 is observed in MS cases [81, 108]. Third, a homologue was recently found between HHV-6 U24 product and myelin basic protein (Table 2) [109]. Some studies suggest that HHV-6A but not HHV-6B is involved in MS [82, 110, 111]. It is noted that HHV-6 DNA was detected in 70% brain samples from healthy controls and HHV-6 could be a commensal virus of the brain. The observation that HHV-6 proteins are expressed in MS brains was not confirmed in some individual studies [112, 113]. Controversial results in addressing the association between HHV-6 and MS have been reviewed recently [114, 115]. In twenty-eight studies retrieved, 4 of 12 different experimental techniques provided evidence for a relationship between HHV-6 and MS, but none was able to show a causative relationship [115]. *C. pneumoniae* has recently been linked to the pathogenesis of MS [83, 84], but neither this organism has been shown to have a causal relationship [116-119].

Table 2. Homologues observed between human viruses and myelin basic protein (MBP)

Name of protein or virus	Amino acid sequence*	Reference
MBP Adenovirus 2, 5 late 100K	DHARHGFLPR NKARQEFLLR	[103]
MBP Adenovirus 7 terminal	TAHYGSLPQK RAGYQNLPAR	[103]
MBP Measles virus nucleocapsid	SRFSWGAEGQ SRFGWFENKE	[103]
MBP Measles virus P3	EISFKLGQE EISDNLGQE	[120]
MBP Influenza A/NT/60/68 NP	GILDSIGRFF KMIDGIGRFY	[103]
MBP Influenza B/Lee/40 NS1	SLSRFSWGAE CYERFSWQRA	[103]
MBP Epstein-Barr virus EC-RF2	RGLSLSRFSW RHGELFRFIW	[103]
MBP Epstein-Barr virus EC-LF2	GYGGRASDYK GYGNHAGDYH	[103]
MBP Hepatitis B virus ORF1	ASQKRP TLQKRP	[104]
MBP Hepatitis B virus polymerase	TTHYGSLPQK IGCYGSLPQE	[100]
MBP Japanese encephalitis virus	ASQKRP ICQKRP	[104]
MBP Herpes simplex virus 1	ASQKRP QDQKRP	[104]
MBP Coxsackie virus B4	ASQKRP KDQKRP	[104]
MBP Human herpesvirus 6 U24	PRTPPPS PRTPPPS	[109]

*Amino acid sequences underlined were documented to induce EAE in animals.

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1.2.7 Animal models

Experimental autoimmune encephalomyelitis (EAE) is a widely used animal model for studying MS. EAE can be induced by immunization with myelin antigens or peptides or by transfer of myelin-reactive T lymphocytes [121, 122]. EAE is characterized by inflammatory infiltration of the CNS, associated with lesions within the CNS. EAE-like disease or myelin damage can also be induced with viruses in animals, for example, murine coronavirus [123], mouse hepatitis virus [124], Theiler's murine encephalomyelitis virus [102], semliki Forest virus [125] and measles virus [126]. The virus-induced demyelination is most likely to be mediated by an autoimmune response [127], and not by a direct effect of the virus [125]. Viral infection of the CNS can enhance the susceptibility of the CNS to autoimmune T cell attack [126]. The data obtained by EAE should be interpreted with caution for any deduction of MS in humans because of the potential dissimilarities between EAE and MS [128].

1.3 RNA INTERFERENCE (RNAi)

RNA interference (RNAi) is a process of gene silencing induced by double-stranded RNAs [129]. The first manifestation of RNA interference was observed in plants and was termed co-suppression because of the degradation of the RNAs that were derived from both the transgenes and the homologous endogenous genes [130]. A similar phenomenon can be induced by viruses, known as virus induced gene silencing (VIGS) [131]. Such gene silencing has been found to occur at a post-transcription level [132], therefore termed posttranscriptional gene silencing (PTGS). Our primary understanding of RNAi is mostly based on the data obtained in experiments using *C. elegans* [129, 133] and *Drosophila* [134]. The RNAi in vertebrate was first demonstrated in zebrafish [135, 136], then in Xenopus and mouse embryos [137, 138]. RNAi has recently been demonstrated in cultured mammalian cells [139]. RNAi is a natural defense strategy of protecting host cells from invasion by mobile genetic elements, such as viruses. RNA interference is conserved in species from plants to mammals.

1.3.1 RNAi and double-stranded RNA

The first direct evidence of dsRNA inducing RNAi was shown in *C. elegans* by Guo and Kemphues, when they found that either of RNA with antisense or sense polarity

was effective in knocking down gene expression [133]. Fire and colleagues then demonstrated that dsRNA was substantially more effective than either strand individually to suppress gene expression. Only a few molecules of injected dsRNA were required per affected cell. Corresponding gene products disappeared from both the somatic cells of the organism as well as in its F1 progeny [129]. Duplex siRNAs are more potent, compared with the corresponding single-stranded siRNAs, in most experiments using mammalian cells [140-146]. RNAi has also been induced by hairpin siRNAs or U6 promoter-driven siRNAs in cells [147, 148]. It is generally believed that the antisense strand of a siRNA duplex plays a key role in RNAi action. The assumption is supported by a recent study, which suggested that antisense-stranded RNA guide target mRNA cleavage in HeLa cells [149].

1.3.2 Mechanisms of RNAi

One of the most important findings in study of RNAi mechanisms is the identification of RNA species of 21-23 nucleotides (small interfering RNAs, siRNA) as being the RNAi triggers. siRNAs are processed by cellular proteins and enzymes from input dsRNA. siRNA plays a key role in the process of degrading target mRNA in cell-free system [150, 151] and intact cells [152]. Based on those findings, a two-step model of RNAi action has been proposed: 1) long dsRNA molecules are diced into 21-23nt siRNAs by a dsRNA-specific nuclease; 2) these siRNAs guide a nuclease-containing protein complex (RNA-induced silencing complex, RISC) to the target mRNA, leading to the cleavage of the mRNA in a sequence-dependent manner (Figure 4A). Dicer, a RNAase III like enzyme, has been found to be involved in the process of forming siRNAs from long dsRNAs in *Drosophila* [153]. Dicer is evolutionarily conserved with homologs present in fungi, plants, worms and mammals. It needs ATP in the processing double-stranded RNAs into siRNAs in a *Drosophila* embryo lysate system [153, 154]. Human Dicer has been recently identified and cloned. The human Dicer appears to act without the requirement of ATP in the process [155, 156].

It is unknown how a RISC recognizes its target sequence in the mRNA. One hypothesis is that siRNA duplexes participate in the second step of RNAi process [157, 158], as shown in figure 4A. Another hypothesis is that the antisense strands of the siRNA duplexes guide the recognition and cleavage of target mRNA [159-161], as shown in figure 4B. The first hypothesis is suggested by the observations that dsRNAs are usually more potent than single-stranded RNA of antisense or sense polarity, and a

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single dsRNA can induce degradation of both sense and antisense targets [162]. The possibility of a strand exchanging between the duplex siRNA and its mRNA target has been proposed [157]. The later hypothesis was supported by several lines of evidence: 1) single-stranded RNAs can serve as primers to transform the target mRNA into double-stranded RNA by RNA dependent RNA polymerase activity (RdRP). The nascent dsRNA is degraded, generating new siRNA, in a cycle [163, 164]; 2) modification or mutation on the antisense strand of a siRNA has a negative effect on the activity, whereas those in the sense-strand are tolerated [140, 165]. 3) RISC was found to contain a single-stranded siRNA and a single-stranded siRNA alone can guide the cleavage of the target mRNAs in HeLa cell extract [149]. However, the putative RdRP appears to have little effect in human cells, as blocking of the 3'-OH positions of siRNA with FITC did not reduce the RNAi effect in human cells [165]. Therefore, the underlying mechanisms of RNAi action, especially in mammals, remain to be elucidated in details.

1.3.3 Features of RNAi

RNAi has a high specificity in a sequence-dependent manner. Even one mismatch in a siRNA duplex can abolish its effect [165-167]. RNAi also shows a high potency to inhibit gene expression. Only a few molecules of injected double-stranded RNA are required per affected cells to induce gene silence [129]. Besides those striking feactures, RNAi shows two other remarkable features: transport between cells and tissues and long-term of action in *C. elegans*. The interfering activity of RNAi can be transported across cell boundaries in *C. elegans*. Feeding the worms with *E. coli* expressing gene-specific dsRNAs can produce populations of RNAi-affected animals with phenotypes that were comparable to the corresponding loss-of-function mutants [168]. RNAi has a remarkably long term of action. It can last for several days after the initial injection of double-stranded RNAs and its effects are observed not only in the injected animal but also in all the injected animal's progeny. It is found that the red-2 and mut-7 genes are required for the transmission of RNAi in *C. elegans* [169]. The potency and duration of RNAi also suggests the existence of a catalytic or amplification step in the RNAi mechanisms in the animals [129]. However, such transport of RNAi between tissues or cells and the amplification mechanism has not been observed in mammals.
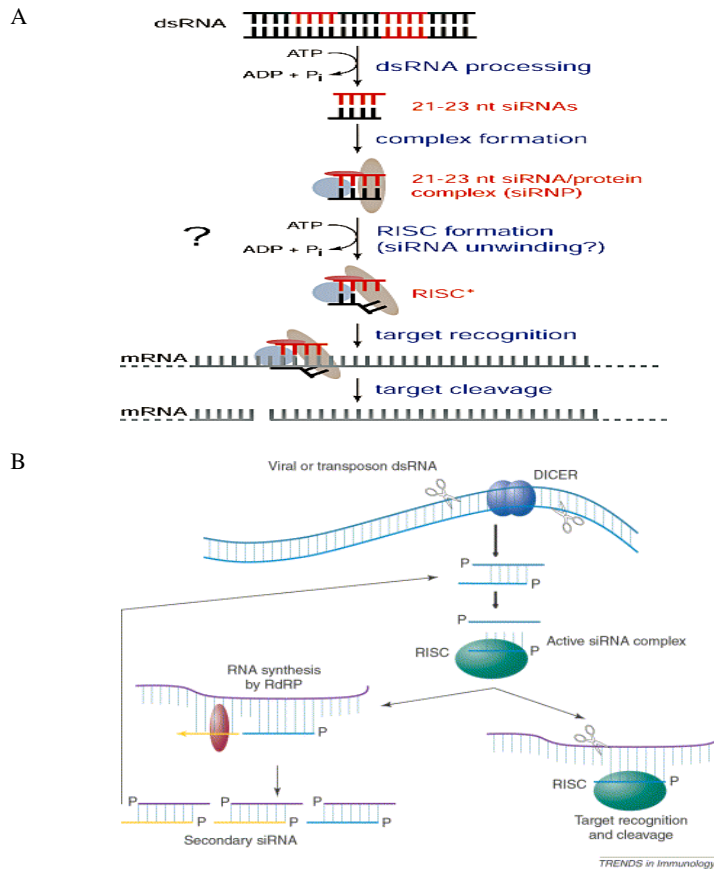
Figure 4. Two models for RNA interference (RNAi) in a cell. A) A model hypothesizing that both strands of a siRNA duplex contribute to the cleavage of the mRNA target [158] (Zamore P.D., Nat Struct Biol, 2001;8(9):746-750, reproduced with permission from Nature Publishing Group). B) A model hypothesizing that the antisense strand of a siRNA duplex directs the recognition and cleavage of the mRNA target [161] (Martinez, M. A., et al., Trends Immunol, 2002;23;(12):559-561, reproduced with permission from Elsevier).

1.3.4 Small interfering RNAs (siRNA)

It is well known that long dsRNA can induce a nonspecific effect through a protein kinase-mediated pathway in mammalian cells [170]. dsRNAs of >30 base pairs can bind and activate the protein kinase PKR [170] and 2', 5'-oligoadenylate synthetase [171].

Those features of long dsRNAs prevent them from being used as a specific tool for gene silencing in mammals. This obstacle has been overcome by using small interfering RNAs (siRNA) [139]. siRNAs of 21-nt with 2-nt 3' overhangs are most effective among the different siRNA constructs tested by Elbashir et al. [166]. The position of the cleavage site in the target RNA is defined by the 5'-end of the antisense strand of a siRNA duplex [166] and free 5'-OH groups on the antisense strands of the siRNA duplexes are required for RNAi in vivo [140]. Synthetic siRNAs have been successfully used to silencing exogenous and endogenous genes in mammalian cells.

1.3.5 Alternative approaches of generating siRNAs in cells.

The inhibitory effects of synthetic siRNAs are usually transient because mammalian cells lack the mechanism to support the amplification of siRNA mediated gene silencing as observed in *C. elegans* and *Drosophila* [172]. To generate stable and long-term RNAi in mammalian cells, alternative approaches have emerged, such as DNA plasmid –based techniques [145, 147, 148, 173-177] and virus-based systems [178-181]. Lentiviruses have two key advantages over other gene delivery systems. First, they can infect non-cycling and post-mitotic cells [182, 183]. Second, transgene expression from lentiviruses is not silenced during development and can be used to generate transgenic animals through infection of embryonic (ES) cells or embryos [184, 185]. Lentivirus-mediated RNAi is one of the promising tools for gene therapy, especially if an inducible system can be designed to allow temporal silencing.

1.3.6 Applications of RNAi

RNAi is promising for wide employments, e. g. for probing of gene functions and for the treatment of human diseases. About 885 of the 19,427 predicted genes in *C. elegans* were inhibited in function by RNAi induced with a bacteria library of 16,757 clones. Such genome-wide RNAi analysis would not only provide a key resource for studying gene function in *C. elegans*, but also provide insights into human gene function since more than half of the genes in *C. elegans* have human homologues [186]. By this strategy, a genome-wide RNAi analysis has recently been made for the fat regulatory genes in *C. elegans* [187]. A core set of fat regulatory genes as well as pathway-specific fat regulators have been identified in the animal. Over 50% of the *C. elegans* fat regulatory genes identified have mammalian homologues that have not been previously implicated in regulating fat storage. Homologues to the newly identified *C. elegans* fat regulatory genes may also control mammalian body weight. Those genes identified in

C. elegans could help us to identify candidate obesity loci in humans, which could be targets for the treatment of obesity and its associated diseases. By RNAi, 61 genes that contribute to genome stability in somatic cells have been identified in *C. elegans* [188]. Those genes have presumed roles in DNA repair, replication, chromatin organization, and cell cycle control. Of these genes identified, 82% have clear orthologes in the human genome. Those observations might help identifying potential tumor suppressors in higher species, including humans.

Primary data have shown the possibility of using RNAi in the treatment tumors of specific genetic abnormalities. The proteins encoded by RAS genes (K-RAS, H-RAS, and N-RAS) regulate a broad spectrum of cellular activities, including proliferation, differentiation, and cell survival. K-RAS is necessary for early onset of tumors, and its continued production for maintenance of tumor viability. A virus-based RNAi system against the K-RAS led to the loss of tumorigenicity (CAPAN-1 human pancreatic carcinoma) in nude mice [178]. Chromosomal translocations frequently lead to the generation of chimeric fusion oncoproteins that trigger malignant transformation. A siRNA duplex targeting an oncogenic hybrid gene (BCR/ABL rearrangement) killed the human leukemia cells (K562) containing, but not the cells without the gene [143]. Transfection of four myeloid leukemia cell lines (HL-60m Y937, THP-1 and K562) with siRNA duplexes corresponding to *c-raf* and *bcl-2* genes decreased the Raf-1 and Bcl-2 expression. Combined siRNA duplexes for *c-raf* and *bcl-2* induced apoptosis in HL-60, U937, and THP-1 cells and increased chemosensitivity to etoposide and daunorubicin [189].

RNAi is a natural defense, like the immune system, protecting host cells from invasion of mobile genetic elements, such as transposons and viruses. Several lines of evidence have shown the possibility of using RNAi for treatment or prevention of viral infections, including HIV infection. Transient transfection of siRNA directed to HIV genes, such as p24, the HIV long terminal repeat, vif, nef, tat and rev, induced pre-integrated HIV-1 RNA degradation and a consequent reduction of HIV-1 antigen production in infected cells [142, 190, 191]. siRNA can also have an effect on a later step in the HIV life cycle, the post-integration degradation of HIV RNA transcripts [145, 190, 191]. Virus receptors or co-receptors are alternative targets for siRNA to block viral infections in cells. Expression of the HIV receptor (CD4) was reduced by eight-fold by specific siRNAs, and HIV entry and virus production was consequently inhibited [191].

The siRNAs that target chemokine receptor genes (CXCR4 or CCR5) specifically impeded them in acting as HIV-1 co-receptors [192]. RNAi has also been used to block other human virus infections by inhibiting viral protein expression in vitro, such as poliovirus [193], respiratory syncytial virus [194], human papillomavirus [195], and hepatitis C virus [196]. Virus variants may be resistant to siRNA in some cases. One strategy to minimize such virus variants is using of siRNA duplexes or alternative approaches directing against multiple conserved sequences in the mRNA targets.

2   AIMS OF THE STUDY

1. To elucidate the antigenity of p41 in HHV-6 variants and use the recombinant p41 proteins in HHV-6 serology.

2. To investigate the potential link between HHV-6 and the pathogenesis of MS by measuring human serum IgG and IgM responses against p41.

3. To get better understanding of the RNAi mechanisms by analyzing accessible sites in the mRNA to siRNAs and antisense DNAs and by comparing of double- and single-stranded siRNAs in inducing RNAi in mammalian cells.

4. To establish a CD46-specific RNAi model in human cells to provide a tool for investigation of the roles of CD46 in human diseases.

3 RESULTS AND DISCUSSION

3.1 Differentiating between HHV-6 variants A and B with monoclonal antibodies against p41 (Paper I):

3.1.1 Specific background

HHV-6 early protein p41 is encoded by the gene U27 and conserved in HHV-6 A and B. p41 is a DNA-binding protein and a putative DNA polymerase stimulatory factor. Two monoclonal antibodies (mAb) against the protein (mAb to p41 and mAb to p41/38) are available. Both have been claimed to react with both HHV-6A and HHV-6B [197, 198]. However, our primary IF experiment indicated that the mAb to p41/38 recognized a nuclear antigen in the cells that were infected with HHV-6A but not those infected with HHV-6B, while the mAb to p41 recognized the cells infected with either of the two strains. That observation suggested that this mAb might be a HHV-6 variant specific monoclonal antibody. Using this mAb, p41 expression was observed in oligodendrocytes of MS patients, but not in controls [80]. It indicated a link between HHV-6 and the pathogenesis of MS. However, the protein p38 may not be encoded by the viral genome [199]. Identification of the specificity of the two monoclonal antibodies against p41 available at present could be of importance to correctly interpret the observations using the monoclonal antibodies against p41 in previous reports and to use them in future studies.

3.1.2 Methods

U27 genes were cloned from the HHV-6A strain GS and the HHV-6B strain Z29. p41 was expressed in HEK-293 cells and as glutathione-S-transferase fusion proteins (GST-p41) in *E. coli*. BL-21 cells were transformed with the recombinant PGEX-4T-3/U27 plasmids from the HHV-6A strain GS and HHV-6B strain Z29. Positive clones were isolated by PCR and restriction enzyme cleavage. The BL-21 cells harboring the recombinant plasmids were propagated in LB medium. The expression of GST fusion protein was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) in the cell culture medium. GST fusion protein was purified using glutathione-sepharose 4B from the bacterial lysate. Both HHV-6 infected cells and the recombinant p41 fusion proteins were assessed with the mAb to p41 and mAb to p41/38 in immunofluorescence (IF) and Western blot. (see materials and methods, Paper I).

3.1.3 Results and discussion

A nuclear fluorescence was observed by IF using the mAb to p41/38 in HEK-293 cells transfected with the U27 gene from HHV-6A GS but not that from the HHV-6B Z29, while the nuclear fluorescence was observed using the mAb to p41 in the cells transfected with either of the two U27 genes. Those results suggested that the mAb to p41/38 recognizes only HHV-6A, unlike previously reported [198]. To verify our observations we tested the recombinant p41 fusion proteins (GST-p41) with the two monoclonal antibodies in Western blot. The mAb to p41 recognized the GST-p41 fusion protein from either of the two HHV-6 strains whereas the mAb to p41/38 reacted with only the GST-p41 fusion protein from the HHV-6A strain GS (Fig. 1, Paper I). A polypeptide of about 41 kDa was detected using either of the two mAbs in HSB-2 cells infected with the HHV-6A strain GS. The polypeptide was detected in Molt-3 cells infected with the HHV-6B strain Z29 with the mAb to p41, but not the mAb to p41/38 (Fig. 1, Paper I). Thus, it can be concluded that the mAb to p41/38 reacts with only HHV-6A and mAb to p41 reacts with both HHV-6 A and B. The mAb to p41/38 can be used to differentiate between HHV-6A and HHV-6B.

3.2 Identification of an epitope of p41 that allows discrimination between HHV-6A and HHV-6B (Paper I):

3.2.1 Specific background

The HHV-6A [5] and HHV-6B genomes have been sequenced [6, 7]. Published data indicate a high homology between the U27 genes from HHV-6A and HHV-6B. Our observation showed for the first time different antigenicity of p41 in the two HHV-6 variants. Identification of the epitope recognized by the mAb to p41/38 could provide evidence for whether the mAb can be generally used to differentiate between HHV-6A and HHV-6B. A synthetic peptide containing that epitope could have potential utility in measuring HHV-6 variant specific antibodies. p41 antigen prepared from HHV-6 infected cells was used to measure p41 specific human IgG or IgM antibody responses in patients with MS or other diseases [81, 108, 198, 200]. However, a synthetic probe for the p38 protein reacts with uninfected cellular DNA, and the sequence of a peptide fragment of p38 shows a high similarity to human beta-actin (Accession No. Protein AAH02409).

3.2.2 Methods

The U27 genes from HHV-6A strain GS and HHV-6B strain Z29 were sequenced and translated into amino acid sequences. To identify the epitope recognized by the mAb to p41/38, peptides with sequences not conserved between the two strains (Table 2, Paper I) were synthesized. Those synthetic peptides were used as antigens in ELISA, and tested with the mAb to p41 and mAb to p41/38. To further verify that the epitope recognized by the mAb to p41/38 is HHV-6A specific, CSF samples from patients with HHV-6A or HHV-6B infection were sequenced for the U27 genes using nested PCR. The recombinant p41 proteins (GST-p41) from HHV-6A and HHV-6B were used as antigen in ELISA. Serum IgG and IgM antibody reactivities to p41 were measured using the ELISA in MS patients and controls (see materials and methods, Paper I).

3.2.3 Results and discussion

Out of the first ten synthetic peptides listed in Table 2, Paper I, one peptide, G-p41-5 (DDGKGDRSHKNEDESALASK) was recognized by the mAb to P41/38 (Fig 2, Paper I). G-p41-5 was derived from the HHV-6A strain GS. The corresponding synthetic peptide Z-p41-5 (DDGKGDRNHKNEDGSALASK) in the HHV-6B strain Z29 was not recognized by the mAb (Fig 2, Paper I). The mAb to p41 did not react with any of the 12 synthetic peptides, indicating that its cognate peptide is located elsewhere in the p41. There were two substitutions (S328N; E334G) in the peptide Z-p41-5, compared with the peptide G-p41-5. Those observations confirmed the findings using IF and Western blot in the previous experiments. To further determine which of the two amino acids (S328 or E334) was critical, another two peptides (G-p41-7 and G-p41-8) were synthesized Table 2, Paper I). The G-p41-7 differs from the G-p41-5 by substitution E334G and the G-p41-8 differs from the G-p41-5 by substitution S328N. The mAb to p41/38 reacted with the G-p41-7 but not with the G-p41-8, demonstrating that S328 is critical for the antigenic discrimination by the mAb to p41/38. The amino acid residue S328 is conserved in the p41 amino acid sequences of HHV-6A strains GS (Protein JQ2007, GenBank) and U1102 (Protein CAA58407, GenBank). The substitution S328N is conserved in the p41 amino acid sequences of HHV-6B strains Z29 (Protein AAD49641, GenBank) and HST (Protein BAA78248, GenBank). The same sequences were also observed in the patient derived genomes of HHV-6. S328 was present in the HHV-6A genomes (CSF 1 from patient 1, Fig. 3, Paper I) while the two HHV-6B genomes (CSF 2 and 3 from patients 2 and 3, Fig. 3, Paper I) had N328.

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Thus, we identified for the first time an HHV-6A specific p41 epitope and proved that the mAb to p41/38 is an HHV-6A specific monoclonal antibody that can differentiate between HHV-6A and HHV-6B.

3.3 No significant difference in the IgG and IgM responses to p41 between MS patients and controls (Paper II):

3.3.1 Specific background

There is no reliable serology technique that can measure HHV-6 variant-specific antibodies due to the cross-reactivity between HHV-6A and HHV-6B. The p41 antigen that was prepared using the mAb to p41/38 from HHV-6 infected cells might be contaminated by cellular proteins, as described above. An alternative p41 antigen is needed. A recombinant p41 antigen may reduce the risk of such cross-reactivity and might be used to measure HHV-6 variant specific antibodies. Recombinant GST fusion antigens containing viral proteins have been used in measuring virus specific antibodies in literature [201, 202]

3.3.2 Methods

The purified GST-p41 fusion proteins from HHV-6A and HHV-6B were used as antigen in ELISA to measure p41-specific IgG or IgM antibodies. Human sera from 19 patients with MS, 20 patients with optic neuritis, 20 patients with other neurologic diseases and 13 asymptomatic, age matched healthy individuals were tested using the ELISA. To reduce the risk of false positive results, GST protein was used as antigen control for all samples in each experiment. The serum p41-specific IgM and IgG reactivities were analyzed statistically using the multiple comparison test (WINKS software, USA). The ELISA is described in detail in materials and methods, Paper II.

3.3.3 Results and discussion

Elevated OD values were observed in some sera from patients with MS, optic neuritis or other neurologic diseases compared with the healthy controls (Fig 1, Paper II). However, the differences found between any of the three patient groups and the healthy control group were not significant (p>0.05). Individual serum from the three patient groups and the control group showed similar IgM or IgG reactivity to the recombinant p41 antigens from the HHV-6A and the HHV-6B with a strong correlation (Figure 2, r

= 0.95, p<0.001, Paper II). These results suggest that HHV-6 variant-specific antibody reactivities were not detected with the recombinant p41 antigens. Hence, p41 is not an ideal target for HHV-6 serology. The p41 specific IgG antibody responses were not significantly different between these groups either (Fig 3, Paper II).

Serum IgM or IgG reactivity to p41 has been assumed to be a sign of active HHV-6 replication [79, 81, 108, 198]. However, two positive controls for HHV-6 IgM generated similar OD values as the sera from the healthy controls in the ELISA using the GST-p41 antigens. These results suggested that serum IgM reactivity to p41 is not a reliable sign of active HHV-6 infection. Ideally, sample sets from previous reports should have been used in the present study, but unfortunately such samples were not available. No significant difference in IgM reactivity to p41 was found between patients with MS and healthy controls (Fig 1, Paper II). Our data do not indicate a pathological link between HHV-6 and the MS, unlike previously reported results[81]. Those discrepancies might reflect a varied etiology of MS or a difference in methodology in individual studies.

Different experimental techniques have been used in investigating a link between HHV-6 and the pathogenesis of MS [114, 115]. None was able to show a causative relationship and controversial results were obtained in individual investigations. Those discrepancies in individual studies could be due to differences in reagents, methods or patients enrolled in studies. To make a reliable conclusion, adequate controls are needed. The methods should be verified and the data should be analyzed statistically. MS could be regarded as a syndrome that has varied clinical features, diverse etiology and similar pathologic features (multiple plaques of demyelination). Most of the MS cases are diagnosed based on their clinical histories. The primary clinical symptoms of MS may be mild and varied, leading to an early diagnosis of MS only in few cases. Therefore, it may be difficult to identify infectious agents that might trigger the process of MS in an early stage. Presence of evidence does not mean evidence of presence, vice versa, absence of evidence does not mean evidence of absence. This may reflect the status of the studies of MS etiology. A patient with MS may have encountered several infectious agents before a clinical diagnosis is made or typical clinical symptoms occur [203]. In conclusion, a serologic evidence is not sufficient for identifying a pathologic agent, especially when the suspected agent, e.g. HHV-6, is ubiquitous. Presence of an infectious agent in an MS brain may not be a

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reliable evidence for making a causative relationship, since the agent, such as HHV-6, could be commensal [80]. Another possibility that should be kept in mind is that an autoimmune response induced by a virus may occur after the causative virus has been cleared ("hit and run" event) from the affected organ. Damage can occur in a brain also without a virus invading, but via a "molecular mimicry" or "bystander activation". Those discussions may explain why many human viruses have been assumed to be causes of MS (Table 1), but none of them has been verified with consensus in individual investigations.

3.4 Effective siRNAs and antisense DNAs have different target sites in the mRNA (Paper III):

3.4.1 Specific background

Like antisense DNAs, siRNAs induced target mRNA degradation in a sequence-dependent manner [165, 166]. siRNAs are generally more potent to inhibit gene expression, compared with phosphorothioate antisense DNAs. However, not all siRNAs or antisense DNAs are effective, in another words, not all sequences in the mRNA are accessible to siRNAs or antisense DNAs. It is believed that antisense DNAs recognize target sequences by Watson-Crick base pairing. Antisense DNA target sites can be selected by experimental or computational methods [204-207]. It is suggested that siRNAs recognize target sequences via their antisense strands [149]. It is unknown if an antisense accessible site is also a good candidate site for a siRNA. Few parallel analyses of antisense accessible sites and siRNA accessible sites have been reported [208].

3.4.2 Methods

Twelve pairs of siRNA duplex and antisense DNAs were synthesized, directed towards the Renilla or firefly luciferase mRNA molecules (Table 1, Paper III). The siRNA duplex and antisense DNA in a pair were directed to the same site in the mRNA target. One luciferase gene was used as reporter and another as control. All the siRNAs and antisense DNAs were tested in cultured HEK-293 cells and NIH/3T3 cell in triplicate. An experimental technique for finding antisense accessible sites in the mRNA (designated RiboTAG) and siRNA Target Finder and Design Tool (Ambion, USA) were used to select target sites for antisense DNAs and siRNAs. The inhibitory effects

were expressed by normalized ratios between reporter and control genes as reported [139]. Inhibition by >75%, 51-75% and ≤50% were defined as thresholds for being highly effective, effective and ineffective, respectively.

3.4.3 Results and discussion

Out of the nine sites targeted in the Renilla luciferase mRNA, four sites turned out to be accessible to the siRNA duplexes (siRL82-100, siRL375-393, siRL501-519 and siRL548-566, Fig 1A, Paper III) and two sites were accessible to the antisense DNAs (RLas21-38 and RLas121-139, Fig. 1B, Paper III). The antisense DNA (RLas21-38, inducing 75% inhibition, Fig. 1B, Paper III) targeting the site that RiboTAG predicted was effective, whereas the corresponding siRNA duplex (siRL21-39, inducing 28% inhibition, Fig. 1A, Paper III) was ineffective. The construct siRL121-139 was ineffective as observed in a previous report [139]. Interestingly, the cognate antisense DNA (RLas121-139, inducing 68% inhibition, Fig. 1B, Paper III) was effective. No overlapping was found between the four sites targeted by the effective siRNAs and the two sites targeted by the effective antisense DNAs, suggesting that effective siRNAs and antisense DNAs have different target sites in the mRNA. To verify the hypothesis, we tested another three pairs of antisense DNA and siRNA against the firefly luciferase mRNA. One siRNA (siFL867-885, Fig. 1C, Paper III) was highly effective, whereas the corresponding antisense DNA (FLas867-885, Fig. 1D, Paper III) was ineffective. The FLas961-979 inhibited the reporter gene activity by 44% (Fig. 1D, Paper III), which was close to the threshold of "effective", whereas the siRNA961-979 was definitely ineffective (Fig. 1C, Paper III). Therefore, our results strongly indicate that effective siRNAs and antisense DNAs have different target sites in the mRNA. Our results are in contrast to a recent report that suggested that a site available for antisense DNAs is also available for the cognate siRNA duplex [208]. It should be noted that the antisense DNAs targeting the sites with starting nucleotides at 314, 671, 891 and 2098, respectively, in the PTEN mRNA were the most effective of the 36 antisense DNAs tested by Vickers et al. [208]. The siRNAs targeting those four sites had apparently low effects or no effect, compared with the RNase-H-dependent antisense agents or other siRNAs in that report. This suggests that the most effective target sites for antisense DNAs may not be good candidate sites or conversely bad candidate sites for siRNAs, which is consistent with our findings.

The discrepancy between our study and the previous report [208] could be due to the differences in mRNA targets and methods in individual studies. We used a dual luciferase assay system in assessing siRNAs and antisense DNAs. That system has been used in studying of RNAi mechanism in previous reports [139, 150, 162, 166]. The dose-dependent, highly potent inhibitory effects of siRNAs and antisense DNAs indicated that the agents were of good quality, and the cells and transfection protocol were optimal in our experiments. Identical inhibitory effects for either of antisense DNAs and siRNAs were obtained in two commonly used mammalian cells, suggesting that our observation is a general phenomenon, and not a cell-type related behavior. Our data showed that siRNAs are more potent compared with antisense DNAs. In contrast, the report by Vickers et al. suggests that antisense DNAs (chimeric 2'-*O*-methoxyethyl/ deoxyphosphorothioate modified oligonucleotides) and siRNAs had comparable potencies and durations of action [208]. Another previous report [209] showed results in agreement with ours and in contrast to Vickers'. In our report, one set of threshold was used for analyzing the potency of siRNAs and antisense DNAs. Cut-off values for potent agents varied between siRNAs and antisense DNAs, and among the siRNAs of different mRNA targets in the previous report [208]. It is feasible to use one set of threshold for both antisense DNAs and siRNAs when they are compared for the potency to inhibit the same gene.

Association with cellular proteins and enzymes, or secondary or tertiary structure of the mRNA renders most sequences of a given mRNA inaccessible to intermolecular base pairing with complementary DNAs. Our observations of different accessibilities of a mRNA site to siRNAs and antisense DNAs suggest that those unfavorable factors for antisense DNAs may be favorable or essential factors for siRNAs to bind on the target. siRNAs may recognize its target sequences by an alternative complex mechanism to Watson-Crick base pairing. A recent report indicated a relationship between the potency of siRNAs and the predicted mRNA structure using GENTYX-MAC 8.0 [210]. In our report, five highly effective siRNA duplexes targeted the sites of different mRNA structures predicted by mFOLD. Three siRNAs (siRL82-100, siRL375-393 and siRL548-556) targeted the predicted stem regions, whereas two siRNAs (siRL501-519 and siFL867-885) targeted the predicted loop structures. Our data didn't indicate a correlation between the siRNA potency and the predicted mRNA structures, which is consistent with a previous report [165]. Given that there is a relationship as suggested by that report [210], mFOLD doesn't seem suitable for the

purpose. Few effective siRNAs that were documented in literature were selected with a method that is established for determining antisense accessible sites in the mRNA [145]. Our results don't favor the use of such methods to predict siRNA target sites.

3.5 Duplex siRNAs are more potent and may act differently compared to antisense-stranded siRNAs (Paper IV):

3.5.1 Specific background

It is unknown how a duplex siRNA recognizes the target sequence in the mRNA. One hypothesis is that the antisense strand of a siRNA duplex guides the recognition of the target site, while the sense strand plays a role only through protecting the antisense strand from degradation by ribonucleases [159-161]. That hypothesis is supported by the finding of RdRP activity in C. elegans, by which antisense siRNAs, serving as primers, transform the target mRNAs into dsRNAs [163, 164]. It appears that there is weak or no such RdRP activity in human cells [165]. It is well documented that double-stranded RNAs, compared with antisense- or sense-stranded RNAs, are more potent in inducing RNAi in mammalian cells. The lower potency of single-stranded siRNAs are generally assumed to be due to their lower persistence in cells, nevertheless, this assumption has not been proved by convincing evidence obtained in experiments using intact mammalian cells.

3.5.2 Methods

Different constructs of siRNAs (dsRNA, asRNA or ssRNA), targeting exogenous luciferase or endogenous CD46, were tested in HEK-293 cells and NIH/3T3 cells. The sequences of those siRNAs are shown in Table-1, Paper IV. To compare double-and single-stranded siRNAs in inducing RNA interference in mammalian cells, the cells were transfected with siRNAs by different protocols (materials and methods, Paper IV). The luciferase activity was measured in a dual-luciferase assay (Promega). The CD46 expression was measured by IF and FACS using monoclonal antibody against CD46. To verify the specificity of CD46-specific-siRNAs, CD55 expression was measured in the transfected cells in a parallel experiment.

3.5.3 Results and discussion

A previous report concluded that antisense-stranded siRNA was as potent as siRNA duplex in inhibiting target gene expression [149]. In contrast to this conclusion, the data obtained by fluorescence assay demonstrated that duplex siRNAs were more potent than the cognate antisense-stranded siRNAs in inhibiting lamin A/C expression in the HeLa cells. It is also noted that reconstitution of RISC from single-stranded siRNA required 10- to 100-fold higher concentrations compared to siRNA duplex in that report [149]. In our experiments, using siRNAs against the exogenous luciferase or the endogenous CD46, siRNA duplexes appeared more potent than cognate antisense- or sense-stranded siRNA alone in mouse (NIH/3T3) or human cells (HEK-293) (Fig. 1, Fig. 4, Paper IV). Combination of complementary single-stranded siRNAs generated identical inhibitory effects as duplex siRNAs (Fig. 2, Fig. 5, Paper IV). These results indicated that the complementary single-stranded siRNAs formed into siRNA duplexes and induced RNAi in the transfected cells. Our results and the data reported previously suggested that dsRNAs are more potent than antisense-stranded RNAs as being RNAi triggers [140-146] in mammalian cells. To further compare double- and single-stranded siRNAs we transfected cells by a two-step protocol, transfecting with complement single-stranded siRNAs, sequentially.

Our experiments suggested that complementary single-stranded siRNAs formed into duplex siRNAs in the cells and the dsRNA formation is important for inducing effective RNAi in the cells (Fig. 3, Fig. 5, Paper IV). Our results argue for the hypothesis that both strands of a siRNA duplex contribute to the process of inducing RNA interference in cells [157, 158]. It is not surprising that the siRNA duplexes showed stable for more than 9 hours in the transfected HEK-293 cells since siRNA duplexes have been found to be able to persist for more than 24 hours in pure calf serum [209]. It is striking that synthetic single-stranded siRNA persisted for 6-9 hours in the transfected HEK-293 cells (Fig. 3, Paper IV). The long duration of single-stranded siRNA was observed in individual experiments with different siRNAs. Thus, it is a general phenomenon, and not a sequence-related behavior. Our observations suggest that intracellular duration may not be the main reason for the fact that the antisense-stranded siRNAs had no effect, while the duplex siRNAs generated significant RNAi in mammalian cells [140-146]. This point is also supported by the findings that the DNA vector-based systems plasmids that express only sense- or antisense-stranded siRNA had no effect in the transfected cells [148]. In our

experiments, some antisnese-stranded siRNAs targeting the luciferase mRNA were effective, however their efficiency was not always related to the activity of the cognate duplex siRNAs. Those accumulated data suggest that double- and single-stranded siRNAs may act in cells by different mechanisms.

3.6 Silencing of CD46 expression in human cell lines with duplex siRNAs (Paper IV):

3.6.1 Specific background

CD46 isoforms due to alternative splicing of mRNA are observed in individual tissues [211, 212]. Besides protecting host cells from homologous complement attack [15, 88], CD46 may play a biologic role in fertilization in human [212, 213]. In addition to serving as a receptor for HHV-6 entry [4], CD46 also serves as a cellular receptor for measles virus, group A streptococcus and pathogenic Neisseria [85-87, 214-217]. A RNA interference model is useful to further identify and elucidate the biological roles of CD46 variants in those infections.

3.6.2 Methods

Three siRNA duplexes directed against the conserved regions 6 and 9 of the CD46 gene were synthesized [212]. Two of the siRNAs (Table 1, Paper IV) turned out to be effecitve in inhibiting CD46 expression, and are reported here. The siRNAs were transfected into three human cell lines (HEK-293, SK-N-MC and HeLa-ATCC). CD46 expression in the transfectd cells was measured by fluorescence activated cell sorting (FACS) and IF. For measuring CD46 expression, the transfected cells were incubated with a mouse monoclonal antibody against CD46, and FITC-labeled rabbit anti-mouse secondary antibody. NIH/3T3 cells were used as a CD46-negative control for CD46 is not expressed in mouse cells. A siRNA duplex against the Renilla luciferase mRNA was used as an unrelated siRNA to test the specificity of the gene silencing induced with CD46-specific siRNAs. CD55, a member of the complement regulatory proteins [88], was chosen as a target protein control in this study.

3.6.3 Results and discussion

CD46 expression was suppressed by the CD46-specific siRNA duplexes but not by either of the corresponding asRNA or ssRNA (Fig. 4, Paper IV). The NIH/3T3 cells

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were 100% negative for CD46 by FACS, indicating that the assay is CD46 specific. The unrelated duplex or single-stranded siRNAs had no effect on CD46 expression in the transfected cells, indicating that CD46 expression was inhibited by the CD46-specific siRNA duplexes but not by other factors. The specific duplex siRNA suppressed the CD46 expression in a dose-dependent manner in the transfected HEK-293 cells, while the unrelated siRNA had no effect in series of concentration of siRNA (Fig. 6, Paper IV). The CD46-specific siRNAs had a significant inhibitory effect on the CD46 expression, and no effect on the CD55 expression, in the transfected cells (data not shown). Our results showed that the CD46 expression can be specifically and efficiently suppressed by siRNA duplexes. The double-stranded siRNA formation is essential for silencing the CD46 expression in human cells. To inhibit a stably expressed protein, such as CD46, potent RNAi triggers, such duplex siRNAs, are needed. In our experiments, the luciferase specific-RNAi reached maximum effects in the transfected cells about 20 hours after transfection, while the effects of the CD46-specific RNAi peaked at the third day after transfection. The different RNAi behaviors may reflect the rate of the target gene product turnover in the cells.

Significant gene silencing was induced with CD46-specific siRNAs in three human cell lines (Fig. 7, Paper IV), indicating that the gene silencing is a general but not a cell-type related phenomenon. In order to knock down all isoforms of CD46 we designed the siRNAs targeting the conserved regions of CD46 isoforms [212]. This strategy turned out to be successful in our experiments. CD46 expression was blocked by the specific siRNA in 82-91% human cells (HEK-293, SK-N-MC and HeLa-ATCC), measured by FACS or IF with optimal transfection parameters (Fig. 7 and 8, Paper IV). We tried to increase the CD46-specific RNAi effect by administration of two CD46-specific siRNAs of different target sites, and didn't get an improved result. A minority of human cells escaped from the gene silencing generated by the CD46-specific siRNA, which might be due to existence of sub-groups of cells that are resistant to transfection. Such an assumption was consistent with the findings that more potent RNAi effects were observed in the cells that were transfected with siRNAs using Lipofectamine 2000 at a higher concentration (Fig. 7, Paper IV). Similar CD46-specific RNAi models have not been reported in literature. CD46 variant-specific RNAi models can be established in the same way as in this study. Such models could be used in study of the biological roles of CD46 in human

diseases and the interaction between cells and microorganisms using the CD46 receptor.

4 CONCLUSIONS

1. The mAb to p41/38 is HHV-6A-specific and can differentiate between HHV-6 A and B.
2. p41 is not an ideal target for HHV-6 serology studies.
3. Investigation of serum IgG and IgM responses to p41 in MS patients and controls didn't show evidence for a link between HHV-6 and the pathogenesis of MS.
4. Effective siRNAs and antisense DNAs have different target sites in the mRNA.
5. The activity of antisense-stranded siRNAs doesn't always relate to that of the cognate duplex siRNAs and double-stranded RNA formation is important for effective RNA interference in mammalian cells.
6. CD46 expression can be specifically silenced by synthetic siRNAs in human cells.

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