



Please mail or FAX the completed report by **August 15, 2004** to:  
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### **Pilot Project Research Report**

Investigator: Konstance Knox, Ph.D.  
Institute for Viral Pathogenesis  
From 06/01/2003 to 05/31/2004  
Title: *Human herpesvirus six and multiple sclerosis: molecular mimicry*

Pilot Project: PP0919  
Report Date 8/20/04  
Award: \$44,000

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#### Part 1. Scientific summary of research progress (do not exceed 3 pages)

Human herpesvirus six (HHV-6) is a beta herpesvirus that has been implicated in the pathogenesis of multiple sclerosis. As a herpesvirus, HHV-6 establishes a life-long latent infection in its host, and CNS and lymphoid tissues are well established sites of HHV-6 latency. Within cells latently infected with HHV-6 viral gene activity is limited to a single protein designated U94 which is constitutively expressed in the infected cell. The U94 protein's function appears to be the establishment of "active latency" of HHV-6, i.e. as long as it is expressed in the infected cell, the virus remains in its latent or inactive state. Therefore, in patients with MS, a viral protein, U94, is constitutively expressed in both CNS and peripheral tissues that is apparently highly immunogenic. The possibility of the HHV-6 U94 protein playing a role in the induction of autoantibodies reactive with myelin is raised.

In order to address the possible role of U94 in a molecular mimicry mechanism in MS, its amino acid sequence was compared to that of human myelin basic protein (MBP), a major constituent of myelin that is known to be a target of autoantibodies in patients with MS. When the sequence alignments were analyzed, a strong homology was observed between peptide 296 - 311 of the U94 protein and the 78 to 98 region of MBP. This region of MBP is of special interest since it contains an immunodominant epitope known to be a major target of autoantibodies in patients with MS as well as an important T cell epitope. The marked similarity (63% identity with 25% conservative amino acid changes) of the U94 peptide and the ENPVVHFF peptide of MBP suggests the existence of a cross reacting epitope in the two proteins.

In the study that we have now completed, we:

- 1) used synthetic peptides in an enzyme linked immunoassay (ELISA) to determine the prevalence of MBP reactive autoantibodies in the sera and cerebrospinal fluids (CSF) of MS patients and healthy controls
- 2) used synthetic peptides in an ELISA to determine the prevalence of U94 reactive antibodies in the sera and cerebrospinal fluids (CSF) of MS patients and healthy controls, and
- 3) determined the prevalence of dual positivity for antibodies reactive with the MBP and U94 peptides in MS patients and healthy controls.

#### **Summary of Patient Samples and Specimens from Healthy Control Individuals**

One set of serum and CSF samples were obtained from Drs. George Ellison and Lawrence Myers of the Department Neurology of the University of California at Los Angeles ("UCLA" samples). These were:

- 32 matched (i.e. both CSF and serum samples were from the same patient) serum and CSF samples from patients with MS
- one unmatched CSF sample, and
- 18 matched serum and CSF samples from healthy control individuals.

A second set of samples were obtained from Dr. Lorri Lobeck of the Medical College of Wisconsin and Froedtert Memorial Lutheran Hospital (FMLH) in Milwaukee, Wisconsin. These were 37 serum samples obtained from MS patients at the time of clinical relapse ("first samples") and 35 serum samples obtained from the same patients after the disease relapse had resolved (mean interval of 68 days) ("second samples"). Second samples were not obtained from two patients because they were lost to follow-up.

A third set of samples were comprised of 47 serum samples obtained from healthy laboratory personnel or purchased from Analytical Biological Services, Inc.; Wilmington, Delaware.

### **Development of Peptide Specific Enzyme Immunoassay (PSEIA)**

The basic design of the PSEIA system used can be summarized as follows:

- the Multiscreen Millipore Vacuum Manifold system was used for specimen application and washing during the immunoassay
- Millipore Immobilon-P Membrane 96 well plates were used due to their low intrinsic background and high protein binding characteristics
- the IgG concentration of all serum and cerebrospinal fluid (CSF) specimens was determined using an in-house EIA system, and all samples were analyzed at an IgG concentration of 10 ug per milliliter (CSF samples having IgG concentrations below 10 ug/ml were analyzed undiluted)
- it was empirically determined that optimal coating of the assay plates occurred at a peptide input of 3 ug per well in a volume of 100 microliters
- all samples were run in duplicate
- binding of IgG to bound peptides was detected by treatment with a peroxidase labeled anti-human IgG antibody with tetramethylbenzidine (TMB) as a substrate. Optical density of the reaction product in each well was determined by means of an ELX800 Universal Microplate Reader.
- specificity controls included the use of peptides with the same amino acid compositions as the MBP and HHV-6 U94 peptides but in randomized order ("randomized peptides").

Initially, detection of antibodies reactive with the MBP and U94 peptides (and their random sequence counterparts) was attempted using the optical density (OD) as the end result. Representative results obtained with serum samples and the MBP peptide are summarized in Table 1.

All four sets of samples gave similar OD results, and all gave a broad range of values. Similar results were obtained for all four peptides, i.e. MBP, MBP random, U94, and U94 random. Also, it was observed that each serum sample gave similar OD values for each of the four peptides.

It was therefore determined that, for each sample, the ratio between the OD obtained with a specific peptide and its randomized counterpart would serve as a better detection system. Representative data obtained for the MBP peptide and its randomized counterpart using healthy control serum samples are shown in Figure 1. In the figure showing data from all patients (left figure), it can be seen that all but 5 subjects gave ratios lying within the two standard deviations. When these individuals were excluded and the mean and standard deviation were recalculated, the results in the right hand figure were obtained. It was concluded that a ratio of 1.15 could appropriately be used as an upper cutoff value, i.e. samples giving a ratio above 1.15 would be considered positive for antibodies reactive with the MBP peptide. A similar analysis of the U94-U94 randomized peptide system indicated that its ratio cut-off value should be 1.20.

### **Analysis of Serum and CSF Samples from MS patients and Controls for Antibodies Reactive with MBP and U94 Peptides**

When this PSEIA system was applied to the various sets of samples, the results summarized in Table 2 were obtained.

**Table 1. Optical density results of PSEIA analysis of serum sample reactivity with MBP specific peptide.**

<b>Patient Group</b>	<b>Number of Subjects</b>	<b>Mean MBP Peptide Optical Density</b>	<b>Standard Deviation</b>	<b>Maximum Minimum</b>	<b>p Value Compared to Controls</b>
<b>Healthy Controls</b>	<b>65</b>	<b>0.68</b>	<b>0.28</b>	<b>1.68 0.19</b>	
<b>FMLH MS Patients: First Sample</b>	<b>37</b>	<b>0.63</b>	<b>0.32</b>	<b>1.29 0.15</b>	<b>0.240</b>
<b>FMLH MS Patients: Second Sample</b>	<b>35</b>	<b>0.67</b>	<b>0.34</b>	<b>1.55 0.18</b>	<b>0.753</b>
<b>UCLA MS Patients</b>	<b>32</b>	<b>0.70</b>	<b>0.16</b>	<b>1.00 0.42</b>	<b>0.256</b>

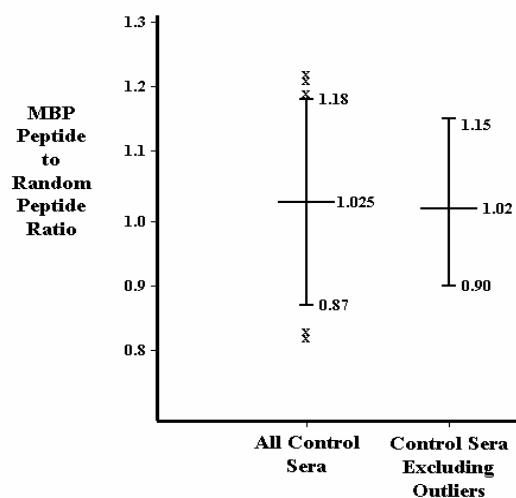


Figure 1. Ratio of MBP peptide OD and MBP randomized peptide OD for serum samples from healthy controls. Large bars indicate the mean values obtained and the short bars show the mean value plus and minus two standard deviations.

**Table 2. Positivity Rates for the MBP and HHV-6 U94 Peptide Specific Antibodies**

Antibody Assay	Patient Group					
	Healthy Control Sera	FMLH MS Patient First Sera Samples	FMLH MS Patient Second Sera Samples	UCLA MS Patient Sera	Healthy Control CSF Samples	UCLA MS Patient CSF Samples
<b>MBP Peptide</b>	9% (6/65) [1.15-1.22]	16% (6/37) [1.15-1.34]	9% (3/35) [1.16-1.41]	16% (5/32) [1.15-1.29]	6% (1/18) [1.20]	3% (1/33) [1.81]
<b>HHV-6 U94 Peptide</b>	14% (9/65) [1.20-1.42]	14% (5/37) [1.33-1.46]	14% (5/35) [[1.23-1.31]	3% (1/32) [1.21]	11% (2/18) [1.20-1.34]	3% (1/33) [1.26]

Positivity rates for reactivity with the MBP peptide ranged from 3% to 16% with no significant differences being observed among the different sets of samples, and no significant differences were seen between serum and CSF samples. Similarly, the positivity rates for the HHV-6 U94 peptide ranged from 3% to 14% with no significant differences being observed among the various groups.

When the matched serum and CSF samples from UCLA were considered, three showed concordant positivity. One control subject was positive for MBP peptide antibodies in both serum and CSF, and one control subject was positive for U94 peptide antibodies in both serum and CSF. Also, one MS patient had MBP peptide reactive antibodies in both serum and CSF. Interestingly, this patient had markedly high ratios for both samples, i.e. serum ratio of 1.29 and CSF ratio of 1.81 (see Table 2).

When the first and second samples from the FMLH patients were analyzed, three were seen to be concordant. Two patients had both their first and second serum samples positive for MBP peptide reactive antibodies, while the third patient had both serum samples positive for U94 peptide specific antibodies. Simultaneous positivity for both the MBP peptide and the U94 peptide was observed in only one subject, a healthy control.

### Conclusions

- The PSEIA system developed in these studies is effective, the use of peptide to randomized control peptide ratio as the major end point provides a high degree of specificity in the detection of peptide specific antibodies, and this system should be of value in the study of other autoantibody systems.
- Antibodies reactive with both the MBP and HHV-6 U94 peptides are present in serum and CSF samples from both MS patients and healthy controls.
- Positivity rates for antibodies reactive with the MBP and U94 peptides do not differ between healthy controls and MS patients.
- Dual positivity for the MBP peptide and the U94 peptide in the same serum or CSF sample is rare and was observed in only one serum from a healthy control subject.
- Molecular mimicry between the ENPVVHFF peptide of MPB and HHV-6 U94 protein is unlikely to be of pathogenic importance.

Part 2. Brief summary for **release to the lay public** (do not exceed space on this page)

Human herpesvirus six (HHV-6) has been implicated as the cause of multiple sclerosis. As a herpesvirus, HHV-6 establishes a life-long latent, i.e. inactive, infection in people infected with it. The brain and spinal cord are known to be sites of latent HHV-6 infections. Within cells latently infected with HHV-6 viral gene activity is limited to a single protein designated U94 which is constantly expressed in the infected cell. This raises the possibility that the HHV-6 U94 protein plays a role in the induction of antibodies that react with and normal myelin, which is believed to be the target of autoimmunity in patients with MS.

In order to address the possible role of U94 in such a process, its amino acid sequence was compared to that of human myelin basic protein (MBP), a major constituent of myelin that is known to be a target of autoantibodies in patients with MS. A strong similarity was observed between a specific portion of the U94 protein and a portion of MBP. This region of MBP is of special interest since it is known to be a major target of autoantibodies in patients with MS.

In the study that we have now completed, we:

- 3) used artificially produced protein pieces to determine the prevalence of MBP reactive autoantibodies in the sera and cerebrospinal fluids (CSF) of MS patients and healthy controls
- 4) used artificially produced protein pieces to determine the prevalence of U94 reactive antibodies in the sera and cerebrospinal fluids (CSF) of MS patients and healthy controls, and
- 3) determined the prevalence of dual positivity for antibodies reactive with the MBP and U94 proteins in MS patients and healthy controls.

The major conclusions drawn from these studies are:

- The assay system developed in these studies is effective, provides a high degree of specificity in the detection of protein specific antibodies and should be of value in the study of other autoantibody systems.
  - Antibodies reactive with both the MBP and HHV-6 U94 proteins are present in serum and CSF samples from both MS patients and healthy controls.
  - Positivity rates for antibodies reactive with the MBP and U94 proteins do not differ between healthy controls and MS patients.
  - Dual positivity for antibodies reactive with the MBP and HHV-6 U94 proteins in the same serum or CSF sample is rare and was observed in only one serum from a healthy control subject.
  - The similarity between the regions of MBP and HHV-6 U94 proteins studied here is unlikely to be important in MS.
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Part 3. Program Evaluation

a. Publications

Give the number of each of the following types of publications that used data from this project:

- \_\_\_ manuscripts
- \_\_\_ meeting abstracts
- \_\_\_ peer reviewed papers

Please enclose **one** copy of each **meeting abstract or peer-reviewed paper** with your report.

b. Further Research

Please list all grant applications you have made using data from this project

Title of Application	Where Submitted	Amount Requested	Status
			<input type="checkbox"/> Pending <input type="checkbox"/> Funded <input type="checkbox"/> Not funded
			<input type="checkbox"/> Pending <input type="checkbox"/> Funded <input type="checkbox"/> Not funded
			<input type="checkbox"/> Pending <input type="checkbox"/> Funded <input type="checkbox"/> Not funded
			<input type="checkbox"/> Pending <input type="checkbox"/> Funded <input type="checkbox"/> Not funded
			<input type="checkbox"/> Pending <input type="checkbox"/> Funded <input type="checkbox"/> Not funded

List additional applications you plan to submit using data from this project:

The results of these studies were negative in that no significant differences were found between healthy controls and patients with MS with respect to antibodies reactive with the MBP and HHV-6 U94 peptides used. However, the peptide specific EIA system developed is a powerful and highly specific assay system, and we plan to use in in similar studies of autoantibodies in MS and other autoimmune diseases. Specifically, we envision a project in which we analyze the large and well characterized collection of control and MS sera and CSF samples gathered for these studies for the existence of antibodies reactive with an encephalitogenic epitope of myelin oligodendrocyte glycoprotein (MOG) that cross react with a specific, homologous HHV-6 peptide that we have identified.

Part 4 Report of expenditures

Budget Category	Original Budget	Actual Expense
Personnel (list names, institutional title, and role in project)		
Personnel subtotals:	\$	\$
Equipment		
Equipment subtotals:	\$	\$
Consumable Supplies		
Supplies subtotals:	\$	\$
Travel		
Other Expenses		
Other Expenses subtotals	\$	\$
Total direct costs:	\$	
Indirect cost*		
Total cost	\$	

Balance of award minus total cost due to the National Multiple Sclerosis Society: \$ \_\_\_\_\_

Investigator: Konstance Knox, Ph.D.

Financial Officer

Name:

\_\_\_\_\_  
(signature)

\_\_\_\_\_  
(date)

\_\_\_\_\_  
(signature)

\_\_\_\_\_  
(date)

\*For institutions in the USA, the **maximum** indirect cost is 10% of the direct cost **minus** equipment and patient costs. Indirect costs are **not provided** for institutions outside the United States.

**Investigator: Dr. Knox**

**Instructions for completing a progress report for a Pilot Research Award from the National Multiple Sclerosis Society.**

As you know, the National Multiple Sclerosis Society requires a report of the scientific progress and the expenditures made for every grant that we award. The report of a Pilot Research award has four parts. The first three are on the scientific progress. These are due at the Research Programs Department within 15 days of the end of the grant. The fourth part is the report of expenses for the grant. It is due 30 days after the end of the grant.

- Part 1 This is a scientific summary of the results of your research. Unpublished data **should be identified as such** and will be treated as confidential until you publish it. This section should not be longer than the first page and two continuation pages.
- Part 2 This is a summary written so that someone interested in multiple sclerosis, but with no scientific or medical background, can understand it. The first paragraph of this section should describe the goals of the project; the other paragraph(s) should describe the major result(s). Information in this section **may be released to the public**, either in the form you provide, or as edited by the Research Programs Department. This section should be limited to the single page provided.
- Part 3 This is a brief questionnaire to help us compile statistical information about the Pilot Research Program.
- Part 4 This is a report of the expenses charged to your grant. Please have it signed by the financial officer of your institution.

Return the completed report by mail or FAX to:

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