Potential Role of Persistent Paramyxovirus Infection in Chronic Fatigue Syndrome

Interim Progress Report and Research Proposal

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CFS is a debilitating illness associated with persistent severe fatigue, a variety of physical and neuropsychological signs and symptoms, and frequently, an unusual susceptibility to a variety of infections. The study of intracellular signal transduction pathways may have provided a key insight into the immunological defect operative in CFS patients Signal transducers and activators of transcription (STAT) are a family of proteins that play central roles in the responses of cells to cytokines. Specifically, the protein STAT1 is intimately involved in the response of cells to type I (alpha and beta) and type II (gamma) interferons.

Previous work by our laboratory has demonstrated that a subset of patients with CFS have a significant deficiency in the expression of STAT1 in their peripheral blood mononuclear cells (PBMC). Recent work has demonstrated that the STAT1 abnormalities present in the PBMC of CFS patients are stable over long period of time and that artefactual destruction of STAT1 protein during shipment of blood samples is not involved in the STAT1 deficiency that is observed. These data are shown in **Appendix 1**.

Since members of the paramyxovirus family are known to induce destruction of STAT 1 in the cells that they infect, a logical hypothesis would be that the STAT1 deficiency observed is due to a persistent paramyxovirus infection. Such persistent infections by paramyxoviruses are well known.

As a test of this hypothesis PBMC samples from the same patients and controls included in the previous study were evaluated for paramyxovirus proteins and RNA. Details of these studies are presented in **Appendix 2**. Using a highly sensitive and very specific polymerase chain reaction (PCR) assay, the RNA of one particular paramyxovirus (simian virus 5) was detected in the PBMC sample from one CFS patient. All of the other patients and healthy control subjects tested were negative for the viral RNA. In other studies, using an antiserum that recognizes proteins from two subfamilies of paramyxoviruses, we found that 16% of the CFS patients had paramyxovirus proteins detectable in their PBMC compared with 0% of the healthy control subjects. When the paramyxovirus reactive serum was studied in detail, it was observed that it was probably reacting with the hemagglutinin-neuraminidase (HN) proteins of the various paramyxoviruses.

Thus, it was concluded that many of the CFS patients were infected with a paramyxovirus, and in some cases the virus was closely related to (or identical to) SV5, a member of the rubula subfamily of paramyxoviruses.

Since the antiserum used in these studies reacts very strongly with human parainfluenza virus 3 and weakly with the other viruses and the viral protein involved in the broad reactivity appeared to be the viral HN protein, the HN proteins of the various viruses were analyzed in detail. These comparisons are presented in detail in **Appendix 3**. The goal of these comparisons was to identify a region of the HN proteins that were identical, or nearly identical, in all of the virus strains. Once found, that particular protein region could be used to produce an antiserum that would react strongly with numerous strains of paramyxoviruses.

A region of the HN protein (designated HN 160-199) was identified that was identical in 80% (32/40) of its amino acids within the rubula subfamily of paramyxoviruses. The same protein region is 25% identical in the respiro subfamily of paramyxoviruses. Thus, this protein region is an attractive candidate for a pan-paramyxovirus reagent if it is antigenic and specific for paramyxoviruses. In other words, will it stimulate the production of specific antibodies when used to immunize an animal and will those antibodies react only with paramyxovirus HN proteins?

The questions concerning the antigenicity and the specificity of the HN 160-199 peptide was specifically addressed as is explained in detail in **Appendix 4**. When compared with all human protein sequences in a national biotechnology depository, the HN 160-199 peptide showed significant homology with a single protein, the human mucin 13 protein (Muc13). Mucins are a large family of proteins that are expressed by cells of the intestines and other organs. One group of mucins is termed "secretory", and they are secreted from cells. These proteins comprise the

mucous that coats and protects the bowel wall. The other type of mucin (such as Muc13) is termed "transmembrane" mucins. These proteins are expressed on the surface of intestinal cells and play important roles in the interaction of the cells with their local environment. With respect to disease pathogenesis, the transmembrane mucins are important because many forms of inflammatory bowel disease have been associated with autoimmune reactivity towards them. Therefore, if a paramyxovirus expressing the HN 160-199 peptide is involved in the pathogenesis of CFS, an autoimmune reaction aimed at Muc13 may be triggered by a mechanism known as molecular mimicry leading to inflammatory bowel disease, an illness quite common in patients with CFS.

When the antigenicity of HN 160-199 was analyzed, two highly antigenic amino acid sequences were identified. The first of these is comprised of amino acids 9 through 15 which encompasses the Muc 13 homologous sequence. The antigenicity of this area makes a possible molecular mimicry reaction much more likely to occur. The second antigenic sequence is located toward the C terminus of the peptide and is composed of amino acids 21 through 32. We propose the production of two HN-160-199 specific antisera. The first will be made using a peptide composed of amino acids 1 through 20 which contains the Muc13 homologous sequence. This antisera will be used in experiments aimed at determining whether or not a molecular mimicry mechanism may exist in patients with CFS. The second antiserum will be raised against the C terminus half of HN 160-199, i.e. amino acids 21 through 40. This antiserum will be highly specific for the paramyxovirus HN proteins and will be used to search for the virus that appears to be involved in many patients with CFS. Details of our research proposal are contained in **Appendix 5**

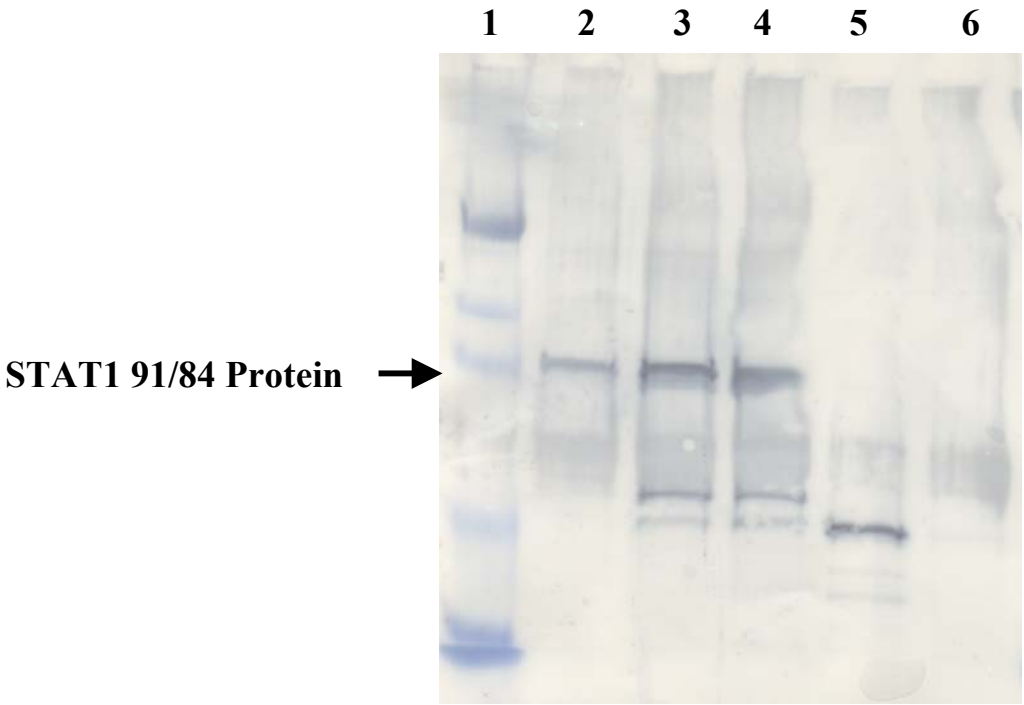
Appendix 1

Stability of STAT1 Protein Abnormalities in the Peripheral Blood Mononuclear Cells from CFS Patients Over Extended Periods of Time

Stability of STAT1 Protein Abnormalities in the Peripheral Blood Mononuclear Cells from CFS Patients Over Extended Periods of Time

Previous work from our laboratory has shown that a subpopulation of patients with CFS have significant abnormalities in the expression of STAT 1 protein.[1] In light of these findings, two important questions have arisen. First, how stable are the abnormalities in the STAT1 91/84 protein, i.e. the form of the protein directly involved in the interferon signaling system? Second, could the deficiency of the STAT1 91/84 protein observed in some patient samples be an artifact caused by proteasomal protease during overnight shipment of the blood sample to the laboratory for testing?

In order to answer these questions, two of the CFS patients studied previously were chosen, one (patient 2051) with an abnormally high expression of STAT1 91/84 protein and one with an abnormally low expression of STAT1 91/84 (patient 2036). Two blood samples were obtained from each patient on October 13, 2004. To one sample from each patient was added a proteasomal protease inhibitor (MG132 with a final concentration of 20 uM). All four blood samples were then shipped by overnight courier to the laboratory where the expression of STAT1 91/84 protein in the samples was assessed by immunoblotting with an antiserum specific for STAT1 protein. Identical numbers of cells were analyzed for all samples. Results are shown in the figure below.



Lane 1: Molecular weight markers

Lane 2: PBMC sample from healthy control individual

Lane 3: PBMC from CFS patient 2051 (no proteasomal protease inhibitor)

Lane 4: PBMC from CFS patient 2051 (with proteasomal protease inhibitor)

Lane 5: PBMC from CFS patient 2036 (no proteasomal protease inhibitor)

Lane 6: PBMC from CFS patient 2036 (with proteasomal protease inhibitor)

As can be seen in the figure, patient 2051(who showed an abnormally high level of STAT1 in her original PBMC sample obtained on November 17, 2003) still expresses an abnormally high level of STAT1 91/84 protein when compared to the healthy control individual. Thus, expression levels of STAT1 91/84 protein remains essentially unchanged for a period of almost one year. Also, the addition of the proteasomal protease inhibitor to the blood sample had no effect on the STAT1 91/84 protein.

With respect to patient 2036 (who showed an essentially undetectable level of STAT1 91/84 in her original blood sample obtained on November 4, 2003) still expresses an undetecable level of STAT1 91/84 protein in her PBMC. Thus, her STAT1 91/84 protein status also has remained stable for almost one year. Similar to the findings with patient 2051, addition of the proteasomal protease inhibitor to the blood sample had no effect on the detection of the STAT1 91/84 protein.

References

1. Knox KK, Cocchetto A, Jordan E, Leech D and Carrigan DR. Deficiency in the expression of Stat1 protein in a subpopulation of patients with chronic fatigue syndrome. The Seventh International Conference on Chronic Fatigue Syndrome, Fibromyalgia and Other Related Illnesses. October 8-10; 2004. Madison, Wisconsin.

Appendix 2

Assessment of Peripheral Blood Mononuclear Cells from CFS Patients for Paramyxovirus Antigens and RNA:Relation to STAT1 Protein Status

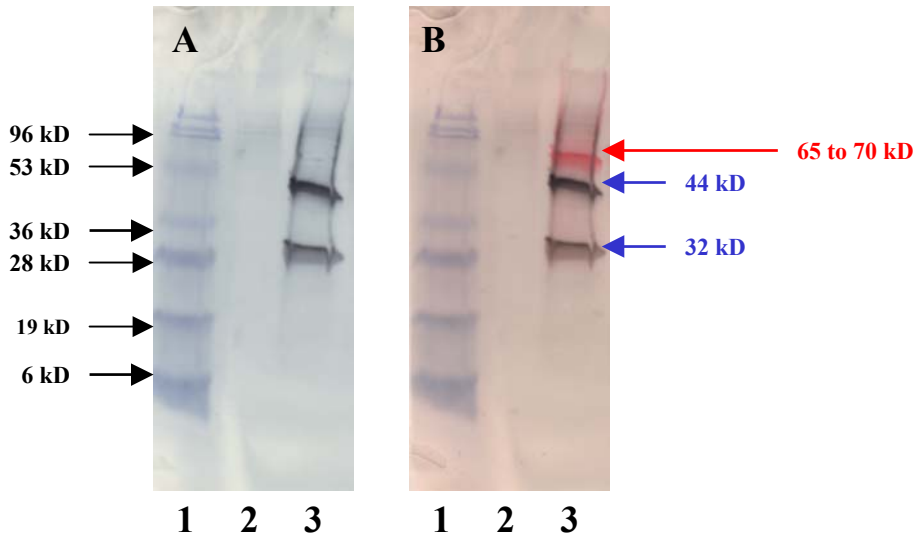
Assessment of Peripheral Blood Mononuclear Cells from CFS Patients for Paramyxovirus Antigens and RNA:Relation to STAT1 Protein Status

Previous work by our laboratory has demonstrated that a subset of patients with CFS have a significant deficiency in the expression of one member of the signal transducers and activators of transcription (STAT) protein family in their peripheral blood mononuclear cells (PBMC).[1] This protein, specifically STAT1, plays a central role in the response to both type I and type II interferons.[2] Since members of the paramyxovirus family are known to induce destruction of STAT 1 in the cells that they infect,[3] a logical hypothesis would be that the STAT1 deficiency observed is due to a persistent paramyxovirus infection. Such persistent infections by paramyxoviruses are well known.[4,5]

Viral antigens. To test this hypothesis, PBMC samples from the same cohort of CFS patients showing the STAT1 deficiency were assessed for the presence of paramyxovirus antigens by indirect immunofluorescence (IFA). The antiserum chosen for these initial studies, serum AB1070 from Chemicon International Inc., was a hyperimmune serum raised against human parainfluenza type 3 virus (HPIV3), a member of the respirovirus genus of paramyxoviruses. Using an IFA procedure, it was demonstrated that this serum reacted very strongly with HPIV3 infected cells and that it weakly cross-reacted with HPIV1 (a respirovirus), simian virus 5 (SV5, a member of the rubulavirus genus of paramyxoviruses) and HPIV2 (a rubulavirus).

When the patient's PBMC samples were analyzed 16% (3/19) were found to be positive for viral antigens compared to 0% (0/24) of PBMC samples from healthy control subjects. The pattern of the IFA staining was most consistent with a cell surface viral glycoprotein since intracellular inclusion bodies failed to stain while the infected cell surface demonstrated prominent staining. Since the antiserum used broadly cross-reacts with several members of the paramyxovirus family, it remains unclear as to which member or members of the paramyxovirus family is involved with these patients.

As a first step in determining the identity of the virus being detected in the CFS patient PBMC samples, the reactivity of the antiserum with cells infected with SV5 was analyzed by Western Blot. These results are shown in the figures below.



LLC-MK2 monkey kidney cells were infected with SV5 and incubated for 7 days at 37°C at which time they and uninfected control cells were processed into cell lysates. Proteins in infected and uninfected lysates were then separated by electrophoresis in a 15% polyacrylamide gel. After electrophoresis the protein were transferred to a sheet of nitrocellulose. Panel A shows the immunostaining result obtained when the blot was stained using a murine monoclonal antibody specific for the P protein of SV5 and a peroxidase enzyme substrate that produces a black reaction product. The two forms of the viral P protein[4] are clearly visible in the infected sample (lane 3) but not in the uninfected control sample (Lane 2). This demonstrates the specificity of the system and shows that adequate viral proteins are present for detection. Panel B shows the same blot after staining with the HPIV3 antiserum using an alkaline phosphatase based detection system with a substrate that produces a red reaction product. A single red stained band is present with a molecular weight of 65 kD to 70 kD. This result, when combined with the pattern of IFA staining seen with the HPIV3 antiserum with SV5 infected cells, suggests that the reactive SV5 protein is the viral hemagglutinin since it is expressed on the surface of infected cells and has a molecular weight of 70 kD.

Therefore, it is most likely that the viral protein detected in the CFS patient PBMC samples is the hemagglutinin of a paramyxovirus related to SV5. Further work using reagents of higher reactivity with the viral proteins of rubulaviruses will be needed to confirm and extend these findings.

Viral RNA. The detection of paramyxovirus antigen in the PBMC samples of the CFS patients suggested that viral RNA may also be present in those cells. In order to test this prediction a nested reverse-transcriptase based polymerase chain reaction (RT-PCR) assay was designed. Total RNA was extracted and purified from PBMC samples by means of the Qiagen "QIAMP RNA Blood Mini Kit" system. RT-PCR was performed using the "Access RT-PCR System" developed by Promega Corporation; Madison, Wisconsin. Adequacy of RNA recovery for each sample was ascertained by amplification of the cellular messenger RNA encoding the constituative enzyme ornithine decarboxylase. Nested primers were designed using the fusion protein gene sequence of SV5. The specific PCR primer sequences were:

Outer Sense: F Sense 305 Primer: 5' - G G T T T G C A G G G G T G G T G A T T - 3'

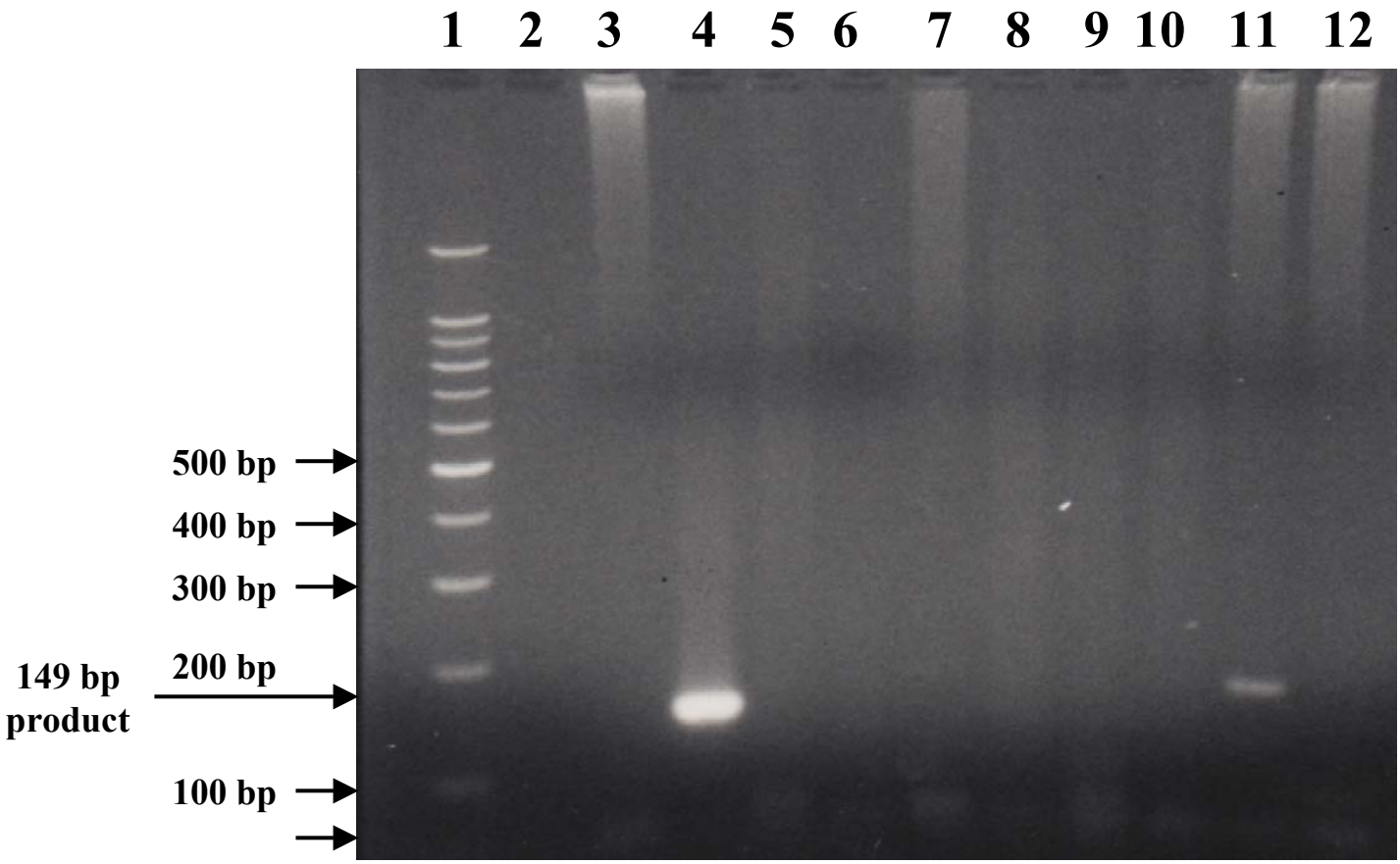
Inner Sense: F Sense 248 Primer: 5' - T T G T A A G G C C C A A G A T G C T A T C A -3'

Outer Antisense: F Anti 762 Primer: 5' - C C C A C A A T C T G G C C T G T C A A T 3'

Inner Antisense: F Anti 376 Primer: 5' - T C G G C A A G G T A C T C C C C A G T A - 3'

The inner sense and inner antisense primers produced a an RT-PCR reaction product that was 149 base pairs in length as determined by agarose gel electrophoresis and ethidium bromide staining A representative gel is shown in the figure on the following page.

Of the 22 CFS patients for which adequate PBMC RNA was available, only one (5%) was positive in the RT-PCR assay. The patient is a 44 year old woman with long-standing, severe CFS.



Ethidium bromide stained agarose gel analyzing an RT-PCR assay for the fusion protein RNA of simian virus five (SV5).

Lane 1: Molecular weight markers (100 base pair ladder)

Lane 2: No RNA negative control

Lane 3: RNA from uninfected LLC-MK2 monkey kidney cells

Lane 4: RNA from SV5 infected LLC-MK2 cells

Lane 5: RNA from CFS Patient 2081

Lane 6: RNA from CFS Patient 2076

Lane 7: RNA from CFS Patient 2071

Lane 8: RNA from CFS Patient 2066

Lane 9: RNA from CFS Patient 2061

Lane 10: RNA from CFS Patient 2056

Lane 11: RNA from CFS Patient 2051 (Positive for RT-PCR reaction product)

Lane 12: RNA from CFS Patient 2041

The possible implications with respect to STAT1 protein expression of detecting SV5 RNA in the PBMC sample of a patient with CFS was explored by attempting to reproduce the findings of other workers that suggest that SV5 and other rubulaviruses can cause the degradation of STAT1 protein in infected cells. To this end, a line of monkey kidney cells (LLC-MK2) were infected with the W3 strain of SV5, and seven days after infection identical numbers of infected and uninfected control cells were assessed for expression of STAT1 91/84 protein by immunoblotting. Results are shown in the figure below. The SV5 infection dramatically suppressed the expression of STAT1 91/84 protein.

Lane 1: Molecular weight markers

Lane 2: SV5 infected LLC-MK2 cells

Lane 3: Uninfected LLC-MK2 cells

Relationship Between Virologic Findings and STAT1 Protein Status. These virologic observations made it of interest to examine the relationship between the detection of viral antigens or RNA and the expression of STAT1 protein in the patients' PBMC. A Table summarizing the results of that examination is presented below.

Subject Group	STAT1 Protein Species		
	91/84 kiloDalton	56 kiloDalton	51 kiloDalton
Healthy Controls	303 +/- 217[1]	226 +/- 146	238 +/- 136
Virus Positive CFS Patients	781 +/- 411[2,3]	252 +/- 118	166 +/- 160
Virus Negative CFS Patients	190 +/- 279[4]	215 +/- 213	287 +/- 90

[1] **Mean Luminosity +/- 1 Standard Deviation**

[2] **p = 0.05 compared to controls by Mann-Whitney Test**

[3] **p< 0.03 compared to virus negative CFS patients**

[4] **p< 0.05 compared to controls**

The virus positive CFS patients had a significantly elevated level of STAT1 91/84 kD protein expression compared to both the virus negative CFS patients and the healthy controls. Interestingly, the STAT1 56 kD and 51 kD protein expression was similar in all three of the subject groups.

The reason for the elevated expression of the STAT1 91/84 kD proteins in the CFS patients with detectable paramyxovirus infections is unclear. However, it is tempting to speculate that the viral infection is causing an on-going down regulation or destruction of the STAT1 91/84 kD protein leading to an over-all partial loss of interferon reactivity. The increased expression of STAT1 in the remaining uninfected cells may reflect a homeostatic compensation aimed at maintaining adequate interferon responsiveness. Further investigations, especially those aimed at identifying the specific virus involved in these infections, may yield important clues as to the pathogenic mechanisms responsible for this abnormality in the expression of STAT1 91/84 kD proteins.

References

1. Knox KK, Cocchetto A, Jordan E, Leech D and Carrigan DR. Deficiency in the expression of Stat1 protein in a subpopulation of patients with chronic fatigue syndrome. The Seventh International Conference on Chronic Fatigue Syndrome, Fibromyalgia and Other Related Illnesses. October 8-10; 2004. Madison, Wisconsin.
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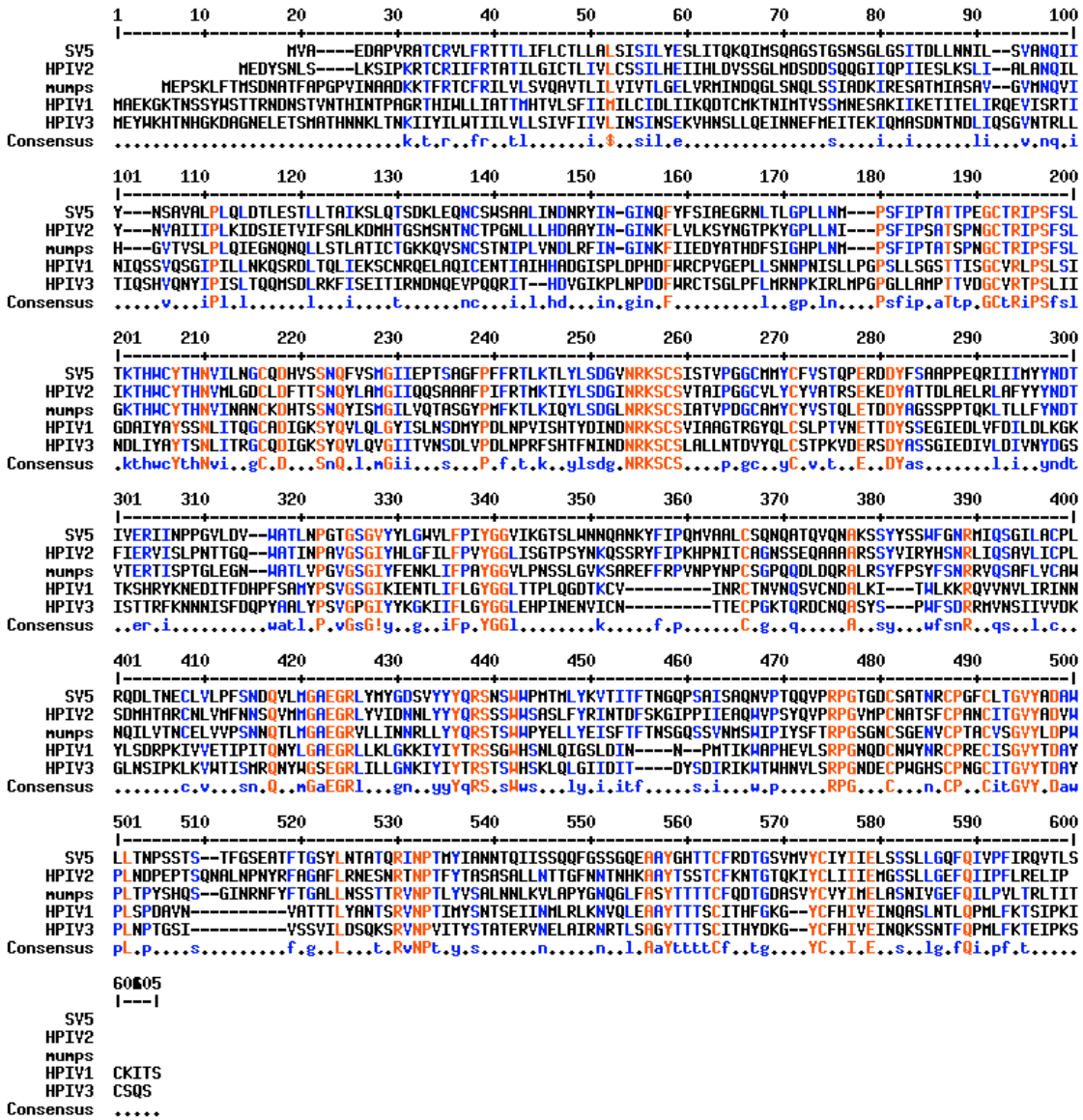
Appendix 3

Alignment of the Hemagglutinin Proteins of Rubula and Respiro Subfamilies of Paramyxoviruses

Alignment of the Hemagglutinin Proteins of Rubula and Respiro Subfamilies of Paramyxoviruses

The observations that (1) an antiserum raised against human parainfluenza virus 3 (HPIV3), a respirovirus, cross-reacts with HPIV1, a respirovirus, and two rubulaviruses (HPIV2 and simian virus five (SV5) by indirect immunofluorescence and (2) the cross-reactivity with SV5 appears to involve the hemagglutinin (HN) protein of SV5 by Western Blot suggested that comparison of the HN proteins of the different viruses might reveal conserved sequences appropriate for the production of a pan-paramyxovirus antiserum. Such a broadly reactive reagent would be an invaluable tool in identifying the virus involved in the CFS patients that have been studied. This would be especially true of the CFS associated virus is related to but distinct from the known human paramyxoviruses. An alignment of the HN proteins of several human respiroviruses and rubulaviruses is shown in the figure below.



Identical amino acids are shown in red, and more than 50% conserved amino acids are shown in blue. Several areas of significant sequence conservation can be identified. Perhaps the most striking area is the sequence NRKSC at position 254 through 259. This sequence is known to be the sialic acid binding site as is indispensable for the enzymatic activity of the neuraminidase function of the HN protein.[1]

A second region of conserved amino acids, especially with respect to the three rubulaviruses, is the sequence bounded by amino acids 180 through 224. The identification of SV5 RNA in the PBMC sample of a CFS patient suggests that the rubulaviruses may be especially relevant in this context so the figure below shows the alignment of their HA proteins.

The sequence conservation from amino acid 160 through 199 is striking. The probable reason for its conservation probably is the fact that it, along with two other regions of the protein, forms the enzymatic active site of the HN protein's neuraminidase activity. In fact, the arginine at position 180 is one of three arginines that are absolutely required for the neuraminidase activity.[2] Therefore, this region of the protein was chosen for antigenicity analysis since it is likely to stimulate antibodies that recognize all human rubulaviruses, and perhaps respiroviruses due to the appreciable conservation of the protein region in them.

References

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2. Taylor, G. Sialidases: structures, biological significance and therapeutic potential. Curr Opin Struct Biol 1996; 6:830-837.

Appendix 4

Antigenicity Analysis of the Mumps Virus HN Protein and the HN 160-199 Peptide

Antigenicity Analysis of the Mumps Virus HN Protein and the HN 160-199 Peptide

As a first step in such an antigenicity analysis, a protein BLAST (Basic Local Alignment Search Tool[1)] search was performed for the HN 160-199 peptide of mumps virus against all primate protein sequences in GenBank (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/). The only significant homology was found with the human mucin 13 protein, and this homology is shown below.

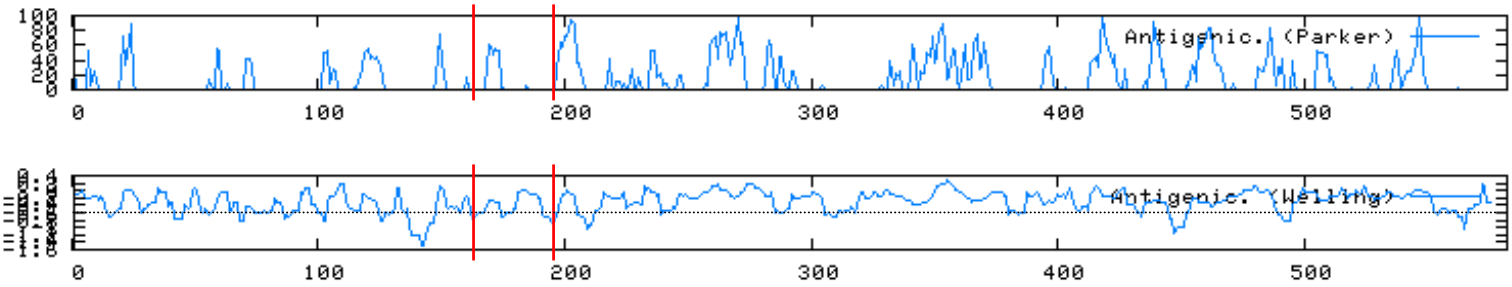
mumps HN amino acid 164: NMPSFIPTATSP

N PSF PTATSP

human MUC13 amino acid 59: N PSF PTATSP

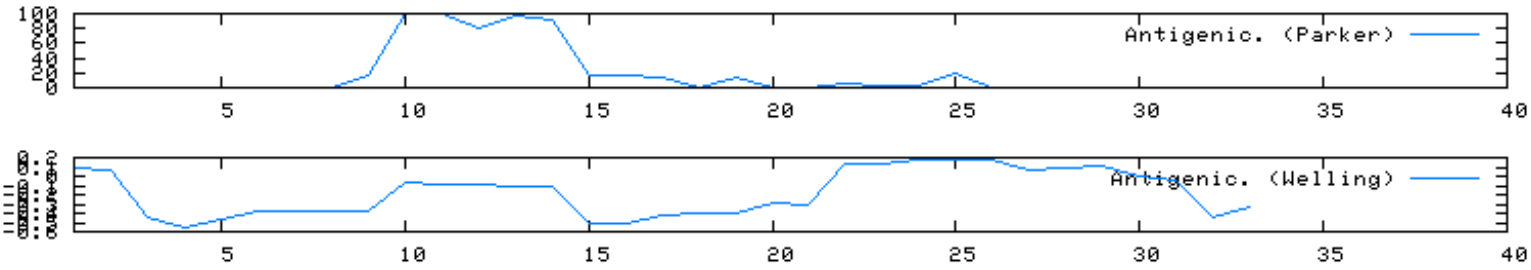
This potential cross-reactivity between rubulavirus HN and MUC 13 may have implications concerning the occurrence of inflammatory bowel disease in CFS patients since mucins are known to be involved in such diseases.[2,3,4 ·]

The antigenicity of the mumps HN protein and the HN 16-199 peptide were assessed by means of a computer program developed by the Pole BioInformatique Lyonnais in Lyon, France.[5] This program utilizes methods developed by JM Parker et al.[6] and GW Welling et al.[7] for the prediction of antigenicity from the primary amino acid structure of proteins. In the Parker et al. method, the protein is assessed for its hydrophilicity, its surface availability and its mobility. In contrast, the Welling et al. method compares the protein under study to the amino acid compositions and sequences of other proteins of known antigenicity. The results for the HN protein obtained by the two methods are summarized below. The higher the value the more antigenic is the protein sequence. The location of the HN 160-199 peptide is shown by the vertical red lines. In the Welling et al. results , the antigenicity cut-off value is indicated by the dashed line.



Amino Acid Number

The results shown in the figures were amplified by applying the antigenicity analysis the the consensus HN 160-199 peptide sequence. These results are summarized in the figures below.



Amino Acid Number

It is apparent that the two antigenicity prediction methods agree that a strong antigenic site exists in the peptide between amino acids 9 and 15. However, the Welling et al. program predicted a second antigenic site between amino acids 21 and 32. Since the two methods differ in their approaches to the amino acids involved, the predicted N terminus and C terminus antigenic sites are indicated in the sequences below by underlining and italics, respectively.

```
SV5          GPLLNMPSFIPTATTPEGCTRIPSFSLTKTHWCYTHNVIL
HPIV2        GPLLNIPSFIPSATSPNGCTRIPSFSLIKTHWCYTHNVML
Mumps        GHPLNMPSFIPTATSPNGCTRIPSFSLGKTHWCYTHNVIN
Consensus    GPLLNMPSFIPTATSPNGCTRIPSFSLGKTHWCYTHNVIL
Human MUC13  STTANTPSF-PTATSPAPPIISTHSSSTIPTPAPPIISTHS
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Since both antigenicity prodiction methods are well established, and have their its own intrinsic strengths, peptides will be chosen for use in the preparation of HN reactive antisera according to them both. First, the N-terminals 20 amino acids [**N - GPLLNMPSFIPTATSPNGCT- C]** will be used for immunization of rabbits in order to obtain an antiserum with potentially important cross reactivity with human MUC13, a protein of potential importance in cancer and the pathogenesis of inflammatory bowel disease.[4] Second, the C-terminal twenty amino acids [**N - RIPSFSLGKTHWCYTHNVIL - C**] will be used to immunize rabbits in order to obtain an antiserum specific for the HN proteins of the rubula subfamily of paramyxoviruses. This antiserum should show little, if any, reactivity with MUC13.

References

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