**Integrative Neural Immune Program Intramural Research Award**

# Role of chronic inflammatory and immune stimulation by active herpesvirus infection in development of immune dysfunction and Mantle Cell lymphoma in Chronic Fatigue Syndrome patients


Francis W. Ruscetti

HEAD, LEUKOCYTE BIOLOGY SECTION

CANCER AND INFLAMMATION PROGRAM

CENTER FOR CANCER RESEARCH

NCI-FREDERICK

DENNIS D. TAUB

HEAD, CLINICAL IMMUNOLOGY SECTION

NIA, BALTIMORE MD

## 1. Project Abstract

In elderly humans, notable changes observed in several components of the immune response. Natural killer (NK) cells and dendritic cells (DC) play a critical role in the innate immune response against infections and tumors and providing links to the adaptive immune response. In younger patients with chronic fatigue syndrome (CFS), similar immune aberrations including, low NK activity, oligoclonal expansions of T cells, and activation of 2-5A synthetase and RNase L antiviral pathway have been noted (1). Dr. Daniel L. Petersen has found a significant incidence of CFS patients with active HHV6A infections, a virus associated with many diseases with neurologic components. In this cohort, 13 out of 50 patients examined demonstrated a clonal T cell receptor-γ chain gene rearrangement with seven of these developing lymphoma, five being mantle cell lymphoma (MCL) (2). Previous studies have shown that CFS patients have an increased incidence of lymphoid malignancy compared to the normal population (3). We hypothesize that chronic inflammatory stimulation from active and recurrent infections of multiple viruses leads to the pathogenesis characterized by CFS with increased incidence of rare lymphoma.

Using this unique cohort of patients, we propose an IRA research project in collaboration with Dr Dennis Taub, Head, Clinical Immunology Section, NIA in which the same CFS pathological materials will be analyzed for viral and immune parameters. The patient samples will be provided by Drs. Dan Peterson and Judy Mikovits of the Whittemore Peterson Institute for NeuroImmune Disease. This research project will provide new insights into the pathogenesis and may reveal novel therapeutic approaches for CFS.

## 2 Specific Aims

The major aim of this project is to define viral and immunologic parameters that correlate with progression of disease in a single cohort of CFS patients, who appear to have defined stages of disease. We have identified a cohort of CFS patients, subsets of whom have progressed to developed T cell abnormalities defined by a clonal rearrangement of TCRγ. A significant proportion of this subgroup has gone on to develop the relatively rare and aggressive MCL. We will compare samples from normal individuals with CFS patients, CFS patients, CFS patients with clonal T cell receptor-γ chain gene rearrangements (cTCRG) and CFS patients with cTCRG who develop MCL. Previous studies have shown that viral specific immune responses and immune abnormalities play critical roles in the pathogenesis of neuroimmune diseases, including CFS (1,4). The specific aims of the project are:

1) Because of the constant threat of emerging pathogens and limitations of most approaches, we will utilize a DNA microarray-based platform for viral

identification and characterization to determine viruses expressed in CSF patients at various stages of the disease.

2) Studies of immune abnormalities in this CFS cohort will involve phenotypic analysis of NK, DC and T cell populations, either from PBMC or cloned cell populations. State of activation and differentiation will be studied; whether the cells are immune activators or suppressors due to response to infection

3) Microarray technology is an efficient means by which the molecular differences between control and diseased tissues can be identified. We will focus on the correlation of viral and immunologic gene expression. The state of inflammation will be studied by an analysis of cytokine and chemokine production using patient sera, CSF, PBMC or cloned immune cells.

4) A cell biological approach to identifying viral and immune defects will be undertaken by creating immortalized cell lines from the CFS patients using human telomerase reverse transcriptase.

For this collaborative project, Drs. Petersen and Mikovits will provide the clinical resources. Dr Ruscetti will provide virological characterization, cytokine assays and associated cell line development support. Dr. Taub will provide the immune cell characterization and immune gene microarray technical support. We are confident that this research project to investigate the pathogenesis of CFS in collaboration with Drs. Taub, Petersen and Mikovits will provide useful new insights into CFS.

## 2. Review of background/preliminary data

In elderly humans, notable changes are observed in several components of the immune response. One of the most striking is the accumulation of oligoclonal expansions of CD4 and CD8 T cells that can comprise a significant component of the T cell repertoire. Natural killer (NK) cells which play a critical role in the innate immune response against infections and tumors are often compromised in the elderly. Herpes viruses that elicit vigorous T cell responses such as CMV, EBV and HHV6 are never cleared from the host following infection. Co-infections with herpes viruses have been shown to impair the immune response to co-resident herpes virus infections (5). We have previously reported a significant incidence of CFS patients harboring monoclonal TCRγ receptor rearrangements and active HHV6 infections and and cancer. (2, 6). Importantly, the rearrangements and tumors are manifested in a younger population, harboring other immunological defects including reduced NK cell function and RNAseL activity suggesting a correlation between long term expression of viruses such as HHV6 and EBV and chronic immune stimulation resulting in immune dysfunction and cancer.

Human herpesvirus-6 (HHV-6) infects several lineages of immune cells including $CD4^+$ T cells and macrophages and is associated with lymphoproliferative and neurological disorders (7). Two variants of the HHV-6, A and B, are associated with disease. The majority of the population is infected with HHV6B with the virus before the age of 2, causing a latent infection. However, a small proportion of the population experiences reactivation of HHV6A and B and other co-resident herpes viruses. We hypothesize that the chronic inflammatory stimulation from low-level active and recurrent infections leads to pathogenesis characterized by CFS and to an increased incidence of rare lymphomas including MCL. No causative association has been established for CFS (1) but HHV-6A has been commonly found in this CFS patient cohort (Table 1). Previous studies have shown that CFS patients have an increased incidence of lymphoproliferative malignancy compared to the normal population (8).

| # | ID # | Age | Sex | HHV-6 PCR | HHV-6 Viral Loads | TCR-γ Clonality | Lymphoma/ Cancer | CIHHV 6 PCR | Viral Subtype |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 3005 | 10 | M | + | | | | + | HHV-6A |
| 2 | 3001 | 55 | F | - | | | | - | |
| 3 | 3002 | 54 | M | + | | | | + | HHV-6A |
| 4 | 3004 | 16 | M | - | | | | - | |
| 5 | 3003 | 24 | M | + | | | | + | HHV-6A |
| 6 | 1379 | 25 | F | + | 2000000 | + | | + | HHV-6A |
| 7 | 1904 | 41 | M | + | | | | - | |
| 8 | 987 | 53 | M | + | 8658 | + | | - | |
| 9 | 1566 | 53 | F | + | 105780 | | | - | |
| 10 | 1282 | 58 | M | + | 187 | + | MCL | - | |
| 11 | 1023 | 67 | F | - | | | | - | |
| 12 | 1736 | 64 | M | + | 22 | | | - | |
| 13 | 1078 | 60 | M | + | 928000 | | | - | |
| 14 | 1987 | 85 | M | - | | + | MCL | - | |
| 15 | 1726 | 64 | F | + | 63000 | + | | + | HHV-6B |
| 16 | 1014 | 58 | M | + | 6660 | | | - | |
| 17 | 1761 | 49 | M | - | | | | - | |
| 18 | 1631 | 45 | F | + | 7160 | | | - | |
| 19 | 1094 | 54 | F | + | 4232 | | | - | |
| 23 | 1281 | 68 | M | + | 29210 | | | ND | |
| 24 | 1614 | 69 | F | + | | + | | ND | |
| 26 | 1815 | 50 | F | - | | + | | ND | |
| 27 | 1689 | 48 | M | + | 39759 | | | ND | |
| 28 | 1380 | 77 | M | + | 6827 | | | - | |
| 29 | 1164 | 55 | F | + | 932 | | | - | |
| 30 | 1143 | 66 | F | + | 50770 | + | | - | |
| 36 | 1857 | 51 | M | + | 9807 | | | - | |
| 38 | 1777 | 49 | M | + | 1309100 | | | - | |
| 42 | 1127 | 62 | F | - | | + | MCL | - | |
| 43 | 2591 | 42 | F | - | | | Parotid tumor | ND | |
| 44 | 2152 | 75 | M | | | + | MCL | ND | |
| 45 | 1674 | 66 | F | - | | + | MCL | ND | |
| 46 | 1849 | 56 | M | - | | + | Hemangioma | ND | |
| 47 | 1369 | 39 | M | + | 6277 | | | ND | |
| 48 | 1581 | 60 | F | + | 5162 | | | ND | |
| 49 | 1933 | 53 | M | + | 244000 | | | ND | |

(ND) represents a sample that was negative for Globin PCR and thus no hair follicle was present.

While the incidence rate of non-Hodgkin's lymphoma is 0.02% in the United States, nearly 5% of the CFS patients positive for HHV-6A have developed the disease (1.8). Additionally, 26% (13 out of 50) of those examined showed cTCRG rearrangement (Table 1). Patients with persistent HHV-6A infection and viral co-infections, such as Epstein-Barr Virus (EBV), might have increased risk of developing clonal rearrangement and lymphoma (6). EBV and EBV-encoded small nuclear RNAs have been found in lymph nodes of all patients with angioimmunoblastic T-Cell lymphoma (6).

Several previous studies have reported an increased incidence of tumorigenesis in CFS patients. One such study reported an increased incidence of primary brain and lymphoid tumors in CFS patients followed longitudinally (8). Herpesviruses have been associated with lymphoma by a variety of mechanisms including gene intergration and viral co-activation. We hypothesize that co-infection of unknown viruses and HHV6A can trigger persistent CFS and result in a dysregulated immune response to viruses as evidenced by cTCRG rearrangements. This chronic immune dysfunction may lead to the development of lymphoma. Moreover, these studies may lead to the identification of a novel virus

associated with MCL. A preliminary viral microarray experiment was conducted in 32 patients of the cohort according to the methods detailed in the Experimental design section. A total of 1608 viral transcripts, microRNA or endogenous viral elements (Sines, Lines) were observed. The top 300 to 600 transcripts were reported for each subject. Herpes viruses predominated among cTCRG and MCL patients with a much lesser extent of expression in control patients. Adenoviruses and rhinoviruses were the predominant viruses expressed in healthy controls. Human endogenous retroviral elements were also expressed. Since the envelope protein of HERV-W can cause neurodegeneration (9), the expression of these elements in CFS will also be studied

## 4 Experimental Approach

Here we propose to investigate the association of Herpesvirus co-infections and immune status in a cohort of CFS patients with clonal TCRγ rearrangements and an increased incidence of MCL. Specific aims to be addressed are as follows:

**Aim 1. Microarray-based detection and verification of viral pathogens in CFS patients at different stages of disease.**

A viral detection DNA microarray composed of oligonucleotides sequences of all known viruses will be used to identify any viral pathogens in the CFS patient cohort. The analysis will be preformed in conjunction with the Molecular Diagnostics Group, Laboratory of Molecular Technology, NCI-Frederick. This viral gene microarray is a highly sensitive method to detect as few as 200 copies of expressed virus of more than 100 viruses including all human herpesviruses and human endogenous retroviruses (10) and has been used to identify a novel gammaretrovirus in prostate tumors (10, 11). Because simultaneous expression of several viruses may activate endogenous viruses or suppress immune response to co-resident viruses, these data can provide significant insight into the pathogenesis and immune dysfunction seen in CFS and CFS associated tumorigenesis. HHV6 has recently been shown to accelerate AIDS in Macques (12).

Approximately 75 human samples will be used in the study. Their age, gender clinical parameters, and other demographics will be recorded. Samples will be coded for anonymity and identities, associated with codes will not be known to the investigator. RNA and DNA will be prepared using Trizol (Invitrogen, Carlsbad, CA). Total RNA extracted from PBMC will be amplified and fluorescently labeled in a sequence-nonspecific method (by the MTL in Frederick). The amplified and labeled fragments, which contain host as well as potential viral sequences, will then be hybridized to a DNA microarray bearing the most conserved sequences. A positive hybridization will determine which viruses are harbored and expressed by each patient in the study at the time the sample was prepared. In this way longitudinal samples can be tested to correlate viral gene expression with disease onset.

Viral sequences chosen for verification will have positive hybridization signal for a substantial number of patients samples, and present in samples prepared at two different times and arrays to eliminate "batch effects". To obtain viral clones, a direct microarray recovery technique (DNA eluted from array spot will be re-amplified and plasmid libraries constructed) will be used (10,11). Specific primers will be used to determine the homology of the virus recovered to previous isolate from the same viral genus.

**Aim 2. Immortalizing clonal NK and T cell lines from the CFS patients using human telomerase reverse transcriptase (hTERT) for viral and immune studies**

Another method of isolating novel viruses would to cloned directly from patient samples but it is more practical to establish cell lines. Transduction of human T cells with xlox(LNGFR)hTERT by magnetofection has resulted in immortalization of both normal CD4 and CD8+ humans T cells which remain IL-2 dependent (13). T cells have to be actively proliferating as MuLV-based retroviral vectors transduce only dividing cells. The materials for this protocol come from OZ BioSciences. A detailed protocol is available at http://www.ozbiosciences.com/English/viromag.htm. After overnight incubation the cells are taken off the magnetic plate and incubate for 48-72 hours to allow for retroviral expression before analyzing the NGFR expression by FACS, which is routinely between 30-50%. The cells will be sorted for this expression followed by limiting dilution cloning for T cell clones. We will also try to establish MCL cell lines. These immortalized cell lines can be usual for examining viral production and be used as a source of material for identification of viral pathogens (14). Immortalized normal cell lines will be used to attempt to isolate viruses from these CFS patients (15)..

**Aim 3 Studies of immune abnormalities in this CFS cohort will involve phenotypic analysis of NK, DC and T cell populations from PBMC or cloned cell populations.**

Heparinized PBMCs will be isolated from peripheral blood by ficoll density gradient separation for the number of cells of the innate and adaptive immune susyem will be determined using commercially available mABs and standard procedures for flow cytometry analysis. T cell differentiation will be measured by intracellular flow cytometry for IFN-γ (Th1) IL-4, (Th2), IL-17 (Th17), foxP3 (T-regulatory cells). Since NK cells are primarily responsible for host defense against viral infection. NK deficient individuals are particularly susceptible to recurring viral infections (16, 17) . The large diversity of killer cell immunoglobulin like receptors (KIR) and NK cell and some T cells have led to studies of there involvement in human disease (18). The inhibitory and activating roles of KIR in viral and inflammatory diseases are now emerging. The distribution of KIR on NK cells in these patient populations will be important to determined. In addition, the activated and/or tolerized state of the dendrtic cells will be determined though the production of IL-12 vs IL-10.

**Aim 4. Microarray technology focusing on immune gene expression will be used to identify molecular differences between control and CFS tissues.**

Different approaches will be used to identify genes whose expression is dysregulated in CFS. Microarray analysis has proven useful in comparing normal and pathological tissues in neurological (19), viral (20) and MCL (21) . Total RNA will be isolated from tissue samples of the CFS cohort at the different stages of the disease. RNA will be labeled and hynbrized to the Agilent 44K oligomicroarray kit according to the manufacturer's protocol. Genes of interest from the microarray will be sequenced verified. Gene specific primers will be constructed for real time SYBR green PCR. In addition, the state of inflammation will be studied by an analysis of cytokine and chemokine production using patient sera, CSF, PBMC or cloned immune cells. Pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α will be assayed in multiplex suspension arrays using Luminex technology. The cellular sources for aberrant cytokine or chemokine production will be studied using intracellular cytokine staining. PCR for IgG heavy chain and TCR-γ rearrangement and chromosomal translocations will be performed by multiplex PCR analysis (In VivoScribe, San Diego, CA).

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