FY 2010 BENCH-TO-BEDSIDE PROPOSAL

XMRV, a member of gammaretrovirus family known to cause neoplasia in animals, was previously found in tumor-bearing prostate tissues of individuals homozygous for a variant of RNase L (R462Q) (1). We have recently reported in *Science* the isolation of infectious and transmissible XMRV from the blood of CFS patients, implicating XMRV in the pathology of CFS (2). XMRV nucleic acids were detected in the PBMC of 68 of 101 (67%) of CFS patients whereas 8 of 218 (3.7%) regional, healthy controls contained XMRV DNA. Infectious virus was transmitted from activated primary PBMC as well as from purified B and T cell cultures and plasma derived from CFS patients by establishing a secondary infection in uninfected primary lymphocytes and LNCaP prostate cancer cell line. **Our discovery opens up a new area of research with many unanswered questions: How does XMRV infection affect innate immune response? What are the key immune cell targets? It is present in other immunocompromised individuals? The goal of this proposal is to determine the incidence of XMRV in neuroimmune disease and lymphoma and identify potential therapeutic targets of the innate immune response.** We and others have developed quantitative assays to detect XMRV replication and methods for infection in culture. In addition, we have access to 3 large cohorts of CFS patients and 3 cohorts of cancer patients in which to investigate**. This project and resources will contribute to advancing our knowledge of the immune system's relationship with XMRV, and contribute to understanding the pathogenesis of CFS, which may lead to new therapeutic interventions. Successful completion of these aims will identify patient populations enable clinical trials of both anti-retrovirals and immune modulating therapies in Phase II.**

Aim 1 Determine the incidence of XMRV in additional neuroimmune diseases and non-Hodgkin's lymphoma (NHL) and prostate cancer. Determine which antivirals can control XMRV expression.

Aim 2. Determine the effects of XMRV infection on NK or other members of the innate immune systems; 2a) Evaluate phenotypic and functional responses to XMRV infections of primary NK, dendritic and gamma-delta T cells in vitro. 2b) Evaluate innate immune cell responses to immune modulators in XMRV infected CFS patients ex vivo. 3.a) Determine the ability of immune modulators such as Ampligen™ and other TLR agonists and antagonists to restore NK cell responses without increasing XMRV expression.

BACKGROUND and SIGNIFICANCE

Recent data have led to the concept that constant exposure of retroviruses (HIV, HTLVII) to the cells of innate immune system results in their chronic hyperactivation with harmful effects on the T cells which drive adaptive immune response. In particular, constant stimulation of plasmacytoid dendritic cells (pDCs) and NK cells in humans drives immune system dysfunction underlying AIDS progression (3).

CFS is a disease characterized by severe chronic fatigue lasting at least 6 months which is accompanied by symptoms such as impairment in short-term memory or concentration, sore throat, tender lymph nodes, and muscle pain (4), with an incidence of 4 adults per 1,000 in the United States. Among other components chronic immune cell dysfunction and activation has been demonstrated by several groups (5-8).More recently, CFS subjects were reported to have significantly fewer $CD3^{+}CD25^{-}$ T cells and significantly more $CD20^{+}CD5^{+}$ B cells (9), a subset associated with auto-antibodies. Significantly fewer $CD56^{+}$ NK cells were also found (6) as well as reduced NK cell activity in CFS, associated with low levels of intracellular perforin (5). Down-regulation of TGF-β and IFN-α, and up-regulation of IL-6, TNF-α and of the CXC chemokines IL-8, MIP-1α and MIP-1β has been observed (10,11**). The mechanism(s) underlying the dysregulation of the immune system in CFS are consistent with our hypothesis that XMRV infection results in the establishment of an immune deficiency in part by directly or indirectly altering NK cell function or phenotype**.

Many Cells of the innate immune system, NK cells, dendritic cells, gamma delta T cells and macrophages have evolved specifically to the front line of host defense against microbial infection. The innate immune system initiates and regulates the adaptive immune response through interaction with T lymphocytes. Viruses have devised numerous strategies to evade the activation of NK cells and this way escape NK cell recognition and suppress the natural anti-viral immunity of the host. (12)

In recent years, data have accumulated on the association of viruses with immune deficiencies and the development of cancer. Among other virus (i.e. EBV, HPV, HCV, etc), the retrovirus HIV and Human T-lymphotropic virus Type I (HTLV-1), are known to cause immune deficiencies and be associated to the development of Kaposi's sarcoma, NHL and T-cell leukemia/lymphoma, respectively (13). In 1998, Levine et. al. reported NK cell dysfunction in a family with CFS. In this family 5 of 6 siblings and 3 other family members developed CFS as adults. All of the affected family members had persistently low NK cell activity and unaffected family members had intermediate NK cell function compared to healthy controls. In this family of 5/6 siblings with CFS 2 of their offspring developed pediatric malignancies (14). Thus, CFS patients with persistent viral infections might have increased risk of cancer. **This study supports our hypothesis that XMRV infection of B, T, NK and other immune cells causes the chronic inflammation and immune deficiency resulting in an inability to mount an effective immune response to opportunistic infections, as seen in CFS, and development of malignancy**.

SIGNIFICANCE

The detection of XMRV in 3.75% of the control population in our recent study suggests that ten million Americans could be infected with this recently identified human retrovirus of unknown pathogenic potential. These studies will pave the way

for clinical trials of antiretroviral and immune modulating therapies in XMRV associated neuroimmune disease and cancer

PRELIMINARY DATA

Previous studies have shown that CFS patients have an increased incidence of lympho-proliferative malignancy compared to the normal population (15). While the incidence rate of non-Hodgkin's lymphoma is 0.02% in the United States, nearly 5% of the CFS patients developed the disease. Additionally, development of cancer coincides with an outgrowth of gamma delta T cells with specific cTCRG rearrangements and XMRV infection (Table 1 below).

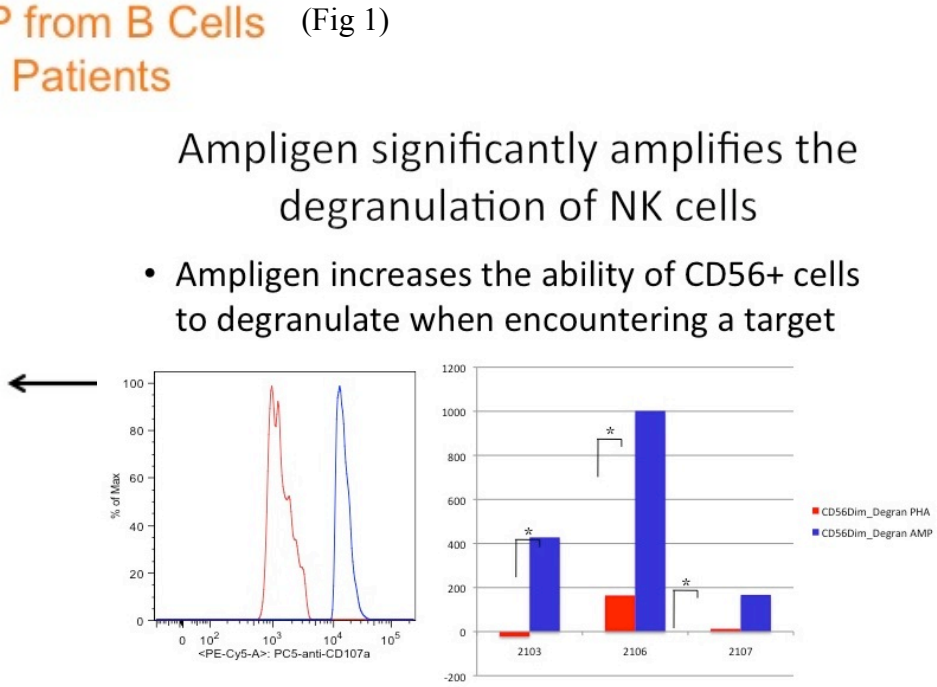
Nevada CFS Patients with Cancer

ID#	XMRV status	Clonal TCRγ	Lymphoma/cancer
1103	positive	positive	MCL
1109	positive	negative	Thymoma
1118	positive	negative	myelodysplasia
1125	positive	Positive + IGH	MCL
1186	positive	positive	Lymphoma
1199	positive	positive	Previous Lymphoma
1150	positive	positive	Lymphoma
1320	Not tested	Not tested	Thymoma
1321	Not tested	Not tested	MCL
1174	positive	positive	Thymoma
1205	positive	Not tested	lymphoma
1172	positive	positive	MCL
1135	positive	positive	suspicious
1204	positive	Positive + IGH	suspicious
1113	positive	positive	CLL
1322	Not tested	Not tested	MCL
1181	positive	Not tested	CLL
1188	positive	positive	CLL
1189	positive	positive	MCL
1190	positive	positive	suspicious

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 (15). We hypothesize that XMRV and perhaps co-infection of unknown viruses can trigger persistent CFS and result in a dysregulated immune response to viruses as evidenced by out growth of cells with specific cTCRG rearrangements. This chronic immune dysfunction may lead to the development of lymphoma.

In the course of our studies, we have developed B cell lines from 2 MCL and 1 CLL patients with a long standing history of CFS. Each of this B cell lines are producing infectious XMRV.(Figure 1, above) HIV depression of immune system in AIDS leads to the development .of AIDS associated Lymphoma. Patient 1125 B cell line positive for XMRV was XMRV positive in 1984 (Fig 1, above) was seen for CFS at the NIH in 1988. He was also seen at the NIH for lymphoma in 2000. The development of lymphoma more than a decade later does not invalidate the original CFS diagnosis. XMRV has been

associated with prostate cancer and we can isolate from the plasma of these patients (Fig 1)



In other recent studies, we have observed that peripheral blood $CD56^{+}CD3^{-}$ NK cells from healthy donors show increased CD107a expression compared to CFS patients when PBMCs were incubated with the HLA class I-deficient K562 targets and anti-CD107a Ab for 4 hours (data not shown). CD107a is a surrogate marker of degranulation and NK cell cytotoxicity. One immune modulator, Ampligen™, has shown efficacy in ~13% of CFS patients tested in clinical trials for more that a decade (unpublished data, Hemispherx) but the mechanism or target is as yet unknown. We hypothesized that one mechanism determining efficacy may be the ability to upregulate NK cell function as measured by degranulation as discussed above. To test this hypothesis, we isolated PBMC from patients harboring NK cells with decreased functional capacity and incubated them ex vivo with Ampligen™ and showed increased degranulation in 28 of the 30 patient cells tested (Figure 3 above)

EXPERIMENTAL DESIGN

Aim 1 Determine the incidence of XMRV in additional neuroimmune diseases and non-Hodgkin's lymphoma (NHL) and prostate cancer.

We have established collaborations with clinicians with CFS, NHL and prostate cancer corts, each with extensive clinical data on these patients including:

- Drs. Paul Cheney (NC), David Bell (NY) and Eric Gordon (CA): CFS
- Drs. Richard Burack, (NY), Michael Snyderman (NY) and Nam Dang (FL): NHL
- Dr William Dahut (NIH): Prostate Cancer

We will determine the status of XMRV infection in these patient populations (50 minimum form each population) using the quantitative assays to detect XMRV replication and methods for infection in culture, which we and others have developed. Thus, we can determine if the ability to detect XMRV has any impact on anti cancer response to protocol based therapy (NIH clinical center in Phase II) and identify patient populations for antiretroviral clinical trials in CFS patients (to be done at WPI in collaboration with GSK and Gilead). The preclinical studies are in progress and sponsored by the pharmaceutical companies and are not part of this phase I proposal but the patient populations and therapeutic interventions identified can be part of phase II the

clinical component of this proposal. The WPI is under contact with Phlebotomy Services international, a HIPAA compliant provider for NIH grants. The immunological and XMRV infection data, which will be coded, will be maintained in a secure database. If investigators become aware of data with exceptional potential clinical significance, they will determine how best to proceed, in consultation with the NIH and ethics advisors. While benefits to the patients are unpredictable, new knowledge about the pathophysiology of XMRV infection in CFS, cancer and other neuroimmune diseases will be directly applicable to the development of novel therapeutic strategies for these complex and poorly understood diseases.

Aim 2. **Determine the effects of XMRV infection on NK or other members of the innate immune systems**

We will correlate levels of viral replication with degrees of NK cell-mediated inhibition of XMRV replication in the presence and absence of immune modulators. XMRV replication as well as number of infected cells will be quantified as previously described (2). We will monitor changes in phenotype (CD56, CD94, NKG2A, NKG2D, and KIR). Reduction in the number of infected CD4+ T cells by NK cell-mediated cell death (membrane damage) will be evaluated by using Annexin/PI staining (flow cytometry) using Abs against CD56, CD3 and CD4 to distinguish populations. We will also determine levels of cytokines and CC chemokines in all cell cultures supernatant by Luminex. We hypothesize that IFN-β produced by virus-infected CD4+ T cells and NK cell-produced IFN-γ inhibits XMRV replication. The percentage of different cell populations of NK cells secreting IFN-γ will be measured by flow cytometry using IFN-γ-specific Catch Reagent. We will evaluate the effects of blocking IFN-α, IFN-β and/or IFN-γ in the co-cultures on the ability of NK cells to inhibit XMRV replication using specific neutralizing Abs against these cytokines (Research Diagnostics). infection and correlate with sensitivity of targets to NK cell-mediated lysis To determine whether NK cells require contact with infected cells to mediate viral inhibition, NK cells and XMRV-infected CD4+ T cells will be separated in transwell experiments. We will perform blocking inhibitory KIR studies using anti-KIR Abs to block the interaction between KIR and MHC class I to further determine which KIR are determinant in anti-viral responses mediated by NK cells.

STATISTICAL METHODS

We will use AS, SAS-JMP version 9.1.3, and GraphPad Prism Version 3.0cx software to manage and analyze the data. Differences between parametric and non-parametric data will be made using the Shapiro-Wilks test. Parametric data will be analyzed using the Student's t test and non-parametric data will be analyzed using the Mann-Whittney test. Kruskal-Wallis, Analysis of variance with Tukey's correction, non-linear regression, linear regression, logistic regression will be appropriately used. Additional data analysis methods may be necessary upon completion of exploratory data analysis.

Importance of knowledge to be gained:
The patients will be part of a groundbreaking biological study that will help investigators understand the underlying immune deficiency associated with XMRV infection. XMRV is the first known human gammaretrovirus infection shown to infect T, B cells in vivo. Knowledge of the effect of XMRV interaction/infection of NK cells may uncover the mechanisms of pathogenesis of XMRV associated neuroimmune disease and cancer and lead to novel treatment statement strategies that prevent disease progression in XMRV infected individuals.

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