Productive Replication of XMRV in Peripheral Blood Mononuclear Cells of Chronic Fatigue Syndrome Patients.

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XMRV sequences have previously been identified in prostate tissue biopsies of patients with familial prostate cancer. We have identified similar sequences in 29 of 42 (69%) peripheral blood mononuclear cell (PBMC) samples from a well-defined cohort of patients diagnosed with Chronic Fatigue syndrome (CFS). Viremia was identified in both plasma and cerebrospinal fluid from these patients. In addition, XMRV production in primary patient PBMCs (17/20), in conjunction with PCR data from archived samples, revealed infection of more than 75% of this CFS cohort with XMRV. Infectious virus, produced from primary purified B and T cell cultures and present in plasma derived from CFS patients, was demonstrated by establishing a secondary infection in both normal primary lymphocytes from healthy donors and indicator cell lines. Budding retrovirus particles were identified by Transmission Electron Microscopy (TEM) in primary and secondary infections developed from CFS patients. Full length clones(?) revealed 98% homology with previously isolated clones of XMRV. These data show the first PBCs productive infection of XRMV in a human disease population and support a role for XMRV infection in the pathogenesis of CFS.

The recent identification of XMRV genomes in a subset of men with prostate cancer harboring a specific mutation in the antiviral endoribonuclease, RNase L, suggests that other human diseases may involve infection by XMRV particularly in populations with defects in antiviral response pathways (1,2). Chronic Fatigue Syndrome (CFS) is a multi-system disorder, manifested by inflammatory sequelae including, innate immune activation, RNaseL dysfunction and low Natural killer cell (NK) number and function, prompting us to test the hypothesis that XMRV is associated with CFS.

To investigate prevalente of XMRV infection in CFS, we first screened retrospective samples from our repository, by nested PCR for the XMRV gag gene as previously described (3). These samples were collected from more than 100 patients of a well-characterized CFS cohort between 2006 and 2008 and stored in trizol (Invitrogen) prior to nucleic acid isolation. Of the retrospective samples analyzed 69% contained sequences 96% homologous to XMRV (Fig. 1 A,B). To examine the degree of XMRV sequence diversity in different patients, we sequenced the amplified fragments from 10 of the 27 samples, which were positive by the nested gag RT-PCR. The amplified gag fragments were highly similar with >98% nt and 98% aa identity to each other. All CFS patient derived gag sequences were more similar to each other than they were to any other sequence. Analysis of fresh patient material sent to the NCI in 2007, confirmed identical gag sequences from the same patients (data not shown). In addition to the gag gene, we also examined the same patient samples for sequence variation in the pol and env genes. We sequenced PCR fragments obtained with a set of primers targeting a >2500 nt stretch of the gag-pol gene (in progress Fig.1C). Like the gag fragments both the env and the gag-pol fragments were highly similar…..(finish these data).

We next cloned the full length virus from the PBMC of 3 patients. XXXX (Finish these data) (Fig 1C,D). These findings suggest that the observed XMRV sequence variation is a result of natural sequence diversity consistent with the virus being independently acquired by the affected patients. These findings in different laboratories argue against laboratory contamination as a source of XMRV in the CFS patient PBMC.

To further confirm that the XMRV sequences detected in the CFS PBMCs were part of an ongoing productive, transmissible infection, we developed intracellular flow cytometry and western methods using monoclonal antibodies to SSFV gag and Rausher Viral

gp70,p10,p30,RT to detect viral proteins in PBMCdrawn fresh from patients blood, whether or not they contained XMRV gag sequences by PCR. PBMC were isolated from heparinized whole blood by ficoll hypaque density separation according to standard techniques. PBMC were activated using phytohemagglutinin (PHA, Sigma) at 10ug/ml in RPMI complete media. After 3 days, recombinant human interleukin 2 (rhIL2) was added to the cultures at 20ng/ml. Three of 11 patients PBMCs tested positive for multiple viral proteins (Fig. 2A).Two tested positive only for p30 (Fig 2B) and nine healthy control PBMC cultures tested negative (Fig 2C). Little is known about the tissue tropism of XMRV, therefore we purified B and T cells from one of the patient's PBMC and demonstrated that both T and B cells were infected with XMRV (Fig 2D). A B cell line developed from one of the patients in 2007 also tested positive for multiple viral proteins (2E). These results were confirmed by western analysis using antisera to purifed viral proteins (in progress 2E)

Since it well established that efficient retroviral production requires proliferating cells and some human retroviruses such as HTLV-1 are not directly observed to be producing virus in peripheral blood without stimulation, we therefore postulated that stimulating cells to divide using PHA and IL2 would result in the production of infectious XMRV that could be transmitted to indicator cell lines. Activated PBMC cells expressing XMRV proteins were co-cultured with two indicator cell lines, LNCaP and Human foreskin fibroblasts (HFF). LNCaP is a metastatic androgen sensitive prostate cancer cell lines with defects in both the JAK STAT pathway and the RNaseL pathway. The HFF cell line is normal with respect to the levels of phosphorylated STAT 1 in response to interferon beta stimulation (data not shown). Interestingly, none of the 42 patient samples initially tested positive for env PCR. However upon activation and co-culture with the indicator cell lines, both HFF and LNCAP co-cultures expressed env sequences (Fig 3A and multiple viral proteins as detected by western analysis (Fig3B). Cell free supernatants form the co-cultures were filtered through0 0.1uM filter to remove cells and cellular debris and virus was concentrated by ultracentrifugation over a 20% glycerol cushion producing abundant90-*100nM particles consistent with a type C retrovirus (Fig 3C).*

To investigate possible routes of transmission, we next examined plasma from heparinized blood that had been frozen in liquid nitrogen within one hour of blood draw and shipped on dry ice. Viral RNA was isolated from 300ul of plasma and the XMRV gag gene amplified in a one step quantitative RT-PCR procedure. Plasma viremia was detected in 4 of the five patients tested and 1 of the 10 regional controls with copy numbers ranging from 100-5000 copies per ul (table 2 1?). To determine if XMRV detected in plasma was infectious , we employed a virus Isolation spinning protocol which has previously shown to greatly amplify ability to isolate clinical isolates of CMV( ). Plasma and 0.22uM filtered cell free supernatant were diluted 1:1 and added to a six-well culture plate with the HFF indicator cell line or primary PBMC isolated from healthy donors. The plate was centrifuged at 1500 rpm in a table top centrifuge for 5 min, turn plate 180, and centrigfuged again for 5 min. The supernatant with virus was decanted immediately and cells cultured and subcultured for 10-14 days and the cells tested by FCM for the presence of viral proteins (Fig 4A) amd confirmed by western (inprogress Fig 4B) and PCR (in progress 4C). We confirmed cell free transmission to the T cell line SupT 1 by placing cultures of patient PBMC in the top of 0.2 uM transwells in 6 well plates. After 3 days the transwells were removed and SupT1 assay for XMRV gag sequences by RT-PCR (Fig 4 D). These results strongly support routes of both cell associated and cell free transmission of XMRV in this patient population.

The prevalence of XMRV among the general population is not yet known. Our results showing only 1/100 normal PBMC positive by PCR and lack of viral expression in 20 activated normal PBMC samples (ip) suggests that the prevalence is low. Since XMRV was originally discovered in a subset of prostate cancer patients withthe R462 Q variant of the RNase L viral defense

gene, we investigated the association of XMRV infection and the R462Q RNASEL genotype of the CFS patients and found there was no correlation (data not shown). This does not rule out other defects in the interferon pathway are related to infection and spread.

The molecular clone isolated from the peripheral blood of CFS patients is most closely related to the molecule XMRV clones contructed from the patients with familial prostate cancer. Our demonstration that infectious virus is present inT and B lymphocytes, cells of the immune system well known to be major targets of human retroviral infection. In contrast to the close homology between the close of XMRV found in cancer and neuroinflammatory diseases, the seguences had <XX% nt and< XX identity to the most related exogenous sequence of MuLV in DG-75 , a human lymphoid cell line.

Retroviruses such as several MuLVs, primate retroviruses and HTLV-1 are not only associated with cancer but also associated with neurological diseases. Investigation of the molecular mechanism of retroviral induced neurodegenration in rodent models ( ), vascular and inflammatory changes mediated by cytokines and chemokines are noted before any neurological changes. Viral copy number has been reported to play a role in the development of HTLV-1 associated myelopathy ( ). Retroviral involvement has been suspected not only for CFS but also for other neurological diseases such as Multiple Sclerosis (MS) and amyotropic lateral sclerosis. McCormick et al recently explored the candidacy of XMRV of XMRV in ALS ( ) They did not find XMRV in the blood or CSF of the 25 ALS where reverse transcriptase (RT) was detected. The finding of a novel infectious retrovirus in a majority of patients with a chronic inflammatory, immune suppressed and cognitive dysfunction syndrome suggests that XMRV plays at least a partial role this pathogenesis. Furthermore, we hypothesize that a higher incidence of neoplasia might be associated with chronic XMRV infection

Fig 1. Identification of XMRV in CFS patient PBMC by PCR (A) Results of nested PCR specific for XMRV gag gene amplifed from cDNA or DNA of 75 CFS patients vs. 5 of 98 healthy controls. Amplified gag PCR fragments along wih corrsponding human globin amplification controls were separated by gel electrophoresis. Representative results form one of the groups of 20 patients and controls is shown. (B) PCR products were excised form the gel, purified and sequenced. The sequence was consistent with XMRV. The sequence was 96% homologous with XMRVS. Sequence variation in the CFS Patient cohort is consistent with the infections being independently acquired. (C) Multiple-Sequence alignment of 5'gag nucleotide sequences from XMRV and patient viurses

A.

MW 1551 1674 1185 1942 plasmid control

B. Sequence alignment XMRV gag and env genes from 5 CFS patients.

Figure from VCL here
1185 gag PCR product
…

1942 gag PCR product
…

C. Genome sequence similarity plots comparing XMRV VP62, MULVDG-75, MTCR and sequences of complete genomes from WPI1185,WPI1942, WPI1282. The alignments were made using XXXX ( ) and plots were generated using XXX ( )

Fig. 2 Identification of XMRV viral proteins in CFS PBMC
A Intracellular FCM using goat anti-Rauscher viral proteins. Blue line normal goat sera; black antiviral proteins
1282 B cells 997 T cells normal T cells

B. Viral proteins p10 and p30 are present in both T anc B cells of CFS patients

997 CD4+ T cells

1282 CD19+ B Cells

Normal CD4+ T cells

Fig3. Transmission of XMRV from CFS PBMC

Figure 4. Cell free transmission of XMRV.

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MW 1 2 3 4 5 6 7 8 9

1 Marker 100 bp, 2 Raji, 3., 2740, 4. 2902, 5. H1566; 6. H2740
7., H1815 (no transwell), 8 SupT1 ; 9. H2740 (no transwell)