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Abstract Book

1st International Workshop on XMRV

Pathogenesis, Clinical and Public Health Implications

7 -8 September 2010, Betheseda, USA

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Abstracts

Oral Presentations

Abstract: O_01

Basic virology

Screening mouse genomes for XMRV-like elements

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Background: XMRV represents the first reported case of a gammaretrovirus infection in humans. It bears very close resemblance to some of the endogenous xenotropic MLV (Xmv) proviruses found in large numbers in the genomes of inbred mice. XMRV was initially isolated from a subset of prostate cancer patients; it was then detected in a number of chronic fatigue syndrome patients as well as in some healthy subjects. Although its association with human disease is currently the subject of ongoing debate, the implication of cross-species transmission has prompted us to study its possible origins.
Despite the high degree of sequence identity, the XMRV genome is clearly distinct from all Xmvs described so far. Over the course of evolution Xmvs have successfully invaded the genomes of wild and inbred mice and some of these elements have the ability to produce infectious virus. Given the high degree of similarity between XMRV and endogenous Xmvs, we hypothesized that there could be a reservoir of XMRV or an ancestral virus that gave rise to XMRV in mice.

Materials & Methods: Although a provirus identical to known XMRV isolates is not present in the sequenced mouse (C57BL6) genome, there exists the possibility that other strains of laboratory or wild mice might harbor XMRV-like sequences. We sought to determine whether we could find such elements in various mouse genomes. We used four different approaches to search for XMRV-like endogenous elements: 1) A highly sensitive PCR-based assay, 2) Single genome amplification, 3) Genomic hybridization blots, and 4) *In silico* searches of sequenced mouse genomes. Initial studies focused on a characteristic deletion in the *gag* leader region not found in any of the sequenced proviruses.

Results: We screened genomic DNA samples from 75 different wild and inbred mouse strains representing a total of 18 *Mus* subspecies with XMRV-specific primers. This study was complemented by a single genome amplification (SGA)-based approach from the genomic DNA of a number of selected mouse strains. We have found evidence for the presence of an XMRV-specific subsequence in at least one strain, although the full genome sequence for the provirus is not yet available. We then analyzed total genomic DNA from different strains of interest by hybridization with XMRV-specific probes, along with *in silico* searches of whole genome shotgun reads from publicly available sequencing projects.

Conclusions: Our findings suggest that there might be endogenous proviruses present in various mice that harbor sequences previously described as XMRV-specific. The nature and prevalence of these elements or whether they have in any way contributed to the evolution of XMRV will require further characterization.

No conflict of interest

Abstract: O_02

Basic virology

A novel gene product of the prostate cancer associated retrovirus, XMRV

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Xenotropic Murine Leukemia Virus-Related Virus (XMRV) is a retrovirus initially identified in human prostate cancer specimens through its sequence similarity to gammaretroviruses. Several subsequent studies have confirmed the presence of XMRV transcripts or proteins in

biological fluids and cells of prostate cancer patients, whereas others have not. While extensive sequence homology exists between XMRV and Murine Leukemia Viruses, the most divergence is found in the envelope (env) coding region.
Here we report the identification of a novel alternatively spliced subgenomic transcript produced early during XMRV viral infection.
By RT-PCR and northern blotting, we show that various XMRV infected cell lines display different degrees of expression of this novel transcript when compared to the known singly-spliced env transcript of XMRV. Cloning and sequencing analyses of the novel cDNA product reveals that the novel splice variant is 1.2-kb in length and lacks most of the env coding region. We also show though protein expression studies in vitro and mass spectrometry analysis of XMRV infected cells that the novel transcript encodes a putative protein of ~11 kDa. Fluorescence microscopy was performed to determine subcellular localization of the novel protein in vitro.
Multiply-spliced viral transcripts in other complex retroviruses are known to encode proteins (e.g., HIV Rev, HTLV Rex, and MMTV Rem) that bind unspliced and singly-spliced viral RNAs in the host-cell nucleus and facilitating their export to the cytoplasm. Multiply-spliced viral subgenomic RNA transcripts that give rise to various regulatory and accessory proteins is the defining feature of complex retroviruses.
The existence of a novel spliced transcript in XMRV-infected cells implies that the diversity of transcripts and proteins from XMRV is greater than previously supposed.

No conflict of interest

Abstract: O_03

Therapeutics/ Vaccine (animal models)

Wild-derived mouse (Mus pahari) as a small animal model for XMRV infection

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Introductions: A novel human gammaretrovirus, xenotropic murine leukemia virus-related virus (XMRV), has been identified in patients with prostate cancer and chronic fatigue syndromes. However, the etiological link and the mode of transmission remain to be determined. For better understanding the XMRV transmission, tissue tropism and pathogenicity, the development of a small animal model for XMRV infection is crucial. Although standard laboratory mice have provided important small animal model systems for many human diseases, they cannot support XMRV infection due to the lack of a functional receptor. Previously, Dr. Kozak and colleagues have demonstrated that XMRV can use $Xpr1^{p}$ from Gairdner's Shrew-mouse (*Mus pahari*) for infection. In this study, we examined whether *Mus pahari* can support XMRV replication *in vitro* and *in vivo*.

Materials and methods: *Mus pahari* mice were purchased from Jackson laboratories. Cell-free XMRVs were harvested from a prostate cancer cell line 22Rv1 and from 293T cells transfected with an infectious molecular clone pVP62 (kindly provided by Dr. Silverman).

Results: When infected with a XMRV Env-pseudotyped, GFP-expressing retroviral vector, primary *Mus pahari* fibroblast cells were efficiently transduced to express GFP, confirming the expression of a functional XMRV receptor in this wild mouse species. Upon infection with cell-free XMRV, primary fibroblasts expressed XMRV proteins and produced cell-free virions, indicating that *Mus pahari* cells support early and late phases of XMRV replication. *In vivo* XMRV infection study showed a single XMRV injection induced high levels of XMRV neutralizing antibodies after 2 weeks injection. Western blotting revealed the presence of XMRV Env- and Gag-specific antibodies in plasma at 2 and 8 weeks post infection, respectively. XMRV proviral DNA was frequently detected in spleen, blood and brain, suggesting that XMRV is lympho- and neuro-tropic in *Mus pahari*. Notable G-to-A hypermutations in the viral sequences isolated from spleen suggested the possible role of innate restriction factor APOBEC3 in restricting XMRV replication *in vivo*. Except for panleukopenia in a subset of XMRV-infected mice, no notable clinical signs or pathological changes were observed in our short-term infection study for up to 2 months.

Conclusions: *Mus pahari* can support XMRV replication *in vitro* and *in vivo*. *Mus pahari* may be used to study the possible pathogenesis of XMRV, to address the mode of XMRV transmission, or to evaluate the efficacy of antiviral interventions.

No conflict of interest

Abstract: O_04

Therapeutics/ Vaccine (animal models)

XMRV induces a chronic replicative infection in rhesus macaques tissues but not in blood.

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Background: Xenotropic MuLV-related Retrovirus (XMRV), a gammaretrovirus, has been identified in association with familial cases of prostate carcinoma and in patients with Chronic Fatigue Syndrome, although an etiological link remains to be established. To address this issue, we established an animal model to study XMRV dissemination, tissue tropism and pathogenicity was essential for understanding its role and infection transmission.

Methods: Five rhesus macaques were experimentally infected with XMRV IV and followed for various periods of time post infection and euthanized during acute infection (n=2) or during chronic infection on days 146 and 289 post infection. The animals were monitored for immune parameters and viral replication as well as extensive tissue collections and in situ analyses performed at necropsy

Results: XMRV infection although successful in macaques, did not show evidence of pathogenesis during the 9 months follow-up. The infection raised relatively low antibody response as well as mostly undetectable cell mediated immune responses to the virus, suggesting limited antigenic stimulation. This correlated with a rapid clearance of virus from the blood after infection. Surprisingly though, upon detailed in situ analysis of the various organs and tissues at various times post infection, virus replication was clearly detected in all animals by FISH and IHC techniques. Evidence of fully disseminated infection was confirmed by the sensitive FISH although the levels of FISH and IHC signal varied greatly by organ. XMRV is lymphotropic in lymphoid organs, including spleen, blood, lymph nodes and the GI mucosa, although in lung, macrophages were the predominant target. Of interest was the finding of XMRV replication in reproductive organs, such as prostate, seminal gland, testis as well as vagina and cervix, suggesting a potential for sexual transmission. Unique among these sites, prostate showed extensive foci of infected acinar epithelial cells during acute but not chronic infection during which IHC results were negative but low level signals were still detected by FISH. Virus replication in other tissues appeared to vary less between acute and chronic infection.

Conclusions: An animal model of human XMRV infection has been established with which long-term chronic infection, pathogenesis, immunity and the validation of future vaccines can be studied, although expansion of the model is urgently needed.

No conflict of interest

Abstract: O_05

Host restriction factors

Evolution and species distribution of functional and sequence variants of the mammalian XPR1 receptor for XMRV and mouse xenotropic gammaretroviruses

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Introduction: Genetic conflicts between retroviruses and their receptors result in the evolution of novel host restrictions on entry and novel virus envelope variants. The xenotropic/polytropic mouse leukemia viruses (X/P-MLVs) rely on the XPR1 cell surface receptor for entry into host cells. In this study, we examined the evolution of *Xpr1* in rodent species and the co-evolution of *Xpr1* and X/P-MLVs in *Mus* species, and we extended this functional and sequence analysis to nonrodent species to uncover additional determinants for virus entry and to identify entry restrictions that may modulate trans-species transmissions.

Materials & methods: We screened rodents and other mammals for *Xpr1* sequence variation and for susceptibility to 7 X/P-MLVs we previously determined to have 6 distinct tropisms in rodent cells (Retrovirology 6:87,2009). The X/P-MLVs included 2 X-MLVs, 2 P-MLVs, 2 wild mouse isolates and the human-derived XMRV. Mammals selected for analysis include species that serve as pets, farm animals, primates and other zoo animals. Residues contributing to host range restrictions were identified by site-specific mutagenesis and by phylogenetic analysis of *Xpr1* sequence variants for positive selection using the PAML suite of programs.

Results: Among cells of mammalian species, we identified 10 distinct phenotypes distinguished by resistance to different X/P-MLVs; these include the 4 known *Xpr1* variants in *Mus* and a novel 5th *Xpr1* found in *M. molossinus* and *M. musculus* that is highly restrictive. We describe the geographic and species distribution of the 5 functional *Xpr1* variants in *Mus* and their evolutionary association with endogenous X/P-MLVs. All non-*Mus* mammals tested are susceptible to mouse-derived X-MLVs, but some are resistant to P-MLVs or wild mouse X/P-MLVs, and two species are uniquely resistant to the human-derived XMRV. Mutational analysis identified 6 specific residues important for mouse X/P-MLV entry and demonstrated that XMRV relies on X-MLV entry determinants but uniquely requires at least one additional residue.
Phylogenetic sequence analysis of the two extracellular loops (ECLs) critical for virus entry demonstrated that the rodent *Xpr1* is under positive selection, indicative of the diversifying selection that can mark antagonistic host-pathogen interactions; one of the two ECL3 residues under strong positive selection is critical for virus entry. The ECL4 sequence is hypervariable, but contains 3 nonvariant residues; mutation of these residues does not affect virus infectivity indicating that the evolutionary constraint on these 3 residues is unrelated to receptor function. All tested mammalian cells carry functional *Xpr1* receptors suggesting that ECL4 can sustain significant hypervariability without losing receptor function. Interestingly, the 3 virus-restrictive *Xpr1* variants in MLV-infected mouse species show an unusual pattern of variation; these species uniquely carry different deletions in ECL4 suggesting that loop size may be important for receptor function.

Conclusions: The XPR1 receptor is highly polymorphic, and although all mammals carry functional receptors, these differ in their ability to restrict the various human or mouse derived viruses due to sequence substitutions or deletions in the two ECLs that carry receptor determinants. These findings illuminate the evolution of these gammaretroviruses, their co-evolution with host cell receptors, and their transmission to other species.

No conflict of interest

Abstract: O_06

Prostate cancer

Variant XMRVs in clinical prostate cancer

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Background: While XMRV has been linked to prostate cancer there has been little analysis of variant forms of integrated pro-viral sequences in clinical samples. There is a single intriguing report of a high-transforming variant of XMRV that contained additional host genomic sequences that resulted from multiple passages in mammalian cell culture. No reports have yet documented additional sequences being carried by XMRV in actual clinical specimens.

Materials & methods: Using whole viral genome amplification protocols that our group has used extensively in defining HIV variant sequences we performed whole-XMRV amplifications from prostate cancer tissues. Amplicons of at least 8kb were gel purified and sequenced.

Results: Three separate tissues from three separate prostate cancer specimens revealed whole-XMRV amplicons of greater than the predicted molecular weight for XMRV. One specimen consistently produced an amplicon of approximately 9kb. Multiple partial sequences confirm that the amplicon contained XMRV sequences.

Conclusions: Clinical prostate cancer samples contain integrated XMRV pro-viral sequences, which can be amplified in nested PCR approaches with approximately 1000 more bases than can be attributed to XMRV alone. These additional DNA sequences, if contained within the XMRV genome, could contain DNA that is relevant to either the promotion of prostate cancer or the survival of XMRV in the malignant prostate.

No conflict of interest

Abstract: O_07

Prostate cancer

Failure to detect XMRV in prostate cancer and benign prostatic tissues

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Introduction: Xenotropic Murine Leukemia virus Related Virus (XMRV) was first identified in human prostate tissues from prostate cancer patients. Subsequent studies on the prevalence of the virus have produced inconsistent results, leaving the link between the virus and prostate cancer unclear.

Materials and methods: We applied a quantitative real-time PCR assay and two well-controlled immunohistochemistry (IHC) assays for the detection of XMRV in freshly obtained and archival prostate tissue specimens.

Results: Our real-time PCR assay reliably detected XMRV DNA from a single cell's worth of genomic DNA (gDNA) from 22Rv1 cells, an XMRV-infected cell line, even in the presence of a vast excess of uninfected cell gDNA. Our IHC assays utilized two rabbit polyclonal antisera, specific for murine leukemia virus $p30^{CA}$ and $gp70^{SU}$. Both antisera showed robust immunostaining of 22Rv1 cells and XMRV-transfected cells, but did not stain a number of uninfected cell lines. We tested 161 prostate tumor-derived DNA samples by real-time PCR and 596 prostate tumor tissue specimens by IHC. Our tumor tissues were enriched for cases with high Gleason score (e.g., ≥ 7) and included a number of metastatic lesions. We also tested 452 prostate tissues containing a variety of benign pathologies by IHC. We did not detect XMRV in any of the samples tested.

Conclusions: The findings suggest an extremely low prevalence, or absence, of XMRV in benign and malignant prostate tissues in men living in North America.

K.S.S. and A.L.A. contributed equally to this work.

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No conflict of interest

Abstract: O_08

Prostate cancer

XMRV prevalence in prostate cancer tissue and the role of the prostate compartment in XMRV infection

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Background: XMRV is the third retrovirus after HTLV-1 and HIV identified in a bonafide human infection. The virus has been identified in prostate cancer samples from patients with familial prostate cancer who additionally carry mutations in the RNase L gene (R462Q) (Urisman, Molinaro and Fischer et al., 2006). The XMRV provirus is not endogenous to the human genome and there are no similarities to human endogenous retroviral sequences, suggesting that XMRV is transmitted by exogenous infection.
XMRV has gained significant interest due to recent studies reporting viral protein expression in up to 23% of all prostate cancer cases and detection of XMRV specific sequences in PBMCs of 67% of patients with chronic fatigue syndrome (CFS) (Schlaberg et al. 2009; Lombardi et al. 2009). Studies conducted in Europe, however, failed to detect or showed only a low prevalence of XMRV in prostate cancer samples. Differences in the patient cohort, geographical restriction and non standardized detection may be responsible for the observed differences.
It is conceivable that XMRV-infected stromal or epithelial cells could produce paracrine signals that stimulate cell proliferation in the surrounding epithelia. The present study addresses the following important questions: (1) Prevalence of XMRV in prostate cancer in Northern Europe. (2) In vivo restriction of XMRV to prostate cells. (3) Putative role of XMRV infected prostate cells (stromal fibroblasts and epithelial cells) in tumorigenesis.

Methods: (1) tissue samples (n=355) and PBMCs (n=40) from PCA patients in Northern Europe as well as control samples (n=70) from men without evidence of PCA were analyzed for the presence of XMRV specific sequences by nested RT-PCR. In addition, XMRV protein expression was analyzed in prostate cancer tissue sections (whole sections and tissue microarrays) using XMRV-specific antisera.
(2) infecton, replication and spread of replication competent XMRV was tested in vitro.
(3) Primary human stromal fibroblasts, isolated from prostate tissue, and prostate cancer epithelial cells were examined for changes in gene expression as well as release of cytokines following XMRV infection.

Results/Conclusions: (1) XMRV was detected in two tissue samples; all tested PBMCs were negative for XMRV sequences. The two XMRV positive patients were wild type or heterozygous for the R462Q mutation and thus carried at least one fully functional RNase L allele. (2) XMRV efficiently infects and spreads in prostatic cells, XMRV-LTR activity is significantly increased and retroviral restriction factors are expressed at low levels. Prostate epithelial cells or a population of prostatic stromal fibroblasts could be one important reservoir for XMRV replication in vivo.(3) On a global level, gene expression measured by whole human expression arrays show only subtle changes after XMRV infection. Interestingly, however, cytokine antibody arrays revealed significant differences in cytokine release of XMRV infected stromal fibroblasts and prostate epithelial cells. These results are strengthened by migration/invasion assays. Our findings suggest that XMRV-infected cells may contribute to the pathogenesis of prostate cancer via paracrine mechanisms, which may explain the finding that XMRV-infection is frequently restricted to a subset of fibroblasts in cancerous tissues.

No conflict of interest

Abstract: O_09

Prostate cancer

XMRV infection of prostate cancer patients from the Southern United States and analysis of possible correlates of infection

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Background: The prevalence and geographic distribution of xenotropic murine leukemia virus-related virus (XMRV) among prostate cancer (PCA) patients remains unknown, and reports regarding the association of XMRV infection with the ribonuclease L (*RNASEL*) R462Q polymorphism are conflicting. We therefore investigated whether XMRV could be detected in PCA patients from the southern United States (U.S.), and sought to verify the association of XMRV with the R462Q polymorphism.

Methods: Prostatic tissue specimens of 144 PCA patients from the southern U.S. were genotyped for R462Q by real time PCR allelic discrimination and screened for XMRV proviral DNA by nested PCR targeting the variable region of the *env* gene. Detection of XMRV-specific DNA was confirmed by sequencing and phylogenetic analysis. For 55 of the 144 patients, both tumor and normal prostatic tissue was available for screening.

Results: The R462Q polymorphism was present in the patient population with an allelic frequency of 33%, which is a representative number for cohorts of unselected or sporadic PCA cases. XMRV was detected in 32 (22%) of the 144 patients. XMRV was detected in normal tissue, and patients were significantly more likely to test positive for XMRV in both tumor and normal tissue rather than either alone ($\kappa = 0.64$). Positivity for XMRV was not significantly correlated with the R462Q polymorphism ($p = 0.82$) or clinical pathological parameters of PCA, including Gleason score ($p = 0.29$), seminal vesicle invasion ($p = 0.33$), extracapsular extension ($p = 0.59$), or surgical margin invasion ($p = 0.89$). Successful detection of XMRV by PCR was dependent on the quantity of patient DNA used as template and on the viral gene targeted for amplification.

Conclusions: XMRV is present in PCA patients from the southern U.S. It can be detected in both normal and tumor prostatic tissue, and infection is independent of the R462Q polymorphism and clinical pathological parameters of PCA. The presence of XMRV in normal tissue suggests that infection may precede cancer onset.

No conflict of interest

Abstract: O_10

Chronic Fatigue syndrome/ neuro immune diseases

Prevalence of XMRV in CFS patients and healthy controls

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Background: In 2006 a novel gammaretrovirus, XMRV (xenotropic murine leukemia virus-related virus), was discovered in some prostate tumors. A more recent study indicated that this infectious retrovirus can be detected in 67% of patients suffering from chronic fatigue syndrome (CFS), but only very few healthy controls (4%). However, four groups have published to date that they could not substantiate the reported data in other cohorts of CFS patients. Since there is a high degree of similarity between XMRV and abundant endogenous murine leukemia virus (MLV) proviruses, it is important to distinguish contaminating mouse sequences from true infections.

Materials and methods: DNA was prepared from peripheral blood lymphocytes and tested for XMRV by the following methods: A Taqman qPCR assay (1) was used to detect a sequence in the IN portion of the XMRV pol gene, and two different nested PCR assays (2, and newly

developed by our group) were used to detect sequences in the XMRV gag gene. Contamination of mouse DNA was monitored with a Taqman qPCR assay for murine mitochondrial cytochrome oxidase (Switzer, CDC, personal communication), and a conventional PCR assay for sequences in the highly abundant intracisternal A-type particle (IAP) long terminal repeat sequences (developed by our group).

Results: While the assay for XMRV integrase sequences was able to detect a single XMRV-infected cell (6 pg DNA) in 5 micrograms of human genomic DNA, it yielded negative results in the test of 600 ng genomic DNA from the peripheral blood of 184 CFS patients and 25 healthy controls. On the other hand, positive results were obtained with some of these samples, using either one of the two nested PCR assays for the XMRV gag gene. DNA sequencing of the PCR products revealed identical sequences as described from prostate cancer and CFS patients, as well as sequences that were more closely related to known endogenous MLVs. However, all samples that tested positive for XMRV or MLV DNA were also positive for mouse IAP and mitochondrial DNA, using either assay. The source of this apparent contamination is under investigation.

Conclusions: Mouse cells contain upwards of 50 copies each of endogenous MLV DNA. Even much less than one cell's worth of DNA could yield a detectable product using highly sensitive PCR technology. It is, therefore, vital that contamination by mouse DNA be monitored with adequately sensitive assays in all samples tested.

References: *Schlaberg, R., D. J. Choe, K. R. Brown, H. M. Thaker, and I. R. Singh. 2009. XMRV is present in malignant prostatic epithelium and is associated with prostate cancer, especially high-grade tumors. Proc Natl Acad Sci U S A 106:16351-16356. 2. Urisman, A., R. J. Molinaro, N. Fischer, S. J. Plummer, G. Casey, E. A. Klein, K. Malathi, C. Magi-Galluzzi, R. R. Tubbs, D. Ganem, R. H. Silverman, and J. L. DeRisi. 2006. Identification of a novel Gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant. PLoS Pathog 2:e25.*

No conflict of interest

Abstract: O_11

Chronic Fatigue syndrome/ neuro immune diseases

XMRV in Chronic Fatigue Syndrome: A Pilot Study

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Background: In October 2009, Lombardi et al reported detection of XMRV in blood of persons with CFS. Since then, four studies that performed PCR analysis on DNA from blood of CFS patients failed to detect XMRV. The present blinded study was undertaken to determine whether XMRV could be detected in peripheral blood mononuclear cells (PBMCs) from three small groups of subjects from a single geographic area.

Materials and methods: The 30 adult subjects of this study were divided into three groups of ten persons each: severely ill with CFS, recovered from CFS, and a control group lacking a CFS diagnosis at any time. Inclusion did not require that they became ill in the time frame or in the exact location of a cluster of CFS in Lyndonville, NY. All patients in group 1 ("severe CFS") currently meet Fukuda criteria. The individuals in group 2 ("recovered CFS") had met Fukuda criteria for CFS at one time in the past, but presently considered themselves recovered. One patient in group 1 and seven in group 2 are considered part of the "Lyndonville outbreak". The ten persons included in group 3 "healthy, non-household contact controls" have never experienced a CFS-like illness, are healthy, and have never lived with persons with CFS. Average ages of the three groups are 42.2, 39, 49.8, respectively; and M:F ratio is 2:8, 6:4, 2:8. All thirty subjects completed seven different clinical survey instruments including the SF-36.
Blood was collected in EDTA tubes and PBMCs and plasma separated within 24 hrs of draw. Plasma from some samples was incubated with LNCaP cells. The Invitrogen SuperScript® VILO™ cDNA Synthesis Kit was used to

produce cDNA from RNA isolated from PBMCs or LNCaP cells. Nested PCR with USB Hot-Start IT FideliTaq was performed using the Gag O-Gag I primers originally described by Urisman et al. PCR products were separated on gels and any bands of approximately 400 bp were sequenced and analyzed by BLAST for similarity to the XMRV *gag* gene. PCR with mouse COX2 primers was performed on all cDNA preparations to rule out mouse cell contamination.

Results: XMRV *gag* sequences were detected in eight of the "severe CFS", three of the "recovered CFS" and in one of the controls. Plasma from six blood samples, five CFS and one control, was incubated with LNCaP cells; the six cultures were passaged six times. The five cultures found to exhibit XMRV *gag* sequences were those inoculated with CFS patient plasma. Although group 2 members described themselves as recovered, their scores on the SF-36 were significantly lower than the healthy control group, according to Hoteling's T2 test. Tukey's multiple comparison of means indicates that there are highly significant differences between the scores of the "severe" and controls on all 7 instruments.

Conclusions: XMRV *gag* sequences were detected in a higher percentage of severe or recovered CFS cases (55%) compared to controls (10%) with one-sided p-value = 0.0118. Human plasma samples from CFS cases contain infectious XMRV. Our results corroborate those of Lombardi et al.

No conflict of interest

Abstract: O_12

Chronic Fatigue syndrome/ neuro immune diseases

Detection of MLV-related virus gene sequences in blood of patients with chronic fatigue syndrome and healthy blood donors

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Background: Chronic fatigue syndrome (CFS) is a debilitating disorder defined solely by clinical symptoms and the exclusion of other diseases; its distribution is wide and its cause(s) unknown. A number of objective immunological and neurological abnormalities have been found more often in patients with CFS than in healthy controls or in patients with other fatigue-inducing illnesses. Various microbial and viral infections have been implicated as possible triggers of CFS. A recent study identified DNA from a xenotropic murine leukemia virus-related virus (XMRV) in peripheral blood mononuclear cells (PBMCs) from 68 of 101 patients (67%) by nested PCR, as compared with 8 of 218 (3.7%) healthy controls (Lombardi et al., 2009). However, four subsequent reports failed to detect any murine leukemia virus (MLV)-related virus gene sequences in blood of CFS patients

Methods: By nested PCR assays targeting the MLV-related virus *gag* gene, using both the previously described primer sets (Lombardi et al., 2009; Urisman et al., 2006) and an in-house-designed primer set with highly conserved sequences from different MLV-like viruses and XMRVs, we examined DNA prepared from the blood samples of 37 CFS patients for the presence of MLV-like virus *gag* gene sequences. In addition, RNA was prepared from the deep-frozen plasma samples of these patients and analyzed by RT-PCR assay. DNA extracted from frozen PBMC samples of 44 healthy volunteer blood donors, was tested in parallel

Results: 41 PBMC-derived DNA samples from 37 patients meeting accepted diagnostic criteria for CFS were examined by PCR. We found MLV-like virus *gag* gene sequences in 32 of 37 patients (86.5%) compared with only 3 of 44 (6.8%) healthy volunteer blood donors. In 42% of samples, we also detected and sequence-confirmed the presence of MLV-related viral RNA in the frozen plasma samples of these CFS patients, using RT-PCR. A highly sensitive and specific semi-nested PCR assay targeting mouse mitochondrial DNA was developed to detect trace amounts of mouse DNA. No evidence of contamination with mouse DNA was detected in the PCR assay system or any of the

clinical samples. Seven of 8 *gag*-positive patients were again positive in a sample obtained nearly 15 years later. However, in contrast to the previously reported findings of near-genetic identity of all XMRVs, we identified a genetically diverse group of MLV-related viruses. The *gag* and *env* sequences from CFS patients were more closely related to those of polytropic mouse endogenous retroviruses than those of XMRVs, and were even less closely related to those of ecotropic MLVs

Conclusion: Our results support the report of finding XMRVs or MLV-related viruses in high frequency in patients with CFS. However, further studies are needed to determine whether the same strong association with MLV-related viruses is found in other groups of patients with CFS, whether these viruses play a causative or secondary role in the development of CFS, as well as the implications of the viral diversity.

No conflict of interest

Abstract: O_13

Chronic Fatigue syndrome/ neuro immune diseases

Detection of infectious XMRV in the peripheral blood of chronic fatigue syndrome patients in the United Kingdom

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Introduction: Since publishing the detection of infectious XMRV in CFS patients, several studies failed to detect XMRV in CFS or prostate cancer. More sensitive and specific methods for the biological and molecular amplification of XMRV in unstimulated blood cells and plasma have since been developed and we used these to determine if XMRV was present in UK CFS patients.

Materials and methods: Peripheral blood was drawn from fifty CFS patients under informed consent in Ashford England by Phlebotomy Services International Inc. Blood was shipped to NCI-Frederick where plasma and PBMC were isolated in a non-retrovirus lab. Serology and virus isolation were performed in two different labs. Since the most important variable in detecting XMRV in CFS is patient selection, the patients in the study all met the requirements for CFS based on, the most rigorous criteria used worldwide. We performed multiple methods for XMRV detection on these samples, the most sensitive blood-based assays for detection of XMRV in decreasing order are: 1) performing nested PCR for gag or env sequences or western analysis from LNCaP cells that have been co-cultured with subject's cell-free plasma or with cell-associated or cell-free supernatant from PHA/IL-2 activated PBMCs, 2) the presence of antibodies to XMRV Env in subject's plasma, 3) presence of gag products by nested PCR on stimulated PBMCs or detection of viral proteins expressed by activated PBMCs with appropriate antisera, 4) nested RT-PCR of plasma nucleic acid and 5) PCR of DNA from unactivated PBMC prepared from subject's blood. Some form of amplification, either molecular or biologically increases XMRV detection.

Results This study demonstrates that infectious virus was present in >60% of infected CFS patients in a UK cohort. XMRV could be transmitted either cell-associated or cell-free from both activated lymphocytes and plasma from infected individuals by passage to LNCaP. Cell-free supernatant from these infected cultures contained infectious virus, which could be passed to other permissive cell lines. Using an newly developed ELISA for detecting XMRV antibodies, plasma from of individuals with infectious virus, was reactive to the envelope protein of XMRV, showing that XMRV elicits an immune response in these patients. We developed a new set of primers for XMRV ENV, which allows detection of both XMRV and polytropic MULV sequences, we were able to detect env sequences in PBMC of seven patients before co-culture. Sequencing showed that it is identical to XRMV in this region of Env.

Conclusions: Demonstration that XMRV can be detected in a subset of CFS patients in England indicates there is a wider distribution of XRMV in the world. The study of XMRV is in its infancy, and much more information is needed concerning replication and pathogenesis of this virus in humans. Priorities include the absolute need for assay validation between groups to be used clinically, including the development of sensitive nucleic acid and serologic tests for high throughput screening and the development of therapeutics for clinical testing. Validated positive and negative panels are urgently needed and we hope to provide them.

No conflict of interest

Abstract: O_14

Assay development & Screening

Development and optimization of a multiplex serological assay to detect XMRV antibodies

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Introduction: XMRV studies in prostate cancer or chronic fatigue syndrome (CFS) have provided conflicting evidence for the detection of the virus in these conditions. There are also limited and variable reports of the prevalence of the virus in healthy donor populations. As an initial step in characterizing the sero-prevalence of XMRV in disease states and in healthy populations, we initiated the development of a multiplex, ELISA-based test for antibodies to XMRV in human plasma.

Materials & methods: Our approach was to individually clone, express and purify each XMRV antigen in different systems to simultaneously evaluate the optimal production criteria for each antigen evaluated in this platform. The individual assays were developed on the Meso Scale Discovery (MSD) platform to facilitate multiplexing. Using normal unscreened donor plasma (N=77) and clinical specimens from WPI-CFS patients XMRV-positive (by a qPCR, nested PCR, virus culture and/or antibody test) (N=39), assay conditions were rigorously defined. Non-parametric Receiver Operator Characteristic (ROC) of each assay/antigen was evaluated and the relevant area under the curve recorded, utilizing the DeLong method for calculating standard errors. To identify potential assay cutoffs in each assay, two criteria were utilized, minimizing misclassification or maximizing correct classification. For each potential cutoff, sensitivity, specificity and likelihood ratios were calculated. Refinement of assay parameters and cut-offs are ongoing through testing and evaluation of >750 normal donor plasmas including confirmatory assays including Western blots.

Results: Reactivity to XMRV recombinant antigens is statistically higher in XMRV positive subjects than in normal donor plasma. Subjects that had been determined to be XMRV positive by another test are most reactive in the recombinant SU and TM antigen tests. Antigens with antibody reactivity were down-selected and a scoring algorithm has been developed and optimization of the assays continues.

Conclusions: These assays and platform have demonstrated utility in the screening of donor plasma for antibodies to XMRV. Continued identification of XMRV positive subjects is necessary to the development and optimization of any clinical assay and remains of critical importance.

No conflict of interest

Abstract: O_15

Assay development & Screening

Development of a GFP-indicator cell line for the detection of XMRV

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Introduction: Human immunodeficiency virus (HIV) titer can be estimated using indicator cell lines, such as GHOST cells, within days of infection. HIV indicator cells rely on production of Tat to transactivate expression of a reporter gene under the control of HIV LTR sequences. Simple retroviruses typically do not encode transcriptional transactivators. For simple retroviruses that lack transformational or cytopathic activity, their titers are often measured by infection of cells after end point dilution and assaying for virus proliferation after weeks of culture. Replication-dependent vectors have been leveraged to assay the mobilization of retrotransposable elements and the replication of retroviruses. Here we describe an indicator cell line for the detection of infectious xenotropic murine leukemia virus-related virus (XMRV) that relies on the propagation of a vector, which leads to expression of a GFP reporter.

Materials and methods: We constructed an MLV vector encoding puromycin resistance and a CMV enhancer/promoter driven GFP reporter gene whose transcription was antisense to the vector mRNA. The GFP reporter sequence (iGFP) was interrupted by an intron placed in the sense direction relative to the vector. The prostate cell line, LNCaP, was stably transfected with the above construct, and puromycin-resistant cell clones were obtained and assayed for sensitivity to XMRV infection via transduction and activation of GFP. GFP was detected by fluorescence microscopy or FACS analysis of paraformaldehyde-fixed indicator cells. XMRV was obtained from supernatants of CWR 22Rv1 cells or HEK 293T cells transfected with the pVP62 molecular clone.

Results: Several LNCaP-iGFP cell clones displaying sensitivity to XMRV infection after end point dilution were isolated and designated Detectors of Exogenous Retroviral Sequence Elements (DERSE) cells. GFP signal could be detected within three days of infection, with the number of GFP-positive cells increasing over subsequent days. Infection was enhanced by the addition of polybrene to cultures. Plasma and serum samples from persons previously found positive for XMRV detectably infected the DERSE cells; whereas samples from XMRV-negative individuals did not induce GFP expression in the cells even after weeks of culture. Compared to detection of infection using LNCaP parental cells, XMRV from patient samples was observed two to three times faster in DERSE cells. GFP signal after virus inoculation was dose-dependent and could be impaired by heat inactivation of virus stocks or the addition of AZT to cultures at the time of infection.

Conclusions: Here we describe DERSE cells, a new indicator cell system for the sensitive and rapid detection of replicating XMRV. In principle, DERSE cells should also detect other gammaretroviruses capable of infecting human cell lines. Because this indicator cell system utilizes GFP as a reporter, infection can be monitored in live cultures by fluorescence microscopy. Consistent with prior reports using LNCaP cells, DERSE cells detect virus from human blood samples.

Abstract: O_16

Assay development & Screening

Development of XMRV immunoassays useful for epidemiologic studies

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Background: Xenotropic Murine Leukemia Virus-related virus (XMRV) is a human gammaretrovirus identified in prostate cancer tissue and in lymphocytes of chronic fatigue syndrome (CFS) patients. To establish the etiologic role of XMRV infection in human disease requires large scale epidemiologic studies. Development of highly sensitive XMRV antibody and confirmatory assays would provide useful tools to facilitate such studies.

Materials and methods: Recombinant XMRV proteins, p15E, gp70 and p30, were used to develop three direct chemiluminescence immunoassays (CMIAs) on the automated ARCHITECT® instrument system. Sensitivities of the assays were evaluated with seropositive serial bleeds from XMRV-infected rhesus macaques, goat antisera to Friend-MuLV or to envelope of Rauscher-MuLV. Specificities of these assays were evaluated with 2262 US blood donor samples and 110 HIV or HTLV seropositive samples. In addition a Western Blot (WB) confirmatory assay was developed using purified XMRV viral lysate as well as recombinant proteins.

Results: The p15E and gp70 CMIAs demonstrated 100% seroconversion sensitivity by detecting all seropositive primate bleeds: specificity was 99.9 and 99.5%, respectively, for blood donors. Furthermore, none of the 110 HIV or HTLV seropositive samples were reactive in either assay confirming absence of cross-reactivity. Both assays exhibited excellent discrimination between the seropositive primate bleeds and the negative blood donor population. The anti-p30 response was lower in the XMRV-infected primates, leading to a p30 CMIA seroconversion sensitivity of 92%; specificity for blood donors was 99.4%. Based on end point dilution of primate bleeds, assay sensitivity could be ranked as gp70 CMIA > p15E CMIA > p30 CMIA. In addition, antibodies to Friend-MuLV or the envelope of Rauscher-MuLV were detected by the prototype assays with end point dilutions of 1:10,000 -1:64,000. The WB confirmatory assay detected antibodies to all XMRV structural proteins and showed good seroconversion sensitivity.

Conclusions: Three automated high-throughput assays were developed to detect the predominant antibody responses to XMRV infection. Based on the excellent sensitivity and specificity of the gp70 and p15E CMIAs, these prototype assays provide important new tools for large scale epidemiologic studies of XMRV infection in humans.

Abstract: O_17

Assay development & Screening

Validation of XMRV single-copy assays (X-SCA) to detect xenotropic MLV-related virus in human blood products

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Background: The human retrovirus XMRV (xenotropic murine leukemia virus-related virus) was recently identified and reported to be associated with prostate cancer and chronic fatigue syndrome. To clarify the public health impact of this new virus, it is necessary to develop reliable, specific, and sensitive assays for its presence in blood cells and plasma.

Methods: We developed and validated quantitative PCR assays called XMRV single-copy assays (X-SCA) (similar to the HIV single-copy assay, SCA) that can detect XMRV DNA and RNA in whole blood, PBMCs, and plasma. These new assays will be used to assess the prevalence of XMRV and its associations with human disease. A blinded panel of XMRV-spiked patient samples was generated by the Blood XMRV Scientific Research Working Group and was used to assess the performance of X-SCA methods for detecting XMRV DNA in whole blood and RNA in plasma. Whole blood was spiked with half-log serial dilutions of XMRV-infected Cw22Rv1 cells to final concentrations of 9900 to 0.5 cells/ml. Plasma was spiked with 1:5 dilutions of XMRV-infected Cw22Rv1 cell supernatants to final concentrations of $2.5x10^5$ to 0.128 virus copies/ml. Six XMRV-negative samples were included in both the whole blood and plasma panels. Samples were assayed in triplicate.

Results: All XMRV-unspiked whole blood and plasma samples were properly identified as XMRV negative by X-SCA. These results demonstrate that X-SCA did not result in false positivity due to either nonspecific amplification or DNA contamination of assay materials. XMRV DNA was detected in 0.5ml of whole blood spiked with each dilution of infected cells from 9900 to 0.5 cells/ml. Samples were assayed in triplicate and XMRV DNA was detected as expected by Poison distribution. Sensitivity of X-SCA for detecting XMRV RNA was limited to >3.3 virus copies/ml due to low volume of plasma availability (0.3mL for XMRV detection). All dilutions containing >3.3 XMRV copies/ml were reported as positive by X-SCA.

Conclusions: X-SCA results from a blinded panel of XMRV-spiked and unspiked patient samples showed that these methods are able to discriminate between XMRV-infected samples with 100% specificity and high sensitivity. These data provide evidence that X-SCA is a reliable method to begin evaluating the prevalence of XMRV in relevant cohorts.

No conflict of interest

Abstract: O_18

Assay development & Screening

No evidence for XMRV in CFS and MS patients in Germany despite the ability of the virus to infect human blood cells in vitro.

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Background: Retroviruses are able to induce immunodeficiency, malignant transformation and neurologic diseases. In addition to HTLV and HIV evidence for a third exogenous human retrovirus named XMRV has been found in prostate cancer patients. Recently an association of the same virus with chronic fatigue syndrome (CFS) has been published. The prevalence of the novel retrovirus and its role in both diseases is presently a matter of controversial debate. We have investigated a well characterized German cohort of CFS patients from the Charité clinic in Berlin for evidence of XMRV and a group of patients suffering from relapsing remitting multiple sclerosis (MS) with and without MS related fatigue, an MS related symptom complex sharing many hallmark troubles with CFS. In addition, the capability of XMRV to infect and replicate in PBMCs from CFS patients and healthy blood donors has been investigated.

Material & Methods: Blood samples were from patients of a cohort fulfilling the Fukuda/CDC criteria (CFS), from patients with an established MS diagnosis and healthy controls. Fatigue severity in MS patients was assessed using the Fatigue Severity Scale (FSS). PBMC were activated by PHA and cultured for seven days in the presence of IL-2. RT-activity in the supernatants was measured with the Mn+ C-type activity kit (Cavidi).

Results: Antibody responses particularly to Env and Gag proteins are salient characteristics of retroviral infections. We therefore set out to screen sera from 36 CFS and 17 healthy individuals in a blinded fashion with Gag- and Env-ELISAs. In addition 50 multiple sclerosis patients with fatigue symptoms (FSS Mean 4.7 +/- 1.07) were tested for serological evidence of an XMRV infection. In none of the sera XMRV specific antibodies were found. Moreover, DNA isolated from stimulated PBMCs of 39 CFS and 50 MS patients and 30 healthy controls has been tested negative by diagnostic nested-PCR for XMRV proviruses. XMRV is assumed to productively infect dividing cells only. Therefore activation and proliferation of PBMCs should result in a higher detection probability. Consistent with the PCR, no RT activity was measured in supernatants of the cultured PBMC on day 7. Co-culturing of a randomly chosen subset of the PBMC samples resulted in no XMRV transmission to susceptible LNCaP indicator cells. However, blood cells from healthy donors and from CFS patients can be infected with XMRV produced by the 22Rv1 cell line and release infectious viral particles.

Conclusion: We have used antibody ELISAs, sensitive PCR methods and co-culture with

indicator cells to detect XMRV infection in a cohort of CFS and MS patients with or without MS related fatigue or healthy controls. No evidence for the retrovirus was found in any of the serum or activated PBMC samples. However, we confirm that PBMC cultures from healthy donors and CFS patients can be (experimentally) infected by XMRV and release transmittable virus.

Abstract: O_19

Assay development & Screening

Search for XMRV in Swedish patients with myalgic encephalitis/chronic fatigue syndrome (ME/CFS) and prostate cancer; methods and results.

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Background: We wanted to search for XMRV and related viruses in Swedish ME/CFS/FM and prostate cancer patients, as well as Swedish blood donors using newly developed broadly targeted nucleic acid, virus isolation and serological techniques.

Materials & methods: Broadly targeted real-time QPCRs: Integrase, Gag and Env PCRs for RNA and DNA were developed. They were able to detect 1-10 copies of viral nucleic acid. The PCRs were designed for variation tolerance by targeting conserved domains and the use of Megabeacon (Muradrasoli et al, 2009) probe technology. They detected murine XMRV-related ERVs but not human ERVs. Specificity was evaluated versus human, mouse and XMRV plasmid DNA. Repeatability was required for a positive result. Around 100 ng of nucleic acid was used per QPCR reaction. The amount of nucleic acid, and its amplifiability, was ascertained using a histone 3.3 QPCR (Andersson et al, 2005), and the Nanodrop spectrophotometer.
Virus isolation: Plasma from 40 ME/CFS patients was inoculated onto LNCAP prostate cancer cells, and were tested for XMRV by QPCR after 5 days. Suspected positive cultures were passaged onto fresh cells and inoculated for another five days.
Serology: Multiepitope serology with 22 synthetic peptides from Gag and Env of XMRV bound to colour-coded Luminex beads. Peptides were designed to react broadly by conserved sequence selection and degenerated positions.
Patient and control samples: RNA and DNA was extracted from PBMC of 50 ME/CFS patients. The patients were selected according to the Fukuda criteria. DNA from trans-urethral resections (TURPs) from prostate tissue of 400 prostate cancer patients from Umeå University Hospital. All human samples were obtained after appropriate ethical permission.

Results: None of the 50 ME/VFS/FM patients were positive. None of 400 prostate cancer samples were positive. None of 200 blood donor sera were positive.
Virus isolation: Three cultures from 40 ME/CFS plasmas reacted with a few copies in the integrase PCR. One of the three could be passaged twice, but not further. The other two could not be passaged. The weakly positive LNCAP cultures had more cells floating in the medium than the negative ones. We were however not able to recover virus and viral nucleic acid from these cultures.
Serology: Sera from 60 ME/CFS patients and 100 blood donors were tested with the multiepitope assay at 1/10. All sera were simultaneously tested with peptide-coated and noncoated bead (background). Sera which reacted over background with at least three peptides were provisionally labelled “positive”. One blood donor serum and two ME/CFS plasmas reacted in this fashion.

Conclusions: XMRV, and putative related viruses, seem to be rare or nonexistent in Swedes, using our novel methods. The few weak and uncertain results should be further investigated.

No conflict of interest

Abstract: O_20

Assay development & Screening

Multi-laboratory evaluations of XMRV nucleic acid detection assays

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Background: Detection and isolation of XMRV from blood cells and plasma has been demonstrated in a high proportion of chronic fatigue syndrome (CFS) patients and in over 3% of healthy controls. If corroborated, these findings may have important implications for the safety of the blood supply. The Blood XMRV Scientific Research Working Group was established to design and coordinate collaborative studies to standardize existing assays and to investigate the prevalence of XMRV in blood donors using standardized XMRV assays.

Materials and methods: A four phase study has been designed to (i) evaluate XMRV nucleic acid (NAT) detection assays in terms of sensitivity, specificity and reproducibility; (ii) assess assay performance on various specimen types represented in existing blood donor/recipient repositories, and (iii) determine the prevalence of XMRV nucleic acids in blood donors. Phase I involved production of whole blood and plasma analytical performance panels comprised of pedigreed negative blood and plasma spiked with serial dilutions of XMRV infected cells (22Rv1) and supernatants, respectively. These panels were tested in a blinded fashion using XMRV NAT assays developed by the six participating laboratories. Phase II is comparing XMRV nucleic acid detection in frozen PBMCs, whole blood and plasma derived from CFS patients identified as XMRV viremic in a previous study. Further, replicate blood specimens have been processed at different storage intervals to determine whether the 2-4 day delay in processing common to many of the existing blood donor repositories adversely affects assay performance. Phase III will involve further evaluation of the clinical sensitivity and specificity of candidate assays by using a blinded panel of pedigreed XMRV positive and negative samples. Phase IV will test a blinded panel of at least 300 blood donor samples collected from apheresis blood donors located in the western United States. Phase III and IV studies will also include the evaluation of several antibody detection assays.

Results: In phase I, all laboratories detected at least 136 proviral copies/ml and four out of six assays demonstrated even more sensitive limits of detection. Four out of five plasma RNA assays performed similarly, with limits of detection of 80 RNA copies/ml or less. Phase II results will be available in August, and phase III and IV later in 2010.

Conclusions: The Blood XMRV SRWG has established a collaboration between many of the US laboratories conducting research into XMRV and its detection in blood and has initiated steps to compare performance of XMRV assays using analytical panels and clinical panels comprised of blood samples from XMRV-pedigreed CFS patients and untested blood donors. Results from this initial four phase study will guide further investigations into donor prevalence and XMRV transfusion-transmission rates using existing donor/recipient repositorie

No conflict of interest

1st International Workshop on XMRV

Pathogenesis, Clinical and Public Health Implications
7 – 8 September 2010, Bethesda, USA

Abstracts

Poster Presentations

Abstract: P_01

Basic virology

Assessing the potential for XMRV endogenization

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Background: Exogenous gammaretroviruses such as XMRV can maintain their existence by moving from cell to cell, and organism to organism via serial infectious events. However, occasionally, exogenous retroviruses gain the ability to infect germ cells. If this happens, a profound shift in the dynamics of retroviral spread occurs. If germ line-integrated provirus gains a foothold within the species of the infected individuals through vertical transmission to offspring, an endogenous retrovirus comes into existence. This has happened often within mammalian and proto-mammalian species. The most recent example is that of the koala retrovirus (KoRV). KoRV employs PiT1 a receptor found on germ cells.

Materials and methods: Human semen samples were obtained from healthy donors according to World Health Organization standards. After complete liquefaction, semen samples were subjected to spermatozoa selection using the swim-up assay. Motile cells were resuspended in alpha-MEM medium supplemented with 3 mg/ml BSA. Soluble A-MuLV, GALV, or X-MLV envelope SU proteins encoding the receptor-binding domain (RBD) fused to a double HA epitope tag was used to detect the binding affinity of each of these RBDs to sperm. One million sperm cells were incubated with HA-tagged RBDs at 37°C for 30 minutes. Fluorescence-activated cell sorting (FACS) was performed to detect the binding of HA-tagged RBD to sperms using HA.11 monoclonal antibody.

Results: We have determined using HMMTOP, an automatic server for predicting transmembrane helices for multiple membrane spanning proteins, that XPR1, the receptor for X-MLV and XMRV, is structurally similar to the receptor PiT1. PiT1 and XPR1 are assumed to be members of a class of ubiquitously expressed transmembrane solute transporters. Germ cells that express PiT1 are susceptible to KoRV. To assess whether germ cells express XPR1 we undertook binding assays with envelope derived receptor binding domains (RBDs) containing an HA epitope tag at their carboxyl terminus and purified human sperm. These RBDs retained the ability to specifically bind cells expressing XPRI but not cells lacking this receptor. Results obtained with RBDs derived from gammaretroviruses that use PiT1, as expected, bind to sperm. X-MLV RBD also specifically bound sperm.

Conclusion: The results of these experiments show X-MLV can bind to sperm cells and suggest XMRV can infect germ cells and therefore has the potential to become an endogenous virus of humans. The development of an animal model system for XMRV endogenization is an essential next step in determining that XMRV can endogenize.

No conflict of interest

Abstract: P_02

Basic virology

Understanding the entry of XMRV

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Xenotropic murine leukemia virus-related virus (XMRV) is a new human retrovirus that is associated with prostate cancer and chronic fatigue syndrome. The genome structure and sequence of XMRV is similar to that of other gammaretroviruses, and as xenotropic and polytropic murine leukemia viruses (X/P-MLV) XMRV uses the cellular protein Xpr1 for its entry into host cell.
Using an infectious XMRV reporter virus or XMRV/MLV pseudotypes, we examined the

entry of XMRV in a variety of cell lines derived from humans and several other species, and found that the XMRV entry differs substantially, and with no perfect correlation with the Xpr1 expression. While transduction of these cells with a retroviral vector encoding human Xpr1 conferred XMRV infection in certain cells, no or little increase in some other cell lines was observed.

We also examined the role of XMRV envelope (Env) in membrane fusion and entry, and observed that XMRV Env does not induce a detectable level of syncytia formation or cell-cell fusion, even in the cells that have been engineered to overexpress human Xpr1. Noticeably, progressive truncation of the XMRV Env cytoplasmic tail at or beyond the 21 amino acid residues from its C-terminus induced a substantial level of syncytia and cell-cell fusion as measured by our quantitative flow cytometry assay. The increased fusion activities of these truncation mutants correlated with their increased levels of SU shedding in the culture media, suggesting a conformational difference between these truncation mutants and wildtype in the extracellular domain. Synthetic peptides homologous to the N and C-heptad repeats of XMRV were also synthesized, yet no significant inhibition of these peptides on XMRV fusion and infection was found.

To investigate the possible role of endocytosis and/or low pH in XMRV entry, we treated cells with low pH or inhibitors that block cellular endocytosis, and we found that while low pH failed to increase the membrane fusion of XMRV Env, endocytosis inhibitors or lysosomaltropic agents did not block, but rather, enhanced the XMRV infection.

Taken together, we conclude that XMRV uses a pH-independent pathway for fusion and cell entry, and that endocytosis is not involved in the productive entry of XMRV into host cells.

No conflict of interest

Abstract: P_03

Basic virology

XMRV productively infects primary antigen-presenting cells

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Introduction: Last year, we reported that infectious XMRV was present in the peripheral blood of infected individuals with chronic fatigue syndrome (CFS). PBMCs isolated from peripheral blood could, following activation and culture, transmit XMRV to susceptible prostate and T cell lines, and to primary T cells. XMRV was also transmitted from T and B cells purified from activated PBMCs, and from plasma from infected individuals. For a number of individuals with both prostate cancer and CFS, we have been able to isolate virus from the plasma, but not from T and B cells from the same individual, suggesting that T and B cells present in the peripheral blood are not the major reservoir for XMRV. For a number of other viruses, antigen-presenting cells play a role in the dissemination of the virus in the host. We have recently initiated studies to examine whether antigen-presenting cells can be infected with XMRV.

Materials & Methods: Studies to determine the susceptibility of different cell types to XMRV were performed using mouse leukemia virus (MLV)-based reporter constructs expressing GFP, pseudotyped with either independently expressed XMRV Env proteins or with infectious XMRV. These viruses were used to infect established cell lines or primary cells. The primary cells were isolated from the peripheral blood of normal blood donors, or, for cell types derived from monocytes, cultured under the appropriate conditions for 7-10 days. The phenotype of the cells were verified by FACS analysis for appropriate cell surface markers. The relative titer of the viruses on different cell types was determined from the percentage of cells that expressed GFP four days after infection, as determined by FACS analysis. To

determine whether XMRV-infected primary cells produced infectious with XMRV, supernatants from these cell cultures were harvested, and assayed for the presence of infectious virus by culture with DERSE cells, a newly developed indicator cell line for XMRV (see abstract by Lee et al).

Results: We observed that, in addition to T and B cell lines, cell lines derived from other immune cells could be transduced by XMRV-pseudotyped indicator viruses including monocyte/macrophage (THP-1) and pDC-like (CAL-1) cells. Further studies revealed that both monocyte-derived macrophages and monocyte-derived dendritic cells, as well as freshly isolated pDC, could be infected with XMRV, and that treatment of the cells with AZT blocked the infection. Moreover, supernatant from the untreated cultures, but not the cultures treated with AZT, were able to infect the DERSE indicator cell line, demonstrating that these three cell types were infected with XMRV and were producing infectious virus. Since gammaretroviruses including MLV have been shown to be unable to infect terminally differentiated, non-dividing cells including macrophages and dendritic cells, we are currently investigating whether the infection observed represents the infection of a small number of dividing cells in these cultures, or whether XMRV is not subject to the same restriction as MLV in these cells.

Conclusions: XMRV can infect primary antigen-presenting cells, suggesting that they may play a role in viral dissemination.

Abstract: P_04

Pathogenesis

XMRV infection induces host genes that regulate inflammation and cellular physiology

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Background: XMRV is a novel human retrovirus associated with prostate cancer and CFS/ME. Although other retroviruses of the same genus as XMRV (gammaretroviruses) cause cancer and neurological disease in animals, it remains unknown if XMRV is a cause of either disease. However, indirect or direct modes of carcinogenesis by XMRV have been suggested depending on whether the virus was found in stroma or malignant epithelium. Immune dysfunction mediated by XMRV has been suggested as a possible factor in CFS/ME. To gain insight into the possible role of XMRV in these diseases we have identified genes that are induced in response to XMRV infection.

Methods: Prostate cancer cell line DU145 was infected for 8, 24, 48 and 120 h with XMRV. A comparison to uninfected DU145 cells cultured for the same periods of time served as controls. A population of total RNA was isolated using Qiagen RNeasy Mini Kit followed by digestion of DNA with DNAse treatment. XMRV infections at the different time points were monitored using real-time RT-PCR for *env* XMRV RNA. The RNA samples were analyzed for gene expression using Sentrix humanRef-8 v3 expression bead chips from Illumina (Cleveland Clinic Genomics Core). To verify the results obtained by the array experiment, we determined induction of a subset of the regulated genes. Total RNA was reverse transcribed to cDNA using iScript Select cDNA Synthesis Kit from Bio-Rad (random primers method). Induction of selected genes by XMRV infection was verified by qPCR (Relative Quantitation) from the cDNA pool using SYBR Green master mix. Fold-induction at each time point for the individual mRNAs was determined. In addition, pathway predictions were determined using Ingenuity Systems (content version 3002) software for genes induced by more than 2-fold following XMRV infection.

Results: In gene expression profiling, we observed maximal gene induction between 24 and 48 h post-infection. For example, the pro-inflammatory cytokine IL8 gene, a potential contributing factor to androgen independent growth of late-stage prostate cancer, was consistently induced by XMRV infection by up to 6-fold. Of the XMRV induced genes, pathway analysis indicated 10 genes are implicated in cell morphology, 11 genes in cellular development,

12 genes in cell-to-cell signaling and interaction, 11 genes in cellular movement and 13 genes in cellular growth and proliferation. Thirteen XMRV induced genes are also implicated in cancer.

Conclusions: The chemokine IL-8 is one of the most highly induced genes in response to XMRV infection of prostate cancer cell line DU145. XMRV induction of the 30 host genes identified in this study suggests a profound effect of the virus on fundamental cellular physiology and inflammation. These findings could be relevant to the possible pathogenic effects on XMRV in prostate cancer and CFS.

No conflict of interest

Abstract: P_05

Pathogenesis

Detection of XMRV sequences in EBV-transformed B cell lines

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Background: XMRV infection has been found in humans linked with prostate cancer and chronic fatigue syndrome (CFS). XMRV is able to infect a wide range of cells and has been found in a variety of cell types both in Vitro and in vivo, including B cells and other immune cell types.

Materials and methods: In order to determine the presence of XMRV sequences in B cells, we have screened 21 B cell lines available in our laboratory. These cells were generated by EBV immortalization of PBMC obtained from 11 CFS affected individuals (fulfilling both Fukuda and Canadian criteria), 5 healthy donors, 4 HIV infected individuals and 1 prostate cancer patient. DNA was extracted from dry cell pellets and XMRV sequences were amplified using a real-time PCR covering a 150bp pol sequence and using a nested approach in both gag and Env genes.

Results: Envelope amplification yielded positive bands in 4 out of 21 individuals tested, 3 CFS affected individuals and 1 healthy donor. However, gag amplification yielded only 3 positive samples (1 CFS affected individual, 1 healthy donor and one HIV+ patient). In contrast, Real-time PCR of Pol fragment detected 7 positives samples in 14 individuals tested (4 SFC , 2 donors and 1 HIV+ individuals). To confirm the presence of XMRV sequences we performed sequence analyses of gag and env amplicons. The analysis of the three available gag sequences confirmed the XMRV characteristic 24-nt deletion, which is not found in any known exogenous MuLV. Sequences were 100% identical to reported XMRV sequences. Furthermore, envelope sequences were also homologous to previously described XMRV sequences. In this case, sequence variability was low or absent. Interestingly, most of changes observed corresponded to G to A mutations that were accumulated in one positive sample.

Conclusions: Despite the discrepancies observed in the different PCR approaches using gag, pol or env sequences, our data suggest that EBV transformed B cell lines harbor XMRV specific sequences, and therefore this cell type may represent a reservoir for XMRV contributing to its potential pathogenesis.

No conflict of interest

Abstract: P_06

Pathogenesis

Host factor involvement in XMRV infection-analysis of gene expression profiling using PCR array

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Background: In order to study the host response to XMRV infection at the RNA level, we used PCR array to analyze the expression of a focused panel of genes related to the inflammatory response involved in XMRV infection. The PCR array is a set of optimized

real-time PCR primer assays on 96-well plates for pathway or disease focused genes together with appropriate RNA quality controls that are capable of performing gene expression analysis.

Methods: DU145 cells were infected with XMRV and total cell RNA was isolated 24 and 48 hours post infection. Total cell RNA was used as a template for the PCR ARRAY real-time assay. The expression profile of genes involved in mediating immune cascade reactions during inflammation, chemokines, cytokines, interleukins and their receptors involved in the inflammatory response, and signal transduction pathways were analyzed.

Results: Cytokines, chemokines and chemokine receptors involved in host inflammatory response like IL-1 (alpha and beta), IL-13, IL-17C, TNF-alpha, CCL5, CCL17 and CCL19 were found to be upregulated at the time points tested. The gene expression profiles of IL-8, CCL2, CCL29, CXCL3, CXCL5 and CCR7 were found to be significantly down-regulated. Other genes involved in signal transduction pathways like FOS, IGFBP3, EGR1 and WNT were differentially regulated at the time points tested.

Conclusion: The gene expression profiling identified a number of differentially expressed chemokines, cytokines, interleukins and their receptors that are involved in the inflammatory response. In addition the analysis revealed differential regulation of genes involved in signal transduction pathways.

No conflict of interest

Abstract: P_07

Pathogenesis

Xenotropic murine leukemia virus-related infection of human lymphoid tissue ex vivo

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Background: The gammaretrovirus xenotropic murine leukemia virus-related virus (XMRV) has been recently associated with prostate cancer and chronic fatigue syndrome. In patients with both diseases, the virus has been found in a variety of cell types, including T and B cells. Moreover, in XMRV-infected rhesus macaques there is evidence of viral replication in lymphoid organs, suggesting that lymphocytes are a primary target for XMRV. Histocultures of tonsils support productive infection with various lymphotropic viruses, including HIV and HHV-6. In this study, ex vivo lymphoid tissue was used to investigate the pathogenic mechanisms of XMRV

Materials and methods: Human tonsils from 2 healthy individuals undergoing tonsillectomy were collected in PBS and cultured in small pieces (2mm3) over gelfoam soaked in RPMI 1640 medium. Small tissue blocks were left uninfected or were infected ex vivo with a XMRV viral stock obtained from a 22Rv1 cell culture supernatant. Culture medium was replaced every 3 days. After 14 days in culture, uninfected and infected tissues were mechanically homogenized and cells were isolated. Viral infection was evaluated at different times in the cells migrating out of the tissue and at day 14 in tissue cells, by analyzing the presence of viral DNA by PCR. In addition, tissue cells were immunophenotyped and analyzed by flow cytometry

Results: At day 7 post-infection cells migrating out of the tissue were positive for XMRV DNA. After 14 days of culture, tissue cells were also highly positive, confirming that XRMV (as other viruses) was able to infect human tonsil tissue fragments in the absence of exogenous stimulation. Despite this apparent efficient infection, cells showed similar percentages of T and B cells in uninfected and infected tissues. XMRV infection did not modify the percentage of CD3 (76 and 75% in XMRV+ and XMRV- tissue, respectively), CD4 (53% vs 52%), CD8 (39% vs 40%) or CD19 cells (3% vs 1%). A deeper analysis of T cell subsets showed that XMRV infection did not modify the naïve/memory cell ratio, as evaluated by CD45RO staining, or immune activation markers, as evaluated by the expression of HLA-DR and CD38 in both CD4 and CD8 T cells

Conclusions: The development of in vitro models to study XMRV pathogenicity is essential

for understanding its role in human diseases. Our data show that histoculture of human lymphoid tissue is a suitable model for the analysis of XMRV pathogenesis. In this system we observed a infection by XMRV although this infection did not result in depletion of T or B cells nor an immune activation, suggesting the absence of evident depleting effect of XMRV in these cells in lymphoid tissue.

No conflict of interest

Abstract: P_08

Pathogenesis

Host Factor involvement in XMRV infection-Analysis of Gene expression profile using Agilent microarray

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Background: In order to analyze the effects of XMRV infection on the expression of host factors in the prostate cancer cell line DU145 at the RNA level, we probed the Agilent Whole Human Genome Oligo Microarray comprising of about 41,000+ unique human genes and transcripts representing all known genes and transcripts in the human genome.

Methods: DU145 cells were infected with XMRV and total RNA isolated 24 hr and 48 hr post infection. The RNA samples that passed quality control on the Nanodrop ND-1000 and Bioanalyser2100 were amplified and labeled using the Agilent Quick Amp Labeling Kit and hybridized to Agilent whole genome oligo microarray in Agilent's SureHyb hybridization chambers. After hybridization and washing, the processed slides were scanned using the Agilent DNA microarray scanner following Agilent Technologies' guidelines. The resulting .txt files extracted from Agilent Feature Extraction Software (version 10.5.1.1) were imported into the Agilent GeneSpring GX software (version11.0) for further analysis. The 4 microarray data sets were normalized in GeneSpring GX using the Agilent FE one-color scenario (mainly median normalization). Genes marked present in all samples ("All Targets Value") were chosen for data analysis. Differentially expressed genes were identified through Fold-change screening. The p-value was calculated using t-test. Unsupervised hierarchical clustering was performed using the Agilent GeneSpring GX software (version 11.0). The analysis of Gene Ontology (GO) and Pathway is based on Fisher's exact test.

Results: A number of host gene expression profiles were significantly changed at all time points tested. Genes involved in immune response, phospholipids metabolism, cellular development, cell death, cancer, and cell cycle were among those whose expression were differentially regulated

Conclusion: The gene expression profiling identified a subset of differentially expressed genes. Pathway analysis and GO Analysis were performed to reveal the biological functions of this subset of genes. These observations will help to further our understanding of XMRV pathogenesis and provide additional insights into the biological pathways involved in XMRV infection and prostate cancer

The findings and conclusions in this abstract have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any Agency determination or policy.

No conflict of interest

Abstract: P_09

Therapeutics/ Vaccine (animal models)

XMRV: virological, immunological and clinical correlations in patients with Chronic Lymphocytic Leukemia and Mantle cell lymphoma (MCL)

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Background: XMRV has recently been identified in patients with prostate cancer and the Chronic Fatigue Syndrome (CFS). CFS patients have an increased incidence of lymphoproliferative malignancy compared to the normal population. 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. To address this we identified several XMRV infected CFS patients who subsequently developed Chronic Lymphocytic Leukemia (CLL) and Mantle Cell Lymphoma (MCL). Treatment of XMRV associated neoplasia has not been previously reported. However, HTLV-1 associated T-Cell lymphoma/leukemia does respond to zidovudine (AZT) and IFNa. In addition, multiple human tumor cell lines including breast cancer show growth inhibition and apoptosis when exposed to AZT. Several groups have reported inhibition of XMRV by FDA approved antiretrovirals including AZT, Raltegravir and tenofovir in cell culture. Our study investigated additional XMRV associated malignancy, including CLL.

Methods: CLL is a disease with circulating tumor cells which can be isolated from the peripheral blood, thus CLL patients are ideal subjects to study the correlation of virological, immunological and clinical parameters associated with XMRV infection and tumor development. Peripheral blood mononuclear cells were isolated and the CLL cells shown to be infected by intracellular staining of antibodies to XMRV gag and Env and infectious virus isolated from blood by methods developed in our lab (Lombardi et. al. Science 2009). Cytokine profiles were determined by multiplex analysis of 30 cytokines chemokines and growth factors on a Luminex platform and immune cell phenotyping was performed my multiparameter flow cytometry on and LSR2 (BD Biosciences)

Results: We have developed B cell lines from 2 MCL and 1 CLL patient with a prior diagnosis of CFS and XMRV infections. Each of these B cell lines is producing infectious XMRV. By flow cytometry, the immunophenotype was compatible with MCL and CLL respectively (i.e. CD5+CD20+CD23-FMC7+) and (CD5+, CD20+ CD23+ FMC7). The first patient to be studied in detail is a 68-year old Oncologist with a three-year history of untreated CLL and symptoms consistent with CFS. He has been identified as having XMRV plasma viremia by the methods reported by Lombardi et al. and his lymphocytes are positive for XMRV. His virological, immunological and tumor cell status is amenable to study using assays described by Lombardi et al. We have also determined integration sites of XMRV and co-expression of Herpesviruses including EBV, CMV, HHV6A,B using a quantitative multiplex detection chip.

Conclusions: XMRV was isolated from the tumor cells of XMRV infected patients with CLL and MCL. Development of cancer coincides with an outgrowth of gamma delta T cells, XMRV infection and a distinct inflammatory cytokine signature but not with co-expression with EBV, CMV, HHV6A or HHV6B. A new patient with CLL and XMRV infection is identified with clinical, virological and immunological parameters measurable with our methodology. Thus a trial of anti-retroviral therapy for this XMRV related cancer appeared justified. After 63 days of well tolerated AZT and raltegravir the previously rising lymphocyte count decreased from 16,348/mm3 to 13,186. Treatment is ongoing.

No conflict of interest

Abstract: P_10

Therapeutics/ Vaccine (animal models)

Therapeutic targeting of TSG101 in XMRV-infected cells

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Work in our laboratory has demonstrated a promising opportunity for therapeutic or prophylactic intervention to combat infections by XMRV. Our approaches take advantage of the well-established fact that viruses "hijack" their hosts and alter the expression or function of particular host proteins to facilitate their propagation.

Our studies have focused on one particular molecule, TSG101, which is a ubiquitously-expressed cytoplasmic protein that is known be "hijacked" upon viral infection. This hijacking of TSG101 is absolutely essential for budding of enveloped viruses. Moreover, targeted inhibition of TSG101 completely blocks propagation of viral infection, suggesting that the virus does not have secondary means for budding and maturation. Moreover, the utilization of host TSG101 is shared by many different virus families and all members within a given virus family.

For example, TSG101 is essential for infections caused by all retroviruses tested to date. Our laboratory has demonstrated that TSG101 is uniquely exposed on the surface of virus-infected cells and that this altered function provides much-needed opportunities for therapeutic targeting of retrovirus-infected cells. Specifically, TSG101 can be targeted using small molecules that prevent viral hijacking or using monoclonal antibodies, which serve to eliminate infected cells via normal host defense mechanisms.

We will present promising preliminary evidence demonstrating that XMRV infection in particular is associated with surface exposure of TSG101. Consistent with prior findings linking XMRV with prostate cancer, we also demonstrate these changes in TSG101 appear to be highly relevant to prostate cancer based on immunohistochemical analyses of patient-derived prostate tumor specimens.

We also provide preliminary insight as to studies meant to demonstrate efficacy of TSG101-targeted approaches for the treatment or prevention of XMRV-associated diseases. Altogether, these findings suggest potential opportunities for TSG101-directed therapeutic in the treatment or prevention of XMRV-associated diseases.

Abstract: P_11

Prostate cancer

Prevalence of neutralizing antibodies against XMRV in clinical prostate cancer

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Background: While XMRV has been convincingly linked to prostate cancer (PC) there has been less investigation of the immune responses against XMRV. One study that attempted to discover anti-XMRV antibodies in PC patients' sera using an ELISA assay was unable to detect XMRV Gag- and Env-specific antibody among PC patients from Germany (Hohn et al. 2009, Retrovirology 6: 92). Recently, we described the adaptation of a single-round reporter gene assay that is broadly used for the quantification of HIV-1 neutralizing antibodies (NAb) as an approach for detecting XMRV NAb (Arnold et al. 2010, Urology 75: 755). The XMRV NAb assay we developed was in concordance with two different methods of XMRV detection (i.e. FISH and PCR).

Materials & methods: Using a single-round reporter gene assay, 258 sera samples from a cohort of PC patients at the Emory Department of Urology were analyzed for neutralizing activity against XMRV and HIV-1 pseudoviruses. Relative neutralization from PC patient's sera (percentage of control) was calculated using a correlation analysis. To exclude non-specific inhibition, serum samples having neutralization activity against the HIV-1 pseudovirus that was

not statistically different from that of the XMRV pseudovirus were scored as nonspecific.

Results: XMRV-specific NAb were detected in 6.2% of PC cases (16 from 258 sera), which is lower than we described previously.

Conclusions: We have further expanded our XMRV NAb dataset to include >200 additional serum samples from a PC cohort. As a result, our estimate of XMRV NAb prevalence in this cohort is strengthened by (1) the increased numbers of samples investigated and (2) applying more stringent statistical analyses. There are however still significant discrepancies between our estimate of XMRV prevalence and that reported in different publications to date, which could be due to the methods being used to detect XMRV and actual differences in the cohorts being investigated. Our data further supports previous studies indicating that XMRV is capable of infecting humans and that a subset of PC patients have been infected with XMRV.

No conflict of interest

Abstract: P_12

Prostate cancer

Prevalence of XMRV in prostate cancer patients at Mayo clinic

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Introduction: A novel human gammaretrovirus, xenotropic murine leukemia virus-related virus (XMRV), was originally identified in prostate cancer tissues. Significantly higher XMRV infection cases were found in men who were homozygous for a variant of RNase L (R462Q), which is associated with inherited prostate cancer. These observations suggest the possible role of XMRV infection in prostate cancer development. However, the etiological link between XMRV infection and prostate cancer remains elusive. Our aim of this study was to determine the prevalence of XMRV in patients with or without prostate cancer at Mayo Clinic.

Materials and methods: Total DNA from 40 normal/benign, 70 intermediate (Gleason Score [GS] 5-7) and 40 high grade (GS 8-10) prostate biopsy samples were tested for *RNASEL* R462Q mutation and XMRV proviral DNA by XMRV *gag* real-time PCR and nested PCR for the XMRV linker-*gag* sequence. For seroprevalence of XMRV, plasma samples from 159 patients with prostate cancer (GS 5-7) and 201 age-matched patients with no prostate cancer or urological disorders were used. For detection of XMRV-specific antibodies, we employed indirect immunofluorescent assays, virus neutralization assays with GFP-expressing XMRV, and Western blotting.

Results: Nested PCR found 1, 5 and 2 samples as XMRV-positive, while real-time PCR detected XMRV DNA in 1, 4 and 1 samples (>1 viral copy per 1 µg DNA) in normal/benign, intermediate and high grade tumors, respectively. We are currently testing the presence of XMRV-specific antigens in the sections of PCR-positive prostate cancer samples using our XMRV-infected mouse plasma and the rabbit anti-XMRV antibody (kindly provided by Dr. Singh).
When seroprevalence of XMRV was examined by indirect immunofluorescent assays using XMRV-infected and –uninfected 293T cells, no sample was found seropositive. No human plasma samples could completely block XMRV infection even in low dilutions, while highly diluted plasma samples (80 to 160-fold dilutions) of XMRV-infected wild mice could completely block XMRV infection. Apart from few exceptions, human plasma samples failed to detect XMRV antigens by Western blotting.

Conclusions: Based on the real-time PCR results, no statistically significant association was observed between the presence of XMRV DNA and prostate cancer, cancer grades, or *RNASEL* R462Q mutation. Our serosurvey results suggested that XMRV is not highly immunogenic in humans, or the virus is not prevalent in the patients at Mayo Clinic.

No conflict of interest

Abstract: P_13

Chronic Fatigue syndrome/ neuro immune diseases

Blood donation and transfusion in CFS patients

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Introduction: The Centers for Disease Control and Prevention (CDC) estimate that between 1 and 4 million people in the U.S. have chronic fatigue syndrome (CFS). The CDC has also shown that middle age women are at 2-4 fold greater risk of developing CFS than men. Further, population based studies have indicated that fewer than 20% with CFS have been diagnosed by a physician. The cause(s) of CFS are not known but several viruses are known to trigger CFS and/or have been detected in CFS patients. In October 2009, a Science publication indicated that 67% of people with CFS and 3% of healthy controls had a retrovirus known as XMRV detected in their blood. Detection rates such as these would pose a serious threat to the blood supply. Objective: To assess blood donation practices and rates of transfusion in people with CFS.

Methods: A web-based survey tool (Survey Monkey) was used to administer a 50-item questionnaire to identify possible CFS risk factors. Four items derived from standardized instruments were related to blood donation and transfusion. These items were:
1. Have you ever received a blood transfusion?
2. Have you ever donated blood or blood products (ex., plasma)?
3. How many times have you donated blood or blood products in the past 10 years?
4. How many times have you donated blood or blood products since being diagnosed with CFS?

Results: There were 1747 respondents and 1441 completed the survey. 90% of respondents had been diagnosed with CFS by a physician. The majority of respondents (86%) were women with an average age of 57 years. Of the 1529 people who answered the question on blood transfusion, 124 (8%) indicated they had a blood transfusion prior to CFS and 50 (3%) received a transfusion after being diagnosed with CFS. 1531 people answered question 2 regarding blood donations and 648 (42%) reported ever having donated blood while 4% indicated they have donated blood one or more times over the past 12 months. 650 people answered question 3 and 30 (4%) have donated blood in the past 12 months and 225 (34%) responded that they had donated blood one or more times over the past 10 years. Finally, 115 (18%) of the 640 people that answered question 4 indicated they donated blood one or more times since being diagnosed with CFS.

Conclusion: Web-based surveys are a cost effective method to collect large amounts of information in a short period of time. 11% of the respondents have received a blood transfusion; slightly higher than the population norm for transfusion. More than 40% of people diagnosed with CFS have donated blood; many of which have made donations more than once during the past 10 years. Blood donations by people with CFS are more common than would be expected for a chronically ill population.

No conflict of interest

Abstract: P_14

Chronic Fatigue syndrome/ neuro immune diseases

Comparison of demographic parameters and health/performance status of XMRV antibody positive vs. negative CFS subjects in a phase III clinical trial

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Background: CFS is a severe disorder consisting of profound fatigue and a variety of other debilitating symptoms that affects up to 4

million Americans. Recently, one of us (JAM) identified DNA from a human gamma retrovirus (XMRV) in 67% of CFS subjects. Evidence also suggested that approximately 50% of the CFS infected subjects mounted a specific antibody response against XMRV (Science 326, 585-589 (2009)). The objective of this study was to compare demographic parameters and health/performance status of XMRV antibody positive vs. negative CFS subjects enrolled in a Phase III clinical trial evaluating the safety and efficacy of a toll-like receptor 3 agonist, rintatolimod (PolyI:PolyC_{12}U, Ampligen®).

Materials and methods: Two-hundred-eight (208) evaluable subjects, who met the 1988/1994 Centers for Disease Control (CDC) criteria for Chronic Fatigue Syndrome (CFS), participated in this randomized, placebo-controlled, double-blinded, multicenter study. Only severely debilitated patients were selected for this study. The primary endpoint was exercise treadmill duration. Subjects received rintatolimod (200-400 mg) or an equivalent volume of placebo (saline) twice weekly by intravenous (IV) infusion for 40 weeks. Baseline (or earliest available specimen) serum samples from all 208 subjects were analyzed for antibodies directed against XMRV.

Results: Seventy (33.7%) of the 208 CFS subjects were positive for antibodies directed against XMRV, while 138 (66.3%) were negative. There was no significant difference in the number of CFS subjects positive or negative for XMRV with regard to age, gender, duration of CFS, cognitive dimension (SCL90-R), exercise treadmill duration, or SF-36 vitality score ($p>0.3$). However, the subjects negative for XMRV had a lower Activity of Daily Living score (66.9 vs. 71.2, $p=0.010$, ANOVA) and a lower overall activity level based upon a lower activity monitor score (183K vs. 210K, $p=0.033$, ANOVA). There was also a trend for a lower KPS score in the negative CFS subjects (49.1 vs. 50.4, $p=0.117$, ANOVA). These results indicate that approximately 1/3 of the CFS subjects have a detectable immune response directed against XMRV.

Conclusions: The results suggest that the XMRV antibody negative subjects have a lower activity level and a lower ability to complete normal daily activities. Additional studies to further evaluate XMRV in this CFS population are underway.

** Authors also include AMP 516 Investigators - L. Bateman, J. Bellesorte, P. Cimoch, J. John, R. Keller, C. Lapp, A. Mercandetti, D. Peterson, M. Papernick, R. Podell, B. Stein, and L. Taylor*

No conflict of interest

Abstract: P_15

Chronic Fatigue syndrome/ neuro immune diseases

Mechanisms of induction of NK cell activity by ampligen® in XMRV-positive chronic fatigue syndrome patients

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Background: Chronic Fatigue Syndrome (CFS) is a debilitating disease of unknown etiology that affects ~17 million people worldwide. Patients with CFS display immunologic abnormalities and experience both cancer and neurological pathology, all of which may be associated with persistent viral infections. We recently detected a new human exogenous retrovirus, XMRV, in the blood of patients with CFS. Natural Killer (NK) cells are the first line of immunological defense against viruses and cancer, and NK cell function is deficient in CFS patients. Ampligen®, also known as PolyI:PolyC_{12}U, is an immunomodulatory synthetic dsRNA drug (Hemispherx Biopharma, Inc.). This drug is currently being tested as a potential treatment for CFS, and several placebo-controlled clinical trials of Ampligen® have reported improvement in exercise performance. Importantly there is no current biomarker to identify CFS patients more likely to respond and the mechanism of action is unknown. In this study, we have investigated the effects of Ampligen® on the activation of NK cells in mixed lymphoctye cultures tested ex vivo from XMRV-positive CFS patients.

Materials and methods: Fresh peripheral blood mononuclear cells (PBMCs) were obtained from 30 XMRV-positive CFS patients with low NK cell cytotoxicity, and were treated with 25µg/mL of Ampligen® for 24 h. Degranulation was determined by externalization of CD107a, a marker for NK cell cytotoxicity, and was assessed on NK cells after exposure to HLA class I-deficient K562 targets. Cell staining for phenotypic markers (CD56, CD3, TRAIL) and cytotoxic proteins (perforin and granzyme B) was performed by multi-parameter flow cytometry using specific mAbs and LSRII cytometer (BD). Cytokine production in the treated cultures was determined using a human cytokine 25-plex panel on a Luminex platform (Invitrogen).

Results: Treatment markedly increased CD107a externalization in the NK-CFS cell population as indicated by 5-fold increases in CD107a-positive cell frequencies and 3-fold increases in their CD107a low mean fluorescence intensity (MFI), with slight positive shifts in intracellular granzyme B and perforin. In contrast, T cells showed little change in CD107a externalization. A significant upregulation of TRAIL, a death receptor ligand that triggers target cell apoptosis, was also observed on the NK cells from XMRV-positive patients. The production of IFN-α and TNF-α was elevated in the Ampligen® treated PBMCs from XMRV-positive CFS patients. The effect of ex vivo Ampligen® treatment on patient PBMCs was variable with regard to infectious virus as measured by QPCR and the DERSE assay and will require further investigation.

Conclusions: Here, we have shown that the increase in degranulation per NK cell indicates a new mechanism by which Ampligen® treatment can improve NK cell function, regardless the levels of cytotoxic proteins. Our results suggest that this drug has potential for use as a therapeutic agent for the treatment of CFS in part by boosting NK cell responses, and that this potential applies to XMRV-positive CFS patients. Further studies are indicated to determine the in vivo relevancy of these findings.

No conflict of interest

Abstract: P_16

Chronic Fatigue syndrome/ neuro immune diseases

XMRV detection in a national practice specializing in chronic fatigue syndrome (CFS)

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Introduction: Chronic Fatigue Syndrome (CFS) is a disabling disorder characterized primarily by post-exertional fatigue . There have been many attempts to link CFS to viruses due to the nature of its clinical onset and various immune aberrancies suggesting a possible viral origin. There have been several epidemics of CFS reported and one report (1991) of a possible link to retroviruses. Recent evidence has emerged that suggests a link to a novel human exogenous retrovirus, XMRV (Lombardi, 2009).

Methods: Forty-seven consecutive patients were selected from a private, specialized CFS-only practice located in Asheville, NC. All patients met criteria for CFS according to the 1994 CDC case definition. The primary symptom in 100% of patients was disabling, post-exertional fatigue which required substantial adjustments in activities of daily living. All were incapable of full time employment or schooling. Patients were categorized according to clinical severity using the physician assigned Karnofsky Performance Scale or KPS score. Age, sex, date of onset, geographic location and length of illness were recorded. The cohort was selected in a consecutive fashion from October 2009 through December 2009. Peripheral blood was submitted to a CLIA certified laboratory (VIP Dx) for XMRV detection according to Lombardi et al. (Science, Oct 2009) including the use of PCR on plasma and cultured PBMC nucleic acids as well as isolation of XMRV to LNCaP.

Results: The average KPS score for the entire study group (N = 47) is 54.8 meaning that the average patient in this study cohort cannot work but is capable of some degree of independent living with assistance. The KPS range is 40-70, average age is 50.2 years with a range of 23-72, gender distribution is 81% female (38/47) and 19% male (9/47). Length of illness of the study

cohort averages 18.9 years with a range of 2.5 to 36 years of continuous illness. Earliest onset date is in 1974 and latest onset date is 2008. Geographically, patients came from 24 states and two foreign countries (Canada and Australia). The US patients comprise 96% of cases with 40% from the Southeast, 33% from the West, 11% from the Midwest and 16% from the Northeast.
Patients reported significant illness in first order family members. These included 21 patients (45%) with CFS or CFS-like illness in other family members, 22 patients (47%) with cancer in family members or recently in themselves (2) and 13 patients or 28% with autoimmune disease in family members. 45 patients (96%) have cardiac diastolic dysfunction with cardiac left ventricular dysfunction linked to cellular energy deficiency which predisposes to orthostatic intolerance common to CFS.

Conclusions: XMRV was detected in 74.5% of 47 consecutive cases of well-characterized CFS from 24 states and two countries. Comparison of positive and negative cohorts shows no significant differences in age, sex, geographic origin, length of illness or clinical severity. There is a high positivity rate (50%) of XMRV in non-CFS exposure controls within family members.

No conflict of interest

Abstract: P_17

Chronic Fatigue syndrome/ neuro immune diseases

Demographics of XMRV: a summary of national and international clinical laboratory testing

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Background: XMRV is a new human retroviral infection of as yet unknown pathogenic potential. We as well as others have reported the prevalence of XMRV as 3% of healthy adults. Several additional studies have reported a significantly higher incidence of XMRV infection disease populations including, prostate cancer (6-23%), Chronic Fatigue Syndrome (CFS, 67%) and immune compromised individuals (10%). Other studies have failed to detect XMRV in prostate cancer; CFS or healthy populations suggesting the distribution of XMRV might be limited to more specific geographical regions as is the case with HTLV1 infection. To begin to investigate this question, XMRV infection rates were correlated with demographic data from clinical laboratory testing of XMRV over the 6-month period following the first isolation of XMRV from blood.

Methods: Between November 2009 and May 2010 a total of 712 EDTA or heparin anti-coagulated blood samples were received by the CLIA licensed clinical laboratory, Viral Immune Pathology Diagnostics (VIP Dx) in Reno Nevada, for XMRV analysis. Plasma and PBMC were isolated and XMRV detected according to the methods described by Lombardi et al. (Science October 2009) using a combination of techniques, including PCR of nucleic acid derived from activated PBMCs, plasma/PBMC co-cultured on LNCaP indicator cell lines as well as PCR of whole blood and plasma. Patient demographic information was provided on each sample, however disease status was not disclosed to the laboratory. In order to evaluate the demographic distribution associated with XMRV status patient information was stratified by geographic location, age and gender.

Results: A total 640 U.S. samples were tested. Of the 640 tested, 413 were found to be XMRV negative (65.5%) and 227 were XMRV positive (35.5% positive). The range of patients tested was 1 year to 88 years of age. Approximately twice as many samples were received from female patients compared to male patients. No statistical significance (nonparametric analysis) was observed in XMRV incidence for gender or correlation between XMRV infection and age. Patient samples were received from all but 4 of the 50 states with XMRV detected in 37 of the 46 represented. Twenty samples were received from Canada, of which 4 tested positive (2 males and 2 females). Fifty-one samples were received from Europe, the majority of which were from the United Kingdom (n = 44). Of the 51 samples received from Europe XMRV was detected in 15 samples (7 male and 8 female). All positive

European samples were from patients residing in the UK.

Conclusion: XMRV is has been detected in men, women and children throughout the United States, Canada and the United Kingdom. Although distribution of XMRV appears to coincide with population distribution, less populated areas provided fewer samples making geographical analysis difficult. Of the samples received, XMRV was found in both men and women with equal frequency and was identified in children and adults. Larger epidemiological studies are necessary to provide a better understanding of XMRV distribution, however, this study firmly established that XMRV is not restricted to discrete geographical locations within the United States.

Abstract: P_18

Chronic Fatigue syndrome/ neuro immune diseases

Altered B, T and NK cell phenotype in chronic fatigue syndrome (CFS) individuals.

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Background: Several studies have reported controversial results on the dysfunction of the immune system in Chronic Fatigue Syndrome (CFS) affected individuals. Since the recently described Xenotropic murine leukemia virus-related virus (XMRV) can infect cells from the immune system (B, T or NK cells), we designed different panels of antibodies to deeply characterize the phenotype of B, T and NK lymphocytes populations in patients with CFS.

Material and methods: We obtained blood samples from 12 individuals affected from CFS (fulfilling both Fukuda and Canadian criteria) and 15 healthy donors (HD). B, T and NK cell populations were phenotyped in fresh blood samples and analyzed by multicolour flow cytometry. Fresh PBMCs were obtained to characterize spontaneous ex vivo apoptosis and NK cytotoxic activity against EGFP-K562 cells, both analyzed by flow cytometry.

Results: No changes in the percentage or absolute numbers were observed in principal populations of B (CD19+), T (CD3+, CD3+CD4+, CD3+CD8+) or NK (CD56+ CD16+) cells. However, significant differences were observed in various subsets of these populations.
B lymphocytes from CFS individuals showed a decrease in marginal-zone marker CD1c, especially in memory IgG population. The percentage of memory IgG+ cells, effector memory CD38high IgG+ cells and plasmatic B cells (CD38high/CD27high) was also reduced in CFS individuals. In contrast, CFS individuals showed an increase in transitional B cells (IgD+CD38highCD5+CD10+), which present lower spontaneous ex-vivo apoptosis.
T-cells from CFS individuals presented increased CD25 levels mainly within CD8+ population. Proliferation marker Ki67 was significantly diminished in CFS CD4 T-cells and a trend was also observed in CD8 T-cells. In addition, individuals with CFS showed increased CD5 levels within CD8 T-cells which could suggest an anergic state. No signs of altered senescence (CD57+ T cells) or activation (CD38+, HLA-DR+ or double positive cells) were observed in CD4 or CD8 subsets, although, CD8 T cells from CFS individuals showed higher expression of FAS and PD-1 and a slightly higher spontaneous ex-vivo apoptosis.
NK cells showed a significant decrease in CD57+ expression and expressed higher levels of CD69 and activating NK Cytotoxic Receptors (NCR) NKp30, NKp44 and NKp46. Nevertheless, this altered phenotype did not impact function, as we did not observe differences in NK cytotoxic activity in CFS patients compared with HD.

Conclusions: CFS patients showed qualitative but not quantitative alterations in all major immune cell types. B cells presented a phenotype partially similar to some autoimmune disorders or viral infections. Interestingly, an anergic phenotype observed in T cells from CFS individuals could be related with an impaired control of viral infections and could explain the lack of increased activation and senescence observed in our patients. Finally, altered NK phenotype did not seem to significantly modify cytotoxic activity. On the whole, the results suggest a global immune dysfunction with an

unknown aetiology as potential contributor to the mechanism of CFS.

No conflict of interest

Abstract: P_19

Chronic Fatigue syndrome/ neuro immune diseases

Detection of infectious XMRV in the peripheral blood of children

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Background: XMRV is a new human retroviral infection of as yet unknown pathogenic potential. Recent reports have found XMRV infection in 3% of healthy adult populations and high percentages in populations of immune compromised individuals and Chronic Fatigue Syndrome (CFS). The prevalence of XMRV infection has not been explored in families with CFS or in children. An understanding of the XMRV infection rate in children may be particularly helpful, given that 1 in 100 children in the US are diagnosed with neuroimmune disorders, including Autism Spectrum Disorder (ASD) and that CFS and childhood neuroimmune disorders share common clinical features including immune dysregulation, increased expression of pro-inflammatory cytokines and chemokines, and chronic active microbial infections. Thus, we hypothesized that XMRV infection may be detected not only in families with CFS but also in children with other neuroimmune disorders.

Methods: 66 subjects participated as family members of a parent or child diagnosed with a neuroimmune disease. Age, sex, date of onset, geographic location and length of illness were recorded. The study group consisted of 29 children, 2-18 years of age and 37 parents. 19 of the adults (51%) have a neuroimmune illness including CFS, fibromyalgia and Lyme disease and 17 of the children (59%) are diagnosed with ASD. One pair of 3 yr old twins have Niemann-Pick type C, a neurodegenerative disease. 10 of the children (34%) were healthy siblings. Geographically, the subjects came from 11 states, 12% from the Southeast, 74% from the West, with 10% from NV, 8% from the Midwest and 6% from the Northeast. Sixteen families had more than one child participating including healthy siblings. Peripheral blood was drawn by a licensed phlebotomist under an approved IRB protocol, and shipped to the WPI for XMRV detection according to Lombardi et al. (Science, Oct 2009) including serology for antibodies to XMRV ENV, using PCR and RT-PCR on cultured PBMC nucleic acids as well as plasma isolation of XMRV to the LNCaP cell line. PCR products were sequenced at the Nevada Genomics Center using the ABI3730 DNA Analyzer.

Results: XMRV was detected in 55% of 66 cases of familial groups from 11 states. Sequencing of PCR products of *env* and *gag* confirmed XMRV. The age range of the infected children was 2-18. 17 of the children (including the identical twins) were positive for XMRV (58%) and 20 of the 37 parents (54%) were positive for XMRV. 14 of the 17 autistic children were positive for XMRV (82%). Of the 17 families, only one had all members of the family test negative for XMRV. In contrast, 16 of the families with neuroimmune disease, 9 families had at least 1 parent and child test positive for XMRV. 4 of the families had a parent test negative with a positive child, and 2 families had a parent test positive with the diseased child testing negative.

Conclusions: XMRV is observed in children with a wide spectrum of neuroimmune disorders and their family members. The significance of these findings is not clear.

No conflict of interest

Abstract: P_20

Chronic Fatigue syndrome/ neuro immune diseases

Is the mechanism of systemic immune activation in XMRV positive CFS patients similar to that observed in HIV ?

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Introduction HIV-1 infection results in chronic activation of the immune system. It was suggested (Brenchley et al., Nature 2006) to occur through a breakdown of the mucosal barrier and stimulation of immune cells by microbial products. CD14+ monocytes/macrophages secrete soluble CD14 (sCD14), which binds LPS and pro-inflammatory cytokines. In the study cited above it was shown that LPS directly stimulates sCD14 production in vivo. Earlier our group reported that serum LPS is significantly higher in Chronic Fatigue Syndrome (CFS) patients compared to contact and non-contact controls. Our hypothesis is that XMRV positive CFS patients also present with immune activation related to mucosal translocation in the gut

Material & methods: Fifteen XMRV positive CFS patients who fulfilled the Fukuda et al. criteria (1994) and matched controls were studied. The detection of XMRV was performed by LNCaP co-culture with PMCs as described by Lombardi et al. (2009). We used commercially available ELISA's to quantify levels of sCD14, C4a and cytokines. Stool IgA was determined by Diagnos-Techs,Inc (Tukwila,Washington,USA). Statistical analysis was performed using an ANOVA T-test

Results: XMRV positive CFS patients showed statistically significantly ($p<0.05$) higher serum levels of sCD14, C4a, IL-8 and MIP1-beta. Stool IgA levels were extremely low ($p<0.01$) compared to those of healthy controls

Conclusion: Although it is too early to conclude that XMRV is a cause of CFS, the similarity with HIV-1 where microbial translocation is a cause of systemic immune activation is striking. A study in rhesus macaques infected with XMRV showed infected CD4+ T cells in the gastrointestinal tract. The frequency of infected XMRV cells in the gastrointestinal tract in these macaques increased from acute to chronic infection (Sharma et al.). The data of our study along with the findings in XMRV infected macaques ask for confirmation by performing gastrointestinal biopsy studies in XMRV positive CFS patients

No conflict of interest

Abstract: P_21

Assay development & Screening

Inactivation of XMRV in platelet and RBC components prepared with the intercept blood system™

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Background: Xenotropic murine leukemia-related retrovirus (XMRV) is a newly identified retrovirus detected in humans. Results from several studies suggest an association of XMRV with prostate cancer while additional studies are ongoing to confirm disease association of XMRV with chronic fatigue syndrome (CFS). XMRV is infectious and blood-borne. Currently there are no regulatory approved blood screening assays for XMRV. To mitigate the potential risk of transfusion transmission, blood services in U.S., Canada, Australia, and New Zealand have decided to either discourage or defer donors with CFS from giving blood. Alternatively, pathogen inactivation (PI) technologies could be implemented to safeguard the blood supply from

XMRV, as is the case for plasma fractions (PPTA Press Release *April 7, 2010*). The INTERCEPT Blood System™ has robust inactivation capacity against a broad spectrum of viruses, bacteria, and parasites as well as leukocytes in platelet (PC), plasma, and RBC components, including two human retroviruses, HIV and HTLV. Preliminary experiments have shown that XMRV was sensitive to INTERCEPT treatment in PC. This study further evaluates the sensitivity of XMRV to INTERCEPT treatment and determines the level of XMRV inactivation in PC and RBC.

Materials and methods: Experiments were carried out by contaminating blood components with a natural isolate of XMRV from a CFS patient and then measuring the level of inactivation using an infectivity assay. The cell-free virus stock in PBS has an RT/PCR titer of ~10^7 copies/mL and an infectivity titer of at least 6 logs IU/mL. Two replicate experiments each were performed for PC and RBC. 30 mL of each PC containing 3×10^{10} platelets in 35% plasma/65% InterSol™ were inoculated with 1.5 mL of stock XMRV and treated with 150mM amotosalen and 3 Joules/cm^2 UVA light. 20 mL of each RBC in SAG-M (50% hematocrit) were inoculated with 1 mL of stock XMRV and treated by incubation with 0.2mM S-303 and 20mM GSH for 3 hours at room temperature. For each experiment, samples were withdrawn before and after treatment and assayed for the level of XMRV infectivity. Viable XMRV was quantified on LNCaP- iGFP indicator cells and analyzed using flow cytometry. Inoculated LNCaP-iGFP cells were passaged at least twice for analysis. The inactivation was expressed as Log_{10} reduction based on the ratio of the infectious viral titer pre-treatment to post-treatment.

Results: The mean pre-treatment XMRV titer was 5.2×10^4 IU/mL in PC and 1.6×10^5 IU/mL in RBC. After treatment, no viable XMRV was detected in PC (200 µL) after two passages on LNCaP-iGFP cells suggesting a residual titer of <5 IU/mL and a log reduction of >4.0. Similarly, no viable XMRV was detected in post-treatment RBC (60 µL) samples after two passages on the indicator cells suggesting a residual titer of <17 IU/mL and a log reduction of >4.0.

Conclusions: This study demonstrated that XMRV is effectively inactivated in both PC and RBC components by treatment with the INTERCEPT Blood System. PI with INTERCEPT has the potential to prevent transfusion-transmitted XMRV infection. Further studies will be performed to confirm XMRV inactivation in plasma with INTERCEPT treatment.

No conflict of interest

Abstract: P_22

Assay development & Screening

Presence of XMRV RNA in urine of prostate cancer patients

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Introduction: Urine specimens from cancer patients are often used as a source of RNA for detecting expression of genes of interest involved in cancer development and metastasis. A noninvasive urine based test for XMRV detection would be useful for screening and monitoring men with prostate cancer.

Methods: We have examined urine samples from 120 unselected prostate cancer patients after prostate massage during digital rectal examination. Also urine samples from 22 normal, healthy control individuals were tested. RNA was extracted from the samples using magnetic bead-based Magmax viral RNA isolation kit from Ambion. Quantitative RT-PCR (qRT-PCR) was used to measure low copies of XMRV RNA in the samples. Standard curves were performed using in vitro transcribed XMRV *env* RNA. Confirmation of the presence or absence of XMRV nucleic acids was obtained by nested RT-PCR (nRT-PCR) followed by sequencing of the amplicon. These methods specifically amplify *env* sequences encoding a region of XMRV gp70 envelope protein (variable regions A and C).

Results: We have identified XMRV specific nucleic acids from prostate cancer patient urine samples. The sensitivity of the qRT-PCR assay was determined to be about 10 copies of XMRV RNA per reaction. About 26% of prostate cancer

patients (31/120) showed the presence of XMRV *env* RNA by qRT-PCR analysis, whereas only 10 (8.3%) were scored as positive by nested nRT-PCR. However, all 10 of the samples that were positive for XMRV RNA by nRT-PCR were also positive by qRT-PCR. In contrast, 1 out of 22 (4.5%) control urine samples from normal healthy individuals were scored as positive for XMRV *env* RNA by qRT-PCR. A subset of positive samples was retested by qRT-PCR for another region of *env* and confirmed as positive. The range of XMRV RNA copy numbers detected was 15 to 60 copies per ml of urine. RT-PCR products of selected samples containing XMRV RNA were confirmed by DNA sequencing.

Conclusions: The presence of XMRV RNA in urine is significant because such data can provide the basis for a urine-based XMRV detection assay that is noninvasive, rapid, and easy to perform, avoiding the difficulty of obtaining blood or tissue biopsies. Results showed that qRT-PCR was more sensitive than nRT-PCR. Because men with XMRV infections, especially those that fail to clear the virus, may be at increased risk of prostate cancer these studies could result in a new diagnostic tool for evaluating risk of prostate cancer initiation and/or progression.

No conflict of interest

Abstract: P_23

Assay development & Screening

Absence of detectable XMRV in plasma or PBMC of human immunodeficiency virus type one (HIV-1) infected individuals in Cameroon and Uganda

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Background: Xenotropic murine leukemia virus-related virus (XMRV) was identified in 2006, and. may be linked to prostate cancer and chronic fatigue syndrome (CFS). XMRV has also been detected in about 4% of healthy individuals in the U.S. Similar to HIV-1, blood transfusion and intravenous drug use may be one potential route of transmission since both peripheral blood mononuclear cells (PBMCs) and plasma were shown to be susceptible to XMRV infection and transmit virus to uninfected cells.. These results highlight the need for studies on blood donors and HIV-1 infected individuals to determine the positive rates for XMRV in these populations. Towards this end, we set out to investigate the prevalence of XMRV infection in blood donors or HIV patients in selected African countries, a geographic distinct region from those already reported.

Materials and methods: A total of 163 plasma samples were tested including 69 HIV-1 antibody positive samples collected from 2006 to 2009 in Cameroon and 94 HIV-1 antibody positive plasma samples collected in 2005 in Uganda. Thirty-eight percent of the Ugandan subjects were on anti-retroviral therapy (ARV) and had fully suppressed HIV viral levels (viral load <400 copies/ml). Nineteen PBMC samples from HIV-1 positive individuals collected in 2007 in Cameroon were also included in the current study. Blood was collected aseptically into an EDTA vacutainer, and PBMC and plasma were separated out using Ficoll gradient centrifugation. Viral RNA and genomic DNA were extracted from 200 µl of plasma or PBMC samples using QIAamp MiniElute Virus Spin kit (Qiagen, Valencia, CA). RT-PCR, DNA PCR and a quantitative PCR (qPCR) were used to amplify XMRV gag, env and pol genes. HIV-1 gp41 gene was also amplified in parallel.

Results: Viral RNA in the plasma samples was used to amplify both XMRV and HIV-1 sequences. HIV-1 RNA was detected in 52% (36/69) and 52% (49/94) of plasma samples from Cameroon and Uganda, respectively. However, none of the 163 plasma samples were positive for XMRV RNA with either the gag or env primer sets of XMRV although the positive control was successfully amplified in each PCR run. Genomic DNAs from PBMCs of 19 HIV-1 infected Cameroonian individuals were also tested but negative for XMRV by both nest PCR and (q)PCR assays. Among them, 42% (8/19) were positive for HIV-1.

Conclusions: We screened 163 plasma samples and 19 PBMC samples of HIV-1 infected individuals from Cameroon and Uganda for XMRV infection. None of the samples were XMRV DNA/RNA positive. Our findings in this limited study population failed to demonstrate the presence of XMRV, in the African countries we evaluated.. To our knowledge, this study is the first attempt to evaluate the presence of XMRV DNA/RNA in plasma or PBMC samples collected from individuals in African countries. More data are needed to understand the epidemiology and geographic distribution of XMRV infection worldwide.

No conflict of interest

1st International Workshop on XMRV

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