An insight into "Investigation into the Presence of and Serological Response to XMRV in CFS Patients" (1)

Method

This next section is from the introduction:

"*It has been suggested that these negative results could have arisen because of a failure to duplicate the experimental conditions described in the original publication by Lombardi et al. To remove any element of doubt, we have repeated our genetic analysis using the same oligonucleotide primer sets described*" (in Lombardi et al).

Given that the last publication by these authors (2) which attempted to replicate the study of Lombardi et al (3) by introducing a large number of variables thus departed almost completely from the methodology of the latter group it would be interesting to investigate whether Erlwein et al have replicated the methodology of Lombardi et al on this occasion. The paper published in 2010 (2) raised a storm of controversy. This situation was largely generated because the lead author (McClure) had claimed that the research had only taken a few weeks and that the study only received 24 hours of peer review prior to publication. Amidst the resulting furore the authors were accused of duplicity and overt manipulation. Although this paper would appear to be the sort of rushed research that Stephen Legrice was so worried about in a recent WSJ article this reviewer would not accuse these investigators of possessing dark motives. The problems inherent in the design of the earlier study and indeed this study might well cause people to think of duplicity but a more simple explanation is that the research has indeed been rushed and the requisite literature searches have not been carried out properly.

Choosing to use 2 ml of whole blood as a source of DNA, and not the PMBCs isolated from 8 ml of blood used in Lombardi, is not an auspicious start for a group determined to engage in replicating methodology. Reducing the concentration of a PCR reactant will obviously reduce the yield as it would do in any reaction. Indeed with a low concentration, a reaction of a reactant may not proceed at all. This error however would seem to be the least of the mistakes made by the authors. To examine the magnitude of the departure from the methodology of Lombardi et al involved in this study a reminder of the conclusions presented in the Lombardi study would seem to be appropriate:

"XMRV-gag specific PCR products and no env specific PCR products following single round DNA PCR of DNA of unstimulated PBMCs. In contrast, when cDNA was prepared from PBMCs, 67% of the nested PCR, which inevitably raises questions of contamination, is not essential to detect XMRV in highly viremic ME/CFS patients. The XMRV-gag specific PCR products and no env specific PCR products following single round DNA PCR of DNA of unstimulated PBMCs. In contrast, when cDNA was prepared from PBMCs, 67% of the nested PCR, which inevitably raises questions of contamination, is not essential to detect XMRV in highly viremic ME/CFS patients." (addendum to 3)

"Using the Whittemore Peterson Institute's (WPI's) national tissue repository, which contains samples from well-characterized cohorts of CFS patients, we isolated nucleic acids from PBMCs and assayed the samples for XMRV gag sequences by nested polymerase chain reaction (PCR) (3). Of the 101 CFS samples analyzed, 68 (67%) contained XMRV gag sequence." (3)

From Urisman et al: "..this nested PCR assay was a reverse transcription PCR using cDNA prepared from patient RNA" (4).

Nested RT-PCR for *gag* sequences was done as described (5), with modifications. GAG-O-R primer was used for 1st strand synthesis; cycle conditions were 52oC annealing, for 35 cycles. For second round PCR, annealing was at 54oC for 35 cycles:

Round 1 PCR:PCR mix 1 (gag):
5.0 ul 10x buffer 2.5 ul 50 mM MgCl2 1.0 ul 12.5 mM dNTPs
0.5 ul GAG-O-R primer
0.5 ul GAG-O-R primer
38 ul H2O
0.5 ul Taq
ul cDNA

Cycles: 2 min 94 °C; [30 sec 94 °C, 30 sec 52 °C, 30 sec 72 °C] x 35 cycles; 7 min 72 °C

Round 2 PCR:
PCR mix:5.0 ul 10x buffer2.5 ul 50 mM MgCl2 1.0 ul 12.5 mM dNTPs
0.5 ul GAG-I-F primer
0.5 ul GAG-I-R primer
38 ul H2O
0.5 ul Taq
2.0 ul Round 1 DNA

Cycles: 2 min 94 °C; [30 sec 94 °C, 30 sec 54 °C, 30 sec 72 °C] x 35 cycles; 7 min 72 °C.

compare the approach of Erlwein et al:

"Reactions were carried out in a volume of 25 µl which contained 0.5 units TaqGold (Applied BioSystems, Warrington, UK), 1× TaqGold reaction buffer (Applied BioSystems), 1.5 mM Mg++, 200 mM each dNTP, 2.5 pmol each primer to which 5 µl DNA extract or control (see below) was added. The PCR conditions were as follows; one cycle at 94°C for 8 minutes, 45 cycles at 94°C for 30 seconds, 55°C for 30 seconds 72°C for 1 minute and a final annealing cycle of 72°C for 7 minutes."

Thus we have a replication approach using different primers, different concentration of primers, different concentrations of nucleotides, different concentrations of magnesium, buffers and cycles, and isolation of a different nucleic acid.

The use of the wrong primers and starting with the isolation of the wrong nucleic acid, from the perspective of the nested PCR assay, could be explained by inadequate care and attention to published research. The addendum to the Lombardi et al study (5) clearly stated that the primers chosen by Erlwein et al were effective in detecting gag and env sequences in seven of the group of eleven patients that displayed viremia. These primers were highly ineffective in detecting XMRV sequences in the other 90 patients investigated. Crucially no env sequences were detected in any of these 90 patients.

Next we turn to the serology section where sadly more glaring flaws in methodology are apparent:

"*gp70 antigen capture ELISA*

Microtiter plates were coated with 83A25 rat IgG2a anti-retroviral gp70 mAb (10 µg/ml), which recognizes xenotropic, polytropic, ecotropic and amphotropic murine leukemia viruses [22]. Serum samples were diluted 1:100 in PBS containing 1% BSA and 0.05% Tween 20 and incubated overnight at 4°C. Virus was detected following a 5-hour incubation with affinity-purified goat anti-Rauscher MLV gp70 or affinity-purified goat anti-NZB xenotropic virus antibodies, labelled with alkaline phosphatase. Results are expressed as absorbance values at 405 nm (A_{405}). In these assays, 2-fold serially diluted NZB virus, obtained from ViroMed Biosafety Laboratories, Camden, NJ, was used as a positive control." (1)

They have chosen the one MLV virus which does not react with the 83A25 monoclonal antibody (6) hence even if XMRV was present no reaction would be seen as the initial antibody antigen complex required for a viable ELISA would not exist.

Erlwein et al have chosen as a control diluted NZB VIRUS so if a virus reacts with an antibody to said virus then this is a measure that the assay will detect XMRV env proteins in plasma. This would seem to be a somewhat optimistic viewpoint.

"XMRV antibody response

Serum IgG anti-XMRV responses were determined by ELISA. Microtiter plates were coated with whole inactivated NZB retrovirus (10 µg/ml), and incubated with 1:100 diluted serum samples overnight at 4°C. The assay was developed following a 5 hour incubation with alkaline phosphatase-labelled goat anti-human IgG antibody (Cappel Laboratories, Durham, NC). The A_{405} values are shown. As there is no defined XMRV-positive human antiserum available, we used 603, a murine anti-Xeno gp70 monoclonal antibody as a positive control."

The assertion that there is no XMRV positive antiserum available as a control is astonishing given its ready availability.

The authors consider an antibody produced against a gp70 produced by mouse ervs as a sufficient measure of their assays ability to detect human XMRV env sequences (7). They also expect antibodies to XMRV env sequences to produce a positive reaction to NZB antigens. NZB has only 67% sequence

homology to XMRV (4).

These assays not only have no provenance of success but have no chance whatsoever of detecting XMRV.

Cohort

The authors clearly believe that the symptoms reported by their patients satisfy the 1994 CDC criteria, known as Fukuda (8). A close examination of the situation however reveals the position that they would certainly not if the meaning of the terminology used was considered rather than regarding the labels ascribed to the patients as objective descriptions when in fact they are not

When used to describe a symptom 'fatigue' is a subjective sensation and has a number of synonyms including tiredness and weariness. A clear description of the relationship of fatigue to activity is preferred to the term fatigability. Two aspects of fatigue are commonly reported: mental and physical. Mental fatigue is a subjective sensation characterized by lack of motivation and of alertness. Physical fatigue is felt as lack of energy or strength and is often felt as discomfort in the muscles.

The use of the Sharpe et al questionnaire (9) produces a cohort of patients who report tiredness, a lack of motivation and alertness and feel as though they have a lack of energy or strength in the muscles.

The following is the position of the authors of the 1994 CDC criteria:

"The chronic fatigue syndrome is a clinically defined condition (1-4) characterized by severe disabling fatigue" (8)

"A case of the chronic fatigue syndrome is defined by the presence of the following: 1) clinically evaluated, unexplained, persistent or relapsing chronic fatigue that is of new or definite onset (has not been lifelong); is not the result of ongoing exertion; is not substantially alleviated by rest; and results in substantial reduction in previous levels of occupational, educational, social, or personal activities;" (9? Haven't got full text)

Thus fatigue in this system of classification has an entirely different meaning and the cohorts are objectively different.

Next we turn to the thorny subject of disability. The authors of Sharpe et al have taken a some what unusual view on this:

"Disability

(i) This refers to any restriction or lack (resulting from loss of psychological or physiological function) of ability to perform an activity in the manner or within the range considered normal for a human being"

This means that anyone referred to a CBT clinic would certainly qualify for the

labelled as being disabled or presenting with a high level of disability because a loss of psychological function, because their loss of psychological function did not enable them to engage in an activity in the manner considered normal by the person assessing the patient. This appears to have the circularity inherent in the descriptive diagnoses which are the hallmark of psychiatry.

On the subject of the difference between objective medical diagnoses used by physicians and descriptive labels applied by psychiatrists, it is possible to classify people as having CFS using the CDC 1994 or to diagnose people as having CFS. To give a medical diagnosis the patient must have objectively measurable underlying abnormality. Patients who exhibit a range of physiologically abnormal responses to exercise (the hallmark symptom of the disease) are medically diagnosed as having what is now termed ME/CFS.

This is what Sharpe et al have to say about abnormal physiological responses to exercise

"The symptom of fatigue should not be confused with impairment of performance as measured by physiological or psychological testing. The physiological definition of fatigue is of a failure to sustain muscle force or power output."

The cohort of patients investigated by Lombardi et al all had physiologically abnormal responses to exercise and displayed a range of neuro-endocrine symptoms. Sharpe et all consider the presence of neuro endocrine symptoms as mandatory exclusion criteria.

Thus the cohorts examined by the authors of this study and that examined by Lombardi et al are different. Moreover none diagnosed by Sharp et al would qualify for a medical diagnosis of chronic fatigue syndrome when the CDC Fukuda criteria are used. Neither do the terms fatigue and disability have the same meaning when applied to the patients used in this study compared to the same terms used in the CDC criteria. Thus the authors have introduced yet another variable which robs their results of any objective meaning

" iiii. To be regarded as a symptom, fatigue must cause:

Disability

(i) This refers to any restriction or lack (resulting from loss of psychological or physiological function) of ability to perform an activity in the manner or within the range considered normal for a human being (ie things people cannot do in the areas of occupational, social, and leisure activities because of their illness.

Mood disturbance

(i) The term mood disturbance has been used to include depression, loss of interest and loss of pleasure (anhedonia), anxiety, emotional lability and irritability.

Myalgia

(i) This refers to the symptom of pain or aching, felt in the muscles

Sleep disturbance

(i) The symptom of sleep disturbance refers to a subjective report of a change in the duration or quality of sleep."

CDC Fukuda defined Chronic Fatigue Syndrome is a clinical condition (1-4) characterized by severe disabling fatigue:

"A case of Chronic Fatigue Syndrome is defined by the presence of the following:

1) clinically evaluated, unexplained, persistent or relapsing chronic fatigue that is of new or definite onset (has not been lifelong); is not the result of ongoing exertion; is not substantially alleviated by rest; and results in substantial reduction in previous levels of occupational, educational, social, or personal activities; and

2) the concurrent occurrence of four or more of the following symptoms, all of which must have persisted or recurred during 6 or more consecutive months of illness and must not have predated the fatigue: self-reported impairment in short-term memory or concentration severe enough to cause substantial reduction in previous levels of occupational, educational, social, or personal activities; sore throat; tender cervical or axillary lymph nodes; muscle pain, multijoint pain without joint swelling or redness; headaches of a new type, pattern, or severity; unrefreshing sleep; and postexertional malaise."

Thus we have a cohort of people disabled by a level of fatigue not produced as a result of exertion, not alleviated by rest, and taking the definitions as a whole produce a different cohort of patients to the one produced via the use of the Sharpe et al questionnaire.

The differences do not end there however. This is Sharpe et al on the subject of disability:

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Thus the cohorts examined by the authors of this study and that examined by Lombardi et al are different. Moreover none diagnosed by Sharp et al would qualify for a medical diagnosis of chronic fatigue syndrome when the CDC criteria are used. Neither do the terms fatigue and disability have the same meaning when applied to the patients used in this study compared to the same terms used in the CDC Fukuda criteria. Thus the authors have introduced yet another variable which robs their results of any objective meaning.

References:

1) Erlwein O, Robinson MJ, Kaye S, Wills G, Izui S, et al. 2011 Investigation into the Presence of and Serological Response to XMRV in CFS Patients. PLoS ONE 6(3): e17592. doi:10.1371/journal.pone.0017592

2) Erlwein O, Kaye S, McClure MO, Weber J, Wills G, et al. (2010) Failure to detect the novel retrovirus XMRV in chronic fatigue syndrome. PLoS One 5(1): e8519.

3) Lombardi V, Ruscetti FW, Gupta JD, Pfost MA, Hagen KS, et al. (2009) Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome. Science 326: 585–589.

4) Urisman A, Molinaro RJ, Fischer N, Plummer SJ, Casey G, et al. (2006) Identification of a novel gammaretrovirus in prostate tumors of patients homozygous for R462Q RNASEL variant. PLoS Pathog 2: 211–225.

5) Mikovits J, Lombardi V, Pfost M, Hagen K, Ruscetti F (2010) Addenda to: Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome. Virulence 1: 1–5.

6) Evans LH, Morrison RP, Malik FG, Portis J, Britt WJ (1990) A neutralizable epitope common to the envelope glycoproteins of ecotropic, polytropic, xenotropic, and amphotropic murine leukemia viruses. J Virol 64: 6176–61

7) Portis J.L., McAtee F.J. Monoclonal antibodies derived during graft-versus-host reaction. II. Antibodies detect unique determinants common to many MCF viruses (1983) Virology, 126 (1), pp. 96-105.

8) Fukuda K, Strauss S, Hickie I, Sharpe MC, Dobbins JG, et al. (1994) The chronic fatigue syndrome: a comprehensive approach to its definition and study. Annals of Internal Medicine 121: 953–959.

9) Sharpe M, Chalder T, Palmer I, Wessely S (1997) Chronic fatigue syndrome. A practical guide to assessment and management. General Hospital Psychiatry 19: 185–199