Briefing notes to the Commonwealth of Australia Parliamentary Committee Hearing, Canberra, 16 April 2009

Defence Sub-Committee's Inquiry into RAAF F1-11 Deseal/Reseal workers and their families

SCOPE OF SPECIALIST QUALIFICATIONS AND EXPERTISE

I am a Specialist medical practitioner registered in the State of Queensland. I hold the qualifications of Bachelor of Medicine and Bachelor of Surgery and a Doctorate in Philosophy (Biochemical Genetics) from the University of Queensland, and Master of Business Administration from the Queensland University of Technology. I am a Fellow of the Royal Australasian College of Pathologists with specialist recognition in Chemical Pathology and in Biochemical Genetics and Fellow of the Human Genetics Society of Australasia with specialist recognition in Biochemical Genetics.
I hold the position of Professor of Medical Biochemistry at the University of Queensland and Director of Biochemical Diseases at the Mater Children's Hospital, Brisbane.

I also have extensive experience in statewide population screening (Newborn Screening) for rare metabolic disorders. Newborn screening programmes must deal with issues of test limitations, specificity (false positives), and sensitivity (false negatives).

My special interests are in the inherited disorders of metabolism. I am the author of 80 research and clinical publications in this field.

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Introduction

In 1999 the Royal Australian Air Force medical section at RAAF Base Amberley noted many members of the fuel tank repair section at 501 Wing had developed a variety of conditions that appeared to be caused by exposure to the fuels and solvents employed in the De-sealing and Re-sealing of aircraft fuel tanks.

A Board of Inquiry concluded that greater than 400 persons had their health permanently damaged as a result of the F111 Deseal / Reseal program.

One such member was identified as suffering from Mitochondrial Encephalopathy and Lactic Acidosis (MELAS), a familial condition that is inherited through the maternal line.

Historical medical documents revealed that this person was asymptomatic up to the time that he started work in the fuel tanks repair section. Soon after commencement of employment in the fuel tanks, he developed a condition similar to epilepsy. His condition steadily deteriorated, developing multiple cerebral (non-haematological) micro-infarcts, resulting in large loss of brain tissue (apoptosis).

Although the member's MELAS condition would most probably have eventually caused his demise, there was some concern that exposure to the various aromatic and aliphatic solvents associated with the Deseal / Reseal programme may have accelerated the development of his condition by compromising his unaffected mitochondria.

A review of medical records and examination of 12 exposed individuals referred to my clinic showed symptoms that are known to be associated with mitochondrial disruption. However, as the symptoms are non-specific, mitochondrial disorders can rarely be diagnosed by symptoms alone. The published consensus for diagnosis of mitochondrial disorders requires a combination of clinical symptoms, patient history, and key major or several minor laboratory findings.

What are Mitochondria?

Mitochondria are small functional bodies (organelles) residing within a living cell. These organelles are responsible for converting the food that we eat into a usable energy source that allows living cells to operate. Without this energy a cell will die, or may not work efficiently if there is a significant reduction of this energy supply. There can be many thousands of mitochondria within a cell, each of these produce a quota of energy. The substance being produced is known as Adenosine TriPhosphate (ATP). On average the human body will generate and consume equal to or greater than 60 kilograms of this substance per day. Mitochondria have their own DNA (mtDNA) independent of the cells nuclear DNA. Nuclear genes code the majority of mitochondrial proteins.

There is a strong relationship between mitochondrial genes/proteins and nuclear genes/proteins. Cells can affect mitochondrial function and mitochondrial function can affect cells. Mitochondria control a programmed

cell death function, called apoptosis. When a cell sustains damage, the mitochondria determine whether the cell should survive or die. Specific mitochondrial pathways for cell repair or cell death are activated. Various cell signals and proteins are then generated to bring about the determined response.

Mitochondria are passed down through generations of cells. In humans, all mitochondria are derived from the egg (and are rarely inherited through the father). In the blood, the mitochondria are derived from stem cells in the bone marrow.

What Can Go Wrong with Mitochondria?

Mitochondria can malfunction or be depleted in numbers by the natural aging process, hereditary disorders, or by exposure to a range of environmental conditions including various solvents. Studies conducted throughout the world have identified a variety of solvents as causative agents in some acquired mitopathies. Changes in mitochondria also occur in response to other types of cell injury.

Investigations into the nature of (cell) injury in exposed individuals.

In 2004, I was commissioned by the Chief of Air Force to study the possible effects on the mitochondria of personnel who were exposed to the F1-11 Deseal / Reseal programs. The purpose of these studies was to identify abnormalities of mitochondria in exposed individuals, both to understand the nature of cell injury following exposure and to identify a possible marker of cell injury.

Summary of previous studies

Initial Pilot Studies.

All studies were conducted in a double masked manner. All controls were matched to subjects for possible confounding factors.

The first study investigated mitochondrial DNA as an index of mitochondrial depletion (which occurs following mitochondrial injury). A small difference in the peripheral blood mitochondrial DNA between samples from exposed and non-exposed was observed, but the difference did not reach statistical significance.

A following study showed an apparent increase in a mitochondrial protein (TOM40) in exposed individuals. TOM40 is a newly discovered protein thought to be involved in mitochondrial maintenance and repair. While the finding was statistically significant, the difference in protein levels was small. The power of the study to identify an exposed sample was low. This could have been due to the small number of (affected) exposed subjects taking part, with considerable overlap between exposed symptomatic, exposed non-symptomatic, and non-exposed samples.

This led to a third study using a proteomics technique where several hundred proteins were assayed and compared with an unexposed cohort. This study appeared to demonstrate an increase in another protein specific to the subject group of exposed individuals. Although this study appeared to confirm that mitochondria were in some way affected in the exposed group, the power of the study was once again low, due to small subject numbers. Also, the protein that appeared to be up-regulated in the subject group was of a nature that has only marginal association with mitochondria (β chain haemoglobin).

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Details of current Pilot Study

1.1 Resulting from the findings of the first studies, a study was commissioned to ascertain how often non-exposed individuals could show protein changes. The study was designed to sample a much larger non-exposed subject group, and a smaller positive exposure group.

Subject Groups

1.2 The Target (unexposed) group consisted of 21 randomly selected civilians from a variety of occupations. Only 2 subjects in this group had connection with the RAAF and all were screened for confounding factors. The Control group of exposed individuals consisted of 5 known heavily exposed individuals and 1 control individual who had ingested Aviation Turbine Fuel under high pressure and is now demonstrating long term effects of that exposure.

Findings

1.3 The 21 members of the subject group were assayed against 5 known exposed individuals in a double blind study. All 6 exposed individuals (5 F1-11 exposed and 1 positive control) demonstrated a small but significant up-regulation of the same five separate proteins (yet to be identified) when compared with the average levels of the unexposed Target group.

Methodology

Study Participants (Annex A)

2.0 Study participants comprised a total of 27 participants divided into two groups, a Target group (unexposed to F1-11 fuel tank maintenance) and a Control group made up of personnel with a history of major involvement with the F111 DSRS process. Both groups were age and sex matched. Both groups were screened by a questionnaire to elicit known confounding factors.

2.1 Target Group (Unexposed Participants): The Target group consisted of 21 unexposed personnel selected at random, mostly from non-RAAF sources. The average age for this population at time of collection was 41 years. The population for this group came from:

** Randomly selected employees from Environmental Toxicology Queensland (ENTOX) comprising 61%*

** Random selected civilians from general public comprising 29%*

** RAAF Associated personnel (non exposed) 10%*

Target Group Confounding Factors examined

2.2 Solvents exposure: 48% of the Target Group did not use solvents while the remaining 52% used a combination of: Isopropanol, acetone, hexane, methanol, ethanol, dichloromethane, white spirits or kerosene on an 'as required' basis. These exposures were typically in a protective environment. All were minimal and without effect.

2.3 Smoking: 38% of the Target group did not smoke, 10% were light smokers, 24% were medium smokers and 28% were heavy smokers

Where up to 5 cigarettes a day was considered light smoking, 6 to 10 cigarettes a day were considered medium smoking and 11 cigarettes a day or more was considered heavy smoking.

2.4 Alcohol: Of the Target population 5% did not drink, 38% drank less than 1standard drink a day, 38% drank 1 standard drink a day and 19% drank more than 1 standard drink a day.

3.0 Exposed Group (F111 Exposed Individuals): The control group consisted of 5 volunteers with an average age of 45 years at time of collection. Members of this group have had major involvement with the F111 fuel tank maintenance programs, plus an internal control made up of a single individual who ingested a large quantity of aircraft aviation fuel (AVTUR) under high pressure and who is suffering major health effects.

Exposed Group Confounding Factors examined.

3.1 Solvents:Five of the exposed group worked within the F111 fuel tanks during some part of the F111 DS/RS programs and exposed to a wide range of solvents used in fuel tank maintenance and to jet fuel.

3.2 Smoking: 83% of the Control group did not smoke, while the remaining 17% were medium smokers.

3.3 Alcohol: 17% of the Control group did not drink, 33% drank 1 standard drink per day whilst the remaining 50% drank more than 1 standard drink per day.

Mitochondrial Extraction

4.0 Mitochondria from both groups were derived from lymphocytes harvested from 40 ml whole blood anticoagulated with EDTA.

4.1 Mitochondrial Extraction from lymphocytes (Annex B):Lymphocytes were harvested from 40ml whole EDTA blood using a modified Ficol Paque Plus gradient technique. After the lymphocytes were isolated, the mitochondria were extracted using a modified "Pierce" reagent based mitochondrial extraction protocol. After extraction, a quantitative analysis of total protein, using a Bradford protein assay was performed. The mitochondrial extract was subjected to a Western Blot analysis to probe for COX 5a (Cytochrome C Oxidase 5a) and GAPDH (a cytosolic protein) to gauge the purity of the mitochondrial extraction.

Proteomics Analysis (Annex C)

5.0 Proteomics analysis was performed by Dr Steven Mason at the University of Queensland. The analysis was performed as a double blind study, using a 2 dimensional gel electrophoresis technique (2DGel). Some two hundred proteins were noted and a proteomics analysis was performed using the Progenisis program. The results of the analysis are at Annex C.

Discussion of study methods and findings

Subject Groups

Enrolment of exposed persons proved somewhat difficult due to the time lag between exposure and participation, therefore only active members of the F111 Support group took part. The Control subjects for this study were selected specifically from personnel that were known to have had heavy exposure to the DSRS process. The internal control for this group had a single major ingestion exposure. to Aviation Turbine Fuel (AVTUR). The selection process of the Control group may bias epidemiological information gained from this study.

The Target (non-exposed) group proved somewhat easier to enrol. Participants were not known to the individual researchers and all were randomly selected. *(However two subjects known to the researchers were included as additional blind internal controls. One of these two subjects suffers type 2 diabetes and the other had received recent chemotherapy. Interestingly, neither of these subjects showed the up-regulation of proteins that are of current interest with the F111 exposed group.)*

Mitochondrial Extraction Process

There was a modification of the mitochondrial extraction process that included a MQ H20 lysis step which was designed to remove haemoglobin from free floating red cells, normoblasts and reticulocytes during the primary lymphocyte extraction. The process also removed any extraneous surface haemoglobin that may be attached to lymphocytes.

The minimum total mitochondrial protein yield requested for 2D Gel analysis was 50 micrograms (allowing for cleanup and the possible necessity for repeat runs). This level of extraction was quite difficult to achieve and difficult to standardise due to differences in total leukocyte and lymphocytes counts between individual subjects. Also the requirement to process all samples immediately after collection restricted the number of samples that could be handled at to two specimens per day. Nevertheless, an average of 100?g of total mitochondrial protein was achieved.

Findings.

This study was designed to test whether previously detected proteins in individuals that were exposed to the F111 DS/RS programs were not present in a larger group of unexposed individuals. The hypothesis for this pilot study was that the unexposed group would not display proteins found in the exposed group. While the previously detected proteins were not evident in this latest study in either the Target or Control groups, there does appear to be a significant if small up-regulation of 5 proteins in the

exposed group measured against the non-exposed group. This up-regulation appears only in the group of exposed individuals.

Because the same 5 proteins are increased in six individuals, the findings are thought to be significant.

In a previous protein study, a pattern of proteins (mainly β chain Haemoglobin) was consistently present in the study group of exposed persons only. This pattern of proteins was not present in any specimens analysed in this current study. There is a suggestion that the haemoglobin fraction detected in the last study may have resulted from a carry over from immature (partially nucleated) red cells in the extraction process. The additional step of a MQH20 appears to have removed that previously detected haemoglobin sub unit; however it still does not answer the reason why this protein was only detected in exposed individuals. (There was no difference in the masked treatment of the samples).

Conclusions

1. The results of these studies implicate changes in mitochondrial proteins in peripheral blood samples in individuals exposed to fuel solvents.
2. The data suggest involvement of immature blood cells (stem cells) in the protein changes seen following fuel exposure. It is my opinion that the mitochondrial changes seen in these pilot studies are an indication of disruption of stem cells in the bone marrow (and possibly in other tissues).
3. One individual who demonstrated a similar pattern had not been exposed to F111 DS / RS solvents but only to Aviation Turbine Fuel (significant accidental ingestion). This indicates that the damaging agent is a constituent of the fuel and not the solvents (used for Re-Seal/De-Seal).
4. The finding of changes persisting in peripheral blood several years after the exposure suggests that the cells responsible for generation of peripheral blood cells (stem cells) in the bone marrow have been affected.
5. The mitochondria in peripheral blood are generated from the mitochondria in the stem cells. Because mitochondria (proteins) are constantly regenerated using mostly nuclear genes and to much lesser extent mitochondrial genes, the most likely explanation is that the changes seen in mitochondrial proteins are a reaction to some disruption in the stem cells.
6. The cohort of individuals involved in fuel exposure are likely to vary considerably in their reponse to the cellular injury. The variation would be due to :-

    (i) differences in exposure,

    (ii) individual genetically determined susceptibilities,

    (iii) individual genetically determined repair abilities, and

    (iv) other lifestyle factors.

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Recommendations

1. Mitochondrial and stem cell derived proteins in peripheral blood have the potential to show cellular injury following exposure to fuel. Before the protein pattern could be considered as a marker of injury following fuel exposure, the mitochondrial and other proteins derived from bone marrow stem cells should be further characterised.

2. There are two investigation strategies that could be considered:-

   (1) Specific protein analysis . A very detailed analysis of a limited number of bone marrow samples from individuals carefully selected to have a burden of disease. This would identify the proteins strongly linked to the cellular pathologies. Bone marrow biopsy is an invasive procedure and volunteers may be unwilling to participate.

   (2) Proteomics approach. More general protein profiling *(Supervised Principal Component analysis)* of peripheral blood cells from a larger number of samples of exposed and non-exposed individuals. A Supervised data analysis of a clinically defined cohort would be used to set the rules for the Principal components to discriminate an unknown data set (of individuals not previously tested). This approach has the advantage that while all exposed individuals would need to be tested, a full clinical history would only need to be ascertained from the cohort used to define the test parameters.

   The proteins should be characterised by a clinical laboratory with experience in human protein abnormalities using state of the art technologies *(such as MALDI-TOF/TOF)*.

3. If the protein profile were to be used as a test for injury following exposure (a screening test) , advice should be sought (from the Committee ?) regarding whether the test should be designed with high specificity *(low rate false positives)* or high sensitivity *(low rate false negatives)*.

4. A mechanism to discriminate (injured from non-injured) would be improved by the combination of indices including:-

   (i) History of Exposure

   (ii) Presence of medical (including psychiatric) symptoms not **fully** explained by other disease causing aetiologies.

   (iii) Abnormal protein profile.

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Annexure: (Available on request)

A1. Participant Information Sheet

A2. Consent Form

A3. Confounding factors Questionnaire

A4. Collection Staff Information and Instruction Sheet

B. Detailed Methodology for mitochondria extraction

C. Proteomics Analysis data

D. Original Research Proposals

E. UQ and Mater Ethics Approvals

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