Oxidation contributes to low glutathione in the airways of young children with cystic fibrosis

Anthony J Kettle[1], Rufus Turner R[1], Catherine L Gangell PhD[2], Irada S Khalilova I[1], D Timothy Harwood[1], Anna L Chapman[1], Christine C Winterbourn[1], Peter D Sly MD[2]

on behalf of AREST CF[3]

AREST CF Early Surveillance Program.

Study population

The AREST CF program covers a geographically-defined population. All children diagnosed with CF in Western Australia are managed at Princess Margaret Hospital, Perth and children diagnosed in Victoria (apart from those in the southern metropolitan area) are managed at the Royal Children's Hospital, Melbourne. The early surveillance program is well received by the clinic populations with >95% of eligible children participating.

Diagnostic Procedures

The newborn screening protocols are as follows:

Perth: immunoreactive trypsinogen (IRT) is measured on the Guthrie blood spot obtained soon after birth. Samples with IRT above the 97% centile for the day are referred for genotyping for Phe508del, Gly551Asp, Gly542X and c.489+1G>T. Children with two CF mutations identified are referred direct to the CF clinic for assessment. Children with a single CF mutation identified are recalled for a sweat test and those with equivocal (35-60mmol/L) or diagnostic (>60mmol/L) sweat chloride levels are referred to the CF clinic for assessment. The CF clinic organises sweat tests on those with two mutations and repeat tests on those with one mutation.

Melbourne: immunoreactive trypsinogen (IRT) is measured on the newborn screening blood spot obtained soon after birth. Samples with IRT above the 99% centile for the day are referred for genotyping for Phe508del, Gly551Asp, Gly542X, Asn1303Lys, c.1585-1G>A, Ile507del, Arg560Thr, Trp1282X, Val520Phe, c.489+1G>T, Arg553X and c.3718-2477C>T. Children with two CF mutations identified are referred direct to the CF clinic for assessment. Child with a single CF mutation identified are recalled for a sweat test and those with equivocal (30-59mmol/L) or diagnostic (≥60mmol/L) sweat chloride levels are referred to the CF clinic for assessment.

Surveillance protocol

The AREST CF surveillance protocol includes an assessment soon after diagnosis (approximately 3 months of age) and then annually, close to the child's birthday. These assessments include:

- Chest CT Scanning using a 3-slice inspiratory (25 cmH_2O) and 3-slice expiratory (0 cmH_2O) protocol under general anaesthesia.
- Bronchoscopy and bronchoalveolar lavage (BAL) follow the CT scan for assessment of pulmonary infection and inflammation.
- Lung function testing using infant lung function tests under chloral hydrate sedation at 3 months, 1 and 2 years; and using preschool techniques (unsedated) at 3 years and older.
- Collection of urine for assessment of biomarkers of inflammation, oxidative stress and lung damage.

Parents are informed that this is a clinically-directed surveillance program with some research aspects. Parents are given the opportunity to consent to each aspect (clinical or research) of the program separately.

Anaesthetic management for CT scanning and bronchoscopy

Anaesthesia is induced with either propofol or sevoflurane according to the clinical judgement of the anaesthetist. The child is then intubated, preferably with a cuffed tube, for CT scanning and a standardised recruitment manoeuvre, consisting of 10 consecutive slow breaths up to total lung capacity (37-40 cmH_2O) over a PEEP of 5 cmH_2O to open up anaesthesia induced atelectasis for 1-2 seconds each inspiration. Then, manual ventilation with a PEEP of 5 cmH_20 is continued. For the inspiratory scans the airway opening pressure was held steady at 25 cmH_2O. For bronchoscopy and BAL the anaesthesia switched to (or continued as) a total intravenous anaesthesia with propofol and remifentanyl as follows: Propofol 12 mg/kg/h for first 10 min, then 9 mg/kg/h for further 10 min, then 6 mg/kg/h; Remifentanyl dilution 20 μg/ml and "Kg body weight" in ml/h of remifentanyl solution as starting dosage, adapted to clinical needs. If anaesthesia needs to be deepened, the remifentanyl is increased rather than the propofol.

For bronchoscopy, the endotracheal tube is replaced by a disposable laryngeal mask airway to facilitate passage of the bronchoscope. Lignocaine is not given before the end of the bronchoscopy as it is bacteriostatic and interferes with the microbiology testing.

Bronchoalveolar Lavage

BAL was performed following the CT. Three aliquots of normal saline (1 ml/kg) were instilled into the right middle or right lower lobe and retrieved using low pressure suction. In March 2007 the protocol was changed to add a single aliquot instilled into the lingula or most affected lobe on CT following those performed in the right lung. The first and aliquot from each site was sent for microbiology and the remaining aliquots from the right lung were pooled and stored on ice until processed (within 1 hour) and frozen at -80°C for later assessment of inflammation. Melbourne samples were transported to Perth for analysis. The samples were frozen and transported to Christchurch in dry ice, by airfreight. In Christchurch, samples were centrifuged at 15,000 x g for 10 minutes at room temperature. Supernatants were aliquoted and stored at -80°C.

Microbiological protocols

Using a sterile disposable pipette a drop (10 – 50ul) of BAL fluid was inoculated onto agar plates and spread for single colonies using a sterile loop. The plates were inoculated in the following manner : Blood agar, Cytosine Lactose Electrolyte Deficient (CLED) agar, and blood agar + ticarcillin (for resistant *P. aeruginosa* identification) were incubated at 35ºC for 48hrs in a CO_2 incubator; PC plate (selective plate for *B.cepacia*) was incubated at 35ºC for 48hrs in a CO_2 incubator, then at room temperature for another 24hrs; MSA (Mannitol salt agar for *S. aureus*) was incubated at 35ºC for 48hrs in an aerobic atmosphere; Sabarouds agar (for fungi isolation) was incubated at 35ºC for 48hrs in a CO_2 incubator, then at 28ºC for 14 days; Fildes agar (for *Haemophilus* isolation) plates were incubated at 35ºC for 48hrs in an anaerobic environment (anaerobic incubation to prevent overgrowth by *P.aeruginosa*)

Gram stain was prepared and examined for bacteria, leucocytes and epithelial cells. Bacteria were identified by colony morphology, gram stain and biochemical tests including oxidase and C390 screening test specifically for *Pseudomonas*. *P. aeruginosa* were classified as smooth, rough or mucoid based on colony morphology on blood agar plates. *P. aeruginosa* colonies were unclassified if colony morphology was ambiguous. Growth was reported as isolated colonies, light, scanty, moderate or abundant growth based on the organism density on the plates which relate to colony counts of $<10^3$, 10^4, 10^5, 10^6, 10^7, respectively.

Sensitivity testing of all pathogens, including *P. aeruginosa* and, *S. aureus* were performed by the agar dilution breakpoint method according to Clinical Laboratory Standards Institute (CLSI) guidelines. Sensitivity of isolates such as *Haemophilus* was performed by disc testing using CSLI guidelines. A wet preparation was made from the BAL fluid and examined for fungal elements.

Immunofluorescence and culture methods were used to identify viral infections. For immunofluorescence, specimens were prepared by washing the cell pellet in PBS until a 'tight' cell pellet was obtained. A glass pipette was used to make a smear of the cells within four wells of an 8-well Teflon coated slide for RSV; Parainfluenzae 1,2&3; influenzae A&B; and adenovirus. The slide was air dried at room temperature and fixed in acetone. Direct Fluorescent Antibody Testing was carried out using commercially available monoclonal antibodies. If no evidence of viral infection was detected then BAL fluid was processed for viral cell culture. Virus was cultured by inoculation of an aliquot of BAL on to cultured mammalian cell lines grown on round glass coverslips. Virus was identified by staining the cultured cells (on the coverslips) with monoclonal antibodies and examination of the cells under a fluorescence microscope.

Assessment of inflammation

BAL fluid was pooled and centrifuged for 5 minutes at 1500 rpm. Aliquots of supernatant were stored at -80ºC until needed for further analysis. The cell pellet was washed if required and resuspended in 1ml PBS. Total cell count using a haemocytometer was performed on 10ul of cell suspension and viability assessed using equal volume of tryphan blue stain. Cytospins were performed using 10^6 cells/ml and stained using

Leishman stain. Differential cell counts were performed on 300 consecutive cells at 100x magnification. The following cells were counted: macrophages, neutrophils, lymphocytes and eosinophils. Samples frozen at -80ºC were transported to Perth from Melbourne overnight in an insulated container packed with dry ice to keep frozen. On arrival in Perth all samples were stored at -80ºC. Frozen samples were also transported to Christchurch at -80ºC for

Analysis of inflammatory mediators IL-1β, IL-12, IL-6, IL-8, IL-10 and TNFα were conducted using a standard cytometric bead array human inflammation kit (BD Biosciences, San Diego, CA) with a working range between 20 and 5000 pg/ml. Analysis of IL-8 was completed using an ELISA (BD Opt EIA, BD Biosciences, San Diego, CA) with a working range between 0.01 and 6.40 ng/ml.

Free neutrophil elastase activity was assessed using an adapted ELISA. BAL fluid supernatant was serial diluted 1:2 with Tris buffer in duplicate. Tris buffer was the negative control and human neutrophil elastase diluted to 25 ug/ml was the standard. Substrate N-methoxysuccinyl-ala-ala-pro-val p-nitroanilide (dissolved in NMP) was added to each well. Activity was read immediately at 450nm. The plate was then incubated at 37ºC in a CO_2 incubator and read again at 20, 30 and 40 minutes. The results were calculated using AssayZap and the best time point taken. The lower limit of detection for this assay was 0.2ug/ml.