

# EXHIBIT W

## Report for the Inquiry into the Convictions of Kathleen Megan Folbigg

1. My name is Professor Robert Llewellyn Clancy AM, BSc (Med) Hons, MB BS (Hons), PhD DSc, FRACP, FRCP(A), FRCP(C).
2. My address is [REDACTED]
3. I attach that part of my DSc thesis that has an explanation and summary of the work I undertook (**Attachment A**).
4. I attach my CV indicating I have specialised knowledge (**Attachment B**).
5. I agree to be bound to the UCPR (signed) (**Attachment C**).
6. Attached are also relevant references (**Attachment D**).
7. I am a mucosal immunologist and Foundation Professor of Pathology at the University of Newcastle.
8. We studied prospectively two hundred and sixty babies born in Newcastle over a period of twenty-one years. We correlated clinical events with parameters of mucosal immune competence.
9. We found that the airways immune system undergoes a dramatic change, weeks to months after birth – a time of unstable immunity when exposure to bacteria and viruses can give an inappropriate and excessive response. We noted leaky mucosa only in those with SIDS and in studies of near-miss SIDS.
10. One of the children in our study was found dead by our nurse and diagnosed as dying of SIDS. We had the only prospective study of SIDS ever and showed a bizarre and inappropriate immune response to a presumed virus infection three weeks prior to death.
11. This was considered to cause a reflex cessation of breathing and death. The stimulus was likely bacteria entering the airways leading to inflammation i.e. there is exudate and bacteria – perhaps a few inflammatory cells within the airways. This is exactly what you find in cases of SIDS at postmortem. N.B. the importance of toxin producing bacteria in SIDS cases was found after our studies, but they complement our immunological findings very well.
12. We then followed twenty cases of ‘near miss SIDS’ (or ALTE’s when the infant stops breathing but is immediately resuscitated) – we found that these infants had the same changes in their saliva at the time of the event (i.e. is it reasonable to extrapolate therefore that there is a common process in most SIDS cases).

## **Review of Post Mortem Examinations**

13. I have read the autopsy reports of Caleb Gibson Folbigg, Patrick Allen Folbigg, Sarah Kathleen Folbigg and Laura Elizabeth Folbigg.
14. I agree with the post mortem diagnosis made by the attending pathologist on each occasion, and agreed by consultants Professor's Cordner and Duflou of SIDS in each of Caleb and Sarah. I agree with the diagnosis made by Professor's Cordner and Duflou in the case of Laura, as acute myocarditis. The diagnosis in Patrick is difficult, but I agree that hypoxic ischaemic encephalopathy resulting from acute life threatening event or ALTE (near miss SIDS) is the most likely diagnosis (see also my report re SIDS and near miss SIDS). I am uncertain why the pathologist (Dr Cala) did not identify myocarditis as the cause of death in Laura.
15. I have looked at the post mortem report of Caleb Folbigg and note within the lungs there is a small amount of eosinophilic exudate in the alveoli. This means that there has been an inflammatory process in the wall of the small airways and that as a result there has been an exudate (i.e. there is protein present) as a response to probable bacteria stimulation and/or there is the 'leaky mucosa' as found in SIDS. Eosinophilic means that the eosin in the stain used has bound to protein.
16. There is in SIDS and near miss SIDS an exaggerated secretion of immunoglobulins (proteins) into mucosal secretions. Thus, this finding of 'eosinophilic exudate' heightens diagnostic confidence of SIDS.
17. The finding of poly bacterial growth in blood culture (Patrick), lungs (Sarah) and Laura (as reported in Professor Blackwell's report) are real findings and again consistent with the recent findings researched by Professor Blackwell. The failure to eradicate contamination of bacteria is consistent with the impaired mucosal immune response at the 'change-over' period discussed in my report. Unfortunately there is no microbiological report for Caleb.
18. I see no evidence that any child has been mistreated or any evidence of external trauma or internal evidence of suffocating or alternate cause of death other than SIDS or myocarditis. Any conviction of Kathleen Folbigg based on medical grounds, in my opinion, would be unsafe.

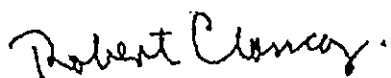
## **Review of Report by Professor Blackwell**

19. I have read the report of Professor Blackwell dated 5 March 2019 and have had discussion with her about same.
20. This is a highly referenced, thoughtful and valuable contribution to elucidating the causes of death in the four Folbigg children.

21. I agree with the comments made and conclusions, which include her review of the post mortem results by anatomical pathologists with expertise in SIDS (Professor Cordner, Professor Duflou).
22. While it is difficult to improve on comments in this report, I will add comment to emphasise aspects I consider important.
23. Page 1: Bacterial isolates and toxins. The foundation studies of exotoxins and bacteria by Professor Blackwell are a major step forward in understanding the pathogenesis of SIDS. They complement the studies of mucosal immunology done by my group and give a likely explanation of the cause of death of Sarah Folbigg.

## Conclusion

24. I conclude that two of the children died from SIDS, one likely had an ALTE leading to brain damage and the fourth child (Laura) died from arrhythmia secondary to significant myocarditis.
25. There is NO evidence of any alternate cause of death.
26. Now we know the developmental 'setting' of SIDS from our immunological studies that there are clearly multiple genetics foci (as well as environmental events such as infections) that come together to cause SIDS. There are many reports of multiple cases of SIDS within a family (here probably three); it was a matter of time before such a case occurred in Australia.



Professor Robert Clancy

13 March 2019



**Submission for Degree of DSc (University of Newcastle)**

Please find attached:

- (i) An extended discourse describing the theme "Chronic Inflammatory Disease as an outcome of antigen-host interaction: with special reference to the mucosal immune system" (4.1 (iii)) (USB – 1)
- (ii) A selection of published works, to support an original, substantial and distinguished contribution to knowledge (USB – 2)

**Statement regarding contribution**

- (i) The only material included that has been submitted for another award (by me) is the identified work in the first part of section 2 (a PhD Thesis). It is included simply to illustrate a continuity of work over 40 years around a central theme. Of course over the years a number of PhD students have participated in my research programme and worked under my leadership and guidance –studies they participated in would be included in their theses.
- (ii) and (iii) The vast majority of the work in this collection was initiated, planned and supervised by me. Where my name is on a paper, I have had a substantial input into its composition. Most of the work was done at the University of Newcastle where I established and ran the 'Newcastle Mucosal Immunology Group,' over in excess of 30 years.

**Section 1(A):**

The RIFFYL Programme was initiated by me to better understand the development of the secretory immune system and to identify laboratory and clinical correlations. This was an unique and major task involving many people (reflected in authorship) over 21 years. Marie Gleeson joined this programme as a PhD student. I added the exercise programme when asked to advise on impaired performance in elite swimmers (Australian Institute of Sports) and 'wintering' in Antarctica (ANARE). In each of these programmes, Marie became a central coordinator.

**Section 1(B):**

Given the central role of T cells in any host response, this was a 'constant' throughout the time frame of study. Gerald Pang joined me as a Post Doctoral student and worked closely with me, contributing particular technical skills.

**Section 1(C):**

This was a connecting theme to much of the work I was primarily responsible for in the programme. Allan Cripps joined me as a Post Doctoral student, but developed as a significant independent research worker (moving to Canberra and Griffiths Universities). He brought particular skills in immunochemistry and would develop an interest in bacterial surface antigens. With respect to my work, he participated in both cellular and humeral aspects of several programmes (as indicated in authorship). Margaret Dunkley was initially a PhD student in my Discipline, becoming involved in translation research and with particular skills in small animal studies.

**Section 2:**

A small section of submitted work, essentially a continuation of my PhD studies, moving to look at control of immune function (important in later mucosal studies). One PhD student was involved in this work.

**Section 3(A) and (B):**

Primarily my work, with a number of PhD students (where indicated in papers) and collaborators relevant to clinical access. The one area where I had little primary involvement was the Queensland component to the candida studies. In the alcohol studies, the animal model was developed and run by R. Batey (a hepatologist). The malaria studies combined several groups, coordinated by PNGIM, targeting development of vaccine. I was responsible for developing studies described here, with a PhD student. All the studies in 3(B) were initiated and controlled by me (with less involvement in the contact lens studies).

**Section (4):**

I was the prime mover in all these studies with collaboration as identified in authorship, with less involvement in the Fish Oil studies (where Dr Pang took a major role). All my senior colleagues would develop their own programmes –sometimes with ideas that began in my studies. Such work is not included here.

I hereby state that –as qualified by the above comments –the body of work within this thesis has been generated and developed and controlled by myself.

A handwritten signature in black ink that reads "Robert Clancy". The signature is written in a cursive style with a small flourish at the end.

**Robert Clancy AM PhD FRACP**



**Chronic Inflammatory Disease as an Outcome of  
antigen - host interaction: with special reference to the  
mucosal immune system**

Robert Clancy AM PhD MB.BS (Hons) BSc (Med) (Hons)

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## INTRODUCTION

This thesis collects studies over 40 years around a central theme of 'Antigen – Host Interaction in Man, and Chronic Inflammatory Disease.' The hypothesis was 'that characteristics of the 'antigen-host relationship' drive chronic inflammation and by extension, chronic inflammatory disease.'

Inflammation is defined as 'the host response to injury.' In the context of this body of work, the 'host response' is "specific adaptive immune response triggered by antigen, linked to non-specific (innate) immune mechanisms such as mast cell degranulation, complement activation or activated macrophages." Inflammation is therefore, a physiological protective mechanism – this reactive process only becomes 'damaging' when the non-specific innate processes lose their tight connection with an appropriate specific adaptive response to antigen, i.e. they are excessive and inappropriate, causing damage to normal host tissues. When the antigen is 'self,' auto-immune disease results, when the antigen is 'non-self,' hypersensitivity disease results (classically recognised in terms of time i.e. immediate (minutes), intermediate (hours) or delayed (days) hypersensitivity). This thin line between protection and damage (i.e. sensitisation and pathogenicity) is the focus of these studies.

Initial studies (in part submitted for a PhD but included here for completeness) examined idiopathic thrombocytopenic purpura (ITP) as a model of human autoimmune disease. These studies would bridge a period from the identification of the 'component parts' of the host-antigen relationship, to an early attempt at recognising impaired immune regulation. These studies were at a time when markers of human T cells were unclear. Studies of ITP illustrate the early limitations with T cell markers, when secretion of macrophage inhibition factor, antigen-induced lymphocyte proliferation and poorly understood markers detected by rosette-formation, were the available tools. The transition towards stable and reliable surface markers identified using monoclonal antibody, reflects the time frame of studies reported here. This evolution of T cell markers is illustrated by studies reported in Section 1(b). A brief comment on this transition period helps to put the included studies into context.

At the outset of these studies, many chronic inflammatory diseases were of uncertain origin - some even retained a classification of "degenerative disease". The idea that

autoimmune disease was caused by 'forbidden clones' lingered. However, a switch in research emphasis from humoral to cellular immunology had begun, initiated by Burnet's clonal selection theory based on cell-bound antibody, an era that would continue without challenge until experiments with knock-out mice restored the value of "whole animal" studies forty years later. Through this period "control" of the immune response was a dominant theme, with the discovery of "T cell help" and "T cell suppression" establishing a framework for discussions. Mossman's recognition in 1986 of T cell clones with stable cytokine secretion patterns and his naming of Th1 & Th2 subsets, was a major advance, enabling dissection of mechanisms of containment, of relevance to host-parasite relationships in chronic inflammatory disease.

By this time (following Leukocyte workshops that began in Paris in 1982) reliable markers of T cell subsets (e.g. CD4+ & CD8+) had become available in man, which when combined with chemical or molecular analysis of cytokine secretion, enabled more powerful analysis of competing pressures in host-parasite relationships in man. Prior to this time, T cell studies in man were limited by technology

In the 1960's two new immunoglobulins were discovered. They were IgA & IgE, which, respectively, became markers for the secretory Immune system (or "mucosal immunology") and allergic inflammation, (a significant chronic inflammatory disease manifest at mucosal and skin surfaces). In 1971, a cellular basis for mucosal immunology began when Cebra's group showed Peyer's patches contained an enriched source of IgA precursor B cells that could populate the intestinal mucosa.

Following my PhD, I worked in John Bienenstock's group at McMaster University. The year after Cebra's discovery of Peyer's patches as a reservoir of B cells destined to secrete IgA (1971), Bienenstock recognised analogous lymphoid collections in the rabbit bronchus which he called Bronchus Associated Lymphoid Tissue (BALT). I became part of the team involved in determining trafficking characteristics of BALT B cells, the results of which were to lead Bienenstock to coin the term, 'common mucosal system.' My interest became the cellular response to local antigen in the airways and human gut (Section 1(b)) about which essentially nothing was known.

Returning to Sydney (1976), I completed studies on autoimmune disease by using methods then available to investigate the idea of B cell control by T cells, using co-cultures and immunoglobulin secretion as the 'read-out.' Similar methods would be

used to examine regulation of mucosal B cell function in man, with demonstration of the expanded population of T suppressor cells (now known as T reg cells). To extend research into examining the presence and relevance of mucosal immunity and the common mucosal system in man, two long-term studies were begun. First, the ontogeny of the secretory immune system, and the significance of early life events would be correlated with disease outcomes, especially recurrent infection and allergic disease. Saliva was used to monitor the secretory immune system in normal infants followed to adulthood. This became known as the RIFFYL study ('Respiratory Illness in the First Five Years of Life') and a single cohort was followed for up to 21 years. The second was the 'Non-typeable Haemophilus influenza (NTHi) in Chronic Bronchitis' study, in which the dynamics of the relationship between NTHi and acute exacerbations of chronic bronchitis were studied, with the thesis that immunity protecting against NTHi-associated acute episodes was generated from specific B cells residing within the Peyer's patches. In the event, significant new discoveries were made. These related to both physiological protection mechanisms of the airways (including control of intrabronchial 'particles' by a T cell – dependent neutrophil clearance system generated outside of the broncho-alveolar system and operating within a mucosal environment dominated by T cell-dependant suppression), and the pathophysiology of acute exacerbations (due to a new form of 'hypersensitivity reaction' involving Th17 cells). Out of these studies, a new form of immunotherapy preventing both chronic symptoms and acute exacerbations in chronic airways disease was discovered and tested in a series of clinical trials. The first publication from this series of clinical trials was in 1985, and the last 31 years later, in 2016.

Through this period, opportunities were sought to study aspects of 'antigen-host' interactions with clinical outcomes. First, the RIFFYL (and linked but later studies in athletes with impaired performance, and in Antarctic expeditioners) study, led to host-parasite studies of Sudden Infant Death Syndrome and Respiratory Syncytial Virus; elite athletes with impaired performance and EBV infection. Second, the chronic bronchitis study focused on the host interaction with NTHi, *Pseudomonas aeruginosa*, and influenza virus. Third, human and animal studies involving chronic infection of mucosal surfaces (*Candida albicans*, *Helicobacter pylori*, Whipple's disease, mycobacterium avium subspecies paratuberculosis (MAP)), and systemic disease (*Plasmodium falciparum* and *Chlamydia pneumoniae*) were studied, as well as interactions with inert antigens (allergens – ryegrass; wheat antigen; and starch particles). Different aspects of these relationships were studied. They included

identifying the pathogen (Whipple's disease) defining the pattern of T cell response (MAP, *Helicobacter pylori*, *Candida albicans*, *Plasmodium falciparum*, *Chlamydia pneumoniae*, and the inert antigens), seeking to understand disease process.

In the course of these studies, opportunities to either develop a diagnostic, or a therapeutic agent that shifts the relationship away from being pathogenic, would arise. These are discussed in relation to the two main models (e.g. A saliva IgA<sub>2</sub> assay to predict SIDS or risk of impaired performance; inactivated bacteria to optimise the Peyer's patches – bronchus 'loop'). Others reinforce protection through the common mucosal system (e.g. probiotics), or shift T cell balance (e.g. conditioning, diet, early pregnancy factor, and penicillamine). The first near – patient 'yes/no' test for *Helicobacter pylori* infection (Helisal) was developed as a result of studies on the immune containment of *Helicobacter pylori*, and the first oral immunotherapeutic to down regulate endobronchitis (Broncostat) was developed as a result of studies seeking evidence to support the idea of a common mucosal system in man.

## SECTION 1(A): THE SECRETORY IMMUNE SYSTEM

### 1. Introduction

The idea of local mucosal immunity dates from observations by Besredka in 1919 following oral immunisation against dysentery in man. However its identity awaited Tomasi's demonstration in 1963 of the predominance of IgA in external secretions, and his postulate that this secretory antibody constituted a 'system' based on selective secretion due to an unique transport site (Reviewed: (42)). This concept would evolve over 50 years to recognise the secretory IgA system as protecting the various mucosal epithelia from toxins, viruses and pathogenic bacteria, and controlling commensal colonising bacteria, reinforcing barrier function, down regulating pro-inflammatory responses, and promoting protective bacterial biofilm formation.

By the mid 1970s much of the chemistry of the IgA secretory system was known, as was the function of IgA. I had established a programme to identify any role played by the early phases of the mucosal immune response in later protection against surface infection. There was interest in a link between mucosal immune competence in early life, and later disease such as allergic disease and recurrent infection, but essentially nothing was known other than some isolated observations of secretory IgA. Environmental influences were postulated but again, little was known.

Stephen Leader (then Professor of Community Health) and I set up a prospective study of normal infants born in the Belmont area of Newcastle, with the intention of following them to adult age. The primary objectives were to identify events or laboratory parameters that influence clinical outcomes, and to assess host-parasite relationships occurring at mucosal sites in the context of such events. However, it was immediately apparent that little was known about the ontogeny of mucosal immunity in man, nor was there an understanding of in-subject or between-subject variability of IgA in saliva, which was critical to interpreting relevance and clinical associations. The literature and thinking around these topics are reviewed in publications by our group on ontogeny between 1982 and 1991 (1-9). Later, we extended studies into assessment of performance in elite athletes, and of the impact of 'wintering' in Antarctica, on the mucosal immune system. As documented in other

sections, variability in the role of cellular aspects of immunity appeared to be more relevant to Host-Parasite Relationships, though quantitation of secretory antibody gave important information of an epidemiological nature as well as the dynamic of mucosal control processes (such as down regulation). An outcome of the group studies on saliva, an IgA<sub>2</sub> saliva test was developed, capable of detecting infants at risk of SIDS, and athletes at risk of impaired performance.

## 2. Ontogeny of the Secretory Immune System

The data regarding the RIFFYL (Respiratory Illness in the First Five Years of Life) project is summarised in the attached papers (1-9). The first report (1) included 63 infants studied over the first year of life. By the first decade of the study (8) 262 healthy infants had been studied for at least five years. The conclusions after the first year were:

- (i) That mucosal permeability (monitored by detection of albumen and IgG in saliva) was increased in the first two months of life i.e. enhanced mucosal permeability early in life is not restricted to the gut.
- (ii) That changes in mucosal permeability did not appear to be linked to weaning or maternal milk.
- (iii) That high levels of IgG in the first weeks –months of life, suggest that maternal IgG is relevant to mucosal protection.
- (iv) That an apparent periodicity of IgA levels may reflect a balance between intense antigenic stimulation in the gut in the first few weeks of life, and immune regulatory mechanisms that were being studied in other models (1(b) and (c)).
- (v) Maternal milk did not modify the pattern of ontogeny. At this time there was much interest in 'protection' from subsequent allergic disease in breast-fed babies, and potential mechanisms were of interest (see also later in this section regarding long-term follow-up).



- (vi) No antibody to E. coli was detected in saliva IgG. This was unexpected given its 'maternal origin' by leakage. Is there a transient phase of 'mucosal IgG' secretion in infants?

We move to 1987(7) and 1991(8) where 263 children have been followed for 5 and 10 years respectively. It is noted that at five years:

- (i) An increase in IgA was delayed in breastfed infants ( $p < 0.02$ ).
- (ii) In most infants the predominant IgA molecule was monomeric in the first 6 months of life – by 12 months dimeric IgA predominated in all children (4), representing a 'maturation' sequence.
- (iii) IgM was detected in 15-25% between one and six months, but was significantly delayed in breast-fed infants ( $p < 0.02$ ). Peaks correlated with formula feeding, suggesting a response to novel antigens (or mitogens). A similar correlation with formula feeds was noted for IgG in the first month of life.
- (iv) Some E. coli antibody was detected especially at 2 months – falling at 12 months. Thereafter there was a gradual increase to 5 years. This was unrelated to feeding patterns.

When reviewed at 10 years (8) most conclusions from the earlier study were reaffirmed but several points can be made:

- (i) A small cohort (2-6%) had consistently low levels of salivary IgA with transient absences during the first four years. At 5 years (i.e. school age) the IgA concentration significantly increased (2mg/l to 27mg/l,  $p < 0.05$ ) (2). After 7 years, levels were constant and at adult levels. Anti E. coli antibody also significantly increased at school age, increasing around 8 years to 'adult levels.' These latter studies (8) reflect on one hand a slow evolution of the common mucosal system, and on the other, an impact of changed environment on the intestinal microbiome (at ages of 5 & 8) and the subsequent mucosal immune response.

- (ii) **IgM:** its appearance in saliva was significantly associated with IgA (6), the level of IgA was significantly higher in those with detectable IgM, up to the age of 4 years – again most consistent with introduction to new food entities (novel antigens or polyclonal mitogens). Other important observations with respect to the co-appearance of IgA and IgM in infant saliva. When IgA is low as a congenital deficiency, IgM is high, as a compensatory response, contrasting with 'physiological low IgA' where the IgA level is coupled to a low IgM level (i.e. a switch defect contrasts with low level stimulation). Also, co-appearance reflects local synthesis, rather than exudation.
- (iii) **IgG:** This has been discussed for the first months of life – review with greater numbers suggest that breastfeeding does impact on the 'closure' of the membrane with less exudation i.e. 'closure' is earlier in the breastfed –this may have importance with respect to 'leaking membrane' and food sensitivity. In summary, breastfed infants have a later stimulation of the mucosal immune system, and an earlier membrane closure (10).
- (iv) **PNG study (unpublished data):** A cross-sectional study of children from birth to five years in Goroka (PNG) examined the effect of nutrition. Children less than 80% weight for age had lower levels of IgA and IgA antibody to E. coli and non typeable H. influenzae (a common respiratory pathogen in PNG) in saliva. Specific antibody differences were greater than those for total IgA when compared with normal weight controls. Children from PNG had 2-3 times higher levels of salivary IgA compared with age-matched Australian children.
- (v) **Environmental exposure:** As discussed above, significant increases in saliva IgA occurred at birth and commencement of school.
- (vi) **Markers of mucosal immunocompetence:**
- Several discussed above (transitory IgA deficiency; failure to develop specific IgA-antibody responses; enhanced mucosal permeability)
  - IgD presence – present in 50% in first month (3)
  - Monomeric IgA: in first 6 months (4)

- IgA specific antibody to E. coli O antigen – maturation process of the common mucosal system. Low levels of antibody till 5 years, then gradual increase to adult levels around 10 years (5)

## Variability

A problem with saliva analysis not generally addressed. Here a careful study of 33 infants showed within-child variability of IgA increased with age, but that between-child variability was 2.8 for log IgA concentration. The autocorrelation for IgA was significantly greater during an infection episode (2).

## Summary

In this section, the ontogeny of human mucosal immune system is studied in a cohort of 263 healthy children followed for around 21 years, using saliva to assess the secretory immune system. The pattern of IgA secretion is described, as is the influence of breastfeeding, and its relationship to IgM detection in saliva. The impact of attending school (aged 5) is described. A 'leaky mucosal membrane' occurs immediately after birth, enabling maternal serum antibody to contribute to mucosal protection. A correlation between IgM and IgA is interpreted as reflecting novel stimuli (foods etc.), and differs from IgA deficiency states where IgA and IgM levels are reciprocal. Analysis of anti E. coli antibody as a surrogate parameter of the physiology of the common mucosal system, suggests a gradual development over years, which has implications with respect to oral immunisation schedules. These data indicate the early appearance of IgA within saliva follows polyclonal activation by mitogens within the gut. Maturation markers identified included salivary IgD and monomeric IgA. This prospective clinical and laboratory database is appropriate to identify correlations with subsequent development of mucosal disease.

### 3. Clinical Correlations

- (a) **IgA deficiency:** IgA deficiency is the most common immune deficiency, with a frequency of about 1:700. The mechanism of IgA deficiency was considered to be a regulatory gene deficit, probably related to "IgM to IgA" switch though how that comes about was unknown. A female infant with a normal IgA development documented with 21 samples developed complete IgA deficiency following two

respiratory tract infections – this became a deficiency monitored over 10 years. Defective regulation at a 'switch level' was supported by high salivary IgM levels. Thyroid autoimmunity in three generations of the family indicated a genetic contribution, with an environmental trigger (later serum samples showed complete IgA deficiency and EBV positivity). This was the first demonstration of an acquired IgA deficiency as a possible outcome of an 'host-parasite' relationship (11).

(b) **Sudden infant death syndrome (SIDS).** The unexpected death (from SIDS) of one infant enrolled and studied from birth, gave an unique opportunity for a prospective study to determine the pathogenesis of the condition. At the time, there was considerable confusion with vigorously held views as to causes of this condition. Inherent in the nature of the condition, there were no prospective studies of SIDS. Airways infections at best were listed as 'risk factors' (reviewed 12 and 13). Our subject was a typical patient with SIDS, with a classical upper airways infection prior to sudden death and early weaning at 5 weeks (12).

Two significant differences from the healthy cohort were recorded. First there was a prolonged period of increased mucosal permeability – indeed up to the last routine saliva collection two weeks before death. Second, an exaggerated and prolonged mucosal immune response following the respiratory tract infection with both IgA and IgM well above the 90<sup>th</sup> percentile (13). An hypothesis was put that infants genetically predisposed to a period of unstable mucosal suppressor mechanisms are at risk for SIDS should certain environmental events occur in the period of mucosal immaturity. An inappropriate and excessive mucosal immune response to antigen (a 'classical hypersensitivity' reaction) may activate vagal afferents or chemoreceptors to induce reflex apnoea.

To test this hypothesis, as it was impractical to prospectively study SIDS, subjects with Acute Life Threatening Episodes (ALTE) or "near-miss SIDS" were studied immediately following an apnoeic event, together with post-infection and normal control groups (13). The ALTE group demonstrated both abnormalities found in the 'prospective' SIDS study i.e. a protracted period of mucosal permeability (with high albumen levels) and post-infection hyper-responsive immunity, when compared with infection and non-infection controls. A major risk factor for SIDS is exposure to smoking, and in this study, those with ALTE's exposed to passive smoking, had higher IgA levels than those not exposed. There was a trend towards higher albumen levels in those ALTE's exposed to smoke. These data

support the hypotheses, and links between RSV infection, passive smoking, genetically influenced 'leaky mucosa' and hyper-responsive immunoglobulin secretion are extensively discussed (13). While many, if not most, with SIDS, would appear to include a 'hypersensitivity' response to an intercurrent viral infection, it is likely that SIDS reflects a syndrome which includes 'non infection' related cases. A patent was filed for a 'near-subject' test based on saliva IgA level to identify infants with an acute airways infection who is at risk of SIDS (but not progressed).

**(c) Correlation of laboratory parameters in First Year of Life with Clinical Outcomes**

(i) **Prolonged 'leaky' mucosa/ Hyperimmune activity** – see SIDS above (13).

(ii) **Transient absence of salivary IgA** (14, 15)

Review of data of 114 of those enrolled in the RIFFYL study showed 18% had episodes of transient absence of IgA in saliva. Assessed later (aged 7.5 – 12 years) there was a significant increase in bronchial hyperactivity (OR 11.6 (2.2 – 60.9)) and a trend, just short of significance, to less atopy (OR 0.35 (0.11-1.11)). However, there was no relationship between absence of IgA and asthma (OR 0.9 (0.2-3.6)). These results emphasise the independence of atopy and bronchial hyperreactivity, but superficially appear to be contradictory. They address the important problem of bronchial hyperactivity without asthma, which had been recently recognised (as is discussed (14)). The current study provides data indicating a key association with mucosal hypoimmunity at a critical stage. An important question for the future, is whether impaired mucosal immunity in the first year of life is a risk factor for later onset non atopic chronic airways disease (COPD), and the importance of 'no smoking' in such a risk group.

**(d) Correlation of Clinical Parameters in the First Year of Life with Clinical Outcomes**

A major objective of the RIFFYL study was to determine what events in the first year of life influenced the evolution of atopy, allergic disease and asthma. This was an unusual opportunity to examine prevalence in the same population, examined repeatedly over time (here, 21 years). The 'cohort' nature of this study,

followed to adulthood was unique. The four occasions of study over 21 years were:

Year	Mean Age (Years)	Age Range (Years)
1985	3.4	1.1-5.7
1991	9.0	6.8-11.7
1993	11.0	8.8-13.7
2001	18.9	16.4 -22.0

The outcome of these analyses has been published (15-19). A summary of the significant associations identified and the study year, over the 21 years is presented (20) – the full thesis is published on the Library web site of University of Newcastle. Main correlations include:

- (i) In the 1985 study of preschool children the particular question addressed was the importance of atopy in the pathogenesis of asthma at that age (16) as there was uncertainty as to the role of allergy in that age group. Conflicting evidence as to whether atopy played a role in the development of asthma in preschool children –data from 116 children (less than 5 years) showed no significant relationship between asthma/ eczema, and atopy (16, 18, 19, 20).
- (ii) Asthma was revisited in 1993 when the mean age was 10.9 years (17). Here recent symptoms of asthma were associated with bronchial hyperreactivity and airways inflammation. Eosinophils in sputum correlated with symptoms, and mast cells with airways hyperreactivity. There was also a positive correlation between male sex and sputum mast cells. These observations extend observations made in adults to children of this age, i.e. 'allergic inflammation' is the underpinning pathology (differing from patterns of dissociation between atopy and bronchial hypersensitivity found in 3-4 year olds studied in the 1985 study). This is consistent with the significant family associations of asthma in the 1993 and 2001 studies (20).
- (iii) **Feeding.** Early cows milk introduction, was associated with atopy in 2-6 year olds, while early solids was associated with more allergic disease in the 7-12 year old study. Breastfeeding was associated with less allergic disease in both the 7-12 years and the 16-22 years age group studies.

- (iv) **Infection:** infection in first year of life was associated with less atopy, especially early in life. It did not protect against 'allergic disease' or asthma. There was no association between infections and asthma in the 1993 study, but when tested in 2001 (age average 19), the OR was 0.16 (0.02 - 1.13)  $P=0.07$ . Numbers were small, possibly accounting for the marginal  $P$  value with respect to asthma protection. This trend is of interest given current interest in relationship of early infection with later asthma.
- (v) **Passive smoking in first year of life.** This has a clear relationship to atopy in older subjects (2001 study) with an OR of 8.8 (1.01 -77.0), but no significant link to allergic disease.
- (vi) **Pets.** The message was the value of possessing a caged bird in the first year of life. This afforded protection only in the older group (2001 study). The OR for allergic disease was 0.18 (0.03 -0.99)  $P=0.04$ .

#### **Saliva IgA levels in high-risk children for atopy**

In a non-selected cohort population of children followed from birth, at risk of atopy with both parents having allergic disease, the question of whether "transient IgA deficiency" at around 3 months in serum is a marker of future atopy and allergic disease as first suggested by Taylor in 1973 (discussed in 21). This is important as our data from an unselected infant population (RIFYYL programme) shows that while 'periods of absent IgA correlate with later hyperbronchial reactivity, the relationship (short of statistical significance) trended towards less atopy, not more. This led to a hypothesis that periods of 'non-detected' saliva IgA reflect a subtle immunodeficiency, predisposing to infection and heightened bronchoactivity as well as a less pronounced IgE response to environmental allergens. As we had insufficient families where both parents had allergic disease, we collaborated with the Children's Hospital in Camperdown, Sydney, to determine whether, in addition to any general relationship between impaired mucosal immunity and reduced atopy, that there is a particular circumstance with genetically influenced regulatory defect characterised by an excessive IgE response to environmental antigen presented to mucosal surfaces (as suggested by the published association of IgA deficiency with atopic disease –see also (11) in this section). Our database of normal subjects was the control data. The postulate of a period of relatively low

saliva IgA levels at that critical period of the first year of life was confirmed with significantly more periods of 'absent' IgA and significantly lower saliva IgA levels at 8 & 12 months (21).

It was concluded that in those with strong genetic tendency towards a regulatory defect exhibited as atopy have impaired mucosal IgA secretion. Isotype 'switching' occurs in a pre-set sequence, through removal of intervening DNA segments. Antigen activation of immature IgM/IgD bearing B cells – if they encounter signalling molecules through CD40 and cytokine receptors (from T helper cells), they undergo antibody class switching restricted to constant heavy chains. In the sequence the epsilon heavy chain precedes the alpha chain and defective switching at a critical period, likely is associated with a reduction in IgA secretion –in some (depending on the nature of the 'switch defect') an absence of IgA occurs (discussed in (21)). Thus, apparent discrepancy between the two studies (11, 21) can be understood in terms of the "physiological immune suppression" associated with low IgE responses to allergens, while those genetically predisposed to defective isotype switching, commonly have reduced IgA secretion especially at that critical stage of development where the infant first meets environmental antigen. In some this may lead to a complete IgA deficiency (see (11)).

#### **(f) Variability**

The increasing interest in saliva IgA as a monitor of mucosal immune function requires an understanding of variability in the system. To quantitate variability consecutive daily concentrations of IgA and other immunoglobulins was studied in 33 infants for 2-4 weeks. The study provided estimates of degree of variability, autocorrelations, detection rate of minor immunoglobulins (in saliva) and the effect of age and respiratory tract infections on these parameters (22). Many of these associations were renewed and correlated (23).

#### **4. The Secretory Immune System – Impaired Performance in Elite Athletes**

In 1989 the Tomasi group described a transient fall in secretory IgA following completion of a marathon. Soon after, I was asked to review an elite swimmer training in the Australian Institute of Sport, who had documented a recent history of recurrent throat infections, impaired performance, and



hypogammaglobulinemia (24). I began a prospective study of all elite swimmers, as an extension of the RIFFYL study described above.

The collaborative programme between the Australian Institute of Sport and the Newcastle Mucosal Immunology Group began in 1990 and continued for 20 years. Continuous studies over this time defined the secretory immune response to training and identified risk factors for impaired performance. Training programmes were modified to limit impaired performance, and studies were extended outside of "elite athlete group" to casual athletes. These programmes involved the elite swimming group involved in the 2000 Sydney Olympics. The research strategy and outcome of study using saliva to monitor mucosal immune competence in elite athletes (swimmers) are included in published papers attached. In brief:

- The initial subject is reviewed in a case report (25).
- The impact of training and training period on salivary immunoglobulins is recorded, showing pre-training IgA as an index of chronic immune suppression over time, and high IgM events, that correlate with performance (32). Correlation with infection risk in the shorter term was less clear (26, 28, 29 and 30) though review of clinical records showed no particular propensity towards upper respiratory tract infections (31).
- IgA subclass (IgA<sub>1</sub>) at the beginning of the training period appeared a better prediction of infection (27).
- Elite athletes responded normally to pneumococcal vaccine (33).
- The variability of saliva immunoglobulin concentrations was greater than found in leisure athletes – the importance of recognising unique variation characteristics for any particular group becomes important in analysis (34).
- Delayed type hypersensitivity was normal in elite athletes (36).

In the context of impaired mucosal immunity EBV sero-positive elite distance runners were studied. There was a significant association between sero-positively and upper respiratory symptoms. A pattern was identified (37):

"Low level of salivary IgA → EBV virus shedding → respiratory tract symptoms"

Treatment with Valtrex (a specific anti-viral) reduced viral shedding but not symptoms (38). A review of 41 competitive – but not elite – athletes presenting with impaired performance, fatigue and recurrent infections found identifiable

causes in two thirds of subjects, emphasising the importance of careful clinical review in athletes (40).

5. Effect of one years isolation in Antarctica on secretory immunity

Anecdotal evidence of impaired immunity in Antarctic expeditioners (related to me as a Scout selected to accompany an ANARE expedition in 1959) led to a formal study using immunoglobulin measurement in saliva samples collected over the period of isolation. The first study (40) over the winter of 1992 at three Australian bases had two outcomes. First, there was a fall in levels of saliva IgA and IgM which returned to normal when expeditioners returned to Australia. Second, although there was a similar pattern in all three bases, concentrations varied significantly between bases. These studies were extended to examine six expeditions at two Australian bases. Consistent results again showing a transient depression of saliva IgA and IgM levels especially between March and May, but with considerable variation between bases and between years (40). A range of variables were assessed as cause of the Antarctica-associated depression of salivary levels of immunoglobulins. Positive correlation was found with a computer recorded psychometric score reflecting stress levels. No correlation was found with any environmental parameter (41).

A review summarising knowledge of the secretory immune system at the onset of these studies is included (42).

**Papers selected to best reflect the narrative:** 1, 4, 5, 7, 8, 10, 11, 12, 13, 14, 15, 17, 18, 19, 21, 27, 37, 38, 40, 41.

## **SECTION 1(B): “CHARACTERISTICS OF MUCOSAL CELL POPULATIONS”**

### 1. Introduction

A major component of studies over the 40 year research period covered in this collection, focusing on ‘antigen-host’ interaction, has been within the mucosal compartment. The key strand that connects most studies has been the T lymphocyte, including those involving mucosal tissues. Studies involving T cells – especially those associated with mucosal immunity have occurred within a framework defined by contemporary knowledge. For example, studies identifying T cell autosensitisation in idiopathic thrombocytopenic purpura in 1970 used functional criteria. Those quantitating T cell subsets in rabbit aggregated lymphoid tissue in the mid 1970s used cytotoxic anti-thymocyte antibody, while those on human tissues in the mid-late 1970s used red cell resetting methods. Recognition that CD<sub>4</sub> T cell subsets (Th<sub>1</sub>, Th<sub>2</sub>, Treg, Th<sub>17</sub>) contributed significantly to the ‘balance’ of any host response – a primary theme of this work – was of particular importance, in informing the post 2000 studies on infections such as candida albicans, Helicobacter pylori and Chlamydia pneumoniae. These CD<sub>4</sub> T cell ‘options’ became defined in terms of their cytokine profile over a 20 year period dating from 1986. It is noted that two of these subsets (Treg and Th<sub>17</sub>) were described here in functional terms in both human and rodent models of mucosal immunity, before their formal recognition with molecular markers. The key contributions of Treg (called here ‘suppressor T cells’) and TH<sub>17</sub> cells (shown here to regulate neutrophil activation and recruitment to mucosal sites), to the current understanding of mucosal immunity, is discussed elsewhere.

### 2. Timeline of T cell markers

To give a context to the methodology of many studies recorded in this thesis, a brief timeline of the evolution of ideas and technology through the period of study (1970-2010) is presented. Today access to molecular genetic assays and detection of surface or secreted protein markers can obscure the challenges of the past. Prior to 1970, both T & B cells were identified by function –secretion of migration inhibition factor (MIF) (1966), antigen-induced proliferation, or collaborative T/B cell experiments. Specific recognition began with Raff’s identification of T & B

lymphocytes in the mouse using a complement-fixing polyclonal antibody against theta antigen in 1969 (T cells) and against immunoglobulin (B cells) (1971). Human T cells were found to bind sheep red cells to form rosettes enabling quantitation of T cell enriched preparations (1972), with separation into 'helper' and 'suppressor' subsets using rosettes based on IgG Fc receptors ("helper" population) or IgM Fc receptors ("suppressor" population) (1977). Others developed T/B cell co-culture assays based on 'low' and 'high' T: B cell ratios to respectively monitor T cell 'help' or suppression (1977).

A sea-change came to T cell biology in the early 1980s. Monoclonal antibodies were developed that recognised 'cluster of differentiation' (CD) antigens on the T cell surface. Cloning technology detected a range of new cytokines, beginning with IL-4, and automated methods based on immunofluorescent markers became available. The CD<sub>4</sub> T cell became a focus of interest when it was recognised as a primary target of the 'new epidemic' virus, HIV. This cell population took centre stage when in 1986 Mossman showed separate T cell clones secreted specific and stable patterns of cytokines, which he named Th<sub>1</sub> (gamma interferon secreting) and Th<sub>2</sub> (IL-4 secreting) cells. Biological significance came once it was recognised that protection against different pathogens was linked to particular T cell subset balances. The CD<sub>4</sub> T cell became of greater interest when it was shown it was capable of differentiating into a series of effector cells. The particular lineage of differentiation was dependent on antigen engagement in close association with exposure to its major cytokine secretion product. Dendritic cells were shown in 1998 by Steinman to be potent antigen processing cells, and by 2000 these cells were recognised as having a significant influence on the differentiation pathway of the CD<sub>4</sub> T cell (secretion of IL-12 biased differentiation along a Th<sub>1</sub> lineage). Differentiation required signalling pathways and a 'master regulator' (T-bet; GATA-3; RORγt; FOXP3). The four differentiated T cell options are Th<sub>1</sub> and Th<sub>2</sub> (1986), Treg (2004) and Th<sub>17</sub> (2006). A particular role of Th<sub>17</sub> cells in mucosal defence was recognised in 2010.

### 3. The Mucosal Immune System: McMaster University

I joined the Bienenstock 'Mucosal Immunology' Group as 'Clinical Immunologist' in 1972. The previous year Craig & Cebra had shown that Peyer's patches contained an enriched source of IgA secreting B cells. Bienenstock had identified aggregated lymphoid tissue in the bronchus mucosa, which he termed 'Bronchus Associated Lymphoid Tissue' (BALT). He postulated that BALT was analogous to Peyer's

patches. In the Bienenstock team, I contributed to studies (i) characterising BALT, and (ii) examining cell traffic (these studies led to the idea of a 'common mucosal system' that became the reason for selecting 'acute on chronic bronchitis' as a model for the study of CMS in man.

Summarised here are the first studies on preparation and characterisation of lymphoid cells from rabbit and human bronchus and gut mucosa.

Initial studies used newly described antisera to rabbit thymic antigen (RTLA) and to membrane immunoglobulins, to characterise mucosal lymphocyte populations (1). The T cell marker was found in 16-18% of lymphocytes prepared from BALT and Peyer's patches. Results using the heavy chain antisera to characterise B cells within BALT and Peyer's patches lymphocyte populations, were consistent with them containing precursor B cells destined to secrete IgA, differing from results in both systemic and gut mucosal cell populations. These results were consistent with BALT and Peyer's patch having similar roles in mucosal protection. In a separate study (2) gut lamina propria lymphocytes were assessed for their capacity to perform in the mixed lymphocyte reaction (MLR). It was shown that gut mucosal T cells could both recognise and respond to cell surface alloantigens in a similar quantitative way to cells from Peyer's patches.

The McMaster Immunology Unit had as its central theme, an understanding of host-parasite relationships at mucosal surfaces – it was this environment that shaped my objective of developing a programme in human mucosal immunology, using infection models. At this time, no successful method of isolating and characterising human gut mucosal lymphocytes had been described. Resected gut was subjected to a series of mechanical processes to overcome problems such as cell-trapping in mucus and contamination with epithelial cells (3). Cell recovery was 15-30 x 10<sup>6</sup> viable lymphocytes per square inch of mucosa with < 1% contamination by epithelial cells. Kinetic studies showed changes in Crohn's Disease characterised by lymphocyte activation, consistent with active inflammation, but without identifying the stimulus.

Twenty-five years later, with technologies such as polymerase chain reaction, mucosal organ culture, and cytokine quantitation using monoclonal antibodies, Crohn's Disease was re-visited, to identify mycobacterium avium subspecies paratuberculosis as one antigen stimulus (see Section 3).

#### 4. The Mucosal Immune System: Royal Prince Alfred Hospital, Sydney (1976-1978)

One popular idea at the time was that mucosal damage in inflammatory bowel disease was mediated by antibody-dependent cell mediated cytotoxicity (reviewed in (4)), based on blood studies. Others have developed methods for obtaining single cell suspensions, but these used mucolytic agents raising concerns with respect to damage of cell surface structures (reviewed in 5). However, the mechanical method developed in Canada, required relatively large sections of gut mucosa, which limited tissue availability. Rectal biopsies (20-40mg) were readily available. The mechanical method was modified to prepare a single cell population of lymphocytes from biopsies with numbers sufficient (upwards of  $10^6$ ) for micro-assays. Chang hepatoma cells were selected in two assays for ADCC, because of their relative specificity for detecting K cell-dependent ADCC (other assays can detect ADCC mediated by cells such as monocytes and neutrophils). No ADCC could be detected using gut lymphocyte preparations from either normal or inflamed mucosa (4), demonstrating an absence of 'K cell' activity in normal or diseased gut (though K cells were detected in autologous blood). This absence of K cell activity was found irrespective of the presence of specific bacterial antigen absorbed onto the Chang indicator target cell. These results do not of course, deny an ADCC effect mediated by cells such as monocytes and neutrophils within the gut mucosa.

At the time, T cell subsets and T-B cell interactions had been a focus of interest in the development, expression and control of the systemic immune response (discussed (5)), but very little was known with respect to the mucosal immune response, especially in man. Therefore, human intestinal mucosal T cells were enumerated using contemporary methods ( $T_{\mu}$  and  $T_{\gamma}$  lymphocytes using ox erythrocytes) coated with IgM or IgG, respectively) (5). There was an enriched number of  $T_{\gamma}$  cells ("suppressor T cells") in the mucosal preparations. This enumeration was consistent with findings from co-cultures of gut mucosal and blood T lymphocytes (6), where suppression of the proliferative response to both PHA and PPD antigen was suppressed. Single cell suspensions obtained from human bronchus (7) showed similar results with suppression particularly noted when the antigen was a 'recall' antigen such as PPD. The other conclusion from the bronchus studies was that 'memory' for re-call antigens was demonstrated, which is an important observation as at that time, there was argument as to whether 'memory' existed within mucosal cell

populations as a result of IgA antibody studies (see also (4,5) in Section 1(c)). Thus collectively, these bronchus and gut mucosal cell studies from human tissues were the first to identify T cell-dependent 'suppression' outside of cells within the blood pool (i.e. within solid tissues), while the response of bronchus T cells to re-call antigens clarified a debate as to the presence or not of secondary immune responses at mucosal sites.

By 1990, cellular immunology had moved to a new level, with PCR technology, antisera available to detect a range of cell surface proteins, with a range of targets for cytotoxicity studies becoming available. These tools were used to further define and characterise cells within the human gut mucosa. The cell fractionation studies used for earlier studies were further developed to obtain cell populations from biopsy of the duodenum at gastroscopy (9, 10, 11, 12).

**(i) CD56<sup>+</sup> CD16<sup>-</sup> CD3<sup>-</sup> large granular lymphocytes (9)**

Considerable interest and study of this heterogeneous lymphocyte population in blood had been taken, with suggestions that these cells could traffic to mucosal sites where they could mediate a range of activities including cell-dependent cytotoxicity and release a variety of cytokines (see discussion (9)). We were able to isolate IL-2 growth dependent large granular lymphocytes from normal duodenal biopsies in pure culture. Their phenotype was CD56<sup>+</sup> but CD16<sup>-</sup> and CD3<sup>-</sup> with the (P70) IL-2 receptor. Strong cytotoxicity was shown for NK cell targets, and genes and secretion product for IL-5, IL-8, GM-CSF and TNF $\alpha$  were demonstrated. The absence of a receptor for Fc fragment of IgG (i.e. CD16<sup>-</sup>) is consistent with the earlier study that failed to demonstrate K cells within human gut mucosa.

**(ii) Cloned NK-2 /B TCR<sup>+</sup> cell line from patient with coeliac disease (10)**

This cloned 'hybrid' T cell/ NK cell was isolated from small bowel, at a time when T cell relationship with NK cells was a topic of interest, with an idea that (at least some) cytotoxic T cells arise from NK cells by the progressive acquisition of clonally distributed TCR, capable of killing specific targets bearing class 1 MHC antigens. This unique cell line could, in this context, represent a 'link' cell, giving an insight into aspects of the evolution of host defence.

**(iii) Gene expression and cytokine secretion in human duodenal fibroblasts (11)**

The complexity of inter-cellular relationships within mucosal tissues was recognised, but though fibroblasts from skin, synovial tissue and the lung had been studied, nothing was known of the nature or role of gut mucosal fibroblasts. Here fibroblasts were grown from normal human duodenal biopsies and found to actively express genes and secrete product for many cytokines (positive mRNA expression for GM-CSF, IL-1, IL-6, IL-8, IL-10 cytokines, and cell-directing molecules (ICAM-I; VCAM-I). Many of these molecules were further stimulated by lipopolysaccharide, all reflecting critical roles for fibroblasts within the gut mucosa in the regulation of inflammation.

**(iv) Human small intestinal epithelial cell lines (12)**

Three 'non-immortal' epithelial cell lines were grown and characterised on morphological, phenotypic and transport criteria. At this time small intestinal epithelial cell lines from man were not available: the pattern of cytokine mRNA expression (IL-6; VCAM-1; EGF) was consistent with a growth promoting and immunomodulatory role. One cell line expressing the IL-2 receptor, when exposed to IL-2 increased cAMP stimulated chloride secretion and cellular proliferation (13). Earlier studies (14) with a normal rat small intestinal enterocyte cell line, using ovalbumin as antigen and primed specific T cells for readout, confirmed that Ia<sup>+</sup> enterocytes could present antigen, but proliferative readout had to first exclude non specific factors secreted from both epithelial cells and T cells.

**(v) Mucosal lymphocyte interactions**

The essence of studies discussed above is the development of single cell preparations and the characterisation of the cells in terms of technologies available. Studies could then progress to those focused on understanding the relationship between these cell populations. Early work using both gut and bronchus mucosa using co-cultures, indicated an enrichment of "suppressor T cells" within the mucosal compartment (5,6,7). Here is discussed a progression of these studies, including an extension of the study of mesenteric lymph node lymphocytes.



**(vi) Pattern of sensitisation of gut associated lymphoid tissue (8)**

The limited studies on patterns of cell sensitisation (and the route of sensitisation) in the mid 1970s, focused on presentation of particulate antigen to Peyer's patches. Rothberg (in Chicago) had developed a model using oral immunisation with a soluble antigen, with subimmunogenic levels of circulating antigen. Studies examined, the earliest phase of cell sensitisation following ingestion (0.1% bovine serum albumen in drinking water of rabbits) i.e. whether this involved lymphocytes within the mucosa or Peyer's patches. Was soluble antigen handled in the same fashion as particulate antigen, or did it 'bypass' Peyer's patches via a 'mucosa-systemic' route?

The results clearly indicated sensitisation via Peyer's patches using lymphocyte stimulation as the readout –at one week with a stimulation index of greater than 1.5 detected in 7 of 8 rabbits. Antibody titre did not begin to rise until week two, and variable sensitisation of both spleen and mucosal lymphocytes followed thereafter.

Thus both particulate and soluble antigen appears to be 'handled' by the Peyer's patch. Studies considered elsewhere (1(c)) will consider handling of particulate (bacterial) antigen by Peyer's patches, in rodent models.

**(vii) Quantitative and functional aspects of T cells within the gut mucosa (5)**

The first study (5) was done at RPAH in the late 1970s, using red cell rosetting methods to quantitate T cells and the subsets characterised as bearing receptors for heavy chains of IgM ("helper" subset) or IgG ("suppressor" subset). The capacity of unfractionated T cells to down regulate the proliferate response of blood T cells to both polyclonal activator (PHA) and recall antigens (e.g. PPD), has been reviewed elsewhere.

Studies examining the relationship between mucosal T and B cells obtained from resected human gut showed significant 'T cell-help' (B:T = 2:1) and 'T cell suppression' (B:T = 2:10) for all immunoglobulin classes, with mucosal lymphocytes obtained from resected intestine. These functional relationships were not so clear using cell populations prepared from gastric mucosa. In addition gastric mucosal B cells did not increase secretion level of immunoglobulin following stimulation with pokeweed mitogen (PWM). So, it remains whether there is a real regional difference between gastric and

intestinal T & B cells, or if the greater degree of interaction (help and suppression) between intestinal T & B cells, reflects a particular pathology.

**(viii) Regulation of IgA secretion by T cells – study using mesenteric node cells (15, 16)**

There are many difficulties working with mucosal cell preparations, which are discussed in the attached papers. Cells migrating from the gut mucosa, transit through the mesenteric lymph nodes.

Two studies were completed. The first used a method developed by Elson (1979) based on co-culture of polyclonally activated T & B cells to examine regulation of T cells on immunoglobulin synthesis. This assay was adapted to study gut mucosal cell populations. The results showed the mesenteric cell population to be technically more reliable, with more reliable quantitative analysis. Both the pattern of 'help' and 'suppression' of immunoglobulin secretion was similar, but more reproducible, to that of the gut mucosal cell studies. In addition there was 'help' for mesenteric node B cells to secrete antibody to E. coli, which was not detected in the supernatants of similarity stimulated blood B cells. Reasons supporting the value of studies using mesenteric node cells as reflecting gut mucosal tissues, is discussed (15, 16). By the mid 1980s, monoclonal antibodies specific for human T cell subsets had been developed that could be used to isolate 'pure' T cell subsets by a panning procedure (reviewed in (16)). The same procedures used for study (15), were repeated, now using T cell subsets defined by monoclonal antibodies OKT<sub>4</sub> and OKT<sub>8</sub>. Results showed that both the amount and isotype distribution of immunoglobulin secreted from mesenteric lymph node B cells, differed from those of blood. Co-culture studies showed that irrespective of whether the B cells came from blood or node, IgA secretion was particularly sensitive to the regulatory influence of T<sub>4</sub><sup>+</sup> helper and T<sub>8</sub><sup>+</sup> suppressor T cells from mesenteric node when compared with that from blood. This was the first evidence of regulatory T cells from mucosa-related tissues in man, having a selective influence on IgA secretion in man, and was consistent with similar observations in a murine model. It was also shown that a 'loop' involving mitamycin – sensitive T<sub>4</sub><sup>+</sup> cells was involved in suppression by the T<sub>8</sub><sup>+</sup> cells (now recognised as T reg cells).

**(ix) Lymphocytes from chronically inflamed human gingiva (17, 18,19)**

The cell extraction and characterisation studies, with some modifications from those using mucosal tissues, were used to recover and characterise T & B

lymphocytes in tissue of patients with chronic marginal gingivitis, obtained from patients having full mouth dental extractions. Culture of cells with or without PWM showed maximum secretion, predominately of IgG, irrespective of the presence of the polyclonal mitogen. Supernatants of cultures (20) showed presence of IL-2 and bone resorptive activity measured as calcium release from mouse calvaria, supporting a role for T cells in inflammation and bone resorption.

## 5. Conclusion

- (i) Extraction, characterisation and functional studies of mucosal lymphocytes began at McMaster University in 1972 and continued for 30 years. These studies included the first attempt at determining single suspensions for gut and bronchus mucosa (using resected gut in Crohn's Disease, with vastly different results to those later studied from 'normal' gut). Over this period, there was an evolution of methodology in preparing cell suspensions just as there was in the methodology for typing and separating T cells (and their subsets). These changes are clearly apparent as one progresses from the rabbit and human studies at McMaster University (1972-76) where the only reliable T cell antiserum was for rabbit T cells (anti-RTLA), to Sydney (RPAH) where rosette formation enabled both T cells (SRBC) and an approximation of T cell subsets using antisera to the heavy chains of, respectively, IgM ( $T\mu$ ) and IgG ( $T\gamma$ ) bound to ox red cells. The evolution of studies continued in Newcastle, culminating in the mid 1980s when monoclonal OK anti  $T_4$  and OK anti  $T_8$  antibodies were available, as were procedures such as 'panning' that allowed relatively pure T cell subsets to be available for co-culture studies involving immune regulation. The continued difficulties obtaining and using human mucosal cell populations, led to a series of studies on normal human mesenteric node cells. Cleaner outcomes were obtained, showing characteristics similar to those found in earlier mucosal studies. Of importance was the first suggestion that down regulation involved  $T_8$  gut-associated positive cells (consistent with the earlier co-culture results using less precise cell populations), and evidence for mucosa-specific regulatory T cells. About this time the 'idea' of suppressor T cells lost favour because of difficulty in identifying a genetic basis for suppressor-specific markers. Two decades later "T reg" cells dominate thinking in immunology, together with the critical role

played by antigen presenting dendritic cells in regulating immunity. What we were calling “suppressor T cells” would today be called “T reg cells.”

- (ii) In the early 1980s I developed a programme centred on oral immunisation in man, with chronic bronchitis as a model. It is apparent tracing studies in this section, that an important objective had become defining ‘rules’ for developing effective oral vaccine outcomes in man (hence selection of acute/ on chronic bronchitis as a model using the MRC diagnostic criteria). The presence of a ‘down regulating’ environment at mucosal sites emerged from the co-culture studies and the limited responsiveness (both T cell proliferation and Ig secretion) of mucosal lymphocytes. This raised concerns regarding the viability of oral immunisation as a way of controlling episodic inflammation in the airways. Clearly more needed to be understood about T cell control mechanisms if an effective oral vaccine was to be developed. Thus the need to understand ‘the rules’ for effective vaccination (see elsewhere). The concerns regarding ‘down regulation’ began with intrabronchial immunisation with inert and infectious antigen in rabbits, while at McMaster University (1(a)).
- (iii) The opportunity to identify (and clone) cells within the mucosa was taken: large granulated lymphocytes with NK cell activity, epithelial cells and fibroblasts. The findings indicated a vastly complicated communicating cell system that requires its own research focus.
- (iv) A review paper published in 1983, highlighted three aspects of our programme addressing the issues of oral immunisation in man, with a particular focus on understanding ‘down regulation’ and control of the mucosal immune response (20). It highlights the importance of ‘responsiveness’ to oral antigen using three contemporary data sets:
  - (a) An oscillatory pattern of the early IgA secretion pattern in infants, driven by repeated cycles of novel antigen (reflected in ‘bursts’ of IgM in saliva) followed by down regulation (1 (a)).
  - (b) Regulatory T lymphocytes in human mucosal tissues (1(b)).
  - (c) Oral immunisation of healthy humans using a polybacterial commercial preparation, where only ‘non sensitised’ individuals responded with an

increase in saliva antibody. These different studies indicate a response/suppression cycle. This suggested there were periods of capacity to respond to oral antigen while at other times, there was suppression. This became a foundation 'rule' in establishing the oral vaccine programme.

**Papers selected to best reflect the narrative: 1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 14, 15, 16.**

## **SECTION 1(C): THE COMMON MUCOSAL SYSTEM – ITS ROLE IN MAN AND ITS MANIPULATION BY A FORM OF IMMUNOTHERAPY IN CHRONIC BRONCHITIS**

### 1. Introduction and early studies

This section traces the theme of the common mucosal system (CMS) and the way it operates to include the bronchus. There is an increasing focus on exploring the connection between the gut and the bronchus, both as further validation of the importance of the CMS in man, and as the value of an innovative therapy for patients with chronic airways disease. Given that in the process of developing oral immunotherapy to control intrabronchial inflammation, a new pathogenic paradigm emerged for 'acute bronchitic episodes' that complicate the natural history of chronic bronchitis, these discoveries form part of the story told in this section. Thus, when chronic bronchitis was selected as a model in man to test ideas about the CMS, the prevailing concept was that damage in chronic bronchitis was due to inhaled toxins –part of that damage involved reduced 'protection' against the downward pressure of bacteria (especially non typeable *Haemophilus influenzae* or NTHi). An intercurrent virus infection precipitates downward migration, with acute bronchitis the result.

Contemporary knowledge encouraged the then hypothesis, that oral immunisation with NTHi would promote the traffic of IgA – containing B cells from the Peyer's patches, secreting specific antibody into the bronchus to prevent the downward migration of upper airways bacteria.

The studies in this section were done over a period of 40 years. As in other sections, each contribution must be seen as a progression of ideas, separately influenced by contemporary knowledge, and the technology then available. Perhaps the greatest change in thinking over this time that would most influence outcome of studies, were the clinical definitions of chronic airways disease. Specifically the clinical diagnosis of 'Chronic Bronchitis' gave way to the functional diagnosis of 'Chronic Obstructive Pulmonary Disease (COPD)' which would have both negative and positive impact on understanding both the nature of the disease and the development of an effective new therapy.

The CMS Programme began with studies of the Bronchus Associated Lymphoid Tissue (BALT); in particular those that demonstrated transfer in the rabbit of allogeneic lymphocytes from BALT or Peyer's patches into x-irradiated recipients, with a predominant repopulation of both the gut and bronchus mucosa, with IgA-containing cells. This was the first use of the term 'common mucosal system' (1). In this study, I was part of the team led by Professor John Bienenstock at McMaster University. From that time, my aim was to take the conceptual framework from this artificial allogeneic system and sort out parameters that may allow development of effective control mechanisms in human disease - in short, an oral immunisation strategy to prevent airways infection. At this early time, nothing was known about directional flow between mucosal sites, the role of cellular immunity within the CMS, nor indeed the relative efficiency of direct versus indirect (i.e. via Peyer's patches) immunisation on bronchus protection. In addition, little was known of cellular immune responses to local antigen within the airways other than that cytokine-secreting cells could be induced within bronchial washings by local immunisation (reviewed in (2)). Two studies were published from work done while at McMaster University. First, using dinitrophenylated human gamma globulin, antigen-specific lymphocyte proliferation was demonstrated following intrabronchial immunisation, selectively in bronchial washing lymphocytes and BALT, before any evidence of antibody containing cells detected by autoradiography (2). Second, the conclusion that there was a selective T cell response was confirmed using a Herpes simplex cytotoxic cell system that excluded antibody-mediated cytotoxicity (3). A surprise in these studies of cellular immune response within the airways was its short-term duration, raising the question that the airways contained down regulating mechanisms, limiting cellular responses (and thus minimising any inflammatory response to inhaled antigens). This question would dominate thinking as a strategy was developed to circumvent suppression to create effective immunity in man. Subsequent studies would support the idea that a connection between the bronchus and the gut, to activate a T cell dependent control of phagocytosis within the airways as the primary protective mechanism. This process cleared antigen from the distal bronchial tree with minimal inflammation (or damage). Recruitment of systemic immune mechanisms would be damaging due to an attendant inflammatory response. Such 'default' mechanisms (e.g. IgG and complement; T cells/granuloma formation) when dominant can lead to parenchymal lung disease.

## 2. Australian Studies

Returning to Australia, there were three important questions to be answered before studies could be designed to formulate an oral “vaccine” (as oral immunisation was then envisaged) (i-iii below):

**(i) What were the functional capabilities of bronchus mucosal lymphocytes – in particular do they express ‘memory’ and is there a dominant T suppression?**

A series of studies of human bronchus mucosal cells showed T cell memory for recall antigens, and also using co-culture with autologous blood cells, showed significant down regulation of blood T cell response to antigen stimulation. Suppression was non-specific and was mediated by T lymphocytes (identified by marker studies) (4,5). This was the first demonstration of “suppressor T cells” in a tissue and confirmed the conclusion from the studies in rabbits (above) of a high level of ‘down regulation’ within the airways mucosa. The results supported the idea that the resident cells within the lining mucosa were unlikely to mount per se, a vigorous, persistent local immune response. Importantly, the human study did indicate that cells were present that could respond to local antigen, which would be of value in re-stimulating specifically activated T cells from Peyer’s patches following oral immunisation, that had ‘homed’ to the bronchus mucosa (the presence of NTHi in sputum, predicts ‘responders’ within COPD to oral NTHi immunisation –see later this section).

**(ii) What is the dominant direction of flow of mucosa – seeking lymphocytes with respect to the gut and bronchus mucosae?**

The respiratory tract of the sheep and the draining lymph nodes contain large numbers of IgA containing cells that can’t be accounted for by local antigenic stimulation (6). Using different immunisation schedules and ovalbumin as antigen, five times the number of specific antibody-containing cells were detected following intratracheal immunisation after priming by intraperitoneal immunisation (i/p antigen) in Freund’s Complete Antigen, compared to the number when priming was intra-tracheal. This enhanced IgA response by antigen delivery to gut-associated lymphoid tissue, was abrogated by continuous intestinal lymph drainage. The sum of these experiments clearly



demonstrated that maximum local IgA response to local antigen in the bronchus occurred when gut-associated lymphoid tissue was initially presented antigen. Otherwise, local intrabronchial antigen stimulated a poor local response. The study also demonstrated a regional variation within the airways, with maximum IgA-containing cells in the upper trachea (6).

**(iii) Do T cells participate in the inter-mucosal cell traffic and can they play a role in airways protection?**

The kinetics of T lymphocyte responses to mucosally presented antigen and the appearance of antigen –reactive T cells in mucosal and systemic tissues were studied to determine whether T cells generated in gut-associated tissues can traffic to distant (including bronchus) mucosal sites. The study measured secondary mixed lymphocyte culture (MLC) reactivity after intestinal administration of alloantigens. The data showed a time sequence of activation (Peyer's patches → mesenteric lymph nodes → thoracic duct lymph → gut lamina propria → bronchus) consistent with a mucosa-seeking traffic for T cells, not unlike that shown for IgA containing B lymphocytes (7). The model developed selectively identified T cells reacting in a secondary mixed lymphocyte culture (MLC), and the response shown was mucosally restricted, and the magnitude of location was influenced by site of antigen administration, with systemic non-reactivity to mucosally presented antigens (7).

**(iv) Can an animal model be developed to determine enhanced clearance of non typeable Haemophilus influenza from the airways and identify mechanisms?**

In a rodent model, presentation of antigen to Peyer's patches (by ingestion or by direct injection) followed by administration of antigen (NTHi) into the bronchus, enhanced clearance of live NTHi. This effect lasted for at least 6 weeks. It was specific and could not be replaced by systemic immunisation. No correlation of enhanced clearance with local or systemic antibody was found. The effector mechanism was a faster recruitment and increased activation of phagocytes, measured by chemiluminescence. Depletion of B lymphocytes from the thoracic duct lymphocyte population did not remove capacity to enhance clearance. Repeating these studies in the nude ('T cell-deficient') Rowett rat, failed to reproduce enhance clearance (8,9,10). Coating 'challenge' NTHi bacteria with bronchial washing antibody did not enhance clearance in naïve rats (11). A series of studies specifically aiming to show that antibody had

a role in enhancing clearance in the rat model, failed to show any direct role in opsonising bacteria for killing or phagocytosis. Rather antibody in bronchial washings blocked cytotoxic activity of serum antibody, and had an anti-inflammatory effect (12). The model was used to show a cross reactivity of enhanced clearance with *Pseudomonas aeruginosa*, which was not mirrored in antibody activity (12).

The studies above, support the importance of local antigen, not regarding the original homing, but retention and expansion of cells be they B or T-lymphocytes, in a similar way to that demonstrated in the intestine using Thiry-Vella loops, with different antigens injected into the proximal and distal loops (13). Using the rat clearance model, purified outer membrane proteins of NTHi were tested. P1 and P6 were effective, showing T cell epitopes relevant to protective immunity, unrelated to antibody stimulation (which in this model, appears irrelevant (14)). Clinical studies that follow use inactivated 'whole' bacteria for oral immunotherapy, but it is likely second generation products will use defined antigens. From that perspective this study is the beginning of the development of antigens that are conserved but effective at optimising protection.

From around 2007 a recently described T cell phenotype (TH<sub>17</sub>) was described in association with the airways in COPD (discussed in (15)) as is the appearance of specific dendritic cells influencing TH<sub>17</sub> cells localising to the respiratory mucosa. In the mouse model (above) intestinal immunisation with inactivated NTHi increased specific TH<sub>17</sub> cells in both mesenteric lymph nodes and in airways (Fig 2 (15)). This T cell subset is contributing to mucosa protection through recruitment and activation of neutrophils, and the secretion of antibacterial factors from the bronchus epithelium. Again discussion is included in (15).

**(v) Can the mouse model be used to study the relationship between infection with NTHi and influenza virus?**

Clinical interaction between bacteria and virus infections within the airways has long been recognised. The mechanisms of such interaction are poorly understood –what is known (such as bacteria liberation of proteases that enhance release of virus from infected cells, and impaired neutrophil function induced by influenza) are discussed in the attached papers (16,17). Relevant to

clinical studies in chronic bronchitis where intercurrent virus infection (including influenza) appears to regularly initiate acute-on-chronic exacerbations, the mouse model described above was used to investigate the outcomes of co-infection and determine if such co-infection simulating a clinical scenario, can be abrogated by pre-oral immunisation with NTHi. The influenza virus A/Qld/6/72 (H<sub>3</sub>N<sub>2</sub>) was used in these experiments. Bacteria used were NTHi; *S. pneumoniae*; *P. aeruginosa*; and *S. aureus*.

Dual infection of A Qld virus and NTHi gave a 10-fold increase in NTHi in bronchial washings and a 5-10 fold increase in the lung homogenate. Oral pre-immunisation cleared NTHi (above) and suppressed NTHi and A Qld in the A/Qld/NTHi co-infection group. As a control for specificity, advantage was taken of a natural co-infection with *Bordetella bronchiseptica*. Here A Qld increased. *B. bronchiseptica* 10-fold in washings and 1000 fold in lung homogenate, but this was not suppressed by pre-immunisation with NTHi. Recruitment of neutrophils –a mechanism shown to be important in control of NTHi (above) – was inhibited by A Qld (16).

A further study examined whether similar relationships existed with other bacteria often associated with influenza infection in man. A/Qld mixed infection caused a significant increase in NTHi and *S. pneumoniae* infection, but not with *P. aeruginosa* or *S. aureus*. Macrophage numbers were reduced in co-infection experiments only with NTHi and *S. pneumoniae*. Virus titre was significantly higher after co-infection with *P. aeruginosa*. These differential effects of influenza/bacterial combinations, may influence clinical outcomes (17).

**(vi) Studies in Man**

The above studies in a mouse model enabled the development of a mono-bacterial oral vaccine used in clinical studies of patients with chronic bronchitis. Studies comparing this oral NTHi “vaccine” were initially compared with a commercially available polybacterial orally administered product (see (18) for details):

- (a) The oral killed polybacterial vaccine given in three courses (each containing a total of 10<sup>10</sup> NTHi) was shown to stimulate a predominately IgA antibody response in saliva in some but not all recipients (i.e. 55%). Non-responders had higher pre-immunisation saliva antibody and ‘non-response’ affected all

antibody classes (18). These observations were consistent with earlier animal and human studies identifying a 'suppression' environment within mucosal compartments of sensitised individuals.

- (b) The dynamics of antibody response were analysed in a complex set of studies in normal subjects, and included a single NTHi oral vaccine containing similar numbers of NTHi to those in the polybacterial vaccine as well as a monobacterial vaccine containing 20-fold as many bacteria (i.e. 10<sup>9</sup> NTHi). Using parameter estimates for the logistic model of best fit, significant antibody was only found when three monthly courses of the polybacterial preparation was taken. Analyses of all studies concluded that the 'added' bacteria in the preparation acted as an adjuvant (19). At no time was mucosal (or systemic) antibody detected following the mono bacterial (vaccine) preparation – a finding also found in subsequent clinical studies in chronic bronchitis (see later).
- (c) The animal model studies suggested that the mechanism of bacterial clearance from the airways involved specific T cells (and not secretory antibody). A complicated study using normal volunteers compared the polybacterial and monobacterial NTHi vaccines (as used in (19) above). Analysis used antigen-stimulated proliferative responses, combined with limiting dilution analysis to quantitate the precursor frequency of antigen-specific T cells and quantitation of IL-2 receptor using an anti-TAC monoclonal antibody (20). In summary the more 'immunogenic' form of NTHi (i.e. the polybacterial compound (19)) induced greater antigen-driven proliferative responses than did the monobacterial vaccine. However limiting dilution analysis showed that the precursor cell frequency was not expanded to account for these differences (as less precursors were detected following the polybacterial vaccine) – with repeated courses, the precursor frequency continued to fall with time. No anamnestic response was detected, consistent with a suppressor activity (as discussed and demonstrated above). Despite the fall in precursor cell frequency, the high affinity receptors for IL-2 (i.e. TAC +ve cells) increased, reflecting the requirement for IL-2 as a growth factor for clonal expansion. Taken together, these results are most consistent with a delayed recruitment of mitogen-activated cells in the polybacterial immunised group, again

consistent with an enhanced response in this group to PHA stimulation (S. Yeung. PhD Thesis, University of Newcastle).

**(d) Protection against acute bronchitis in subjects with chronic bronchitis following 'adjuvanted' and 'non adjuvanted' oral vaccines**

Meta-analyses in the literature of polybacterial vaccines failed to demonstrate clear protection against acute bronchitic episodes in chronic lung disease. Isolated studies however suggested some protection may exist. Thus a study comparing the 'adjuvanted' commercial NTHi vaccine ("Buccaline Berna") with the 'non-adjuvanted' single cell NTHi vaccine was performed (and later described in more detail). Here, polybacterial versus monobacterial (non adjuvanted) vaccine is reported in a study over the 1983 winter period. No significant protection was stimulated in those taking the 'adjuvanted' polybacterial product, while protection afforded by the "less immunogenic" whole cell NTHi oral vaccine was significant –and a significant difference was found between the two vaccines (21). The ultimate clinical test of 'adjuvanted' versus 'non adjuvanted' NTHi vaccine of enhanced mucosal immunity, is consistent with the body of evidence indicating a mucosal immune system geared to minimise damaging inflammation in response to local infection (antigen), by mechanisms of down-regulation.

**(e) Studies Relevant to Host-Parasite Relationship-Luminal events:**

- **T cells**

A considerable data set exists to support important roles played by T cells within the bronchus (reviewed in (22)) especially with respect to regulation of inflammatory events. These studies have used mucosal T cell populations. Yet antigen is often restricted to the lumen, and no studies on intrabronchial T cells were available from human studies. This study developed a method to clone T cells from sputum, then characterise these cells in terms of their cytokine secretion profile and correlate these with mRNA expression. The ratios of CD<sub>4</sub><sup>+</sup> to CD<sub>8</sub><sup>+</sup> T cell clones generated reflected the ratio of T cell subsets in sputum, suggesting little selection bias, and cytokine mRNA gene expression correlated with protein in culture supernatants. Different T cell clone patterns characterised bronchiectasis (Th0 cells) and asthma (Th<sub>2</sub> cells). It was noted that most T cell clones secreted TNF<sub>α</sub> (22).

- **Neutrophils**

Neutrophils isolated from sputum of subjects with bronchiectasis were phenotypically different to those in blood, irrespective of whether they were stimulated by lipopolysaccharide. The numbers of neutrophils correlated with the concentration of IL-8, IL-1 $\beta$ , TNF $_2$ . Cytokine secretion was relatively resistant to IL-10 (which suppresses activated circulating neutrophils) and antibody to IL-1 $\beta$  (but not to TNF) and inhibited both secretion of IL-8 and its message expression. These results indicate that phenotypically modified neutrophils (? due to CD $_4$  +ve T cells) in sputum are a major source of IL-8 and that an autocrine loop involving IL-1 $\beta$  maintains survival and secretion within the bronchus (23).

(f) **IgE anti-H. influenzae antibody**

Reversible airways obstruction is well recognised in patients with chronic bronchitis. In this study IgE anti-outer membrane protein (from NTHi) antibody was measured in serum from subjects in several of the vaccine trials completed (see later). In a study of 'recurrent acute bronchitis' a high level of IgE anti-OMP antibody correlated with a reduction in incidence of acute wheezy bronchitis following oral NTHi immunotherapy. Importantly those in the vaccine group had a different pattern of colonisation with NTHi, a pattern found in the placebo group. Those with established chronic airways disease had high levels of IgE anti-OMP antibody, which remained stable through the study (24). These studies identify a mechanism whereby oral immunisation can reduce symptoms i.e. by reducing colonisation, less antigen is available to drive IgE-dependent mediator release. Allergy to colonising airways bacteria in chronic bronchitis raises new possibilities in both causation and treatment.

(vii) **Direct study of host-parasite relationship in chronic bronchitis**

(a) Patients with chronic bronchitis were identified as 'infection-prone' or 'non-infection prone' based on incidence of acute exacerbations over 3 years. The 'infection-prone' or 'non-infection prone' classification remained stable over the period of observation. The major difference between the two groups was that smoking experience was greater in the 'non-infection prone' group. The infection-prone group had more bacteria

grown from the oropharynx, more bacteria adhering to buccal epithelial cells, and their own buccal cells adhered to NTHi in vitro to a greater extent. In short, this study indicated that the binding capacity of buccal cells from infection-prone subjects was greater than cells from non-infection prone subjects, thus shifting the host-parasite relationship towards acute exacerbations (25). Saliva and sputum sol from this patient population was studied to determine if soluble factors were present that modified adhesion of NTHi to human buccal epithelial cells (26). Saliva and sputum levels of anti-NTHi IgA antibody were inversely correlated with buccal cell adherence ( $r=0.92$ ). Absorption with NTHi removed antibody but only partially removed inhibition of attachment, with IP subjects retaining a greater amount of blocking compared with the non IP subjects. Identification of the 'blocking' factor was considered a possible assay to detect I.P. subjects (26).

Investigation of the soluble factor in saliva and sputum that inhibited binding to buccal cells, showed it to be an aggregator of bacteria (26, 27, 28) – the difference in aggregating capacity from controls was significant ( $p < 0.001$ ). The aggregating factor was identified as lysozyme, which was significantly higher in non-IP subjects ( $P < 0.005$ ). There was a good correlation between aggregating capacity and lysozyme activity. The source of lysozyme was unclear but includes phagocytes (thus participating in the T cell/neutrophil sequence discussed above).

- (b) A double blind placebo controlled study of oral NTHi was completed, to better understand mechanism of action in a disease group (29). Four observations were made, all consistent with the developing hypothesis based on animal studies (and some studies in normal subjects):
  - (i) NTHi-specific T cells increased over the winter period in the placebo group.
  - (ii) The T cell response in the active group occurred earlier and with a different dynamic.

- (iii) A major difference in serum IgG antibody in the placebo group over time correlated with exposure to NTHi determined by culture of throat gargles.
- (iv) A different shape of the lysozyme curve over time correlated with reduction of inflammation in the active group.

The important new conclusion was that the stable serum IgG antibody reflected protection, contrasting with high level variation in the placebo that correlated with exposure of the airways to NTHi. These data collectively support the idea that antigen is aspirated into the gut where it drives Peyer's patches to traffic T cells to the bronchus (see paper for data (29)). They complete a circuit where the physiology of airways protection shifts from potentially damaging inflammatory response to one of primary protection. The proliferative component of this mechanism is not local, but shifts to a more "off site" process with aspiration of antigen into the gut, and the effector limb requiring traffic of TH<sub>17</sub> cells into the bronchus on an 'as needed' basis.

**(viii) H. influenzae**

The model selected for study of the host-parasite relationship identified NTHi as the parasite of interest. A series of studies was completed to determine characteristics of colonisation in normal and chronic bronchitic populations: characterisation of biotypes to show isolates from the upper airways differed from those in sputum. This observation ran counter to the prevailing hypothesis that NTHi descended the airways to cause acute exacerbations. Also, methods were developed to assess specific antibody and details of H. parainfluenzae colonisation in chronic bronchitis (30, 31, 32, 33, 34, 35, 36). As it was anticipated that an oral immunisation study would be carried out in the PNG-highlands where colonisation with NTHi is nearly one hundred percent within a month of birth, and the commonest cause of death in the first year of life was pneumonia with NTHi and S. pneumoniae as the most common bacteria isolated from transthoracic aspirates, understanding the host response in PNG was important. Significant differences from Australian children were found, with a blunted IgA antibody response in healthy PNG children reflecting down regulation, a situation continuing into adult life (37).



(ix) Oral NTHi Immunotherapy in Smoking-Related Chronic Lung Disease

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(Tables 1,2,3, 4)

The model selected was chronic bronchitis as defined by MRC criteria. The intervention was oral immunisation with formalin inactivated NTHi and the primary outcome measure was an acute exacerbation. Thus all patients had chronic cough and sputum, and an exacerbation was defined as 'an increase in volume and purulence of sputum.'

The reasons for choosing an oral antigen preparation and an immunising strategy likely to be effective, was the substance of studies above and elsewhere in this thesis. Given the dominant 'suppressive' environment within both the gut and bronchus mucosa which affects 'responsiveness,' duration, and 'memory,' the immunising course was designed as a series of spaced 'pulses' to minimise the linked down regulation that follows antigen stimulation. Particulate antigen (i.e. whole bacteria) was used as soluble antigen more readily causes tolerance.

The results of clinical trials are presented in tables. Table 1 represents three studies at three sites (Newcastle, Perth and Goroka, PNG) where the study population was selected as 'chronic bronchitis' and the acute exacerbations were defined clinically as 'an increase in volume and purulence of sputum.' Table 2 is a study recruiting subjects prone to recurrent acute bronchitis with no recognised chronic airways disease. Table 3 includes two studies of chronic obstructive pulmonary disease (COPD) for which limited data is available. Table 4 includes two multi-site studies where the entrance criteria was 'COPD.'

In the interpretation of the results of these clinical studies, note that over time the diagnosis of chronic smoking –related airways disease changed from 'chronic bronchitis' to COPD, and the definition of 'acute exacerbation' changed to include a treatment modality (steroids and/or admission into hospital). Both changes had a profound effect on study outcomes, while providing information on clinical benefit.

- **Chronic Bronchitis**

**Table 1**

<b>Study (and duration)</b>	<b>Reference</b>	<b>No. subjects</b>	<b>Age (yrs)</b>	<b>FEV<sub>1</sub> (litres)</b>	<b>% carriage NTHi</b>	<b>% Protection</b>	<b>P value</b>
Newcastle 1985 (3m)	38	50	65	1.0	69	90	0.001
Perth 1991 (6m)	Tandon et al ANZJ Med 21 (1991) 42732	64	72	0.9	29	33	0.024
PNG (12m)	39	62	53	1.4	80	48	0.045

These three studies were identical in structure, at three different single sites. They address the initial hypothesis. All results were significant with respect to the primary outcome. The results support the postulate that presenting antigen to Peyer's patches, activates the 'common mucosal system' in man. They were published between 1985 and 1991. The hypothesis was that the cell traffic included IgA-destined B cells, and that antibody would inhibit 'descent' of oropharyngeal NTHi into the bronchi where they would increase luminal inflammatory exudate. The failure to detect local IgA antibody (38) was at the time surprising, but as has been discussed results of subsequent human and animal studies confirmed that T cell-dependent mechanisms controlled clearance of bacteria within the bronchi. Different methods were used to monitor the impact of oral immunisation on NTHi colonisation. The initial study (38) had early technical problems, but a trend towards a reduction of NTHi in throat swabs was noted. In a second study the culture positive sputum samples were significantly less (50%) in the treated group (Tandon et al.). The third study (39) was the most revealing. Working with the PNG Institute of Medical Research, had significant advantages. First, NTHi is a lifelong colonising bacterium in subjects dwelling in traditional non ventilated thatch houses, where NTHi is transmitted from chronic bronchitic adults to

infants (37) and (R. Grimley (1989) "Cohabitation with an adult complaining of chronic cough as a risk factor for acute respiratory infections in children BSc (Med) thesis. University of Queensland). Adults have significant daily sputum production enabling predictable sample collection. Second, the PNG IMR was an experienced laboratory with respect to NTHi microbiology. Thus, the PNG study an excellent opportunity to quantitate the effect of oral NTHi on the level of airways colonisation.

In Fig. 4 (39) there is a sustained reduction (compared to control) of NTHi density of 1-3 logs. A significant reduction in 'heavy growth' of pneumococci (37% of w 24%  $p < 0.05$ ) is consistent with the hypothesis that activation of the 'protective loop' is specific, but effector function (based on phagocytosis) is non-specific. In other studies with significant pneumococcal growth, similar reductions of carriage in the active group were measured. However, it is the quantitative NTHi reduction in the PNG study that best supports the hypothesis that the key protection mechanism is a reduction in NTHi density, thus buffering the promotion of inflammation following an intercurrent viral infection.

Additional observations that were made in these studies of chronic bronchitis were:

- Clinical benefits included a reduction of admission into hospital (38).
- Reduced use of antibiotics (Tandon et al.).
- Prevention of acute bronchitis, but not pneumonia (39). Nor was there a reduction of upper airways infections (38) i.e. protection is limited to the bronchial tree.
- One immunisation course (i.e. 3 periods over 8 weeks) protected over 12 months (with clinical and bacteriological data in parallel) (39). However, without a second 'immunisation course,' no significant protection was detected in the following 12 months (38).
- There was some evidence that 'day-to-day' or background symptoms (cough, sputum, wheeze, breathlessness) may be less in the active group (Tandon et al.).
- The prolonged protection (e.g. 12 months in PNG study) following one immunisation schedule over 8 weeks is most consistent with immune-based protection, contrasting with protection from polyclonal mitogens

such as Buccaline Bena which have shorter periods of putative benefit.

- **Recurrent Acute Bronchitis**

**Table 2**

	<b>Reference</b>	<b>No. of subjects</b>	<b>Age</b>	<b>FEV<sub>1</sub></b>	<b>Bacteriology</b>	<b>% Protection</b>
Newcastle 1990	40	40	47	2.6	See below	See below

This was a different explorative study aimed at a population of adults with a history of 'recurrent acute bronchitis' without knowledge of any underlying chronic airways disease (40). In the event, 83% were smokers or ex-smokers, 58% had unrecognised chronic bronchitis, and many had COPD. The clinical results were similar to those in chronic bronchitis (Table 1) i.e. there was a 41% reduction in all episodes of acute bronchitis (P =0.016) and antibiotic use was 58% less (P=0.07). However, when acute wheezy bronchitic episodes were assessed, there was a 60% reduction in the treated group (P=0.02).

The bacteriology of this study is of interest, and differs from those with long established airways disease. The 6 month study was over a winter period, and colonisation of the throat in the placebo group with NTHi was progressive, from 17% to 100%. This pattern did not occur in the active group. The second important observation was that the pattern of colonisation with H. parainfluenza did not differ between the groups, providing a control for specificity of protection (given phagocytic effector function is 'non specific.')

This is a different pattern of impact of the oral vaccine on colonisation seen in those with well established chronic bronchitis where a non-specific reduction in sputum bacteria occurs. The specific prevention of colonisation of the upper airways by NTHi suggests an antibody effect –possibly leaked IgG antibody. Two mechanisms way exist for episodes of acute wheezy bronchitis, both mitigated by reduction of NTHi colonisation. The first is as for chronic

bronchitis (above). The second, a reduction in airways colonisation with NTHi, reduces triggering an IgE-dependent bronchial spasm (note – in this study (reported in (24)) IgE anti-OMP (NTHi) antibody was  $79 \pm 14$  compared with healthy controls:  $14 \pm 4$  eu/ml  $p < 0.01$ ).

- **Chronic Obstructive Pulmonary Disease (COPD) (Tables 3 and 4)**

- a) **Single Site Studies**

**Table 3**

	Reference	Number of subjects	Age (mean)	FEV <sub>1</sub> (mean)	% sputum +ve NTHi (%)	Protection
Newcastle 1986 (9 months)	41	109	65	1.4	12	See below
Newcastle 1987 (12 months)	41	103	65	1.6	3	See below

The studies summarised in Tables 1 and 2 were designed and monitored as single site studies, provided by/based on protocols developed from Newcastle. From 1986 funding for trials was provided by biotechnology companies, with protocols and monitoring etc. fitting regulatory packages, while the science remained with my academic group. The first of these was the Perth Study (Tandon et al.) conducted in 1988, with the same protocol used in the initial 1983 study, but of 6 months duration (Table 1). In 1986/87 two studies were completed on the Newcastle site supervised by a biotechnology company –these were hurried trials stressed to produce numbers identified by power analysis at over 100 per trial. Changing ideas regarding inclusion criteria had come from a trans-Atlantic argument. In the UK, the diagnosis was chronic bronchitis based on 'chronic cough and sputum,' while in the USA the focus was on a functional diagnosis of COPD using spirometry and measuring FEV<sub>1</sub> (forced expiratory volume in one second). Clinical trials moved to an entrance diagnosis of COPD, in part because it was appropriate to current trials of bronchoactive drugs, and in part because there was less interest in 'chronic bronchitis,' as there was

confusion over antibiotic value and the role played by bacteria. The impact of this change in diagnosis was not immediately recognised. Thus, studies became a 'mix' of 'chronic sputum producers' and 'non sputum producers.'

At that time, details of both mechanisms of immune protection against intrabronchial infection and the pathogenesis of acute exacerbations of endobronchitis were the subject of investigations (see elsewhere in thesis) and a number of important parameters had not been elucidated. First, the very low levels of colonisation in these two studies (12% and 3%) reflected the low numbers of 'classical chronic bronchitis' patients that were recruited (Table 3, above). Although growth from sputum is likely an insensitive marker of the presence of NTHi, current belief is that it reflects a level required to re-stimulate T cells locating in the bronchus mucosa after oral immunisation.

Second, subjects over 65 years respond poorly to all (including oral) vaccines (see Table 4). Immunosenescence becomes an important influence when the trial population selects few with chronic bronchitis and a low level of sputum colonisation with NTHi (clearly seen in Table 4).

Third, the 'classical' definition of an 'increase in volume and purulence of sputum' was changed in large multicentre trials assessing the value of broncho-active drugs, to include a management strategy (use of corticosteroids or admission to hospital), around 2000. The impact on sensitivity can be seen in our results published in 2010 (42): in Table 2 of that publication, protection using 'traditional criteria' was 16% - when defined as corticosteroid-treated protection it was 63% ( $p=0.05$ ), and when defined as 'admission into hospital' protection was 90% ( $p<0.01$ ). Acute exacerbations were defined in the 1986/87 studies without reference to treatment.

Other confusing factors in the 1986/1987 studies, were that many subjects had previously taken oral NTHi which likely had a 'carry-over' protection effect. Note the incidence of acute infections over a winter period (classically defined) is contrasted with the 2010 study (42) (1.45 acute episodes per subject) – here the rate was 0.45 (1986) and 0.33 (1987).

b) Multi-site studies in COPD

**Table 4: Phase 2 studies of oral NTHi in COPD**

	Reference	No.	Age (mean years)	FEV <sub>1</sub> (% predicted)	% NTHi (sputum)	Protection (%)	P value
2010 STUDY (all subjects)*	Unpublished data	141	68	<70%	25	44	0.005
2010 STUDY (moderate – severe disease)	42	38	68	<50%	30	63	0.05
2016 STUDY (all subjects)	43	320	71	0.98	5.5	0	NS
2016 STUDY (under 65 years)	43	91	59	1.00	6	54	0.001

\*Note: all methods, analysis etc as per published study (42).

\*\* Neon FEV<sub>1</sub> (litres)

These two multi-site studies were sponsored by a biotechnology company and conducted by a clinical research organisation. Details are in attached publications. I was the chief investigator and was responsible for the design and science of the studies. The 2010 study was designed to allow separate analysis of those with more severe disease, for comparison with earlier studies. Both studies were conducted 20 years after the earlier single site studies (Tables 1,2,3), by which time a sea change had occurred in regulatory trials in COPD (see above and in published reviews at end

of this section). Thus, spirometric diagnosis had become the gold standard as discussed above. The diagnostic criteria for an acute exacerbation had changed to include more objective criteria (such as 'use of corticosteroids'; 'admission to hospital') – this gave both greater objectivity and greater discrimination (as shown above) – this was of value in COPD studies with lower levels of sputum colonisation with NTHi (42).

### **2010 STUDY**

A key point (including unpublished data) in this study was that most patients were recruited from the practice of Dr M. Tandon, which included a high proportion of sputum producers, with a carriage rate of 25% of NTHi. The downside was that the success of the 2010 STUDY delayed recognition of the importance of selecting 'sputum producers' (i.e. chronic bronchitis), with consequences for 2016 STUDY (see later).

Points from 2010 STUDY include:

- Protection following oral NTHi immunotherapy is less pronounced (even with a significant inclusion of sputum producers) than found in the earlier studies of clinically defined chronic bronchitis (noting similar degrees of airways obstruction). See table above.
- Protection is noted in those with all degrees of airways disease –again consistent with earlier studies (Tables 1 and 2).
- There is a 'shift' following oral NTHi, away from 'severe' exacerbations towards less severe ones –note particular impact on admission to hospital. Also, reduction in duration of episodes and less use of antibiotics and corticosteroids (42).
- There was no stimulation of IgA antibody in saliva – consistent with earlier results.
- Positive sputum cultures were significantly less in the treated group ( $P < 0.05$ ) and this applied to all pathogens (again, as found in earlier studies).
- In both the total group (141) and in those with positive sputum culture (61 subjects), the number of exacerbations in the active group were reduced by a similar amount (43% and 45% respectively, both  $P$  values at  $< 0.01$ ). One explanation is that the 'carriage' for a particular group is more an indicator of the selected group than of an individual, with variation from sample to sample.



- Age and sex influence: given the profound influence of these parameters in the subsequent study (2016 STUDY), age and sex influence in 2010 STUDY was investigated:

	Number (active/placebo)	Number of exacerbations		Protection (%)	P value
		NTHi Treatment	Placebo treatment		
2010 STUDY all subjects	69/72	0.7	1.25	44	0.005
Aged ≤65	33/41	0.45	1.00	55	0.02
Aged >65	36/31	0.92	1.58	42	0.04
Females	35/31	0.66	1.45	55	0.01
Males	34/41	0.74	1.1	33	NS

These results analysed after completion of the 2016 study, support the results from the 2016 study i.e. the importance of both age and sex in responsiveness to oral immunisation with NTHi. This is also consistent with current evidence that all vaccines including parenteral vaccines are ineffective in COPD in subjects over 65 (discussed in (43)).

#### **2016 STUDY (Table 4)**

This was to be the definitive phase 2 study in COPD. The study was structured on the successful 2010 STUDY (above). When analysed for 'intention to treat,' no protection was found. Analysis of bacteriology in sputum was similar to the results described in the first two studies of COPD (Table 3) with 6% positive for NTHi, and only one third of the study population capable of giving a sputum sample at baseline visit. More careful analysis revealed:

- A major impact of age on outcome –those under 65 had a 54% reduction in rate of exacerbations requiring oral/parenteral corticosteroids or hospitalisation ( $p < 0.001$ ); 57% reduction in hospital admission rate (NS); 60%

less time in hospital; 65% less prescribed corticosteroids; and 22% less antibiotics (with similar differences in other parameters). The statistical rigor of these analyses and the likely causes for the major age effect are discussed (43).

- In the younger group, using the validated St. George Questionnaire, at 12 and 24 weeks, a significant difference in score favouring the active group was detected, with the difference confined to the incidence of symptoms. This was the first quantitative attempt to assess interval symptoms, and the significant finding (in those <65) suggest a continuity of process, supporting the idea that exacerbations represent the incidental infection by a virus, destabilising a host-parasite relationship that was otherwise characterised at a low level of sputum (exudate) production i.e. “chronic bronchitis.”

### 3. Summary of this section:

#### (i) **The normal respiratory tract:**

IgA antibody in mucosal secretions inhibit attachment of bacteria (NTHi) to buccal epithelial cells to limit colonisation. Non-specific inhibition by lysozyme contributes to this process. IgA containing cells populate the bronchus and trachea mucosa following traffic from gut-associated lymphoid tissue. T lymphocytes (including TH<sub>17</sub> cells) participate in this traffic. The bronchus has only a limited capacity in man to respond to local antigen. In animal studies a local cellular response was followed by suppression –this was characterised with human bronchus cells as being T cell dependent. Thus while in ‘normal’ airways immunity to direct stimulation is limited, IgA antibody delivered via Peyer’s patch stimulation, provides a low level ‘non-inflammatory’ response to inhaled antigens. An immune ‘loop’ was completed in a study of subjects with smoking related disease, when evidence was found that antigen is aspirated from the airways into the gut, for uptake into Peyer’s patches.

- #### (ii) The major protective mechanism of damaged airways that controls colonisation of the airways with bacteria (in particular, NTHi) was discovered to be specific TH<sub>17</sub> cells originating from Peyer’s patches, activating and recruiting neutrophils into the bronchus lumen, a state maintained by an autocrine loop involving IL-1. This T cell loop is specifically activated and non-specifically expressed. It would appear to act as a default mechanism, handling antigens that have eluded a primary carpet of IgA antibody. The recent demonstration of a dendritic cell network within the epithelium of

the bronchus (McWilliam et al. Immunology and Cell Biology 73 (1995) 405-413) raises questions related to the relative contributions of the dendritic and Peyer's patch-dependent systems. While the Holt group have focused on the importance of this dendritic cell population in the genesis of allergic inflammation, subsequent studies have indicated a critical role for bronchus dendritic cells in promoting tolerising signals within the draining lymph nodes (e.g. T.C. Condon et al. J Leucoc, Biol 90(5) (2011) 883-895). Given that studies in this thesis have shown in rodents and sheep (e.g. (6) in this section) that intrabronchial antigen induces a poor local protective response—indeed induces down regulation ((2) in this section) – and that a high density of 'suppressor T cells' exists in the bronchus mucosa ((4 & 5) in this section), it is likely that a collaboration between different effector (IgA and Th<sub>17</sub> cells) and regulating cells (T<sub>reg</sub>), maintains a homeostatic function within the airways. Thus active protection involves aspirated antigen activating the common mucosal system via Peyer's patches, while the generation of mucosal down regulation, involves the bronchus dendritic cells and the regional lymph nodes.

### (iii) Acute Bronchitis

The initial hypothesis when chronic bronchitis was selected as a human model was that smoking damages IgA protection, enabling bacteria to descend the airways where they initiate an inflammatory response (clinically diagnosed as 'acute bronchitis'). Studies including a failure to connect NTHi in upper and lower airways (e.g. biotype differences), a connection between carriage of NTHi and sputum production, a different mechanism of protection based on control by neutrophils of luminal bacteria, suggested an alternate hypothesis – that 'acute bronchitis' (and acute exacerbations of COPD), represent a hypersensitivity disease based on TH<sub>17</sub> cells causing an excessive and inappropriate neutrophil response i.e. purulent sputum (analogous to hypersensitivity responses recognised for every other effector immune response – i.e. allergy (IgE), arthus reactions (IgG) and granulomatous reactions (CD<sub>4</sub> T cells). The presence of specific IgE antibody to NTHi antigens and in particular the link between IgE antibody, a pattern of cumulative colonisation with NTHi, and wheezy bronchitis, and its prevention with oral NTHi therapy shown to prevent this colonisation, argues in support of 'allergic' mechanisms contributing to the pathogenesis of acute bronchitis. The topic of the role of IgE-mechanisms in COPD, and the wider role of NTHi in intrinsic asthma, is one earmarked for future study.

### (iv) Oral immunotherapy with inactivated NTHi

These studies began as an experiment to support the idea that the common mucosal system played an important role in man, ended as a series of phase 2 trials to support an oral therapy that could change thinking in terms of chronic airways disease. Through human studies of oral immunisation, both the physiology and pathophysiology of, respectively, airways and airways disease, can be re-considered (above). The human studies were complemented by studies in animal models, which defined 'rules' for establishing an effective oral immunising regime for defining relationships between virus and bacterial infection, and in determining the role of 'homing' T cells. Initial studies tested a commercially available polybacterial product ('Buccaline Berna') which gave data relevant to downregulation and polyclonal activation, helping to define the parameters for formulation of a monobacterial (NTHi) product. This high dose NTHi complex antigen was shown to combine specific immune activation, preventing exacerbations in three studies of chronic bronchitis, while reducing the colonisation density of NTHi, with data consistent with activation of luminal phagocytosis. Subsequent studies continued to support the idea of oral immunisation enhancing bronchus mucosal protection, but were complicated by definitions based on function not sputum production. An upside of these issues was identification of important conditioning factors such as how best to define exacerbations, the impact of gender and age, and the value of sputum cultures in selecting candidates. In summary, oral NTHi in chronic lung disease enhances a physiological mechanism of airways protection, and by improved efficiency connecting a TH<sub>17</sub> response with luminal phagocytosis with reduction in the load of colonising bacteria, buffers against any uncoupling of these processes that would otherwise lead to a hypersensitivity reaction, characterised clinically as an increase in volume and purulence of sputum. It is possible (and isolated results suggest) this form of immunotherapy will be applicable to a range of chronic airways disorders including cystic fibrosis, bronchiectasis and intrinsic asthma.

These laboratory and clinical studies have identified a previously neglected 'second pathway' of damage irrelevant to the specific cause of airways damage, that progresses airways disease even in the absence of the primary cause (e.g. smoking –most patients in these studies had ceased smoking), requiring a different strategy for management based on a new understanding of the physiology and pathology of bronchus disease.

These ideas, plus some data, are included in a series of reviews that progressively chronicle the development of this new framework and the evolution over 25 years of a

putative oral NTHi immunotherapy (44-55). The most recent (61A) covers an overview of all aspects of the oral vaccine development.

### ***Pseudomonas aeruginosa***

The above studies began in the early 1980s on a model for chronic bronchitis and NTHi as the 'parasite.' They have been discussed. While NTHi has been the main colonising bacteria in chronic bronchitis, it is an early player in a sequence of bacteria associated with damaged airways. Thus in patients with longstanding smoking-related chronic airways disease, *Pseudomonas aeruginosa* (Ps.a) was increasingly found (see 'bacteriology' of clinical vaccine studies). A similar situation existed in patients with bronchiectasis, while in cystic fibrosis (CF) colonisation with 'mucoid' Ps.a signalled a poor prognosis, and was present as the dominant colonising bacteria in about 80% of adult patients. Before the current era of intense antibiotic therapy, 50% of CF patients would die within 5 years of onset of chronic Ps.a infection. Though colonisation with Ps.a in COPD was reduced following oral immunisation with NTHi, this bacteria was a relatively poor immunogen and studies were begun in the early 1990s using an isolate of mucoid Ps.a from a patient with CF (serotype II) (62). The background and related literature is discussed and referenced in the published papers (56-67). Highlighted is the failure to develop effective immunity with systemic vaccines especially with respect to lung/airways infection. The animal studies were developed in parallel with the rat/NTHi model, with the two main differences being (i) the challenge dose in the rat of  $\log_{10}$  8.7 live Ps.a was lethal (from pneumonia and septicaemia) by 12 hours, and (ii) that intra Peyer's patch injection of whole cell Ps.a not only enhanced clearance but also protected against lethal infection. Protection was noted with antigen doses less than required to induce systemic (or local) antibody secretion. This was consistent with subsequent cell transfer studies from mesenteric node cells, confirming protection was CD<sub>4</sub><sup>+</sup> T cell-dependent. Immune serum gave some protection, though in the animal model (as mentioned) clear protection could be stimulated in the absence of detectable antibody as with the NTHi /rat model. The mechanism of protection was enhanced clearance mediated by recruitment and activation of neutrophils in a T cell-dependent fashion, less dependent on local production of leukotriene B<sub>4</sub> as found with natural infection. All parameters of neutrophil engagement were enhanced in immunised rats: recruitment, chemotaxis, chemokinesis, phagocytosis (and chemiluminescence). These studies were extended in the rat model to show that enhanced phagocytosis included an

increase in numbers and activity of alveolar macrophages, in part due to release of TNF $_{\alpha}$  (which could be reproduced by instilling TNF $_{\alpha}$  into the trachea at the time of infection). A proof of concept study in patients with bronchiectasis using oral inactivated Ps.a in the same regimen used for NTHi (above), there was no evidence of toxicity. Antigen reactive T cells were detected in blood and a significant reduction ( $p < 0.05$ ) in excess of 90% of bacteria in sputum was recorded (59). In the animal model, protection could also be found when an outer membrane protein (OpTF) which is conserved amongst all serotypes, was injected into Peyer's patches.

It is concluded that the immunology of mucosal protection against Ps.a is similar to that found for NTHi. Ps.a infection in rats is a more virulent infection than that with NTHi, and as a whole cell antigen, inactivated Ps.a appears to be a more powerful oral immunogen. A proof of concept study in patients with bronchiectasis confirms potential in man as a valuable candidate immunotherapeutic.

**Selected papers best covering narrative:** 1, 2, 3, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 36, 38, 39, 40, 41, 42, 43, 55, 57, 58, 59, 63, 64.

## SECTION 2: SELF ANTIGEN AND AUTOIMMUNE DISEASE

The common denominator to studies in this body of work is 'an interaction between antigen and the host immune response,' with the sequelae that any clinical outcome reflects a particular balance within this relationship. Initial studies examined the relationship between antigens of patients and the host autoimmune response, in the human autoimmune disease idiopathic thrombocytopenic purpura (ITP), in two periods (in the early and the late 1970s). The status of understanding of the pathogenesis of ITP (together with discoveries made in the study of this human model) was summarised (1). In short, the single most relevant study supporting an immune pathogenesis remained Harrington's classical study of thrombocytopenic factor in plasma from ITP patients, while the search for a reliable test of autoantibody that correlated with thrombocytopenia gave unreliable results, including then recently published studies using platelet damage detected as release of platelet factor 3 (also reviewed in (1)).

The challenge at the onset of these studies was to understand why there was a discrepancy between platelet count and contemporary autoantibody tests. A recent development by Karpatkin detected antibody as release of PF-3, with 73% of those with chronic ITP being positive. Detection of autoantibody as the cause of platelet damage remained unclear as Karpatkin's data indicated that 85% of those with SLE were positive without correlation with the platelet count, and that platelet damage was caused by a serum factor considerably larger than a 7S molecule (reviewed in (1)). Thus, the relationship of immune complexes and autoantibodies to platelets was confusing and contemporary reviews concluded that, "it was impossible to make a definite conclusion concerning the pathogenesis of ITP" (reviewed (1)).

An opportunity to study the relationship between platelets and autoantibodies came with a patient with longstanding ITP, and who post splenectomy maintained a platelet count between 40,000 and 120,000/cmm. There was easy bruising when the platelet count was 80,000/cmm or above, suggestive of a defect in platelet function. Formal testing of this patient showed significantly abnormal function with reduced adhesion in vivo and to glass, reduced platelet aggregation with ADP, ristocetin, and collagen. ADP-induced aggregation of normal platelets was reduced following incubation with the patients plasma (2). This study was expanded to include 14 patients. Eighty-two

percent with chronic ITP had abnormal aggregation especially in response to ADP and collagen; the degree of dysfunction correlated with clinical disease activity, and the plasma anti-platelet factor bound to platelets and was removed using an anti-IgG antibody (3).

To better define the anti-platelet antibody effect on platelet function, a model was established using rabbit anti-rat platelet antibodies. In this model antibody induced a qualitative platelet defect characterised by impaired platelet aggregation with ADP, and with aggregants that operated in part, by release of platelet ADP. There was no primary inhibition of the platelet release reaction as tested by assay of Platelet Factor 4 availability (4). Structural requirements of antibody –induced thrombocytopeny showed that while an intact Fc fragment was required to induce thrombocytopenia, bivalent antibody fragments without the Fc fragment, retained the capacity to inhibit platelet aggregation (4).

This was a time when the idea of 'antigen-immune response' relationships were emerging with respect to both infections and autoimmune disease. With respect to the latter, organ-specific and non-organ specific groups were recognised, with cell mediated immunity and antibody, respectively, largely responsible for clinical disease. While ITP was classically linked with the 'organ non-specific group,' review of clinical and serological details of our patient cohort found a significant link with the thyro-gastric group (i.e. organ-specific autoimmune group) (5). In this group cell mediated immune mechanisms were considered to be the dominant causes of disease. Given the continued uncertainty regarding correlation of disease activity with *in vitro* tests for autoantibody, patients with ITP were tested for evidence of cell-mediated autoimmunity using *in vivo* and *in vitro* tests available. Six patients had delayed-type hypersensitivity tested by skin tests to autologous platelets –all but one had a dominant perivenular lymphocyte infiltrate on biopsy at 48 hours (5 controls had no evidence of lymphocyte infiltration at 48 hours). The only patient to have no inhibition of leucocyte migration (i.e. a migration index of 0.9-1.0) was the single subject with a negative skin test (unpublished data). These studies were expanded to 10 subjects, 9 of whom had migration indexes of less than 0.9-1.1 (i.e. the normal range) and 6 of 7 had evidence of cell mediated autoimmunity by lymphocyte stimulation (6). Some subjects had a serum factor (that could be absorbed to platelets and was present in purified IgG) that 'blocked' migration inhibition. Numbers are too small to be confident of any biological role for blocking antibody but it was noted that in the two subjects with persistent thrombocytopenia, there was no blocking antibody (6). It was unclear



whether there was identity between 'blocking antibody' and antibody causing a functional platelet defect –there was certainly no correlation of either of these antibody activities with immune-damaging antibodies.

To assess the role the spleen plays in the disease, over and above its known capacity to contribute to the pool of circulating antibody and its 'filter' role removing 'sensitised' platelets. Several observations were made (unpublished data):

- (i) Patients studied before and after splenectomy (2 patients, one studied twice within a week, the other within 2 months) showed percentage aggregation compared with normal, increased for ADP, adrenaline, and collagen (respectively) from 43% to 65%; 0 to 82%; 12% to 80%. The cause of this increase in platelet function could involve a reduction in antibody production. An alternate explanation is that the spleen provides an incubator function through 'pooling.' Platelet aggregation studied in 6 normal subjects before and after 4 hours incubation at 37°C, showed a 60% and 33% reduction respectively for aggregation with ADP and collagen. Taken together, these data are consistent with the spleen providing an environment where platelets can be damaged by antibody and/or cytokines, and thus contribute to dysfunction with impaired haemostasis and premature removal. Study of four ITP patients post splenectomy showed that in three, normal platelet function persisted for at least 6 months. In the fourth, normal function immediately after splenectomy (and while taking corticosteroids) gave way to defective response to collagen at 3 and 6 months, indicated that at least some have persisting instability, perhaps then being susceptible to exacerbations following viral infections.
- (ii) Two populations were followed post splenectomy –those with stable complete remission (12 subjects) and those with incomplete and unstable remission (9). Those in a stable remission, 11 of 12 had normal aggregation with ADP, while 7 of 12 had defective aggregation with collagen. Those with incomplete remission generally had impaired platelet function – 7 of 9 for ADP and 6 of 8 for collagen.

In an attempt to determine whether abnormal function reflects damage that determines accelerated loss, two populations were studied. In the first with patients diagnosed as having acute leukaemia, a correlation of platelet

survival with platelet dysfunction not caused by extracorporeal factors, was found. Thus platelets with abnormal platelet function have a reduced life span.

Second, subjects with a viral illness (infectious mononucleosis) were tested in the acute and convalescent phase, with all having minor functional abnormalities that reversed to normal with convalescence. Those with defective ADP aggregation increase their count by one third in convalescence ( $p < 0.05$ ). These results are relevant to chronic ITP where acute viral infections commonly precipitate rapid falls in platelet count. While several mechanisms may contribute these data suggest additional qualitative changes contribute.

At the conclusion of this first phase of study of the 'Platelet-Host' relationship in the selected autoimmune disease 'ITP,' it was concluded:

- (i) That there is a complex interplay between the host immune response and the platelet. Two new antibody activities ('thrombasthenic' and 'blocking') had been identified, characterised as containing gammaglobulin and binding to platelets, and an additional 'platelet immune-damage' assay developed (Kaolin-based). Cellular autoimmunity was defined using both *in vitro* and *in vivo* methods. Studies and discussion suggested that the spleen provides an unique 'incubating environment' for an interaction between host factors and the platelet. An important conclusion (6) was "that a number of effector mechanisms exist in ITP –some damaging, others protective. Platelet damage would therefore be determined by the balance of the immune response to antigen, rather than any one effector mechanism acting in isolation." Although questions remain as to the extent to which the plasma thrombocytopenic factor in ITP is autoantibody (as opposed to immune complexes), taken together this cluster of studies with cellular autoantibodies and addition of two additional functional impacts of 'autoantibodies,' supports the concept of platelet autoimmunity. Demonstration of qualitative damage in ITP adds to the spectrum of immune-mediated damage (and the complexity of the host-platelet relationship).

The recognition of a pivotal relationship between 'antigen and immune response,' with clinical activity an outcome of a particular 'balance,' became a major determinant of the theme of my future research career (and the bulk of this thesis). The lesson of balance within complexity would become a recurrent theme.

- (ii) Much of the study at this time focused on qualitative platelet damage in ITP –a new concept. Questions raised included:
- a) What is the defect?** The primary defect appeared to be an impaired response to ADP (adrenaline and collagen responses are influenced by release of ADP) with 73%, 91% and 100% of subjects having, respectively, an impaired response to ADP, collagen and adrenalin (3). This was thought to reflect changes found in thrombasthenia (a rare genetic defect of platelet function). Retention of a normal aggregation pattern to Ristocetin in 75% of ITP subjects, supported this supposition. The description of an abnormal membrane glycoprotein in thrombasthenia in 1974, and the cloning and determination of the c DNA sequence for GP IIb and IIIa in the 1980s followed. Later, there was developed alloimmune and murine monoclonal anti-IIb –IIIa complex, leading to successful animal and human studies of humanised ‘abciximab,’ introduced as one of the first monoclonal antibodies in medical practise. In human studies (1994) it was shown to significantly reduce acute infarction and the need for emergency grafting, and mortality, following percutaneous coronary intervention (Seligsohn, J Pathophysiol Haemast Thromb 32 ((2002) 216-17), while autoantibodies to IIb/IIIa platelet glycoproteins were confirmed by Kosugiefal (Thromb Haemost 75 (1996) 339-45).
- b) What is the significance of platelet defects in ITP?** They contribute to defective haemostasis and the data (above) including studies in acute leukaemia and infectious mononucleosis indicate that defective platelets are prematurely removed. Therapeutic modalities were suggested from the human and rat studies. The use of anti-platelet antibody as a therapeutic was discussed in 1973(4), before monoclonal antibodies were developed by Kohler and Milstein in 1975. Because Fc fragment was required in structural studies for thrombocytopenia in the animal model, (Fab)<sub>2</sub> was identified as a logical therapeutic candidate (4). In the event, monoclonal antibodies took centre stage, and abciximab was FDA-approved as the second-indication monoclonal in 1994 (after OKT<sub>3</sub> in 1986).
- c) What significance do qualitative defects have in the history of ITP?** Studies on patients with infectious mononucleosis show abnormal platelet function, suggesting that one mechanism of exacerbation in ITP following a virus infection is added damage. The spleen appears to play a more

complicated role in platelet destruction, through an incubation effect, enabling greater platelet damage from cellular and humoral autoimmunity.

A series of studies carried out at RPAH, Sydney (1976-78) aimed at extending the earlier ones on 'platelet-host' interaction, especially to (i) address the issue of autoantibody versus immune complexes in the cause of thrombocytopenia, and (ii) extend studies on cell-mediated immunity, including any potential role for T cells in control of B cell function, as immune dysregulation in autoimmunity had become a major interest.

A simple, rapid, cheap, reproducible and sensitive  $C_{1q}$  deviation radioimmunoassay was developed, with the capability of identification of antigens within the complex (9). This assay was used in a study of 39 subjects to show 90% had immune complexes binding to  $C_{1q}$  (10). In the same study sensitisation to platelet associated antigen was shown in 80% of subjects, by detecting MIF using guineapig macrophages, while only one third of patients had 'antibody' detected using a platelet-damage assay based on release of  $H^3$ -serotonin. This study strengthened the data set supporting autosensitisation, but left questions over the significance of immune complexes. Therefore, studies were conducted during active and inactive phases of disease, showing an inverse relationship between platelet count and levels of immune complexes. A similar reciprocal relationship was found in both immune and non-immune groups. While the question as to whether immune complexes contribute to platelet destruction remains open, these results indicate a 'buffer' role for platelets by binding complexes, protecting the vascular endothelium (11).

The second objective was to examine the thesis that autoimmunity involved a change in 'balance' between the effector and regulatory cells of the immune system – a popular theory at the time held that B cell secretion of immunoglobulin reflected the balance of  $T_H$  and  $T_S$  cells (the latter now known as Treg cells). We used a method developed by Saxon et al (reviewed in (12)) based on co-culture of purified T- and B-cells driven by the polyclonal mitogen PWM to show parabolic curves when immunoglobulin secretion was titrated against an increasing T/B cell ratio. In normal subjects. However, two thirds of those with ITP failed to significantly suppress immunoglobulin secretion with high T/B cell ratios – a loss of suppression that could be reversed using normal T cells. In a limited study, some 'non-immune' thrombocytopenic subjects failed to suppress IgG secretion at high T/B cell ratios,

making it less clear as to whether the results with the ITP subjects indicated a primary or secondary reduction in T cell-mediated suppression (12).

At this stage, interest shifted to 'host-parasite' relationships, particularly as they operate at mucosal surfaces. Here, the antigen in the interactive relationship was better defined, yet the broad immune mechanisms overlap, with a focus on control.

**Selected papers best reflecting narrative (PhD studies excluded): 10, 11, 12.**

### **SECTION 3: TARGET ANTIGEN IS 'NON-SELF'**

### 3(A) MICROBES

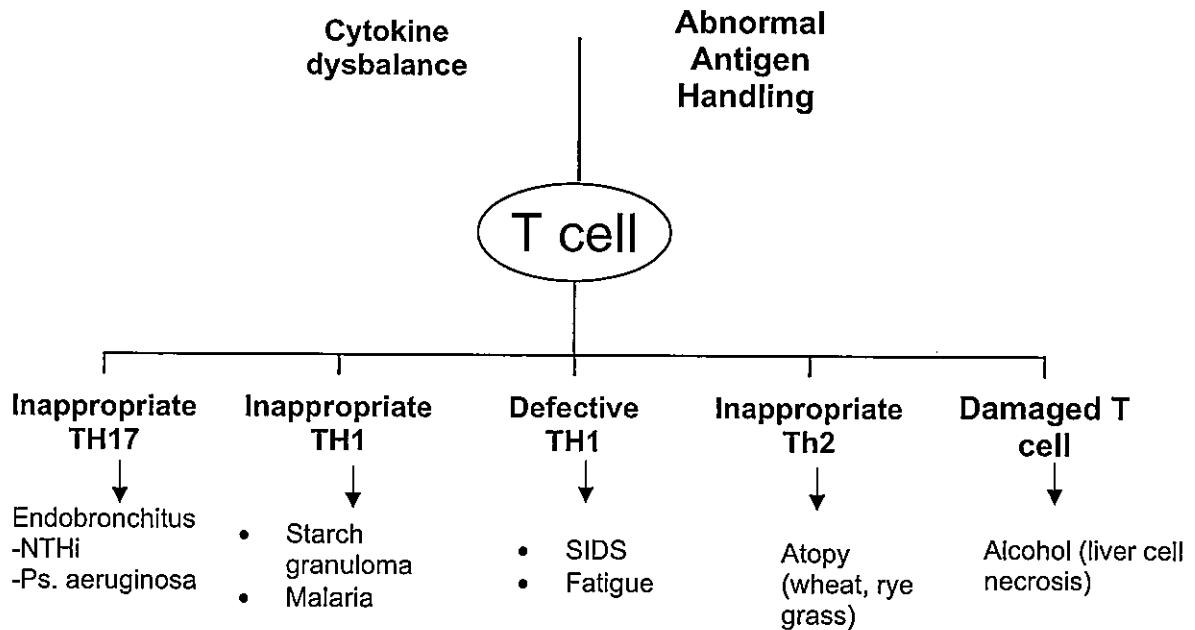
Host-parasite relationships involving 13 human pathogens have been studied over a 30 year period from the mid 1970s. Study objectives ranged from identifying the pathogen (e.g. Whipple's Disease) to analysing the pattern of cytokines secreted from CD<sub>4</sub> T cells and correlating these with outcomes (e.g. *Candida albicans*; *Mycobacterium avium* ss paratuberculosis; *Helicobacter pylori* and *Chlamydia pneumoniae*). A group of 'airways pathogens' (non typeable *Haemophilus influenzae*, *Pseudomonas aeruginosa* and influenza) are studied in relation to pathogenesis of airways disease and its prevention with data in Section 1 (c). The final group (Epstein Barr Virus and Respiratory Syncytial Disease Virus) came out of studies recording changes in secretory immunity during development, and are discussed in Section 1(a). *Plasmodium falciparum* studies were based on development of a micro-merozoite inhibition assay to enable end points of either inhibition of merozoite uptake or inhibition of schizont growth (respectively due to antibody or cytokines) –this allowed identification of protection mechanisms in terms of age and disease category, using serum samples in inhibition assays. The interaction between the intestinal microbiome and alcohol was examined in the context of acute hepatocyte necrosis, with the development of a new hypothesis placing alcohol-damaged T cells within the liver as a key component to development of hepatic cirrhosis.

- (i) Candidiasis (*C.albicans*)
- (ii) Gastritis (*H. pylori*)
- (iii) Atheroma  
(*C.pneumoniae*)

\_\_\_\_\_

- (i) Whipple's Disease
- (ii) Crohn's Disease

**Fig. 1**



1. Whipple's Disease

Whipple's Disease is a systemic illness usually presenting with gut and joint involvement though virtually all organs can be involved with many clinical features. The characteristic pathology is an infiltration of the jejunal mucosa with foamy macrophages with granular cytoplasm staining with PAS. At the time of this study, while indirect evidence supported an infectious etiology, no organism had been identified as the causal agent.

The outcome of the study of a patient with classical Whipple's Disease was (1, 2):

- (i) A gram positive  $\alpha$  haemolytic streptococcus with the cultural characteristics of streptococcus dysgalactiae was isolated from prolonged culture of a resected lymph node.
- (ii) The organism was transferred into a human fibroblast line, where accumulated material stained with PAS and had electron-microscopic

characteristics identical to those in the jejunal biopsy i.e. Koch's postulates had been satisfied albeit in vitro. Growth in hypertonic sucrose suggested identity as a cell wall deficient organism.

- (iii) An IgA antibody reacting with PAS positive material, could be absorbed by the bacteria. Antibody activity disappeared after 12 months of antibiotic therapy as did evidence of T cell sensitisation.
- (iv) Low titre antibody was detected in most controls, indicating either that the organism was commonly encountered or cross reactivity was present.
- (v) Autoantibodies (Smooth Muscle Antibody; Anti-Mitochondrial Antibody) disappeared after 12 months antibiotic therapy.
- (vi) Lymphopenia and cutaneous energy reversed with therapy.

It can be concluded from these studies that Whipple's disease is caused by a gram positive coccoid bacterium with fermentation characteristics of streptococcus dysgalactiae, possibly surviving as a cell wall deficient organism. Systemic infection has a profound impact on the immune system with impaired T cell immunity and autoimmunity both of which reverse with effective antibiotic therapy. Low level antibody in normal controls suggest common exposure, despite the rarity of the disease. It was hypothesised that a specific 'handling' defect leads to persistence of the bacteria with collateral changes in immune parameters, both of which reverse on reduction of bacterial load.

PCR technology became available in 1988, enabling more precise identification of the *S. dysgalactiae* as an Actinomycetes (*Tropheryma whippelii*), though still the microbe could not be cultured (N. Engl. J Med Relman et al. 327 (1992) 293-301). 16s ribosomal RNA from tissues of 5 patients was amplified to determine the nucleotide sequence. It was not until 2012 that autoimmune co-morbidities became recognised (AM J Surg Pathol 36 (2012) E 296-7) and 2000 that culture of *T. whippelii* was again recorded.



## 2. Candidiasis (candida albicans)

This important and common human infection of mucosal sites was studied. A mouse model where the Th<sub>1</sub>/Th<sub>2</sub> paradigm could be validated for subsequent studies in chronic inflammatory disease of man was studied. We also studied circulating T cells in women with Recurrent Vulvovaginal Candidiasis (RVVC) specifically to validate murine studies showing an effect of oestrus on the common mucosal system.

The two most common clinical forms of infection are vaginitis and oropharyngeal infection. While most women will have occasional vaginitis, 3-5% of women have six or more episodes each year, with attendant significant health restrictions. Oral mucositis occurs commonly in older subjects with dentures and in those using regular inhaled steroids. Invasive disease in immune compromised subjects, usually follows infections of the oropharyngeal-oesophageal mucosa. Current views on immune protection against Candida infection are based in part on analysis of inherited immune deficiencies but emphasise the importance of the Th<sub>17</sub> pathway and intact phagocytic mechanism to contain infection at the mucosal surface (Lilic, D; Current Opinion Microbiol 15 (2012) 420-26) (analogous to bronchus protection – see 1(c)). It would not be until 2007 that Steinman recognised that Th<sub>17</sub> cells were the first major revision of the Th<sub>1</sub>/Th<sub>2</sub> paradigm (Nature Medicine 13 (2007) 139-145), and even here, the Th<sub>17</sub> pathway was a mediator of tissue damage in autoimmune disease. In 2009 Khader et al described Th<sub>17</sub> cells as being at the crossroads of innate and adaptive immunity against mucosal infections (Mucosal Immunol 5(2009) 403-11). We showed that T cells participated within a Peyer's patch driven 'common mucosal system' that mediated mucosal protection by recruiting and activating neutrophils in 1989 -18 years before Th<sub>17</sub> cells were identified! These T cells were formally identified as 'Th<sub>17</sub> cells' in our model in 2011.

This broad brush thinking at the time of studies recorded in this thesis, is summarised in a review written with colleagues (1). In brief, CD<sub>4</sub> T cells were seen as important in containing oral infection, linked to phagocytosis as a mechanism limiting extension to systemic infection. A key example of integration of innate adaptive immunity was T cell-dependent recruitment of phagocytic cells from bone marrow reserves.

- (i) **Vaginal candidiasis:** an existing model of experimental murine vaginal candidiasis was converted into a 'continued oestrus' model (2,3,4). A variety of manipulations failed to identify clear evidence of the mechanism of local protection –when athymic 'nude' BALB/C (nu/nu) mice were used, it

concluded that T cells had little influence on local disease, though they did reduce the level of dissemination. Infection susceptibility was not linked to any histocompatibility haplotype tested. Neutrophil depletion had no clear effect on vaginal clearance. Oral immunisation with various candida preparations failed to significantly enhance vaginal clearance of *C. albicans*. It was concluded that the induced continuous oestrus murine model gave little information relevant to the human disease, but did emphasise the importance of hormonal influence on the expression of vaginal candidiasis. McDermatt et al. (J. Immunol 124(1980) 2536-9) has shown that localisation of IgA plasma cell precursors in vaginal tissues was oestrogen-dependent. It had been observed that acute episodes in women with recurrent vulvo-vaginal candidiasis was nearly always immediately before menstrual periods. An hypothesis was developed that in this group, characteristics of the host-parasite relationship that favoured no clinical disease were distorted in the pre-period phase of the menstrual cycle when circulating T cells were less able to access vaginal tissues. This hypothesis was tested in 28 women with RVVC (and 25 controls). A phase-specific reduction in INF- $\gamma$  secretion after stimulation with candida antigen was found in RVVC, but not in controls, when blood was tested in the follicular phase. It was concluded that the most likely explanation was a reduced pool of antigen-reactive T cells, such that during the oestrogen-associated follicular phase, movement from the circulating pool into vaginal tissues resulted in few candida-specific T cells in blood, and insufficient in tissues during the luteal phase to limit candida-associated inflammation. The expanded T cell pool in normal controls was sufficient to buffer against both these outcomes. At the time of these studies (1998), results were interpreted in the existing Th<sub>1</sub>/Th<sub>2</sub> paradigm (7, 8).

- (ii) **Oropharyngeal candidiasis:** Although the studies in the murine model of candida vaginitis were of limited value in identifying a role for T cells in containing infection, study of RVVC in women gave more substantive evidence of a more critical role for INF- $\gamma$  secreting T cells in disease expression. In both models, there was a dominant influence of sex hormones on the host-parasite relationship. To avoid this influence and to directly examine the role of T cells in determining disease outcome, murine models of oropharyngeal candidiasis were studied.

The first series of studies (9, 10, 11) was in conjunction with Dr R. Ashman's group at the University of Queensland, in irradiated or immune manipulated mice using monoclonal antibody to deplete T cell subsets. Studies showed that a Th<sub>1</sub> cell response driven by antigen-presenting cells were critical to mucosal recovery (based on detection of INF- $\gamma$  and IL-12 respectively), and that T cells were connected to recruitment of phagocytic cells.

To date, animal models and study of RVVC had suggested an important role for Th<sub>1</sub> cells in disease expression and recovery with respect to mucosal candidiasis. The murine models all used contrived experimental conditions, which limited interpretation of results. A natural infection model in BALB/c and DBA/2 had been recently described. These strains share H-2 MHC characteristics, but have different rates of spontaneous clearance of candida albicans infection of the oro-pharyngeal space. A series of studies were conducted to dissect T cell contributions to mucosal resistance (12,13,14, 15, 16). The results can be summarised:

- a) A more vigorous T- and B- cell response was detected following oral infection with *Candida albicans* in serum, saliva and regional lymph nodes in the 'infection resistant' BALB/C strain.
- b) Message expression and protein secretion following stimulation of lymph node cells with candida antigen were detected earlier after oral infection (IL-4; INF- $\gamma$ ; IL-12) in 'infection-resistant' mice. Treatment of mice with anti-IL-4 delayed clearance of *C. albicans*.
- c) The mechanism of protection contributed to by IL-4 was an early and vigorous production of nitric oxide.
- d) An oral vaccine (but not a parenteral vaccine) using the blastospore yeast form induced clinical immunity to infection in DBA/2 (infection-prone) mice, associated with an early and sustained production of both INF- $\gamma$  and IL-4 from antigen-stimulated cervical node T cells.

- e) DBA/2 mice pre-treated with an oral probiotic (*Lactobacillus acidophilus*) had significantly shortened duration of colonisation of the oral cavity compared to controls. Enhanced clearance correlated with early mRNA gene expression for IL-4 and INF- $\gamma$  and their secreted products in cultures of cervical lymph node cells stimulated with candida antigen. Rapid clearance correlated with higher levels of INF- $\gamma$  and nitric oxide in saliva.

It is concluded from these studies that BALB/c //DBA/2 mouse model of oro-pharyngeal candida infection is an excellent model to study host-parasite relationships at a mucosal level. It enabled a greater focus on molecular events involved in CD<sub>4</sub> T cell contribution to immunity, to show that a Th<sub>0</sub> (i.e. both Th<sub>1</sub> and Th<sub>2</sub> mechanisms) are required for optimal protection. INF- $\gamma$  inhibits hyphae formation, while IL-4 was essential for nitric oxide production, which inhibits candida growth. Vaccine studies confirmed that the oropharyngeal mucosa participated in the 'common mucosal system' as only an oral vaccine promoted protection. This protection correlated with an early and sustained secretion of both INF- $\gamma$  and IL-4 from antigen-stimulated lymph node cultures. A similar correlation followed ingestion of some but not all probiotics (immunobiotics), providing clear evidence for selective value amongst this category of immunobiotics and focusing attention on important potential roles played by the intestinal microbiome.

### 3. Crohn's Disease (*Mycobacterium avium* ss paratuberculosis or MAP)

In an earlier study (3), (1(b)) data was presented from the first attempt to isolate and characterise gut mucosal lymphocytes in man. In this study, cell populations obtained from subjects with Crohn's Disease, showed high levels of 'spontaneous' lymphocyte proliferation with blast formation in culture, though the stimulus was not clear.

Subsequent studies used co-culture methods to show mucosal cell populations prepared from patients with inflammatory bowel disease appeared to have an expanded population of suppressor T cells (now recognised as T reg cells). The idea of a 'feedback' regulatory mechanism in the gut mucosa, was re-enforced by similar data obtained from resected bronchi suggesting a generic regulatory mechanism operating within mucosal surfaces. These early ideas have evolved and are

discussed in a current review assessing the balance of T reg and Th<sub>17</sub> cells in the context of H. pylori infection (see 'H. pylori' section below).

MAP has been argued as a cause of chronic intestinal inflammation for longer than Crohn's Disease has been recognised as a disease, because of its causative role in Johne's Disease in ruminants (see 1,2). Antibiotics selected on the basis of activity against the MAC group (4) have clinical benefit in Crohns and these include Rifabutin and Clarythromycin. Response to these antibiotics has been used as an argument to support a pathogenic role for MAP in Crohns Disease. Clinical response is not an argument for specificity. Thus studies were designed to specifically examine the relationship between MAP and cytokine secretion patterns in both intestinal mucosal 'organ cultures' and in blood (17,18).

The study using intestinal organ cultures included results from 235 subjects, with 63 having Crohn's Disease (17) against normal and diseased controls. There was significantly more TNF<sub>α</sub> secreted in Crohns Disease. MAP +ve subjects with Crohn's secreted significantly more TNF<sub>α</sub> than did MAP -ve Crohn's, a difference not seen in any other group. Two additional observations were made. First, there was no skew towards a Th<sub>1</sub> response observed from results of mucosa cultures in Crohn's compared with normal controls. Other disease groups showed no such difference (p<0.05). The second study was of circulating T cell secretion patterns (18). Again, large numbers were studied (116), to show no significant secretion profile differences between Crohn's and normal and disease controls. However, when MAP+ and MAP -ve Crohn's were compared, four fold the amount of IL-4 was secreted from MAP +ve subjects (p=0.02) and more IL-2 was also secreted from MAP +ve subjects (p=0.03). No difference between MAP +ve and Map -ve was measured in ulcerative colitis. These results in a large population of both test and control subjects indicate two MAP specific outcomes in Crohn's: an increased TNF<sub>α</sub> secretion in the absence of evidence of TH<sub>1</sub> 'bias,' and distortion of circulating cytokine 'balance' of a 'Th<sub>2</sub>' type. These changes were both restricted to MAP +ve Crohn's Disease. Taken together, studies are consistent with the idea that MAP is a common bacteria in our environment. However, those with CD appear to have a genetically determined defect in 'handling' antigens such as MAP. Macrophages 'loaded' with MAP secrete more TNF<sub>α</sub> and recruit Th<sub>1</sub> T cells to the mucosa which contribute to ineffective 'drive' of the macrophages/dendritic cells. A re-distribution of the Th<sub>1</sub> cells from the circulation would account for the apparent 'Th<sub>2</sub> bias' and detected only in MAP +ve Crohn's Disease. A similar redistribution was demonstrated in sarcoidosis, a systemic

granulomatous disease (19). With respect to aetiology, these results support a contributing role for MAP in the pathogenesis of Crohn's, but not one of being the primary cause. As such, these are the first studies to provide objective data at a mechanistic level supporting a role for MAP in the pathogenesis of Crohn's.

#### 4. Helicobacter pylori

Studies of the host response to infection with H.pylori began around 1990 examining saliva antibody as part of an extension of an interest in the common mucosal system in man (20, 21). Marshall and Warren had described the presence of curved bacteria in gastritis and peptic ulcer disease in 1982, and Borody proved a causative association with duodenal ulcers using effective eradication therapy in 1990. A confidence existed that H. pylori was an established essential link in the chain of events leading to mucosal disease, and that a course of antibiotics would cure ulcers and remove peptic ulcer disease as a significant health threat.

A concern was that this was too simplistic and that understanding H. pylori - associated disease required an appreciation of the host-parasite relationship. This thinking was summarised in a 'current controversies' article (22) which began with the challenging comment "recognition of the H. pylori link with gastric cancer appeared to complete an impressive curriculum vitae for H. pylori. Next disease please!" This statement was followed by the argument that any complacency was poorly placed. Failed eradication was creating an increasingly large pool of resistant bacteria, hope for a vaccine-induced 'sterile' immunity was fading, there was an unexplained change in ulcer epidemiology and there was no understanding of mechanisms of persisting infection and causation of cancer. There had been little to no significant effort to place clinical outcomes as particular balances of a host-parasite relationship at the time our studies began. A current review summarises the status of mucosal immunity and H. pylori, identifying our contribution to contemporary thinking (Section 1 of (23)).

##### (i) **Anti-H. pylori antibody (24-29).**

From the mid 1970s we were using saliva to monitor mucosal events (Section 1(a)). As the significance of H. pylori became recognised through the 1980s, saliva became a logical secretion to study gastric colonisation. At that time, diagnosis depended on invasive tests. In 1988 serum antibody was suggested as a less invasive diagnostic measure (discussed in (24)) but early antigen

preparations gave poor sensitivity and specificity parameters and there was poor discrimination between *H. pylori* +ve and -ve subjects.

We developed antigen preparation from a cell free sonicate fractionated on a superose 6 column to give an unique antigen preparation containing 18 proteins (MW 40-700 Da), several not previously documented. Initial testing gave a sensitivity and specificity of over 90% in a serum ELISA, and 100 and 86% (respectively) for saliva (24). This antigen preparation was incorporated into commercial diagnostics (HELISAL™, Cortecs Diagnostics, Deeside, UK) which performed favourably against other commercial products (reviewed in (24)).

Surprisingly the dominant saliva antibody was IgG immunoglobulin class, not IgA. There was a close correlation with serum IgG antibody activity, suggesting a level of leakage –this, however, was not consistent with a large amount of data on saliva indicating significant ‘leakage’ was restricted to a short post natal period (Section 1(a)). Different kinetics following eradication of *H. pylori* was supportive of local IgG synthesis (25, 26), broadening the concept of ‘the common mucosal system’ which traditionally saw IgA as its centre-piece marker.

On the back of this work the first ‘rapid near patient’ ‘yes/no’ test (HELISAL RAPID, cortecs Diagnostics, Deeside UK) was developed which was the trigger for the development of the ‘test and treat’ strategy used extensively in Europe. Initially the rapid test was planned to use saliva (hence the name ‘Helisal’) but technical issues favoured the use of ‘finger prick’ blood (though the saliva test was used for epidemiological studies) (25, 26). The rapid ‘yes/no’ test was tested against traditional endoscopically obtained markers and found to have a sensitivity and specificity of 90% (e.g. 203 endoscoped patients: Borody et al. *Am J. Gastroent* 91 (1996) 2509-12).

Subsequent studies would show that subjects with dysplasia/carcinoma had different cytokine secretion profiles (see later this section). To test whether this ‘environmental change’ was reflected in a variation in IgG subclass of secreted antibody, IgG subclass anti-*H. pylori* antibody assays were developed and tested (27). It was concluded that low levels of IgG<sub>2</sub> anti-*H. pylori* antibody compared to IgG<sub>1</sub> or total IgG antibody, was a risk factor for

carcinoma, probably reflecting the switch in cytokine dominance from Th<sub>1</sub> to Th<sub>2</sub> pattern (see discussion 27, 28, 29).

It had been noted that H.pylori ELISA kits developed in the west, gave variable results in Asian populations. Asian populations had high levels of H. pylori carriage and a high incidence of gastric carcinoma. Given the data (above), an IgG<sub>2</sub> antibody test was developed (Helirad Alert ELISA kits; VRI Biomedical, Sydney) and tested in a Sydney and a Hong Kong patient group (28). This analysis showed better discrimination of H. pylori status using the IgG<sub>2</sub> assay in the Hong Kong samples, while the 'standard' IgG assay performed better in the Australian samples. To test IgG subclass antibody assays in a European population with a high incidence of H. pylori infection and gastric cancer, Estonian patients with well characterised disease was studied (29). Although a complicated group, there was clear evidence of a fall in both total IgG and IgG<sub>1</sub> antibody in subjects with gastric atrophy and carcinoma, but only those with gastric carcinoma in the corpus of the stomach had low IgG<sub>2</sub> antibody. Further study of host-H. pylori relationships are needed in Estonian vis-à-vis Chinese populations to determine whether similar or different processes operated in complicated H. pylori disease.

Persistent and complicated disease has been associated with a lower density of H. pylori and/or a cytokine switch (as described above). It will be seen in studies discussed later in this section, that an adaptation mechanism of H.pylori appears to involve a loss of urease (and other proteins) and a change towards a 'coccoid' form. Urease is a major protein component of H.pylori antigen preparations, suggesting that current H. pylori antigen preparations may be less valuable in assessing H. pylori positivity in patients with chronic gastritis and gastric cancer. There is a major need for new 'coccoid' antigen preparations to test this possibility.

**(ii) Treatment failure.**

Prior to this study, 'treatment-failure' had been interpreted in terms of drug resistance, not as an outcome of a host-parasite relationship. It was not possible to completely explain failure to respond to antibiotics in terms of antibiotic resistance patterns.



At this time, only scanty data existed related to cellular immunity to *H. pylori* (reviewed (30)). Initial studies compared cytokine secretion in the absence and presence of anti-CD<sub>4</sub> antibody, from gastric mucosal organ culture and a whole blood culture (based on ligation of CD40 ligand by CD40 on platelets) and (for example) IL-4 secretion. The correlation co-efficient between mucosal and whole blood cultures for IL-4 secretion of 0.55 (P<0.001) was satisfactory (30,31,32). Results of secretion studies are summarised:

- (a) Uncomplicated untreated patients with *H. pylori* infection, was associated with a dominant Th<sub>1</sub> response (i.e. antigen stimulated CD<sub>4</sub> circulating cells secreted an increase in  $\gamma$ IF, with no detectable IL-4.
- (b) Following successful eradication of *H. pylori*, there was a switch, to a balanced Th<sub>1</sub>/Th<sub>2</sub> (or a Th<sub>0</sub>) response, with detection of IL-4 in supernatant of stimulated CD<sub>4</sub> T cells in whole blood culture.
- (c) In patients who failed to eradicate *H. pylori*, this 'switch' did not occur, suggesting that a failure to secrete Th<sub>2</sub> cytokines reflected impaired resistance, which contributed to a failure to eradicate infection. This was the first demonstration of a host factor contributing to treatment failure.

N.B. Studies in a mouse model of oral candidiasis, comparing infection-prone and infection-resistant strains, showed that secretion of Th<sub>1</sub> and Th<sub>2</sub> cytokines played critical roles in mucosal clearance of pathogen, that certain oral probiotic preparations could both activate Th<sub>2</sub> secretion in infection-prone mice, and accelerate pathogen clearance (see above). The results with *H. pylori* suggest a similar balance of cytokines is required for effective eradication. The use of selected probiotics prior to 'escape' antibiotic eradication in those who failed eradication with primary antibiotic therapy, is suggested as co-therapy (30, 31, 32). These are ongoing studies.

**(iii) H. pylori and gastric cancer (33)**

The demonstration that gastric cancer was epidemiologically linked to *H. pylori* infection, making *H. pylori* a risk factor for cancer, was a major development in understanding the link between cancer and infection.

However, no mechanism for this association was apparent, somewhat weakening the argument. Collaboration was established with the First Teaching Hospital of Shanxi Medical University, China, to study aspects of H. pylori/ gastric mucosal interaction in patients with documented gastric dysplasia and gastric cancer. The findings can be summarised:

- (a) Patients with uncomplicated H.pylori infection and gastritis, had elevated INF- $\gamma$  secretion and no detectable IL-4 secretion from gastric mucosal 'organ' cultures (consistent with findings (above) in whole blood cultures).
- (b) Patients with mucosal dysplasia (a pre-malignant condition) had increased secretion of IL-4, while maintaining secretion of INF- $\gamma$  i.e. exhibited a Th<sub>0</sub> cytokine secretion pattern.
- (c) Patients with gastric cancer irrespective of detectable H. pylori at the time had a clear and distinctive cytokine secretion pattern. No detectable INF- $\gamma$  was detected (the 'marker' cytokine for a Th<sub>1</sub> response, present in all other patient groups), in both H. pylori positive and negative groups. However, the highest levels of IL-4 secretion were detected in those with gastric cancer.
- (d) **IL-8 secretion** from the mucosal organ cultures (presumably from epithelial cells) showed an increase in H. pylori infection, and in the carcinoma groups, but not in those with dysplasia (possibly reflecting a characteristic of dysplastic cells, though H. pylori had been eradicated in this group).
- (e) **Antibody studies:** The absence of IgG and IgA antibody in both serum and culture supernatants of H. pylori –ve cancer patients (supporting a non H. pylori causation) suggests the mucosal environment within which carcinoma develops, is 'common' to gastric cancer and not specifically a result of the H. pylori infection. The lack of significant increase in antibody in those with dysplasia possibly reflects a group where H. pylori had been eradicated, and who were subject to routine surveillance. A reduction in IgG<sub>2</sub> subclass antibody (compared to those with chronic gastritis) in those with gastric cancer,

likely reflects the near absence of INF- $\gamma$  secretion within the mucosa, as the cytokine contributes to secretion of IgG<sub>2</sub> subclass antibody. Though these data would appear to show a selective IgG<sub>2</sub> antibody deficiency in a Chinese population, a collaborative study that included Estonian patients, suggested that there was also reduced IgG<sub>1</sub> subclass antibody in H. Pylori positive cancer patients, possibly reflecting regional differences.

**(iv) Non-urease producing H. pylori in chronic gastritis (34, 35)**

By the mid 1990s, it was becoming clear that the 'gold standard' clinical parameters of urease testing, histology and culture fell short of detecting all subjects with infection (reviewed (17)). By 1990, the presence of a 'coccoid' form of H. pylori –perhaps an adapted form of the usual helical form –and its contribution to disease, was being discussed, though to many, it was a 'degenerate' form (reviewed). To examine viability and potential pathogenicity of a 'coccoid form,' the viability and capacity to revert to spiral form was studied in culture. Coccoid forms retained DNA integrity, but ceased mRNA production for urease and Cag A, while message for 26 KD protein (a reliable marker protein for H. pylori) and 16 sRNA, was retained. Reversion to urease –secreting helical forms followed acidification of the culture medium (although growth from small number of surviving helical forms can not be completely excluded).

A confusing clinical group was that of non-ulcer dyspepsia and chronic gastritis. We examined the host-H. pylori relationship in 35 patients (35). The important finding was that 26% were negative for urease by CLO test or mRNA but +ve for the backbone 'marker' 26 KD by PCR. All had coccoid forms in biopsies –half only with coccoid forms. These 'urease-negative H. pylori positive' patients had lower levels of inflammation and serum anti-H. pylori antibody, compared to those positive for 'urease positive' H. pylori infection.

**(v) Helicobacter pylori in Intensive Care (36,37,38)**

Earlier studies (above) established the importance of understanding outcomes of H. pylori infection, in terms of the host-parasite relationship. A clinical area of importance, with little study, is the circumstance of the intensive care unit with its seriously ill and stressed patient population. A study was organised

with Drs M. Robertson and J Cade in the Intensive Care Unit of the Royal Melbourne Hospital, testing 100 patients, 100 nurses and 500 blood donors (control group) using the 'near patient' Helisal Alert test described above. Two observations were made:

- (a) in seriously ill patients seropositivity (67%) was significantly higher than controls; a trend towards increased macroscopic gastric bleeding in seropositive patients was noted (100% of those with grade 5 bleeding were seropositive).
- (b) Seropositivity in intensive care nurses was significantly higher than controls and related to duration of work ( $P=0.02$ ).

These observations raise important questions requiring additional study: does the stress associated with severe illness 'switch' dormant *H. pylori* infection (as described above in non ulcer dyspepsia) into one with more active inflammation? Becoming seropositive in the process? Does the inflammatory response contribute to systemic disease associated with severe illness, possibly by changing gut permeability? Can intervention reduce acquisition of *H. pylori* by medical attendants, and nosocomial infection?

The important question of seropositivity and significant bleeding (and its prevention) needs to be settled by increasing numbers studied.

As part of the study of 500 blood donors as a control group, opportunity was taken to determine whether any linkage between ABO blood group and seropositivity existed, given the association between peptic ulcer disease and blood group O subjects and the evidence that *H. pylori* adhesion is mediated by blood group epitopes. No association was found, but seropositivity of 32% was at least as high as any Australian population studied over the previous 19 years (contrasting with a lower incidence of peptic ulcers).

- (vi) **Mucosal Immunisation (39):** There has been a quest for an effective vaccine to act either to prevent or eradicate *H. pylori* infection. Murine models had been used to screen putative antigen preparations. We immunised with a 50/52 –kDa subunit protein (citrate synthase homologue protein) –with 85-90% reduction in infection (21) in a mouse model. Delivery systems were examined but not developed.

**(vii) Conclusion and comment**

A wide ranging progressive programme examining the host-parasite relationship involving *Helicobacter pylori* infection was developed in conjunction with clinical colleagues in Newcastle, the Centre for Digestive Diseases (Sydney) and the Intensive Care Unit (Royal Melbourne Hospital). Collaborative studies in China and Estonia examined serology in gastric cancer and biopsy cultures were also studied in China. At the time of these studies essentially nothing was known of host characteristics, especially in terms of clinical outcomes. The isolated observations in the literature have been documented in the attached papers (1-21). The major findings of these studies (done through the 1990s and early 2000s) can be summarised:

- (i) Saliva IgG antibody was shown to monitor acquisition and eradication of *H. pylori* infection. Both saliva and whole blood anti *H. pylori* antibody assays were developed into the first 'near patient' diagnostics (the whole blood assay became the foundation stone of the 'test and treat' strategy in younger subjects, popular in Europe).
- (ii) Selective reduction of IgG<sub>2</sub> class antibody correlated with *H. pylori*-associated gastric cancer in a Chinese population, likely due to a loss of Th<sub>1</sub> immunity. This selective loss was less clear in a European population.
- (iii) Failed eradication therapy correlated with an absence of 'switch' from Th<sub>1</sub> to a Th<sub>0</sub> cytokine response, showing the importance of host factors in eradication failure. This was consistent with murine models of *Candida albicans* oral infection, where IL-4 deficiency contributed to impaired clearance. Pre-salvage therapy with a probiotic was given to patients with eradication failure (though not studied).
- (iv) *Helicobacter* infection with dysplasia or gastric cancer, was associated with a spectrum change in mucosal T cell balance –Th<sub>1</sub> response was replaced with Th<sub>2</sub> cells. This mucosal setting for gastric cancer was not identified with being *H. pylori*-specific, suggesting that either this was a result of gastric cancer, or that other aetiological agents could induce a similar switch.

- (v) The host parasite relationship in non-ulcer dyspepsia and chronic gastritis, identified a previously unrecognised group linking 'gold standard' criteria for diagnosis, with urease-negative coccoid forms. In a separate study evidence supported viability of urease –negative coccoid forms.
- (vi) Studies in an intensive care environment, flagged possible re-activation of dormant infection in stressed subjects, with both local and systemic outcomes. Nosocomial infection was also linked to exposure to patients in an intensive care unit.

#### 5. Atheroma (Chlamydia pneumoniae)

Atherosclerosis is a 'modern' disease in the sense that it appeared as a major health problem in the western world in the 1930s with epidemiological characteristics of an infection epidemic. One microbe with a significant association with myocardial infarction has been *C. pneumoniae*, but a failure to link eradication with clinical benefit, left doubt as to the validity of this relationship (reviewed 40, 41). There were many problems with the clinical studies, one being the use of acute events as the end outcome rather than 'atheroma growth,' another was that no 'connecting' mechanism was identified. Here, atheroma 'load' was estimated using elective coronary artery angiograms, while the host response to *C. pneumoniae* infection used a whole blood culture with analysis of the cytokine secretion profile. The study included 139 consecutive subjects. A selective increase in IL-4 secretion correlated with *C. pneumoniae* ( $P=0.02$ ) and the amount of IL-4 correlated positively with the extent of coronary artery disease ( $P=0.006$ ). No similar correlations occurred with *H. pylori* infection. Thus, a connection between serological presence of *C. pneumoniae* and atheroma, as well as linkage through a mechanism involving  $Th_2$  cytokine secretion has been documented. Mechanisms are discussed (40, 41). These results encourage a re-look at therapeutic intervention, but using more appropriate antibiotic strategies.

#### 6. Plasmodium falciparum

In the late 1970s four groups agreed to develop a vaccine programme aimed at preventing human malaria (PNGIMR; W & E Hall Institute, QIMR, and the Newcastle Immunology Group). Our interest in Host-Parasite Relationships involving putative

pathogens, focused attention on the development of an assay based on function, that could differentiate between antibody mediated inhibition of merozoite penetration of erythrocytes, and subsequent schizont growth. The former had been developed by Cohen and Butcher, but it was limited using large blood volumes and non-synchronised cultures.

The 1970s were important years regarding malaria in PNG. *P. falciparum* had come to dominate clinical disease, drug resistance was being extensively reported and spraying operations had failed. Initially an epidemiological study of malaria was conducted in an area within a 22km radius of Madang that included 16,500 people, to describe the epidemiology of malaria in a well defined area, and to define endemicity levels within the study area (42).

A microassay of 100 µl volume was established to detect merozoite inhibition antibody based on incorporation of radiolabelled isoleucine into parasite protein –study of healthy adults from Madang showed inhibition in 45% and enhancement with 5% of sera (43). Culture-adapted isolates of *P. falciparum* had different growth patterns when cultured against a standard antibody panel. The assay had the potential for biotyping *P. falciparum* strains, as little variation was detected using indirect immunofluorescent antibody detection.

The microassay was modified to detect either inhibition of schizont growth due to non specific serum factor(s) (but to include TNF<sub>α</sub>) or inhibition of merozoite uptake into a second cohort of red cells. This required morphology –controlled synchronisation of cultures. Age-specific differences in normal subjects were detected. All sera from children inhibited parasite growth by at least 20% an inhibition seen in only 25% of adult sera (44). When schizont-to-ring stage inhibition was measured (to detect merozoite inhibition antibody) half the adult sera were positive compared to 20% of children ( $p < 0.01$ ). These results paralleled the slow acquisition of specific immunity in the community. Schizont growth inhibition in children was stable over time (45). To better understand the nature of inhibition, blood monocytes were co-cultured with parasites –a non dialysable factor was released from monocytes that caused morphological damage to intracellular parasites (46, 47). Subsequent studies would identify TNF<sub>α</sub> as one factor derived from monocytic cells that caused parasite damage.

Acute and convalescent sera from acute episodes were tested to show less merozoite inhibiting antibody in acute phase sera than in controls (48) with no change in convalescent sera. No difference from controls existed for inhibition of parasite growth.

These results contrasted with those using serum from children with cerebral malaria (49), where high levels of parasite growth inhibition were detected. The serum inhibiting factor was non-specific, heat stable, and had a molecular weight about 85,000 daltons (TFN<sub>a</sub> is normally secreted as a trimer of 52 KD<sub>a</sub>). This factor damaged cultured endothelial cells causing release of  $\alpha$  keto PGF, identifying one mechanism to account for attachment of *P. falciparum* –infected red cells to damaged visceral endothelial cells (50).

In summary, the development of a micromerozoite inhibition assay provided for the first time a way of dissecting host mechanisms of protection (and damage) in *P. falciparum* infection. Mechanisms for known clinical events such as slow and unstable acquisition of specific immunity in children and 'crises' disease such as cerebral malaria, were investigated. Important restrictions on future vaccine development were identified. Interisolate variation provides opportunity for bioclassification and accounts for variable results in serum assays.

#### 7. Alcohol modulation of Host Response to Endotoxin (intestinal microbiome)

The idea that liver cell necrosis – the hallmark of acute alcoholic hepatitis and the precursor lesion of cirrhosis of the liver –involved a variable host response was not new as there was no clear evidence that alcohol induced liver cell damage and only a minority of heavy drinkers develop cirrhosis (reviewed in (51)). The hypothesis was that hepatocyte necrosis was an outcome of a host-parasite relationship involving the intestinal microbiome and host T cells, facilitated by alcohol. Thus, T cells were essential intermediates in a sequence of events linking excessive alcohol consumption with hepatocyte necrosis. It was postulated that circulating endotoxin derived from the intestinal microbiome stimulated alcohol-damaged T cells that sequester within the liver from the portal circulation, to release an excessive and inappropriate cytokine response, including TFN<sub>a</sub>. This hypothesis was tested using a rat model with chronic alcohol ingestion, and T cells flushed from the hepatic vasculature. The experimental data supporting this hypothesis is summarised (51-56):



- Long-term alcohol exposure induced a lesion in intra-hepatic T cells, characterised by a reduction in baseline secretion of cytokines especially TNF $\alpha$  (52).
- Alcohol-damaged T cells can be stimulated by polyclonal mitogens such as endotoxin or concanavalin A, to secrete an excessive and inappropriate amount of cytokines (including TNF $\alpha$ )(53) associated with hepatic necrosis (54). Alcohol-damaged T cells transferred Con A –dependent apoptosis and necrosis to normal rats (55). Con A was used in many experiments as while endotoxin can stimulate T cells and Kupffer cells, Con A only stimulates T cells, thus localising target to the T cell (in vivo, both T cells and Kupffer cells are likely to contribute to secretion of TNF $\alpha$ ).
- Why only 10-15% of chronic alcoholics develop cirrhosis is unclear. The postulate that genetic influenced susceptibility of T cells to alcohol-induced damage, is a study to be done.
- This hypothesis and cluster of studies were inspired by an observation by Professor R. Batey that a traditional Chinese herbal medicine (CH-100) reduced hepatocyte necrosis in hepatitis C infection. This herbal mix was tested in the rat-alcohol model. Both hepatocyte necrosis and circulating levels of endotoxin were significantly reduced. Although a primary protection of T cells was considered, the dramatic reduction in the elevation of endotoxin (P = 0.01) in alcohol-fed rats by CH-100, is noted. It needs to be clarified whether the liver cell protection follows a primary effect on T cells, or the primary benefit is due to an effect on the microbiome (56).

## Conclusion

These results provide a new paradigm for considering mechanisms of alcohol cirrhosis, while identifying a framework that is wider, and inclusive of other forms of hepatitis. The dual contribution of an alcohol effect on both circulating levels of endotoxin and T cells within the portal system, gives a novel way of developing both research protocols to further explore mechanisms in liver disease, and therapeutic strategies for managing hepatitis. The CH-100 studies focus attention on the microbiome as a critical determinant in the pathogenesis of chronic liver disease, and studies asking 'what within the microbiome influences endotoxin levels and perhaps enteric T cells,' and 'what is the active ingredient(s) in CH-

100.' Extension of these ideas to a range of chronic hepatic diseases, is encouraged by the results.

**Selected papers best reflecting narrative:** 1, 2, 7, 8, 12, 13, 14, 15, 16, 17, 18, 21, 22, 24, 25, 26, 30, 31, 32, 33, 34, 35, 36, 38, 40, 41, 43, 44, 45, 46, 47, 49, 51, 52, 53, 54, 55, 56.

### 3(B): 'NON-INFECTIOUS' ANTIGEN

#### 1. Immediate Hypersensitivity and Allergic Disease

Host-antigen interaction and allergic disease has a long history, gaining its scientific 'backbone' with the discovery of IgE in the late 1960s. The term 'allergy' was coined by von Pirquet in 1906 to describe the altered reaction to horse serum therapy, while the clinical specialty was rooted in the ground-breaking study of Noon and Freeman in 1911 showing clinical benefit of subcutaneous immunotherapy with grass pollen extracts. At the time our studies began in the mid 1970s examining 'host-antigen' interaction in the thematic frame of 'clinical outcomes reflect a particular balance in a 'stimulus/response paradigm,' there was little agreement with respect to prevention and management of allergy. The basic paradigm was understood and allergic disease was recognised as a 'surface' (skin and mucosa) disease, with atopy (or the predilection to produce IgE antibodies to environmental antigens) having a strong genetic background. There was confusion over the relevance of events such as breastfeeding and infections in the first year of life and the subsequent development of allergic disease, the relationship between atopy and end organ 'responsiveness' (especially brochoreactivity), and whether development of the mucosal immune system influenced subsequent clinical events. Research focus was on IgE –any broader role of the immune apparatus and in particular whether the host response to environmental allergens was a 'normal' one involving T cells as well as B cells, was neglected (reviewed in (1)). Similarly, cells that released mediators other than mast cells had been neglected, including alveolar macrophages which have IgE receptors on their surface (reviewed in (5)). To better understand some of these issues, four areas of study were progressed (over and above the extensive data from the RIFFYL study above). First, specific T cell sensitisation to crude and purified rye grass allergen was demonstrated in RAST positive patients with seasonal rhinitis, with the proliferative response correlating with wheal diameter on skin prick test (1, 2). Antigen groups 1, 2 and 3 of Rye Grass extract were treated with glutaraldehyde and tested in a group of atopic subjects with seasonal rhinitis. The study tested intranasal desensitisation, without benefit demonstrated (unfortunately low pollen count season – study not published). Second, wheat dust-associated disease was studied in a farming community. This condition was confused and poorly understood. This study clearly defined the character of disease, identified it as a major health hazard in wheat farming communities and showed for the first time it occurred in atopic subjects

due to sensitisation to extracted wheat allergens (3). Repeat studies (two either side of harvest and one at the time of harvest), gave data clearly showing a seasonal relationship between exposure to wheat dust and bronchial hyper-reactivity. This is most consistent with the hypothesis that bronchial hyperreactivity can be caused by allergic inflammation (4).

Third, as with other studies of host-antigen relationships in this thesis, the question was asked as to the extent pathogenic mechanisms were 'physiological' as opposed to unique. Here for example the dogma that the mast cell/ basophil was the single source of mediators following allergen-IgE interaction, was challenged by documenting mediators in alveolar macrophages which were known to bear Fc receptors for IgE (5), cells not previously thought to contribute to luminal events involving allergic inflammation.

Fourth, with particular interest in local immunity, the issue of desensitisation in allergic rhinitis was studied by administering glutaraldehyde –modified rye grass antigen intra-nasally in a placebo-controlled study. The glutaraldehyde treatment reduced allergenicity without affecting immunogenicity (2). At this time 'local' desensitisation by sublingual preparations was most controversial, four years before Scadding completed the first controlled study confirming its effectiveness in 1986. In the event, the controlled trial using rhinometry post allergen challenge failed to demonstrate benefit. The 1982 'season' had very low pollen counts and an unexpected low incidence of clinical episodes of allergic rhinitis (data not shown). In recent times 'local' desensitisation has become evidence-based mainline therapy.

## 2. Delayed-type hypersensitivity and Granulomatous Disease

Granulomatous disease was considered to reflect a T helper cell/macrophage response to antigens in genetically susceptible subjects. Little has changed in terms of basic understanding of pathogenesis. Here, two contributions were made. First, the mechanism of 'anergy' in the presence of a 'hyperactive immunity' (i.e. granulomas) was studied in patients with sarcoidosis, generally recognised as the 'signature' granulomatous disease (possibly representing a genetic response to a variety of antigens). Using mixed lymphocyte cultures (MLC) as a probe measuring T cell function, both circulating and lesional T cells were examined (6). Two mechanisms of peripheral anergy were identified. First, a relocation of T cells to areas of disease. Second, circulating lymphocytes from patients with sarcoidosis suppressed MLC

reactions involving cells from normal subjects (considered at the time “suppressor T cells,” and consistent with recent data identifying T reg cell involvement (Kettritz et al Nephrology, Dialysis, Transplantation 21 (2006) 2690-4).

Second, while granulomatous reactions to ‘inert’ material were well recognised, the pathogenesis of such reactions –especially with respect to immune specificity, was unknown. Two subjects with starch-induced granulomatous peritonitis were shown to have a specific T cell immune response as determinant by migration inhibition factor (MIF) secretion and lymphoproliferation. No antibody to starch was detected (7). Of further interest, patients with post-operative band adhesions were also found to have T cell sensitisation to starch. This raised a broader question, as to whether the uncommon acute syndrome of starch peritonitis was connected to the more common clinical entity of bowel obstruction and post-operative adhesions. These data have management implications.

### 3. Response of outer eye to “inert” contact lens

These studies (8,9) aimed at examining the impact of contact lens on the host-parasite relationships in the outer eye. In normal subjects the conjunctival flora increased with age, in terms of colonisation rates and density, accompanied by an increase in IgA antibody (8). In contact lens wearers, while total IgA antibody to colonising flora was reduced, the tear IgA/albumen level was little changed. Colonisation rates and densities increased in contact lens wearers but antibody prevented infection (9).

**Selected papers best reflecting narrative: 1, 2, 3, 4, 5, 6, 7**

## SECTION 4: HOST-ANTIGEN INTERACTION (SHIFTING THE BALANCE)

In the course of study of clinical disease as an outcome of a 'host-stimulus' relationship, opportunities occurred to develop novel diagnostic and therapeutic strategies. In some cases, an idea was just that, while in others products were developed to Phase 2 trials and the market, with others somewhere in the middle. Here is summarised 'ideas to products,' though details and publications for many are in other sections.

The increased recognition of key regulatory and effector roles played by T cells in protection against, or the progression of, disease outcomes of 'host-antigen' interaction, was a focus of studies aimed at identifying mechanisms and opportunities for therapeutic intervention. In summary, the following studies underpinned or clarified therapeutic value:

1. Oral immune enhancing inactivated bacterial products (NTHi; *Ps. aeruginosa*) (see Section 1(c)) act by enhancing a physiological 'loop' whereby Peyer's patches produce specific Th<sub>17</sub> cells that 'home' to the bronchus mucosa.
2. Probiotics act through the common mucosal system to 'adjust' T cell balance at mucosal sites. A window to the intestinal microbiota (1-5).\*
3. Thrombasthenic antibody. Discovered during studies on 'host-antigen' relationships in idiopathic thrombocytopenic purpura, and validated in an animal model, this idea was developed in 1994 into a therapeutic monoclonal antibody (Abciximab) for the reduction of thrombotic complications of coronary angioplasty and acute coronary syndromes (Section 2).
4. Taste aversion conditioning of the immune response. These studies focused on modulating T cell function by demonstrating non-sensory taste aversion having an unique taste (saccharin) with anti-lymphocyte serum is conditioned T cell immunosuppression. This approach has significant clinical opportunity by regulating the immune response, within the larger picture of harnessing 'the placebo response' in targeted therapeutics (6,7).

5. Early pregnancy Factor. An immune suppressant detected through ability to enhance anti-lymphocyte factor activity (and thus a putative suppressant of T cell function), playing an important early role in protecting the conceptus from immune rejection. Here, the biological significance in relation to 'pregnancy survival' was studied. Despite early controversy due to the technical difficulties in the assay, structural characterisation as Heat Shock 10KD<sub>a</sub> protein (HsP10) or Chaperonin 10, validated the assay. Insufficient work on value as an 'immune modifier' has been done (8,9,10,11,12).
6. Pencillamine (13,14). Explorative studies both in vitro and in vivo of this drug which was shown to reduce activity and progression of rheumatoid disease (and titre of rheumatoid factor), and was extensively used clinically as a biomodulating agent in that disease. These studies showed a restricted action against T helper cells, suggesting a possible value in other diseases with similar polyclonally activated B cells (for example, see studies on ITP in Section 2).
7. Non T cell immune modulation. Clinically valuable therapy aimed at preventing antibody-induced damage (occlusion therapy for high-titre sperm antibodies) following demonstration that infertility in a proportion of subjects is due to local anti-sperm antibody. Other studies documented environmental contribution to chronic urticaria with dietary factors modulating mast cell degranulation and potential of antibody therapy for mast cell malignancy. The anti-IgE therapy used preceded by many years the use of monoclonal antibody for 'mast cell disease' (especially asthma and urticaria) (16, 17, 18, 19, 20, 21).
8. Dietary Fish Oil. In the latter part of the 20<sup>th</sup> century there was interest in the use of dietary fish oil and N-3 fatty acids in reducing inflammation in diseases such as rheumatoid disease and colitis, with evidence that T cell secreted cytokines including TNF<sub>α</sub> were reduced (reviewed 22, 23, 24). Concern was raised that patients on such diets were predisposed to infection.

Here interest was two-fold. First to use a mouse/influenzae model to determine whether high N-3 fatty acid diet affected the course of infection or response to immunisation and second, to anticipate a value for such diets in shifting host-antigen relationships towards a position of protection.

In studies to assess effect on immunisation and subsequent protection against challenge, the high N-3 fatty acid diet neither depressed an antibody response to various immunisation routes and doses, nor did it impair subsequent clearing of an infection challenge (22). However, in unimmunised mice fed fish oil, virus clearance was delayed and both cellular (INF- $\gamma$ ) and humoral immunity (specific IgA in airways secretion) were impaired (23). Further study showed that this delay in clearance was due to impaired virus-specific T cell cytotoxicity (24). The practical value of maintaining immunisation status in patients using fish oil or related N-3 fatty acid enhanced diets, is clear.

## **PROBIOTICS**

Interest in probiotics came from a long history of claimed benefit (with isolated observations of enhanced protection such as 'enhanced phagocytosis' and 'increased secretion of INF- $\gamma$ ' (reviewed and referenced in 25, 26, 27, 28, 29). At the time of these studies, 'probiotics' was largely a term and area more of interest to the food industry than to biomedical research. Problems included poor quality control in production, mixed probiotic preparations, and the absence of a unifying concept. Around the late 1990s –early 2000s, interest in the intestinal microbiome was growing, and clinical studies, using probiotics in atopic disease, and gut infections gave encouraging results. Comparative studies showed different probiotics activated different sets of toll-like receptors on antigen presenting cells (reviewed (29)). It was also clear that orally administered probiotics could modulate protection at distant surfaces (e.g. oral mucosa) (Section 3) consistent with activation of the 'common mucosal system' (an objective of studies). An additional incentive was that single live bacterial preparations, safe for human usage, were probes to begin studies on the intestinal microbiome, which appeared to modulate systemic immunity. Studies here used a well characterised lactobacillus acidophilus (LAFTI) isolated from man by the Microbiology Department, UNSW. This isolate adhered to gut epithelium, colonised the human gut, and was resistant to acid and bile. A series of studies were done in an attempt to better define a mechanistic framework for probiotic activity (25, 27). In summary, results were most consistent with probiotics acting through a common mucosal system, to 'adjust' the T cell cytokine balance favouring a Th<sub>1</sub> over Th<sub>2</sub> response (reduced IL-4 secretion and antigen-specific IgE secretion in a mouse model; enhanced INF- $\gamma$  secretion and clearance of NTHi bacteria from the rat bronchus and similar outcome in "candida infection-prone" mice) (Section 3). Other



biological outcomes related to probiotic limitation of liver cell damage in an alcohol/liver necrosis model mediated by a reduction in level of circulating endotoxin, an adjuvant effect when co-administered with oral antigen, and enhanced responsiveness by T cells to a polyclonal mitogen (25, 27). Details of the candida and alcohol studies are included in Section 3. The animal studies were supported by a human study in 'fatigued athletes' (30), where a defective INF- $\gamma$  secretion was reversed following oral *L. acidophilus*. In this human study data suggested that oral *L. acidophilus* may have a more complex role of 'regulating' Th<sub>1</sub> activity i.e. enhancing secretion in those deficient and suppressing high levels (and thus limit damaging inflammation). Further work is needed to confirm the novel idea that certain probiotics can 'regulate' around a mean.

In summary, using a single selected probiotic, a range of studies in animal models and man indicate a significant regulatory impact favouring Th<sub>1</sub> protection at mucosal surfaces, mediated through the common mucosal system. An 'adjuvant' effect with oral antigen suggests an interaction between innate and adaptive immunity. It was concluded that this shift towards a 'medical model,' made sense of a change in terminology to 'immunobiotics,' i.e. to replace the food industry term 'Probiotics' (26). This suggestion is being taken up in the literature. Recently reported studies extend these observations, by demonstrating a protective effect against anaphylaxis in children with peanut allergy.

### Patent profile

Product	Purpose	Patent	Comments
IgA Saliva test	To detect infant at risk of SIDS/ALTIS	EP1185866A4	Especially IgA <sub>2</sub>
IgA saliva test	To detect predisposition to infection associated with stress	US6974674 B1	
Inactivated	Prevent	US487309 OA	Includes range of other

monobacterial non adjuvanted vaccine	exacerbations of chronic airways disease		bacteria including Ps. aeruginosa
IgA <sub>2</sub> anti-H. pylori antibody (saliva or serum)	Monitor eradication of H. pylori	US20040038329 A1	More sensitive than serum test
Oral NTHi vaccine	Prevent/treat asthma	EP211758 6 A1	
Rapid Near Patient Test for H. pylori	Diagnose H. pylori infection 'Test and Treat'	CA2067603 A1	Modified for use with finger prick blood
Lactobacillus acidophilus	Prevent and/or treat chronic fatigue syndrome	WO2006048446A1	<ul style="list-style-type: none"> <li>• Reduce shedding of Epstein Bar Virus</li> <li>• Raise saliva IgA</li> <li>• Limit inflammation</li> </ul>
Lactobacillus acidophilus	Prevent/Treat endotoxaemia	US20040047868A1	<ul style="list-style-type: none"> <li>• Endotoxin-induced liver cell necrosis</li> <li>• Final common path regarding hepatotoxicity is <u>T cell</u></li> <li>• Less liver cell necrosis in rat model (P=0.03)</li> <li>• Lower endotoxin levels in probiotic fed.</li> <li>• Reduced gut colonisation</li> </ul>

Papers best reflecting the narrative: 6, 7, 8, 9, 23, 24, 25, 26, 27, 30.



## Curriculum Vitae (Summary)

### 1. Emeritus Professor Robert Clancy AM

- [REDACTED]
- [REDACTED]

### 2. Awards and Degrees

- BSc (Med) MB BS PhD DSc.
- FRACP FRCP(A) FRCP(c).
- AM.

### 3. Education

- Sydney University.
- Royal North Shore Hospital.
- Royal Melbourne Hospital.
- Alfred Hospital.

### 4. Positions (Post Training)

- a. Assistant Professor Medicine, McMaster University, Canada.
- b. Head of Immunology Unit, RPAH (Syd).
- c. Foundation Professor of Pathology, University of Newcastle (25 years).
- d. Director of Clinical Immunology, John Hunter Hospital, Newcastle (30 years).

### 5. Clinical

Initially trained in gastroenterology, but more recently seen as a clinical immunologist. I was part of the group of three that developed this discipline in Australia. My FRCP(c) (Canada) was the second awarded for clinical immunology. My expertise is mainly adults, but I have seen patients of all ages. Because of my research interest, my main clinical interest have been autoimmune disease and mucosal inflammatory disease (see below and my DSc thesis summary).

### 6. Research

My main research interests have been in mucosal immunology. One of the two main programmes was the RIFFYL study, where we followed about 260 infants from birth to age 21, aiming – for the first time- to determine the relationship of immune development with clinical outcomes. Hence the opportunity to complete the world's first (and only) prospective study of SIDS, as one of our 'RIFFYL Babies' died of SIDS (see copy of paper). Throughout my search period my focus has been on host-parasite relationships, hence the study in near-miss SIDS and virus infection, with apnoea as the clinical outcome.

## **7. Biotechnology**

I have developed a vaccine to protect airways in COPD, a saliva test for SIDS risk, the 'yes/no' test for Helicobacteria infection. I am currently developing an oral vaccine for cystic fibrosis babies.

## **8. Awards**

- a. AM for contribution to immunology and historic cartography.
- b. Initial award for contributions to research and development (University of Newcastle).

## **9. Papers written**

280 (see attached DsC thesis)

## **10. Continuous grant funding for research over 25 years by NHOMRC.**



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### UNIFORM CIVIL PROCEDURE RULES 2005 - REG 31.27

#### Experts' reports

#### 31.27 Experts' reports

(cf SCR Part 36, rule 13C; DCR Part 28, rule 9C; LCR Part 23, rule 1D)

(1) An expert's report must (in the body of the report or in an annexure to it) include the following:

- (a) the expert's qualifications as an expert on the issue the subject of the report,
- (b) the facts, and assumptions of fact, on which the opinions in the report are based (a letter of instructions may be annexed),
- (c) the expert's reasons for each opinion expressed,
- (d) if applicable, that a particular issue falls outside the expert's field of expertise,
- (e) any literature or other materials utilised in support of the opinions,
- (f) any examinations, tests or other investigations on which the expert has relied, including details of the qualifications of the person who carried them out,
- (g) in the case of a report that is lengthy or complex, a brief summary of the report (to be located at the beginning of the report).

(2) If an expert witness who prepares an expert's report believes that it may be incomplete or inaccurate without some qualification, the qualification must be stated in the report.

(3) If an expert witness considers that his or her opinion is not a concluded opinion because of insufficient research or insufficient data or for any other reason, this must be stated when the opinion is expressed.

(4) If an expert witness changes his or her opinion on a material matter after providing an expert's report to the party engaging him or her (or that party's legal representative), the expert witness must forthwith provide the engaging party (or that party's legal representative) with a supplementary report to that effect containing such of the information referred to in subrule (1) as is appropriate.

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## New South Wales Consolidated Regulations

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### UNIFORM CIVIL PROCEDURE RULES 2005 - REG 31.23

#### Code of conduct

#### 31.23 Code of conduct

(cf SCR Part 39, rule 2; DCR Part 28A, rule 2; LCR Part 38B, rule 2)

- (1) An expert witness must comply with the code of conduct set out in Schedule 7.
- (2) As soon as practicable after an expert witness is engaged or appointed:
  - (a) in the case of an expert witness engaged by one or more parties, the engaging parties, or one of them as they may agree, or
  - (b) in the case of an expert witness appointed by the court, such of the affected parties as the court may direct,

must provide the expert witness with a copy of the code of conduct.

- (3) Unless the court otherwise orders, an expert's report may not be admitted in evidence unless the report contains an acknowledgment by the expert witness by whom it was prepared that he or she has read the code of conduct and agrees to be bound by it.
- (4) Unless the court otherwise orders, oral evidence may not be received from an expert witness unless the court is satisfied that the expert witness has acknowledged, whether in an expert's report prepared in relation to the proceedings or otherwise in relation to the proceedings, that he or she has read the code of conduct and agrees to be bound by it.

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## 5 Experts' reports

(1) An expert's report must (in the body of the report or in an annexure to it) include the following:

- (a) the expert's qualifications as an expert on the issue the subject of the report,
- (b) the facts, and assumptions of fact, on which the opinions in the report are based (a letter of instructions may be annexed),
- (c) the expert's reasons for each opinion expressed,
- (d) if applicable, that a particular issue falls outside the expert's field of expertise,
- (e) any literature or other materials utilised in support of the opinions,
- (f) any examinations, tests or other investigations on which the expert has relied, including details of the qualifications of the person who carried them out,
- (g) in the case of a report that is lengthy or complex, a brief summary of the report (to be located at the beginning of the report).

(2) If an expert witness who prepares an expert's report believes that it may be incomplete or inaccurate without some qualification, the qualification must be stated in the report.

(3) If an expert witness considers that his or her opinion is not a concluded opinion because of insufficient research or insufficient data or for any other reason, this must be stated when the opinion is expressed.

(4) If an expert witness changes his or her opinion on a material matter after providing an expert's report to the party engaging him or her (or that party's legal representative), the expert witness must forthwith provide the engaging party (or that party's legal representative) with a supplementary report to that effect containing such of the information referred to in subclause (1) as is appropriate.

## 6 Experts' conference

(1) Without limiting clause 3, an expert witness must abide by any direction of the court:

- (a) to confer with any other expert witness, or
- (b) to endeavour to reach agreement on any matters in issue, or
- (c) to prepare a joint report, specifying matters agreed and matters not agreed and reasons for any disagreement, or
- (d) to base any joint report on specified facts or assumptions of fact.

(2) An expert witness must exercise his or her independent, professional judgment in relation to such a conference and joint report, and must not act on any instruction or request to withhold or avoid agreement.

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EXPERT CERTIFICATE  
S177 EVIDENCE ACT 1995

The Expert Certificate is given by me pursuant to s177 of the Evidence Act that the defendant proposes to tender this Expert Certificate concerning my attached report dated \_\_\_\_\_ which is signed by me as an expert and:

- States my name and address;
- States that I have specialised knowledge based on my training, study or experience as specified in the report attached to this certificate; and,
- Set out an opinion that I hold, and which is wholly or substantially based on that knowledge.

Dated : 12-3-19

Signed: Robert Clancy.

Name: Prof R. Clancy AM

**CERTIFICATE – EXPERT REPORT**

I refer to my report dated \_\_\_\_\_ which is attached to this certificate and certify as follows:

1. I was provided with a copy of the Uniform Civil Procedure Rules 2005 – Expert in Schedule 7 Witness Code of Conduct a copy of which is annexed to my report
2. I have read the Expert Witness Code of Conduct.
3. I agree to be bound by the Expert Witness Code of Conduct.

Dated: 12.3.19

Sign:

Robert Clancy

Name:

Prof. R. Clancy AM



# Mucosal Immune Response in a Case of Sudden Infant Death Syndrome

MAREE GLEESON, ROBERT L. CLANCY, AND ALLAN W. CRIPPS

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**ABSTRACT.** A prospective study to define the normal patterns of development of mucosal immunity in 263 children provided a unique opportunity to study the mucosal immune response in an infant who unexpectedly died from sudden infant death syndrome. The subject initially had a normal pattern of mucosal immune function, which was perturbed after a transient mild upper respiratory tract infection at 3½ wk of age. After the upper respiratory tract infection, there was an increase in mucosal permeability and the appearance of IgA and IgM in saliva. The unusual features in this case were the degree and the duration of the increases in salivary IgA and IgM after resolution of the illness. The marked abnormalities suggested a persistent stimulation of the mucosal immune response. The case provides informative data on potential mechanisms of sudden infant death syndrome and supports a role for involvement of upper respiratory tract infection. (*Pediatr Res* 33: 554-556, 1993)

## Abbreviations

SIDS, sudden infant death syndrome  
URTI, upper respiratory tract infection

SIDS has been reported to occur after a mild URTI (1-4). As there is no epidemiologic, clinical, pathologic, or microbiologic evidence that favors infection by a particularly virulent pathogen, it is likely that a high degree of stimulation of the immune system may be one important link in the chain of events that culminates in respiratory arrest. Previous studies have suggested that secretory component was absent or reduced in bronchopulmonary epithelium in SIDS (5) and that overstimulation of the mucosal immune response occurs in the respiratory and gastrointestinal tracts (6-9). IL-1 has also been suggested as a mediator between sleep apnea and SIDS during URTI (10). However, the limitations associated with retrospective data collected on postmortem material, as well as the quality of control studies, have presented major difficulties with interpretation of SIDS research.

A prospective study from birth of a cohort of 263 children to determine normal patterns of development of mucosal immunity (11, 12) provided an opportunity to study the mucosal immune response in an infant who unexpectedly died from SIDS. We describe in this infant the mucosal immune response after a URTI and postulate that a disturbance in immune regulation and/or mucosal permeability may constitute a link in the chain of events that lead to respiratory arrest.

Received July 7, 1992; accepted January 19, 1993.

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The study was partially funded by the National Health and Medical Research Council of Australia. The data on the incidence of viral illness in NSW, Australia, were kindly provided by Professor Richard Barry (Department of Virology, University of Newcastle).

## MATERIALS AND METHODS

**Clinical history.** The female infant was delivered at term by spontaneous vaginal delivery to a 24-yr-old mother (gravida 4, para 2) after an uncomplicated pregnancy. She weighed 3620 g and had Apgar scores of 8 at 1 min and 10 at 5 min. She was breast-fed until 5 wk of age, but received complimentary formula feeding on the first 2 d of life, and was then totally formula-fed from 5 wk of age. She had not received any childhood vaccinations.

A mild respiratory tract infection (symptoms were a "runny nose" and difficulty breathing) was diagnosed at 3½ wk of age by the local doctor. The subject shared a bedroom with two older siblings, one of whom had a prior respiratory tract infection. Her father was atopic, but otherwise there was no family history of specific illness. Both parents smoked more than 10 cigarettes/d. Her mother did not smoke during the pregnancy but resumed smoking immediately after the birth.

The subject was born at the beginning of the Australian autumn and was followed prospectively during the months of March to May. In March, there was an increase in the incidence of mycoplasma pneumonia in the state of New South Wales, whereas respiratory syncytial virus and parainfluenza virus showed normal background incidence during the same period (Australia Communicable Diseases Intelligence Bimonthly statistics).

The infant died at home at 10 wk of age. The postmortem findings confirmed the infant death classification of SIDS, and the examination showed cerebral and pulmonary edema with some shedding of the alveolar lining cells. No acute bronchitis or bronchiolitis was demonstrated.

**Immunological investigations.** Saliva samples were collected from the SIDS infant 2 d after birth and during wk 2, 3, 4, 6, and 8 after birth. Salivary IgA, IgM, IgG, and albumin were measured by electroimmunodiffusion (13). The reference levels for each analyte were calculated for weekly age groups as the 10th, 50th (median), and 90th percentiles and are represented as shaded areas on Figure 1. Salivary Ig responses during URTI have been previously studied in this cohort of children (14), and the relevant findings have been summarized in this report.

## RESULTS

**Albumin.** The salivary albumin level was initially low near birth (10th percentile for 2 d) but showed a rise and subsequent fall in the 2nd and 3rd wk of life (Fig. 1A); the concentrations remained within the normal age-related reference ranges for wk 1 to 3. The albumin level was significantly increased in the 4th wk, being 7 times higher than the age-related median level. The albumin levels in the 6th and 8th wk declined but remained above the 90th percentile for each age group.

In the control study examining salivary protein responses during URTI (14), the geometric mean albumin levels within any individual child did not differ between periods of URTI and noninfection periods.

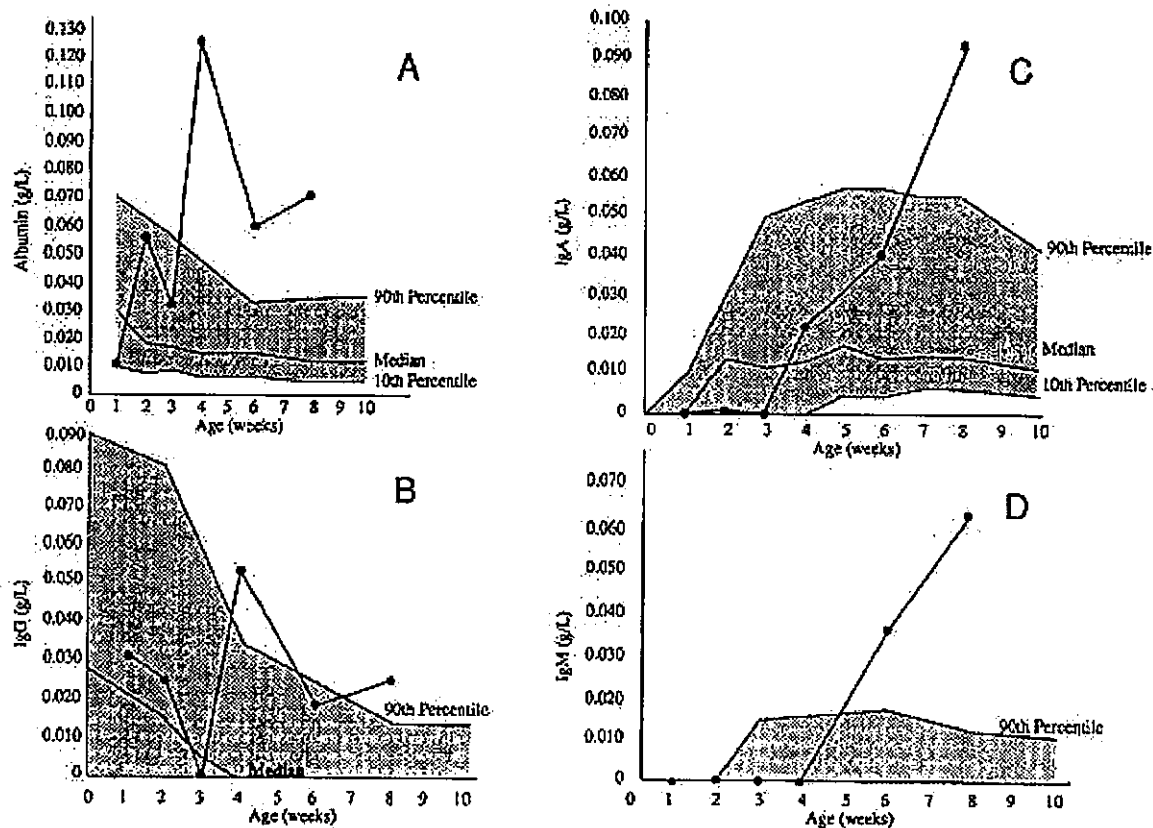


Fig. 1. Albumin (A), IgG (B), IgA (C), and IgM (D) concentrations in saliva collected from birth to 8 wk of age in an infant who died of SIDS. Age-related reference ranges are represented as shaded areas.

**IgG.** The salivary IgG level was within normal age limits at birth (0.031 g/L) and decreased to a nondetectable level at 3 wk of age (Fig. 1B). The IgG level rose at 4 wk of age to 0.054 g/L, a level above the 90th percentile for this age group. The IgG level declined in the 6th wk but at 8 wk was again above the 90th percentile for this age.

In the control study (14), IgG was detected in saliva more frequently in samples collected during URTI (89%) than during noninfection periods (53%), but the concentrations did not exceed the 90th percentile for age for any subject.

**IgA.** Low (0.009 g/L) or nondetectable levels of IgA were measured in the saliva for the first 3 wk of life (Fig. 1C). The IgA level rose in the 4th wk of life (0.022 g/L) and continued to rise in samples collected in the 6th and 8th wk to a level (0.093 g/L) 5 times higher than the age-related median level for 8 wk of age.

In the control study (14), elevations of salivary IgA were observed during 18 of 20 URTI periods studied, and the IgA returned to noninfection mean levels within 10 d of disappearance of symptoms. The IgA peak level during URTI periods in three children studied under 6 mo of age did not exceed 0.020 g/L.

**IgM.** IgM was not detected in the saliva samples collected in the first 4 wk of life (Fig. 1D). IgM was detected in the samples collected in the 6th and 8th wk of life, at levels above the 90th percentile for age, and at 8 wk of age was 10 times higher than the age-related median level.

In the control study (14), there was no significant association between URTI and the detection of IgM in saliva.

#### DISCUSSION

We report results of investigations of mucosal immunity in an infant who died of SIDS. The cohort of 262 healthy infants had been prospectively studied from birth for 5 y, enabling a precise

definition of normal patterns of development of mucosal immunity and factors that alter the ontogeny (11–13), including the influence of URTI (14). Analysis of saliva protein levels in the SIDS infant demonstrated a prolonged period of increased mucosal permeability and an exaggerated and prolonged mucosal immune response after an URTI, both of which persisted until the assessment period 2 wk before the infant's death.

In the immediate postnatal period, the detection of IgG and high levels of albumin reflect a normal transient physiologic state of increased mucosal permeability (15). The subject of this report initially had a normal pattern of mucosal function, with a rapid disappearance of IgG, a decrease in albumin levels in saliva, and an absence of detectable IgA and IgM. The normally evolving saliva protein profile was perturbed after a transient mild respiratory tract infection. The reappearance of IgG and the increase in saliva albumin levels from the time of the URTI reflects an increase in mucosal permeability, which has been noted in normal infants after antigenic challenge, including infection of the respiratory tract (14).

The appearance of IgM and an increase in IgA in saliva is a normal event after URTI (14). The unusual features, unique in our experience in the SIDS infant, were both the degree and the duration of the increases in the salivary Ig levels. We have prospectively followed normal infants with URTI and commonly noted minor increases in IgA, IgG, and IgM, but they have been small in amount and have always returned to normal levels within 10 d of disappearance of clinical symptoms (14). The marked abnormalities in mucosal Ig in the SIDS infant suggested an enhanced immune response, possibly due to increased antigen influx.

Mature mucosal surfaces are down-regulated with respect to immune reactivity to environmental antigen, and control is probably mediated through suppressor T lymphocytes capable of restricting lymphocyte proliferation (16). Little is known about the maturation of mucosal immune control mechanisms, but an

apparent oscillatory pattern of salivary IgA levels early in life (13) would be consistent with a period of instability in the first few months of life. The appearance in the saliva of some infants at this time of IgD (17) and monomeric IgA (18) further suggests a period of mucosal immaturity. Infants genetically predisposed toward lability in the development of normally rigorous mucosal suppressor mechanisms may account for the abnormal response observed in the SIDS infant.

Previous studies have identified a genetic predisposition (19; 20) and a mild URTI (1-4) as risk factors for SIDS, whereas many of the other risk factors identified (20-29), such as social status, environmental temperature changes, infant overheating, and passive smoking (4, 30-33) may secondarily relate to infection or mucosal inflammation. Several of these potentially conditional risk factors were identified in the SIDS infant.

Recent studies (20, 21) have suggested that the pattern of infant feeding is a risk factor, with a decreased incidence of SIDS in breast-fed infants. Formula feeding in early life exerts a significant effect on mucosal function, extending the postnatal period of increased mucosal permeability and causing early activation of mucosal B lymphocytes (12, 15). The SIDS subject in this study changed from breast feeding to formula feeding at 5 wk of age, coinciding with the phase of enhanced mucosal immune response. The possible interaction between the effect of introduced food and a concurrent mucosal infection at a critical stage of mucosal maturation requires further study.

The protein changes in saliva were distant from the site of clinical infection. Whether these changes reflect a contiguous infection of oropharyngeal mucosa or a more generalized mucosal change cannot be stated. Nor can a direct or indirect link with respiratory arrest be proven. We would propose, however, the following hypothesis: infants genetically predisposed to transient labile mucosal suppressor mechanisms are particularly at risk should certain environmental events occur in the period of mucosal immaturity. An inappropriate or persistent mucosal immune response to antigen may activate vagal afferent nerve endings or chemoreceptors to induce reflex apnea.

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## Mucosal immune responses to infections in infants with acute life threatening events classified as ‘near-miss’ sudden infant death syndrome

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### Abstract

This study examined the hypothesis that dysregulation of mucosal immune responses to respiratory infections is a critical event, which could be causal in respiratory arrest of some previously healthy infants. To examine this hypothesis, a prospective study was undertaken of infants presenting to the emergency department of a major teaching hospital with acute life threatening events (ALTE) of unknown cause and classified as ‘near-miss’ SIDS. Salivary immunoglobulin concentrations were measured on admission and again after 14 days. The salivary immunoglobulins were compared with three control groups: infants with a mild upper respiratory tract infection (URTI); bronchiolitis; and healthy age-matched infants. The salivary IgA and IgM concentrations in the ALTE infants at presentation to hospital indicated a significant mucosal immune response had already occurred, with nearly 60% of the IgA concentrations significantly above the population-based reference ranges. The hyper-immune response was most evident in the ALTE infants with pathology evidence of an infection; 87% of these infants had salivary IgA concentrations on average 10 times higher than the age-related median concentration. The most prevalent pathogen identified in the ALTE infants was respiratory syncytial virus (RSV) (64%). RSV was also identified in all subjects with bronchiolitis. Risk factors for SIDS were assessed in each group. The data indicated that the ALTE infants diagnosed as ‘near-miss’ SIDS were a relatively homogeneous group, and most likely these ALTE infants and SIDS represent associated clinical outcomes. The study identified exposure to cigarette smoke and elevated salivary IgA concentrations as predictors of an ALTE. The study findings support the hypothesis of mucosal immune dysregulation in response to a respiratory infection in some infants with an ALTE. They provide a plausible explanation for certain SIDS risk factors. The underlying patho-physiological mechanism of proinflammatory responses to infections during a critical developmental period might be a critical factor in infants who have life-threatening apnoea or succumb to SIDS. The study raises the possibility of using salivary IgA to test infants who present with mild respiratory infections to identify a substantial number of infants at risk of developing an ALTE or SIDS, thus enabling intervention management to prevent such outcomes.

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### 1. Introduction

Study of the pathogenesis of SIDS has been bedevilled by the very nature of the problem – the sudden unexpected death of a previously healthy infant. This has limited the prospective study of underlying

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mechanisms with pathology-based studies restricted to the use of specimens obtained at post mortem. These restrictions have not prevented the completion of valuable epidemiological studies, which identified a number of risk factors for SIDS (reviewed in [1]). Preventative education and management directed at reducing these risk factors has reduced mortality in most countries [1]. There remains, however, an absence of understanding of the underlying pathophysiology. Questions such as the following all remain unanswered: Is there a single cause of SIDS? How do the epidemiology-defined risk factors operate to cause death? What genetic mechanisms contribute to SIDS?

The role of infections in SIDS has long been suggested [2,3], but available data does not favour any one pathogen [4–6]. Data indicates that inflammatory responses to infection play a critical role in the chain of events leading to sudden and unexpected death [7–14], particularly if other risk factors for SIDS are present [13,15,16]. A prospective case study of SIDS [10], though incidental to a birth cohort study of the ontogeny of mucosal immunity, reported a marked inappropriate immune response following an otherwise mild respiratory tract infection with high levels of immunoglobulins in saliva samples collected prior to death. A defect in immune responses following a range of infections during a critical developmental period in infancy would be consistent with the evolving concepts of the pathogenesis of SIDS, at least in a proportion of cases. Many of the risk factors associated with SIDS can also be explained through stimulation of pro-inflammatory responses to infections or toxins if they occur at critical stages of development in infancy [4,11,13,15].

Validation of mucosal dysregulation as an essential link in the chain of events leading to sudden death would provide a focus for more productive research on the pathophysiology of SIDS, and possibly identify tests of value in the detection of infants at risk of developing SIDS. To examine this hypothesis, a prospective study was undertaken of infants presenting to the emergency department of a major teaching hospital with acute life threatening events (ALTE) of unknown cause and classified as “near-miss” SIDS. Several prospective epidemiological studies of apnoea in infancy had identified the risk factors [17] and characteristics of apneic events

resulting in SIDS [18,19], indicating that ALTE in infancy were part of a continuum in the aetiology of SIDS.

## 2. Materials and methods

The study was conducted with approval from the Ethics Committees of the University of Newcastle (Australia) and Hunter Area Health Service. Written informed consent was obtained from the parents of each infant.

### 2.1. Subjects and study groups

Eighty-seven infants, ranging in age from 10 to 346 days, were recruited into the study over a four-year period and classified into one of four study groups (Table 1). Twenty-six infants presenting to the paediatric emergency department with an acute-life threatening episode (ALTE group) for which no identifiable cause could be found on admission or from subsequent investigations were recruited by the attending doctor. The ALTE infants were compared to infants who presented to family practice doctors suffering from either bronchiolitis (BRON group,  $n = 6$ ) or a mild upper respiratory tract infection (URTI group,  $n = 18$ ). Additional comparisons were made with a control group of 37 age-matched, healthy infants recruited from child-health immunisation clinics (WELL group). Infants were confined to the 1–12 month age group. The age and gender distributions for each study group are provided in Table 1.

### 2.2. Study design

The study was designed to test the hypothesis that uncontrolled hyper-immune responses to infections at mucosal surfaces are involved in the pathophysiology of sudden infant death. The mucosal immune status was examined by measuring the immunoglobulin concentrations in saliva. The albumin concentration was measured to assess the integrity of the sample collection and flow rate effects. A saliva sample was collected within 48 hours of recruitment (Sample 1) to determine any immediate immune response to potential infections in the ALTE, BRON and URTI groups, and to compare the

Table 1

Gender distributions and age ranges of infants in each study group and the proportion of sample collections with results suitable for statistical analysis after excluding contaminated or deteriorated samples

Study group	ALTE	BRON	URTI	WELL
No. of subjects	26	6	18	37
Males:females	20:6	3:3	10:8	19:18
Median age (days)	60	40	183	119
Age range (days)	18–235	12–332	40–314	10–211
Suitable Sample 1 saliva collections	22 (85%)	3 (50%)	17 (94%)	30 (81%)
Suitable Sample 2 saliva collections	21 (81%)	3 (50%)	15 (83%)	32 (86%)

'concentrations to the age-matched healthy control infants in the WELL group. Previous research indicated that resolution of mucosal immune responses to respiratory infections typically occurs 6–12 days following the appearance of respiratory symptoms [20,21]. A second saliva sample was collected 14 days after the first sample (Sample 2) to assess any changes in immunoglobulin concentrations over the 2-weeks and to determine resolution of any mucosal immune responses to potential infections. The distribution of SIDS risk factors, and immunoglobulin concentrations in saliva samples collected at recruitment (Sample 1) and after 14 days (Sample 2) were compared between the four study groups.

Separate clinical questionnaires were completed at recruitment by the parents of each infant and the recruiting doctor. A review of all medical records and the discharge summary was undertaken only for the ALTE group infants to assess results of clinical investigations undertaken while in hospital and the infant's final discharge diagnosis.

### 2.3. Parental questionnaire

Parents completed a questionnaire that provided details on family demographics and the infant's exposure to potential SIDS risk factors: gender; a family history of SIDS; parental status; older siblings; socio-economic status; ethnic heritage (particularly Australian Aboriginal-Torres Strait Island heritage); exposure to tobacco smoke; heating sources used in the home; infant feeding practices; usual sleeping posture; immunisation status; and any history of recent respiratory illness. The sleeping posture at the time of the ALTE was also determined for subjects in the ALTE group.

### 2.4. Doctor questionnaire

The recruiting doctor completed a separate questionnaire to confirm the infant's risk factors for SIDS as detailed in the parental questionnaire. The doctor questionnaire also provided relevant medical details, including obstetric history. If a prior infection was indicated, details on the nature and source of the infection were recorded. Extensive medical information was obtained for the ALTE group to exclude any possible identifiable cause of the ALTE due to: airway obstruction; asthma; anaphylaxis; sepsis; congenital malformations; metabolic or cardiac causes; intentional smothering; or shaking of the infant. Infants with any of these causes for the ALTE were excluded from the study, as they did not meet the criteria for 'near-miss' SIDS.

### 2.5. ALTE discharge summary review

The medical records for all ALTE group infants were reviewed after discharge from hospital to provide results

of clinical investigations undertaken while in hospital and to record the infant's discharge summary diagnosis. Particular attention was paid to investigations for infections. Bacterial and viral infections were recorded. Clinical investigations providing evidence of an explainable cause for the ALTE (other than infection) were used to exclude subjects from the study to ensure the ALTE group comprised only infants representing the 'near-miss' SIDS criteria.

### 2.6. ALTE discharge classifications

The discharge diagnosis and clinical investigations were used to classify the ALTE infants into three categories: clinical or pathology evidence of an infection (Infection); evidence of gastro-oesophageal reflux with the potential to cause airway inflammation (Inflammation); 'other' causes consisting mainly of central sleep apnoea (Other). Gastro-oesophageal reflux was determined by barium swallow tests. Central apnoea was determined by a sleep study with respiratory impedance pneumography and defined as an apnoea of at least two respiratory cycles duration in which no respiratory effort was made [22]. The ALTE infants in these discharge classifications were compared for differences in the distributions of SIDS risk factors and immunoglobulin concentrations in saliva samples collected at recruitment (Sample 1) and after 14 days (Sample 2).

### 2.7. Collection of saliva samples

Unstimulated, whole, mixed saliva samples were collected from the buccal cavity using a Specimen Suction Set (Maersk Indoplas Pty Ltd, Sydney, Australia) and an electronic suction pump. All samples were collected at least one hour after feeding to reduce the possibility of contamination of the sample. Saliva samples were immediately frozen and stored at  $-70^{\circ}\text{C}$  until analysis. Both saliva samples from each infant were assayed in the same test batch to reduce inter-assay variations.

### 2.8. Measurement of salivary immunoglobulin and albumin concentrations

IgA, IgG and IgM concentrations in saliva were measured by an enzyme-linked immunosorbent assay [23] using goat anti-human immunoglobulin-specific antisera (BioSource International, Camarillo, CA, USA). Immunoglobulin concentrations were determined by standardisation against a Human Serum Protein Calibrator (Dako Corporation, CA, USA) referenced against BCR-CRM470. All samples were analysed in triplicate in conjunction with high, moderate and low positive controls and saliva from an IgA-deficient

individual. The between-run coefficients of variation (CVs) for the high, moderate and low positive controls were on average 14% for each ELISA (range: 13–16%). The salivary albumin concentrations were determined by electroimmunodiffusion [24], using rabbit anti-human albumin antisera (Dako, Corporation, CA, USA) and calibration against the same Human Serum Protein Calibrator. The between-run CV for the albumin control was 4.5%.

Samples from breast-fed infants were screened for contamination with breast milk by immunoelectrophoresis [24] using rabbit antiserum to human colostral  $\alpha$ -lactalbumin (Dako Corporation, CA, USA). Specimens contaminated with breast milk, and hence containing maternal immunoglobulins, or with low albumin concentrations, indicating sample deterioration [25] were excluded from statistical analyses.

### 2.9. Statistical analysis

Data analysis was performed using the statistical software package Statistics/Data Analysis™ (STATA), Version 6 (Stata Corporation, Texas, USA). Differences in the distribution of suspected SIDS risk factors between the study groups were assessed by a Fisher's Exact test. Immunoglobulin concentrations for each study group were not normally distributed and were log-transformed prior to statistical comparison to correct for the skewed nature of the data. An analysis of variance (ANOVA) was used to assess differences in immunoglobulin concentrations in the study cohort between Sample 1 and Sample 2. Immunoglobulin concentrations were compared within and between the groups by a Student's *t* test. The significance level for all tests was set at  $p = 0.05$ . The salivary immunoglobulin concentrations for each sample were also compared with age-matched reference ranges, previously established for healthy children from the same region [26].

A linear regression model was used to determine the predictive value of salivary IgA concentrations for the risk of an ALTE. The four study groups were modelled as the outcome and salivary IgA concentration for Sample 1, age at recruitment, number of parents, presence of older siblings, immunisation status, exposure to passive tobacco smoke, breast-feeding, the family's economic status and prone-sleeping position were included as variables. The significance level was set at  $p = 0.05$ .

Comparison of data within and between groups is presented in Figs. 2, 3 and 5 as box and whisker plots. The box represents the 25–75th percentiles and the median concentration as the bar within the box. The whiskers on the box represent the 5–95th percentiles and the open circles are outliers for each group.

## 3. Results

### 3.1. Risk factors for SIDS

With the exceptions of incidence of infections and sleeping position there were no significant differences in the distributions of demographic or risk factors for SIDS between the BRON, URTI or WELL groups. The WELL group were selected at recruitment as being free from infections and hence this variable was significantly different when compared with all other groups ( $p = 0.0$ ).

No infants in the ALTE or WELL groups were reported to sleep routinely in the prone sleeping position and none of the ALTE infants were found in the prone position at the time of the event. The prone sleeping position was reported for one infant in the BRON group. The highest proportion of infants routinely sleeping in a prone position was reported in the URTI group ( $n = 5$ , 33%) and was significantly higher than the WELL ( $n = 0$ ,  $p < 0.01$ ) and the ALTE groups ( $n = 0$ ,  $p < 0.01$ ).

The median gestational age was 39 weeks for the ALTE group (range: 26–42 weeks) and 40 weeks for the three comparison groups (range: 31–43 weeks). There was no significant difference between the groups for obstetric history, gestational age, birth weight or Apgar scores. Breast-feeding was very high in all groups (range: 92–100%); on average 93% of infants were breast-fed. On average there was one older sibling for infants in each group (range: 0–5 siblings). The groups were homogeneous for ethnic heritage; the majority of infants were of Caucasian ethnicity (97%). The type of heating used in the home was uniform throughout the Groups; approximately 50% having electric heating and 50% gas or wood heating.

#### 3.1.1. Comparison of ALTE infants with the combined control groups

Data from the BRON, URTI and WELL groups were combined for comparison of the distribution of potential SIDS risk factors with the ALTE group (Table 2). Low socio-economic status ( $p < 0.01$ ), lack of immunisation ( $p < 0.01$ ) and exposure to passive tobacco smoke ( $p = 0.00$ ) all occurred at a significantly higher frequency in the ALTE group compared to the remainder of the study cohort. Male gender ( $p = 0.06$ ) and a family history of SIDS ( $p = 0.06$ ) occurred with greater frequency among the ALTE infants and approached significance when compared to the remainder of the cohort. There were no significant differences between the ALTE infants and the remainder of the study cohort for Aboriginal-Torres Strait Islander heritage, single-parent families, the presence of older siblings, breast feeding practices or routine sleeping position.

Table 2  
The frequency of potential SIDS risk factors in the ALTE group and for the remainder of the study cohort

SIDS risk factors	ALTE group (%)	Combined remainder of study cohort (%)	Significance ( <i>p</i> -value)
Male gender	77	52	0.06
Family history of SIDS	15	3	0.06
Indigenous heritage	8	2	0.21
Older siblings	77	75	0.80
Single parent family	27	8	0.14
Below-average economic status	36	8	<0.01
Passive smoke exposure	62	16	0.00
Prone sleeping position	0	11	0.33
Immunised	42	80	<0.01
Breast-fed	92	93	1.00

Significance levels (*p*-values) are for differences in the distributions between the ALTE group and the remainder of the study cohort.

### 3.1.2. Comparison of the ALTE infants with individual control groups

A significantly higher proportion of the ALTE infants had a family history of SIDS (15%,  $p = 0.03$ ), came from single-parent families (27%,  $p < 0.01$ ) and a lower socio-economic category (36%,  $p < 0.01$ ) when compared to the WELL group (respective proportions were 0%, 3%, 5%). The ALTE group had a significantly lower proportion of immunised infants (42% immunised) compared to the WELL (84%,  $p < 0.01$ ) and URTI (89%,  $p < 0.01$ ) groups, but the proportion was not significantly different from the BRON group (33%,  $p = 1.00$ ). Similarly, the exposure to tobacco smoke was significantly higher in the ALTE group (62%, Fig. 1) compared to the WELL (8%,  $p = 0.00$ ) and URTI (28%,  $p = 0.04$ ) groups, but it was not significantly different to exposure recorded amongst the BRON group (33%,

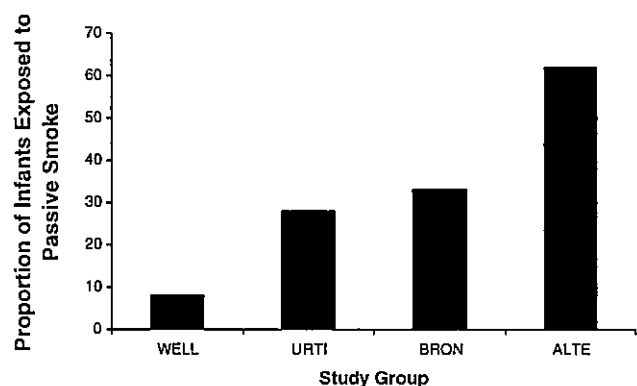


Fig. 1. The proportion of infants exposed to passive tobacco smoke in each study group.

$p = 0.37$ ). The incidence of infections and an acute life-threatening event increased with exposure to tobacco smoke (Fig. 1).

### 3.2. Salivary immunoglobulin and albumin concentrations

#### 3.2.1. Comparison between Sample 1 and Sample 2 collections

The median and ranges of salivary immunoglobulin and albumin concentrations for Sample 1 and Sample 2 for each of the study groups are compared in Table 3. There were no significant differences in immunoglobulin or albumin concentrations between Sample 1 and Sample 2 for the BRON, URTI and WELL infants. The concentrations of salivary IgA ( $p = 0.06$ ) and IgG ( $p = 0.05$ ) in the ALTE group showed a trend for higher concentrations in Sample 1 compared with Sample 2; this trend was not observed for salivary IgM or albumin concentrations. There was a trend for the concentrations of salivary IgA ( $p = 0.06$ ) in the BRON group to be higher in Sample 2 compared to Sample 1.

#### 3.2.2. Comparisons between subject groups

The distributions of immunoglobulin concentrations for Sample 1 and Sample 2 in each study group are illustrated in Fig. 2 as box-and-whisker plots. There were no significant differences in immunoglobulin concentrations between the BRON and URTI (all  $p \geq 0.21$ ), BRON and WELL (all  $p \geq 0.29$ ), and the URTI and WELL (all  $p \geq 0.39$ ) groups for Sample 1.

Sample 1 had: (i) significantly higher IgA concentrations in the ALTE group compared to the BRON group ( $p = 0.04$ ), URTI group ( $p < 0.01$ ) and WELL group ( $p < 0.01$ ); (ii) significantly higher IgG concentrations in the ALTE group compared to the URTI group ( $p = 0.00$ ) and WELL group ( $p < 0.01$ ); (iii) significantly higher IgM concentrations in the ALTE group compared to the URTI group ( $p = 0.01$ ) and WELL group ( $p < 0.01$ ); (iv) significantly higher albumin concentrations in the ALTE group compared to the URTI group ( $p < 0.01$ ) and WELL group ( $p < 0.01$ ).

There were no significant differences in immunoglobulin concentrations between the ALTE and BRON ( $p \geq 0.13$ ), ALTE and URTI ( $p \geq 0.07$ ), BRON and URTI ( $p \geq 0.12$ ), BRON and WELL ( $p \geq 0.13$ ), URTI and WELL ( $p \geq 0.32$ ) groups for Sample 2. There were significantly higher IgA ( $p = 0.04$ ), IgG ( $p = 0.02$ ) and IgM ( $p < 0.01$ ) concentrations for Sample 2 in the ALTE group compared to the WELL group.

### 3.3. Effect of passive tobacco smoke exposure on salivary immunoglobulin and albumin concentrations

Immunoglobulin and albumin concentrations in Sample 1 and Sample 2 for the BRON, URTI and WELL groups were not significantly different between

Table 3

The median (and range) of salivary IgA, IgG, IgM and albumin concentrations for Sample 1 and Sample 2 in each study group

Study group	Saliva collection	IgA (mg/L)	IgG (mg/L)	IgM (mg/L)	Albumin (mg/L)
ALTE group	Sample 1	77.1 (5.5–1500.0)	3.7 (0.0–15.6)	9.1 (0.0–23.6)	20.5 (2.0–52.0)
	Sample 2	29.3 (3.7–230.9)	1.7 (0.0–11.5)	6.3 (0.0–18.5)	13.0 (2.9–34.5)
	<i>p</i> -Value	0.06	0.05	0.70	0.26
BRON group	Sample 1	3.6 (2.7–19.3)	2.5 (0.0–8.3)	1.0 (0.0–13.6)	6.7 (5.3–49.0)
	Sample 2	62.0 (5.8–256.6)	0.0 (0.0–1.8)	2.8 (0.0–24.8)	9.2 (7.4–24.0)
	<i>p</i> -Value	0.06	0.25	0.78	0.87
URTI group	Sample 1	15.3 (0.0–245.3)	1.1 (0.0–2.9)	2.5 (0.0–21.0)	7.1 (1.8–15.0)
	Sample 2	8.9 (1.2–224.8)	0.0 (0.0–3.9)	1.8 (0.0–10.0)	6.2 (4.5–33.0)
	<i>p</i> -Value	0.55	0.06	0.87	0.50
WELL group	Sample 1	12.4 (0.0–71.4)	0.5 (0.0–7.7)	1.3 (0.0–9.9)	6.6 (1.5–25.0)
	Sample 2	9.8 (0.0–222.5)	0.0 (0.0–6.2)	1.7 (0.0–13.8)	7.6 (4.1–40.0)
	<i>p</i> -Value	0.94	0.79	0.71	0.08

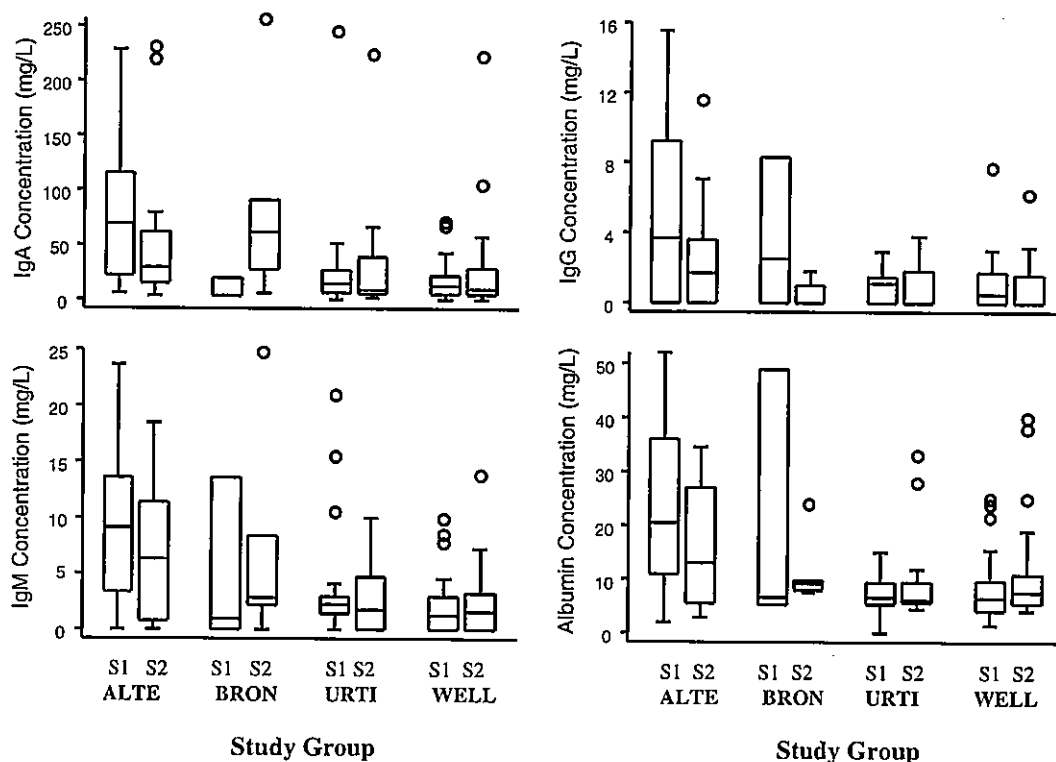
Significance levels (*p*-values) are for differences between Sample 1 and Sample 2 within each study group.

Fig. 2. Salivary immunoglobulin and albumin concentrations (mg/L) for Sample 1 (S1) and Sample 2 (S2) for each of the study groups.

infants exposed to passive tobacco smoke and those not exposed (Fig. 3).

There were significantly higher IgA concentrations in Sample 1 ( $p = 0.01$ ) among ALTE infants exposed to passive tobacco smoke (median = 105.1 mg/L, range: 12.3–1500.0 mg/L) compared to infants not exposed to passive tobacco smoke (median = 25.9 mg/L, range: 5.5–127.8 mg/L). IgG concentrations were also significantly higher in Sample 1 ( $p = 0.03$ ) for ALTE infants exposed to tobacco smoke exposure (median = 3.94 mg/L, range: 0.0–15.6 mg/L) compared to those not exposed to tobacco smoke (median = 0.8 mg/L, range: 0.0–7.0

mg/L). Passive smoke exposure was not associated with elevated IgM or albumin concentrations in Sample 1. There were no significant differences in immunoglobulin or albumin concentrations in Sample 2 among ALTE infants associated with cigarette smoke exposure.

#### 3.4. Comparison of salivary immunoglobulins and albumin with age-related reference ranges

The salivary immunoglobulin and albumin concentrations were compared to age-related reference ranges [26]. The salivary IgA and IgM concentrations for

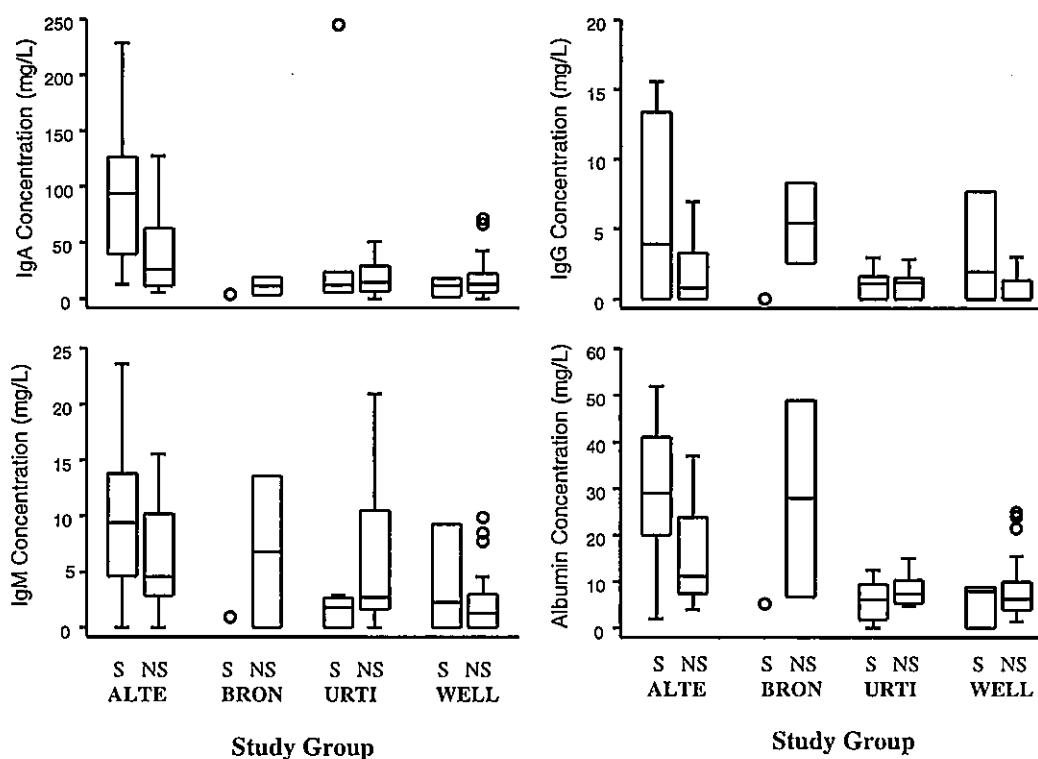


Fig. 3. Salivary immunoglobulin and albumin concentrations (mg/L) in Sample 1, from infants in each of the study groups exposed to tobacco smoke (S) and with no reported exposure (NS) to passive smoke.

Sample 1 for each group are presented in Fig. 4. All IgG and albumin concentrations were within the age-related reference ranges for all sample collections from all groups.

#### 3.4.1. ALTE infants

Twelve ALTE infants (57%) had IgA concentrations above the age-related reference range for Sample 1 and four of these (15%) were still elevated in Sample 2. The IgA concentrations for Sample 1 at recruitment were on average 10.9 times higher (range: 5.7–30.4 times higher) than the age-related median for the reference population [26] and on average 2.9 times higher (range: 1.0–8.1 times higher) than the 95th percentile for age.

Seven ALTE infants (27%) had salivary IgM concentrations above the age-related reference range for Sample 1 and four of these were still elevated in Sample 2; three of these subjects also had persistently elevated salivary IgA in Sample 2.

#### 3.4.2. Non-ALTE infants

The salivary IgA concentrations were significantly elevated above the age-related reference range for one URTI infant in Sample 1 (2% of combined non-ALTE cohort) and for five infants in Sample 2 (two BRON, one URTI and two WELL infants). This represented 8% of the combined non-ALTE cohort. Elevated salivary IgM concentrations were observed for one infant

in the BRON group and two in the URTI group for Sample 1 (5% of combined remainder of study cohort). The salivary IgM for one URTI infant remained significantly high in Sample 2, and elevated concentrations were also observed in Sample 2 for one BRON and one WELL infant (5% of combined remainder of study cohort). The causes of the elevated salivary IgA and IgM values in the non-ALTE infants were associated with a post-immunisation response in the URTI and WELL infants and a post-infection response in the BRON infants.

#### 3.5. Discharge diagnosis for ALTE infants

Clinical investigations of the ALTE infants while in hospital provided evidence for an infection, predominantly upper respiratory tract infection in 11 infants (50%), gastro-oesophageal reflux in seven infants (27%), central apnoea in six infants (15%) and no identified cause of the ALTE for two infants (8%). The pathogens identified in the ALTE infants are listed in Table 4. Respiratory Syncytial Virus (RSV) was the most common pathogen (64%). The two infants classified at discharge as 'unknown cause' of the ALTE both had scanty microbial growth that was reported by the laboratory as insignificant (Table 4). All six infants in the BRON group had RSV bronchiolitis but no evidence of associated apnoea.



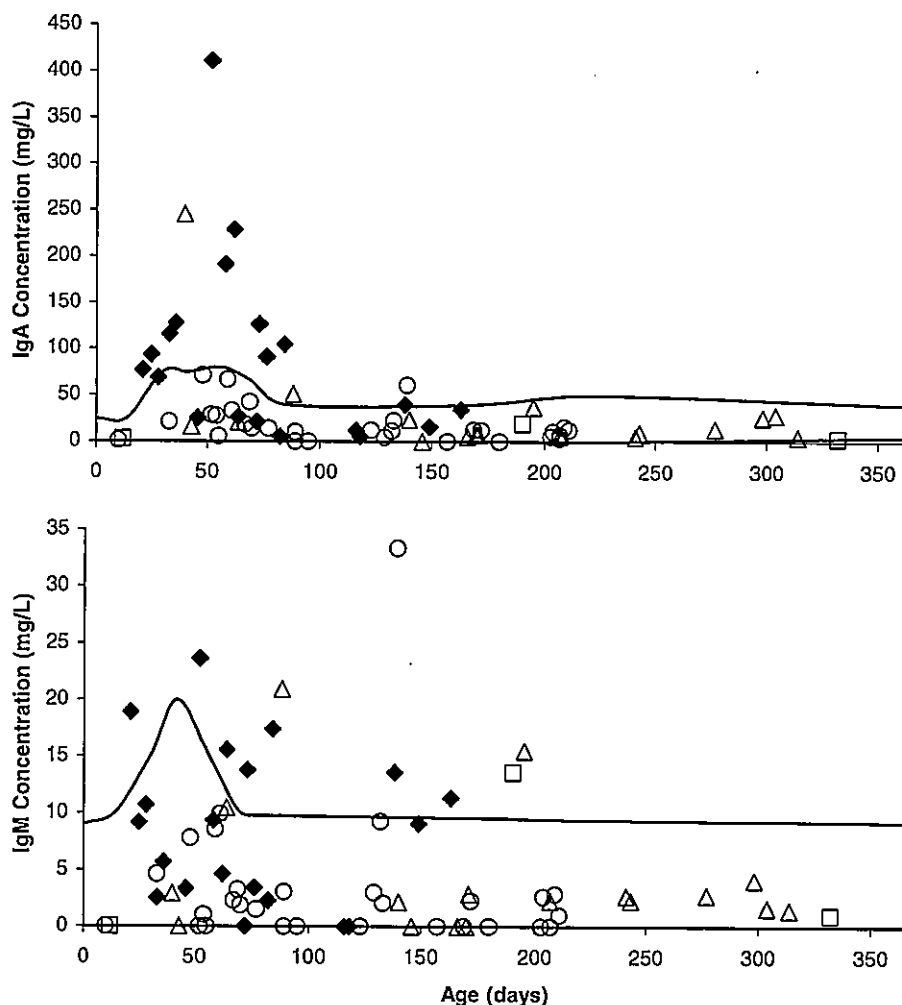


Fig. 4. Salivary IgA and IgM concentrations for Sample 1 from ALTE (◆), BRON (□), MILD (△) and WELL (○) infants plotted against age (days) at sample collection. The IgA concentration of 1500 mg/L for one ATLE infant, collected at 64 days of age, is not shown on the figure. The solid line represents the age related 95th percentiles of the reference range for each age category [26].

Table 4

Pathogens and other clinical conditions identified in the ALTE infants ( $n = 26$ ) following investigations in hospital

ALTE discharge classification	Pathogens identified	Number of subjects infected
Infection ( $n = 11$ )	• Respiratory Syncytial Virus bronchiolitis	7
	• <i>Escherichia coli</i> urinary tract infection	1
	• Streptococcal pneumonia with scanty <i>Staphylococcus aureus</i> , and parainfluenza virus later identified	1
	• No pathology tests performed, URTI with high fever	1
	• No pathology tests performed, viral URTI symptoms	1
Inflammation ( $n = 7$ )	• No pathogens identified in this category. All had evidence of gastro-oesophageal reflux	0
Other causes ( $n = 8$ )	• Scanty <i>Moraxella Catarrhalis</i>	1
	• Scanty <i>Staphylococcus aureus</i>	1
	• All others infants were classified as central apnoea, with no evidence of infections	0

The salivary immunoglobulin and albumin concentrations for Sample 1 were compared between the infants classified on discharge as 'Infection', 'Inflammation' or 'Other' causes of the ALTE, after excluding

contaminated samples (Table 5). The median IgA concentrations for Sample 1 in ALTE infants diagnosed with an infection were significantly higher than those diagnosed with a potential inflammatory cause

Table 5

Median salivary immunoglobulin and albumin concentrations (and ranges) for Sample 1 with the discharge classifications of infection, inflammation and 'other' causes for the ALTE infants

Discharge classification	IgA (mg/L)	IgG (mg/L)	IgM (mg/L)	Albumin (mg/L)
Infection ( $n = 8$ )	115.5 (34.5–1500.0)	4.9 (0–15.6)	9.2 (2.6–23.6)	32.0 (10.0–52.0)
Inflammation ( $n = 7$ )	24.1 (5.5–127.8)	0.9 (0–14.5)	4.0 (0–15.6)	11.3 (2.0–47.0)
Other causes ( $n = 6$ )	39.6 (5.5–191.6)	0 (0–14.3)	9.2 (0–18.9)	23.3 (4.0–41.0)

( $p = 0.01$ ), and also significantly higher than those grouped into the 'Other' classification ( $p = 0.03$ ). Salivary IgG, IgM and albumin concentrations were not significantly different among the ALTE infants based on discharge diagnosis classifications (Fig. 5).

The salivary IgA concentrations in Sample 1 for ALTE infants with different discharge classifications were compared to the age-related reference range [26]. Eighty-eight percent with a discharge classification of 'Infection' (7/8, samples from the other 3 subjects were excluded) had salivary IgA concentrations above the age-related reference range, accounting for 58% of all infants with elevated IgA concentrations for Sample 1 (7/12). The 'Inflammation' classification group accounted for 25% (3/12), and the 'Other' classification group for the remaining 17% (2/12) of the ALTE infants with salivary IgA concentrations above the age-related reference range.

### 3.6. Predictors of an ALTE

With the study group modelled as the outcome, the salivary IgA concentration for Sample 1 was found to

have a significant predictive value for the risk of an ALTE ( $R^2_{\text{adj}} = 0.31$ ,  $p = 0.02$ ). Exposure to passive cigarette smoke also had a significant predictive value for an ALTE ( $R^2_{\text{adj}} = 0.31$ ,  $p = 0.03$ ). The combined predictive value of the salivary IgA concentration and exposure to cigarette smoke was also statistically significant for the risk of an ALTE ( $R^2_{\text{adj}} = 0.29$ ,  $p = 0.01$ ).

## 4. Discussion

The results of this study support the hypothesis that mucosal immune dysregulation in response to a respiratory infection is a critical event in respiratory arrest of some previously well infants. The clinical data indicated that infants with ALTE, diagnosed through strict criteria, as 'near-miss' SIDS were a relatively homogeneous group. Infants diagnosed as 'near-miss' SIDS and SIDS most likely represent associated clinical outcomes in a spectrum of illness. The results support a plausible explanation for certain SIDS risk factors. The underlying patho-physiological mechanism of a proinflammatory

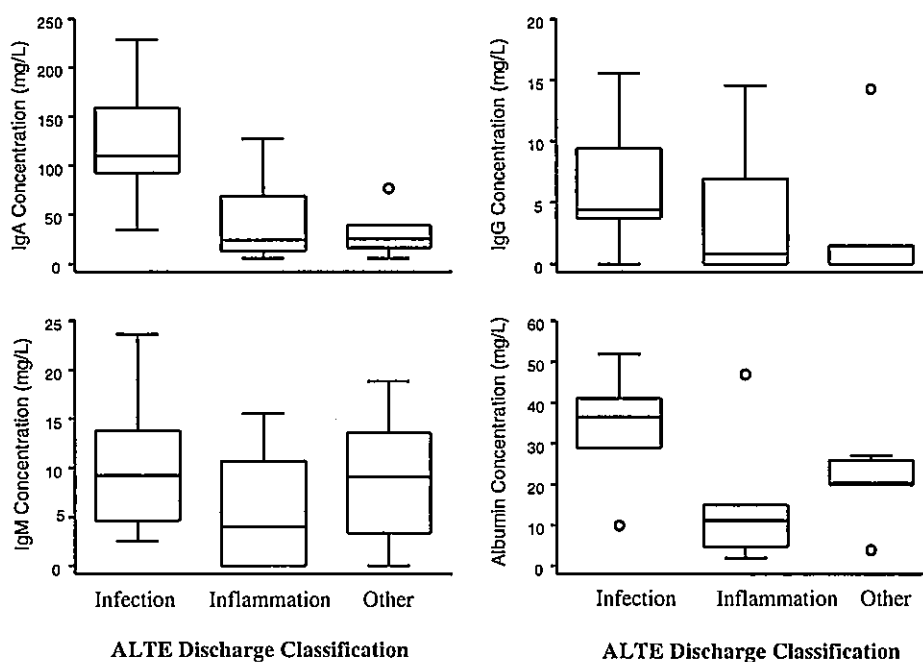


Fig. 5. Salivary immunoglobulin and albumin concentrations for Sample 1 from ALTE infants grouped according to diagnosis at discharge from hospital as infection, inflammation or other causes.

response to infections during a critical developmental period in infancy might be important in the events leading to apnoea-associated ALTE that in some infants could lead to SIDS [17–19]. The predictive value of salivary IgA raises the possibility of using a simple saliva test in infants who present with mild respiratory infections to identify a substantial number of infants at risk of developing an ALTE or SIDS, thus enabling intervention management to prevent such outcomes. The data also support studies indicating exposure to cigarette smoke as a high risk factor for acute life threatening apnoea [17], and potentially SIDS [1].

#### 4.1. Hyperimmune responses to infections

The salivary IgA and IgM concentrations in the ALTE infants at presentation to hospital indicated a significantly exaggerated mucosal immune response had already occurred, with nearly 60% of the IgA concentrations significantly above the population-based reference ranges. The hyper-immune response was most evident in the ALTE infants with clinical and/or pathology evidence of an infection; 87% of these infants had salivary IgA concentrations 10 times higher than the age-related median concentration. The concentrations of salivary IgA and IgM at presentation demonstrated the same gross elevation that was reported for a prospective SIDS case-study [10] and is consistent with reports of inflammatory responses in mucosal epithelium of SIDS infants at post-mortem [7–9,11,13,27–30].

The specificity of the mucosal antibody response was not assessed in this study. The response was most likely linked to the pathogens identified in the ALTE and bronchiolitis infants. The increase in salivary IgA might act as a surrogate marker for a polyclonal response to these infections. There was a high incidence of RSV infections in both groups. A previous study of the IgA antibody response to RSV in nasal secretions of infants showed a four-fold increase in over half the infants studied [31]. The rise in antibody levels corresponded with the disappearance of the virus from the respiratory tract, indicating that IgA neutralisation is important in the control of RSV infection. RSV infection has been identified in ALTE [32–34] and at post-mortem in some SIDS infants [33,35]. RSV-infected infants who suffered an ALTE [34] have been shown to have a significantly reinforced reflex apnoea compared to non-infected infants. The cytokine responses to RSV in infants suffering an ALTE [34] indicated a strong association between the interleukin-1 beta (IL-1 $\beta$ ) concentration and the severity of the apnoea. RSV also fails to induce an anti-viral interferon response in infants [36]. Interleukin-1 has been advocated for some time as a link between prolonged apnoea and SIDS during respiratory infections [37]. The combined findings of these studies suggest that apnoea might be a presenting symptom of RSV infec-

tion and the proinflammatory IL-1 $\beta$  response could be important in the pathophysiology of SIDS. The increase in salivary IgA concentrations in the second sample collected from the bronchiolitis group suggests that these infants presented to their family doctor at an earlier stage in the infection, an intervention that might have prevented subsequent apnoea in these infants.

The mucosal immune response had not resolved in half the ALTE infants with an infection within the 14 days to the second sample collection. In previous studies, examining the response of salivary immunoglobulins to infection in children [20] and adults [21], the salivary IgA concentration returned to baseline levels within a maximum of 12 days of appearance of symptoms of URTI and usually within 6 days. The mucosal immune response in the ALTE infants was significantly different to the response to a mild respiratory infection in the URTI group, in which the salivary IgA and IgM concentrations did not exceed the upper limit of the age-related reference ranges in most subjects. The prolonged period of return to baseline concentrations in the ALTE infants might be due to continued antigenic stimulus; defective dendritic cell handling of the antigens in the respiratory tract of neonates [38]; inappropriate control mechanisms of the hyper-immune response to the infections; and could in part be attributed to the natural 6-day half-life of IgA [39].

#### 4.2. ALTE-associated clinical outcomes

The discharge summaries for the ALTE infants indicated that they were a relatively homogeneous group comprised of infants with evidence of infections, gastro-oesophageal reflux or central apnoea. The discharge classifications were based on strict clinical criteria and thorough investigations in hospital. The study found that mucosal infections, predominantly with RSV, were a significant cause of these ALTE. Clinical evidence of significant infections was identified in 50% (13/26) of the ALTE infants. Two of the ALTE infants were classified as 'unknown-cause' on discharge from hospital, despite pathology findings of scanty *Moraxella catarrhalis* and *Staphylococcus aureus*. While scanty growth might not be clinically significant in most settings, in infants who have a predisposition to SIDS through hyper responsiveness to infections, these bacteria might have been the trigger for the hyperimmune response and a contributing factor to the ALTE.

#### 4.3. Association of ALTE with SIDS risk factors

Many of the SIDS risk factors were found in the ALTE infants in higher proportions than the remainder of the study cohort, and most of the risk factors were significantly higher in comparison with the healthy control infants in the WELL group. This lends weight to

their classification as 'near-miss' SIDS infants. There were more male infants; a significantly greater proportion with a family history of SIDS; more single-parent families of lower socio-economic status; significantly greater exposure to tobacco smoke; and fewer infants immunised in the ALTE group.

The fact that none of the ALTE infants were found in the prone-sleeping position might have been a significant consideration in their survival. There is increasing evidence to support the concept that the reason prone-sleeping is a risk factor for SIDS is this position allows an increase in the nasal cavity temperature to a level that supports bacterial growth and production of some toxins [40]. Had these ALTE infants been lying prone, the infections and the accompanying hyperimmune response might have been even more grossly exaggerated. The combination of an infection and the other risk factors identified in the ATLE infants could act in synergy with prone sleeping and might contribute to the sequence of events that are hypothesised to lead to death in SIDS.

The median age of the ALTE infants was 2 months. This is consistent with the 2–4 month age range associated with the peak incidence for SIDS [1] and supports the possibility that ALTE and SIDS might represent associated clinical outcomes in at-risk infants. The age range for the ALTE infants (1–7 months) is also associated with the peak maturation period of the mucosal immune system [41]. It is feasible that mild, normally non-pathogenic infections presenting for the first time in at-risk infants during this critical developmental period could result in the hyper-immune pro-inflammatory mucosal response evident in ALTE infants with infections and also observed in a case-study of SIDS [10]. This age is also associated with establishment of adult-like circadian rhythms of night-time cortisol levels [42] and it is feasible that if the immunosuppressive cortisol patterns have not been established there is an added risk of an exaggerated proinflammatory response to the infection or associated toxins.

The incidence of SIDS is reduced in infants who have received childhood immunisations [43]. The mechanism of risk reduction is still uncertain. The low immunisation rates in the ALTE infants in this study, in conjunction with evidence of uncontrolled mucosal immune responses, supports the school of thinking that immunisation may be protective against SIDS by induction of protective or cross-reacting antibodies to bacterial toxins identified in SIDS infants [44]. The high immunisation rates in the healthy control group and those who had a mild respiratory infection, compared to the infants who had an ALTE or RSV bronchiolitis (BRON group) suggests that early immunisation might have a modifying effect on how an infant responds to RSV infections during this critical period of immune maturation. Childhood immunisations and infections influence

the Th1/Th2 balance causing a shift away from the predominant intra-uterine/neonatal Th2 cytokine milieu to a more protective Th1 cytokine response [45], providing a potential mechanism for reduced hyper-responsiveness to viral infections. RSV infections induce a Th2 cytokine response in infants [46,47] and are associated with reduced interferon- $\gamma$  levels due to impaired Th1 responses [48], particularly in individuals with cytokine gene polymorphisms [49–51] that mediate respiratory tract inflammation during the acute phase of RSV infection [52,53].

#### 4.4. Exposure to passive cigarette smoke

Exposure to cigarette smoke is a significant risk factor for SIDS [54,55] and shows a significant dose-response relationship [56,57]. Exposure to passive cigarette smoke has also been associated with elevated arousal thresholds in newborn infants [58]. Tobacco smoke exposure was identified in this study as a significant predictor for an ALTE. The proportion of the ALTE infants exposed to tobacco smoke (63%) was significantly higher than any of the comparison groups. The exposure to cigarette smoke was also higher in the BRON and URTI groups compared to the healthy control infants in the WELL group. The data indicates that smoke exposure was associated with an increased risk of respiratory tract infections, particularly RSV. In some infants this might compound the risk of an ALTE if the infant mounts an inappropriate immune response to the infection, or due to an immature immune system that is unable to control the response to the infection.

Increases in secretion of the proinflammatory cytokine tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) were reported following *in vitro* incubation of RSV-infected cells with cigarette extracts [55]. Both RSV infection and cigarette smoke enhance bacterial epithelial binding of bacterial infections associated with SIDS [59,60] and a study of RSV bronchiolitis in children found significantly higher cotinine levels in infected children [61]. These interactions between RSV, bacterial infections and exposure to cigarette smoke suggest possible mechanisms for induction of the proinflammatory responses observed in the RSV-infected ALTE and bronchiolitis infants in this study. These associations might in part explain the seasonal pattern of SIDS [1], as the peak period for RSV infection, as for many other respiratory infections, occurs during the winter months in temperate climates [62] and the rainy season in tropical regions [63]. The transplacental transfer of maternal antibody for RSV also show seasonal variation and the level of antibody is dependent on endemic exposure during pregnancy [64]. Many of the environmental risk factors for RSV are the same as for SIDS [63].

#### 4.5. Identification of infants at risk of ALTE or SIDS

At presentation to hospital, the cause of the ALTE was unknown, doctors having excluded all obvious medical conditions. It was only after extensive clinical investigations that a cause for the ALTE was identified in the majority of the infants. By definition [1] this would exclude these infants from a classification of SIDS had they died from the ALTE, but some of the investigations used to make these diagnoses (oesophageal reflux, central apnoea) would not have been possible at post-mortem.

The identification of two significant predictors for an ALTE was an important finding from this study as both have the potential for intervention to prevent an ALTE, and potentially SIDS. Exposure to tobacco smoke was a significant predictor of an ALTE and is a risk factor modifiable through education programs aimed at changing behaviour. The identification of elevated salivary IgA concentrations as a predictor for an ALTE raises the possibility of using a simple saliva test in infants who present with mild respiratory infections to identify a substantial number of infants at risk of developing an ALTE or SIDS. This would enable intervention management to prevent such outcomes. Measurement of salivary IgA might also differentiate between infants with an infective and non-infective cause of an ALTE at presentation to hospital. Prospective studies in appropriate clinical settings are required to test these hypotheses.

#### 5. Conclusions

This study provides evidence of the role of infections in acute life threatening events in infants who present as 'near-miss' SIDS. The data supports the hypothesis that mucosal immune dysregulation in response to a respiratory infection is a critical event in respiratory arrest of some previously well infants. The study found some ALTE in infancy were associated with many of the known risk factors for SIDS and RSV infection was a significant contributing factor. SIDS and some infants with ALTE most likely represent a continuum of the underlying pathophysiology of inappropriate proinflammatory responses to infections during a critical developmental period in infancy. The results support plausible explanations for some SIDS risk factors. The recently identified genetic differences in cytokine gene polymorphisms among ethnic groups [65] might be a contributing factor to the inappropriate control of the hyperimmune proinflammatory responses to infection in some of the ALTE in infancy.

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MiniReview

# Development of mucosal immunity in the first year of life and relationship to sudden infant death syndrome

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## Abstract

The common mucosal immune system (CMIS) is an interconnecting network of immune structures that provides effective immunity to mucosal surfaces. The structures of the mucosal immune system are fully developed in utero by 28 weeks gestation, but in the absence of intrauterine infection, activation does not occur until after birth. Mucosal immune responses occur rapidly in the first weeks of life in response to extensive antigenic exposure. Maturation of the mucosal immune system and establishment of protective immunity varies between individuals but is usually fully developed in the first year of life, irrespective of gestational age at birth. In addition to exposure to pathogenic and commensal bacteria, the major modifier of the developmental patterns in the neonatal period is infant feeding practices. A period of heightened immune responses occurs during the maturation process, particularly between 1 and 6 months, which coincides with the age range during which most cases of sudden infant death syndrome (SIDS) occur. A hyper-immune mucosal response has been a common finding in infants whose death is classified as SIDS, particularly if in association with a prior upper respiratory infection. Inappropriate mucosal immune responses to an otherwise innocuous common antigen and the resulting inflammatory processes have been proposed as factors contributing to SIDS.

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## 1. Introduction

To understand the impact of infections on the neonate, particularly in relation to the peak period associated with sudden infant death syndrome (SIDS), it is important to know how the immune system develops and what are the significant influences on the ontogeny patterns. In humans the systemic and mucosal components of the adaptive immune system, while interrelated,

develop independently and at different ages. In association with innate immunity, the mucosal immune system provides the first line of defense against infections at external body surfaces.

This review will provide a synopsis of the development of the mucosal immune system in the first year of life and the factors that influence the ontogeny patterns in humans. An understanding of ontogeny, or normal development, provides the foundation for investigations of disease states and factors that influence the maturation of mucosal immunocompetence or modify the mucosal immune response. This review will focus on the development of the mucosal immune system and factors that influence the normal ontogeny patterns in the first year of life and their relationship to SIDS.

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## 2. The mucosal immune system

The human mucosal immune system comprises the lymphoid-associated structures of the nasal, bronchial, gastrointestinal and urogenital tracts, as well as the lachrymal, salivary and lactating mammary glands and the synovium of joints [1–3]. The mucosal associated lymphoid tissues (MALT) form an interconnecting network through which homing receptors allow plasma cells activated at one site to be seeded to distant mucosal sites, thus providing extensive immune protection at mucosal surfaces [4]. Unlike the systemic immune system, the major class of antibodies in mucosal secretions is immunoglobulin A (IgA), and there is a predominance of IgA-secreting plasma cells at all mucosal sites [5]. The mucosal antibodies are predominantly in a secretory (S) IgA form of dimeric IgA linked by a Joining (J)-chain protein that participates in the intracellular polymerisation of IgA and IgM molecules. SIgA is transported across mucosal epithelium by the polymeric immunoglobulin receptor protein, also known as secretory component (SC). SIgM is the other major class of secretory antibodies, and small proportions of IgM, IgG and IgD, but rarely IgE secreting plasma cells are found in mucosal sites. IgD and IgG can be detected during the early maturation process and as compensatory antibodies in subjects with IgA-deficiency, but they do not exist as secretory antibodies attached to SC or J-chain.

The distribution of the two IgA subclasses (IgA1 and IgA2) varies at the different mucosal sites [6]: IgA2 is predominant in the distal gastrointestinal tract (60%); IgA1 is predominant in the salivary glands (60–80%) and nasal associated lymphoid tissue (80–90%, NALT). The concentrations of the two IgA subclass antibodies in the mucosal secretions generally reflect the distributions of the antibody-producing cell populations in the mucosal associated lymphoid tissue at each site and the differences in local antigen types (proteins or polysaccharides) [7]. The antibody responses to protein antigens at mucosal sites are predominantly IgA1; the response to polysaccharides is predominantly IgA2 [7].

## 3. Monitoring mucosal immunity

The gestational age at birth determines the level of immune competence of the neonate, and the degree of passively acquired maternal antibodies that provide effective defense against infections and other antigenic stimuli. The prenatal development of mucosal immune structures will be briefly covered in this context. Prenatal studies have focused mainly on the appearance of anatomical lymphoid structures and expression of lymphoid cells or the proteins associated with secretory immunoglobulins. Prenatal studies have been restricted by the

limited availability of human fetal material and the difficulty in determining the precise gestational age.

In the postnatal period many researchers have used the measurement of salivary immunoglobulins and associated proteins to assess the ontogeny of the mucosal immune network in humans. The salivary glands have long been recognised as part of the common mucosal immune system [1] and salivary antibodies are indicative of mucosal immune competence. Saliva provides a simple non-invasive sample collection procedure, and it is a secretion that can be relatively easily analysed by routine laboratory procedures [8]. A criticism of the use of saliva to draw conclusions about the mucosal immune network has been that the antibodies in saliva most likely reflect the end result of the mucosal immune response following migration of plasma cells from mucosal germinal centers to the salivary glands rather than the induction phase of the mucosal immune response [8]. Another consideration for studies using saliva is the influence of physiological variations such as flow rate on the concentrations of the parameters being measured. Despite these limitations, analysis of salivary parameters has provided considerable useful information on the ontogeny of mucosal immune competence in humans and will be a major focus of this review.

## 4. Prenatal development of mucosal immunity

The mucosal immune apparatus is anatomically in place prior to birth with all components identified by 200 days of gestation. Although the mucosal immune system is not usually activated by antigen challenge until birth, there is good evidence that the fetal mucosal immune system is capable of mounting a response. Immune stimulation in these cases usually arises from intrauterine infection and possibly as an anti-idiotypic response to maternal antibody. Premature babies older than 28 weeks gestation, therefore, have the capacity to mount an effective mucosal immune response at birth.

In the human fetal intestine, the B-cell maturation process commences at about 100 days of gestation with the appearance of IgM/IgD/CD5-positive cells [9,10]. IgA expression occurs from about 120 days of gestation [11]. Discrete primary B-cell follicles, T-cell zones with high endothelial venules, a dome region, and follicle-associated epithelium are observed around 130–140 days of gestation [10]. In the lamina propria, IgA- or IgG-positive cells are not usually present at birth [9,11,12].

The development of bronchus-associated lymphoid tissue (BALT) is dependent on antigenic stimulus, and immunoglobulin-containing lymphocytes do not occur in the lung prior to birth in normal circumstances [13]. IgM-positive cells have been reported in the bronchi and major salivary glands from 110–140 days of gestation and IgA-positive cells at 180 days of gestation [14,15].

The numbers and distribution of IgM- or IgA-positive cells observed at the various fetal mucosal sites examined do not alter after 200 days of gestation [14].

SC can be detected as early as 40 days gestation [15], and by 200 days of gestation, it is expressed in both respiratory and gastrointestinal epithelium [13–16]. The synthesis continues to increase rapidly to adult distribution patterns by 1-week postpartum [12]. The expression of J-chain in the human fetus is also an early event and precedes the expression of  $\mu$ -chain [15]. J-chain is expressed in the fetal liver at 40 days gestation but is not detected in spleen, thymus, lung, or small intestine until 110 days of gestation [14,15].

T-cells are observed in the human terminal ileum as early as 100 days of gestation, and by 140 days they are organised around distinct B-cell follicular areas [9]. The developing T-cell repertoire in the lamina propria includes both helper/inducer ( $CD3^+/CD4^+$ ) and suppressor/cytotoxic ( $CD3^+/CD8^+$ ) phenotypes [9,11], but the helper/inducer phenotype predominates. In contrast to T-cells in the lamina propria, the fetal intraepithelial lymphocytes (IEL) are null cells ( $CD3^+/CD4^-/CD8^-$ ); and in contrast to the postnatal intestine, the T-cell receptor (TCR) is predominantly the  $TCR\gamma\delta$  [17].

## 5. Mucosal immunocompetence at birth

### 5.1. Inmate defenses

In the absence of specific adaptive immunity, innate defense mechanisms provide non-specific protection in fetal life and at the time of birth. Amylase, lysozyme, and lactoferrin are all present in the human salivary gland by 200 days of gestation at levels similar to those observed in the postnatal period [2,16,18]. All three proteins show a dramatic decrease immediately after birth. This is thought to be due to depletion of cellular stores as a result of suckling [16]. After the temporary decrease in concentrations, salivary amylase, lysozyme, and lactoferrin reach peak levels between 6 and 20 weeks of age (covering the 2–4 month age range during which the peak of SIDS occurs) and remain at stable levels after 28 weeks of age [16]. The “window” of depletion of these antimicrobial factors immediately after birth might be significant if the infant is exposed to pathogens during this period when the neonate’s specific immune system is incapable of effective defense mechanisms. In breast-fed infants, this deficit is most likely counteracted by passive maternal antibody protection.

### 5.2. Adaptive immunity

The normal full-term infant is born virtually IgA-deficient. It takes several weeks before gut-associated lymphoid tissue (GALT) responds effectively to anti-

genic challenge. The unresponsiveness of the mucosal immune system is due to the combined effects of several factors: hormonal influences during the birth process; the immaturity of the antigen-presenting cells; and the immunosuppressive effects of maternally derived serum IgG antibodies and colostral IgA antibodies [19].

Studies of intrauterine infections and premature infants who develop pulmonary infections indicate that the mucosal immune system is capable of a rapid response if challenged. IgA- or IgM-containing plasma cells appear in BALT of premature infants at levels equivalent to the 39th gestational week and IgG-containing cells appear 1–2 weeks later in the submucosal layer of the bronchi [13]. The detection of antibodies of fetal origin in the absence of infection has also been reported, but the mechanisms and significance of these antibodies have not been determined [20].

## 6. Postnatal development of the mucosal immune system

### 6.1. B-cell development

In the absence of any intrauterine infection, the mucosal immune system is essentially devoid of IgA-containing lymphocytes, and there are no reactive B-cells in the intestinal lymphoid follicles or BALT at birth. The peak period for maturation of B-cells at mucosal surfaces is from birth to 12 weeks of age. This maturation period coincides with the peak incidence of SIDS deaths between 2 and 4 months.

Germinal centers appear in the intestinal mucosa after birth [21,22], and SC expression in the epithelium increases rapidly to about 8 weeks of age; at this time an adult distribution pattern is observed [12,16]. Prenatally, most of the intestinal germinal center cells stain immunohistochemically for cytoplasmic IgM or IgG, not IgA [23]. This is rapidly reversed after birth in response to antigenic challenge [2]. In the intestine, the number of IgM-containing cells predominates up to 1 month of age [24,25]; thereafter, IgA-containing cells predominate and continue to increase up to 2 years of age [24].

Immunoglobulin-containing cells are not present in bronchial walls of healthy neonates at birth [13]. IgA-containing cells appear around the bronchial glands at 10 days after birth and IgM-containing cells in lesser numbers about 1 month of age. IgG-containing cells are rare under normal healthy conditions [13].

In the parotid salivary gland, IgA- and IgD-producing immunocytes increase in number from about 4 weeks of age; IgG-producing cells initially increase, reaching a peak at about 12 weeks of age and then plateau at lower numbers after 24 weeks of age [16]. The numbers of IgA-producing immunocytes in the salivary glands approach the lower normal adult range at about

15 months, with only small increases throughout early childhood [26]. IgA1-positive cells predominate in the salivary glands in the perinatal period but the IgA2-positive cells increase in the first 3 months after birth to approach adult proportions [16]. This pattern probably reflects the changes in the type and load of antigenic exposure, particularly exposure to polysaccharide antigens.

Plasma cells appear in tonsillar tissue by 8 weeks of age [27]. There is a rise in both IgA- and IgM-producing cells in the appendix between 2 and 4 weeks of age, but after a few months, the IgG-producing cells expand in the appendix lamina propria to represent 50% of the immunocytes [22].

### 6.2. T-cell development

Very few studies have examined the postnatal development of T-cells at mucosal sites in the human neonate. The number of intestinal intraepithelial lymphocytes (IEL) expands after birth, reaching adult levels by 2 years of age. This expansion primarily reflects the 10-fold increase in TCR $\alpha\beta$ -positive cells; however, TCR $\gamma\delta$ -positive cells also expand two- to threefold. It is thought that the expansion of TCR $\alpha\beta$ -positive cells is more dependent on antigenic stimuli than the TCR $\gamma\delta$ -positive cells [28]. Phenotypically, the IEL remain predominantly suppressor T-cells (CD3<sup>+</sup>/CD8<sup>+</sup>) from birth to adulthood [11,28]. The predominance of the TCR $\alpha\beta$ <sup>+</sup>/CD8<sup>+</sup> subset and the location of the IEL support the concept that these T-cells are involved in oral tolerance to dietary antigens [2].

MHC class II antigen expression (HLA-DR), indicating antigenic activation of the immune system, primarily occurs postnatally between birth and 2 weeks of age [11,12]. Small amounts of these antigens are expressed by human fetal tissue [29,30]. The rapid upregulation of HLA-DR expression after birth is consistent with the concept that postnatal exposure to antigen is important in determining the repertoire of immunologically determined epitopes. This upregulation of HLA-DR expression is almost certainly cytokine driven, suggesting that neonatal mucosal T-cells and macrophages can facilitate this maturation process. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interferon- $\gamma$  (INF- $\gamma$ ) can upregulate expression of HLA molecules. Postnatal human mucosal T-cells have not been studied in this context, although fetal lamina propria T-cells have been reported to secrete interleukin-2 (IL-2) and INF- $\gamma$  when stimulated with bacterial superantigens [31]. Other studies on fetal intestinal explants [32] have shown that the adhesion molecules ICAM-1, VCAM-1, and E-selectin can also be induced by activated T-cells in the lamina propria endothelium. At birth, it is likely that the appropriate endothelial adhesion molecules required to direct phagocytes to the intestine are already present.

Dendritic cells, involved in antigen processing are also reported to appear postnatally [33].

## 7. Development of mucosal immune competence after birth

The mucosal immune system is rapidly stimulated at birth by bacterial colonisation of the mucosal and external body surfaces. The development of effective mucosal immunity is essential for protection against infection and allergen exposure in the postnatal period. This period is characterised by two important components of the neonate's immune development: the closure of mucosal epithelial membranes and the appearance of secretory antibodies.

The immediate postnatal period is characterised by intestinal permeability to intact macromolecules [34]. Ingestion of colostrum promotes membrane maturation in the gastrointestinal tract through regulatory factors and results in closure within 48 h of birth [35]. The loss of IgG from mucosal secretions [8,36–38] in the immediate postnatal period is indicative of membrane closure. Rapid closure of membranes is an important process in limiting systemic exposure to antigens, which, if handled inappropriately, can lead to overwhelming infection, atopy, or subsequent tolerance. The membrane maturation factors in colostrum might be a significant factor in the reduced incidence of infections and atopy in breast-fed infants.

### 7.1. Secretory component

The availability of SC is essential for the transport of SIgA and SIgM across the epithelium into secretions, and hence, it one of the prerequisites for the development of mucosal defence mechanisms [39]. Free SC can be detected in the saliva of newborn infants [40]. Coinciding with initial stimulation of the mucosal immune system, SC expression in the salivary gland and intestinal epithelium increases between 1 and 2 weeks after birth [16,41]. Levels equivalent to those in adults continue to be expressed in the intestinal crypts [2], but the expression of SC declines in the salivary glands to the perinatal level around 6 months [16,41].

### 7.2. Secretory IgA

In a healthy neonate, the pattern of appearance of antibodies in mucosal secretions is consistent with the rapid population of the neonatal intestine, initially with IgM and then IgA-containing plasma cells [24] in response to bacterial colonisation. Although SIgA has been reported at birth in saliva [40,42] and nasal secretions [43], most investigators report the first detection of

SIgA in mucosal secretions between 1 week and 2 months of age [20,44–50].

IgA antibodies secreted at extraintestinal mucosal sites begin to increase during the first few weeks of life [36–38,49–53]. Salivary IgA levels (Fig. 1) increase rapidly in the neonatal period to peak levels between 4 and 6 weeks of age [37]. The initial peak in SIgA in saliva has been reported to decline to lower levels between 3 and 6 months of age [37,48,54]; however, this profile has not been reported in all studies of ontogeny in the neonate [20,53]. Prematurity is an important risk factor for SIDS and there is further conflicting data in relation to this group. The appearance of IgA in saliva has been reported to be equivalent to term infants during the first 9 months of life [40], but Kuitunen and Savilahti [55] reported significantly lower levels of IgA in saliva of preterm compared with full term infants. There is agreement that salivary IgA levels remain relatively consistent after 6 months of age until exposure to increased antigenic loads occurs: hospitalisation of children [20]; attendance at daycare centers [20,56]; commencement of schooling at 4–5 years of age [57]. Adult levels of SIgA in saliva are reached around 7 years of age [48,57].

The ontogeny of plasma cells expressing the two IgA subclasses has not been established postnatally in human mucosal tissues, and it can only be speculated that the concentrations in mucosal secretions reflect the plasma cell distributions in these tissues. Studies of the IgA subclasses in mucosal secretions are limited, but both IgA subclasses are present in saliva and the proportions are characteristic of adult saliva by 6 months of age [52,53]. The study by Fitzsimmons et al. [53] indicated that SIgA1 was the dominant subclass in saliva at birth and that the proportion of SIgA2 increased linearly to 6 months of age. The high proportion of SIgA1 in saliva might not necessarily reflect the situation in

other mucosal secretions. The distribution of the IgA subclass plasma cells varies at mucosal sites [6] in response to different antigenic types.

### 7.3. Secretory IgM

SIgM is absent from mucosal secretions at birth in healthy full-term neonates [37] but appears in saliva transiently between 1 and 6 months of age [37,59] and occasionally in nasopharyngeal secretions in the first week of life but is routinely present by seven weeks of age [36]. The concentration of IgM in bronchial washings also increases during the first week of life [38]. Total salivary IgM [59] and specific IgM antibodies [20,52,60] are occasionally observed in infants and adults in a pattern consistent with the concept that IgM antibodies in mucosal secretions reflect immune responses to novel antigens presented at mucosal sites, particularly the gastrointestinal tract. SIgM is usually present in high concentrations in mucosal secretions of IgA-deficient subjects in whom SIgM and IgG appear to play a compensatory role [61–64].

### 7.4. Markers of mucosal immunocompetence

During the first 12 months of life the maturation of the mucosal immune system is dependent on the type and timing of antigenic exposure. In addition to the appearance of specific antibodies, several other markers have been associated with the development of mucosal immunocompetence. During the first six months of life, the immaturity of the mucosal apparatus is reflected by the detection of a significant proportion of IgD-producing cells in the salivary glands [16] and the presence of IgD in saliva of some infants for varying periods of time [40,65]. The inverse relationship between the appearance of IgA and the loss of IgD in infancy [65] and in IgA-deficient subjects [64] probably reflects the role of IgD in isotype switching and the preferential clonal differentiation to IgA-producing cells at mucosal sites.

The most important attribute for assessing immunocompetence is the ability to produce secretory antibodies. A longitudinal study of children indicated the age at which an infant switches from producing monomeric IgA to SIgA in saliva varies during the first year of life [66]. Although a cross-sectional study failed to confirm these findings [58], the conversion to dimeric IgA is probably another marker of mucosal immune system maturation.

Transient absences of salivary IgA in the first year of life have also been observed and shown to be associated with an increased susceptibility to bronchial hyperactivity, but not asthma, later in life [67]. They also had a negative association with the development of atopy [67]. This hyporesponsiveness in the respiratory tract of some infants appears to be associated with defective antigen

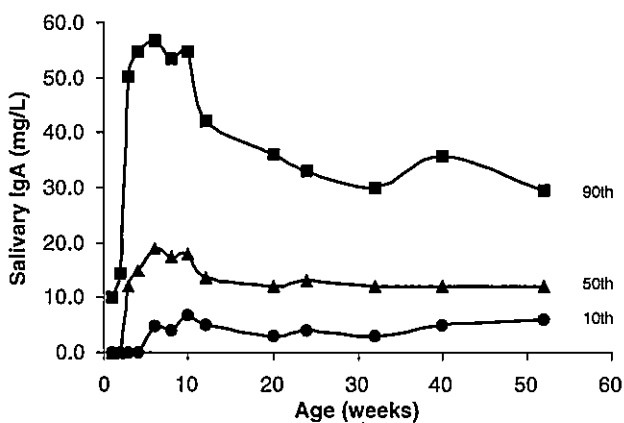


Fig. 1. The development pattern of salivary IgA in infants during the first year of life, represented as the 10th (●–●), 50th (▲–▲) and 90th (■–■) percentiles for each age group. Adapted from Gleeson et al. [37].

processing by the local dendritic cells [68] and is another indicator that the mucosal immune system undergoes a significant variation in the maturation process in the first year of life.

## 8. Factors influencing mucosal immune development

The factors that influence the development of the mucosal immune system can be categorised into those that either provide antigenic stimulation or modify the control of the immune response.

### 8.1. Feeding practices early in life

The role of infant feeding practices in the development of mucosal immunity cannot be underestimated. There are significant associations between neonatal feeding patterns, bacterial colonisation, infection rates and mucosal immunological maturation. Oral feeding per se provides a stimulus for mucosal immune development. Intravenously fed full-term infants are devoid of IgA- and IgM-containing plasma cells in the gut lamina propria at an age when infants receiving oral feeds have adult proportions of immunocytes [25]. This delayed immune development in intravenously fed infants reflects the lack of immune stimulation by food or colonising bacterial antigens.

The initial bacterial colonisation patterns in the gastrointestinal tract differ between breast-fed and formula-fed infants [69,70]. This significantly affects the degree and nature of antigenic stimulation of their mucosal immune systems. Colostrum deprivation also delays closure of mucosal membranes [35,37] and the protracted period of increased membrane permeability might contribute to the higher incidence of infections [39,71–73] and atopic diseases [74] observed in non-breast-fed infants.

Maternally acquired immunity is essential for survival in the neonatal period until endogenous immunity develops. The exogenous antibodies acquired prenatally by transplacental transfer and postnatally via colostrum have a suppressive effect on the development of the infant's mucosal immune responses. In animal models, the presence of maternal IgA in milk delays the onset of maturation of the mucosal immune system [75,76]. In human studies, ingestion of colostrum has been reported to delay the appearance of and level of IgA and IgM in saliva (Fig. 2) of totally breast-fed infants in the first 3 months of life [37,77]. Fitzsimmons et al. [53] reported a more rapid increase in total salivary IgA, and both SIgA1 and SIgA2 concentrations, over the first 6 months in breast-fed infants compared with formula-fed infants. Harrison et al. [78] observed an inverse relationship between infant salivary antibodies to the staphylococcal toxins implicated in SIDS and the levels

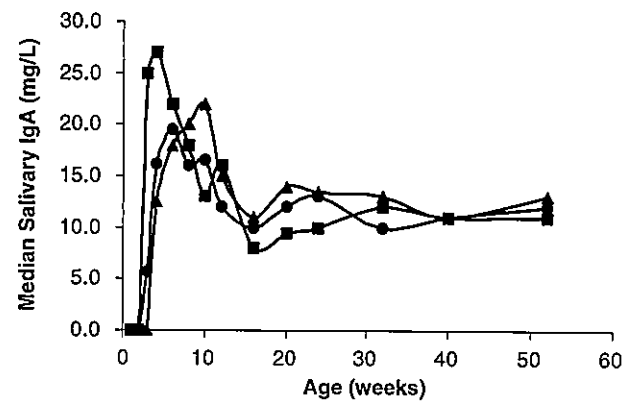


Fig. 2. The influence of infant feeding practices on salivary IgA levels during the first year of life in breast-fed (●-●), formula-fed (■-■) and mixed-fed infants (▲-▲). Adapted from Gleeson et al. [37].

of these antibodies in maternal cord blood and breast milk. These findings most likely reflect the immunosuppressive effect of the passively acquired maternal antibodies on the development of protective antibodies by the infant.

Whether or not colostrum has an immunosuppressive effect in the perinatal period on the initial appearance of SIgA and SIgM, breast-feeding has been shown to enhance vaccine responses to tetanus toxoid and polio [79–81], and breast-fed infants have long-term protection against *Haemophilus influenzae* (*H. influenzae*) infection [82]. Breast-fed infants also produce higher levels of total SIgA in urine compared with formula-fed infants [83]. These studies clearly indicate that breast milk is capable of regulating mucosal immune responses in the infant. While there are conflicting studies, generally breast-feeding is recognised as one of the factors known to reduce the risk of SIDS [84,85]. The mechanisms responsible for the protective effect of breast-feeding are still speculative; however, it is possible that immune regulatory factors in breast milk [86] in some way moderate the mucosal immune dysfunction observed in SIDS infants [87].

### 8.2. Antigenic stimulation – specific mucosal antibodies

The appearance of specific SIgA antibodies in infants is dependent on the degree of vaccination or natural exposure to the antigen. Antibodies to pathogenic organisms such as poliovirus in non-endemic countries only appear after first vaccination [88]; conversely, in endemic countries, the early appearance of mucosal antibodies reflects the early exposure. Another example of these differences is the early appearance of antibodies to *H. influenzae* in infants in endemic areas of Papua New Guinea [89].

Studies on mucosal responses to *Streptococcus pneumoniae* in children aged 2–24 months, have reported that salivary IgA antibodies specific for pneumococcal

capsular types are boosted following secondary systemic immunisation with pneumococcal polysaccharide conjugate vaccines [90,91]. This suggests that some antigens can induce a mucosal IgA memory response when administered systemically. In unvaccinated children, higher levels of antibodies against pneumococcal polysaccharide serotype antigens have been found in the saliva from children who carried these pneumococcal serotypes in the nasopharynx or middle ear compared to those children who did not [92]. These findings indicate that natural colonisation might have a bearing on the efficacy rates of systemic immunisation against pneumococcal otitis media and is dependent on whether the vaccine contains the serotype antigens found within the population to be immunised.

Antigenic exposure is also important in the ontogeny of SIgA responses to common enteric organisms. SIgA specific antibodies to *Escherichia coli* somatic antigens appear in the intestine during the first weeks of life [93]; but, in this period, very low levels of SIgA antibodies against *E. coli* adhesins are detected [94]. Artificial colonisation of the intestine of newborns with non-pathogenic *E. coli* strains stimulated the production of both IgA and IgM antibodies in stool and saliva [95] and reduced the number of infections and mortality in high-risk infants. In a cohort of normal Australian children, SIgA antibodies to *E. coli* O antigen (pool of the six common enteric serotypes) were not detected in saliva until 1–2 months of age and showed a gradual increase with age [57]. Bronchial aspirates from premature neonates showed a similar pattern of appearance of SIgA anti-*E. coli* antibodies [38]. These studies indicate a tightly controlled mucosal immune response to a major group of antigens on bacteria that colonise the intestinal tract. The studies by Mellander et al. [20] in a Swedish community revealed higher levels of *E. coli* antibodies in saliva of children who attended day care centers or who had been hospitalised compared with children cared for exclusively at home. This suggests a greater antigenic load or attenuating factors in environments outside that to which the child is normally exposed.

Salivary antibodies to organisms that colonise the oral cavity are also detected by 1–2 months of age and increase during the first few years of life [52]. Salivary IgA antibodies to streptococci (*S. sanguis* and *S. mutans*) generally appear after tooth eruption and the loss of maternally derived antibody to these microbiota [54,96].

### 8.3. Nutrition and diet

Impairment of systemic immune responses has been well described in children small for gestational age [97], but studies of the effect on mucosal immunity are limited. As children born below 80% weight for age have fewer immunoglobulin-producing cells and lower

amounts of immunoglobulin secreted [97], it is possible that their mucosal immune responses to infections will be compromised. In a cross-sectional study of children in Papua New Guinea [89,98], the total level of salivary IgA and the specific IgA responses to *E. coli* and *H. influenzae* were lower in children below 80% weight for age. The immunological abnormalities resulting from intrauterine malnutrition persist for several months after birth [99] at a time when the mucosal immune system is maximally challenged and maturing.

Protein malnutrition is associated with decreased IgA responses to oral antigens [100] and an increase in eye infections associated with decreased ocular IgA responses [101]. Mild malnutrition reduces the IgA response to dietary antigens but has little effect on the response to common enteric pathogens [102]. In a comparison of British and West African children, the latter having considerably lower nutritional and hygiene status, there was no significant difference in the IgA output in intestinal secretions of well children [103]. During infections, the secretion rates of intestinal IgA and IgM were increased in the West African children with acute watery diarrhea.

Malnutrition during the immediate postnatal period might impair an already immature immune system and further compromise host resistance to infection by reducing the availability of essential vitamins and trace minerals [104]. One of the consequences of the poor nutrition associated with frequent diarrhea is vitamin A deficiency [82]. In rats, vitamin A deficiency results in a 90% reduction in intestinal SIgA responses to oral cholera vaccine [105].

### 8.4. Maternal factors

There are few reports on the effects of maternal stress on the development of mucosal immunity in humans. Many of the conclusions must be inferred from animal models and known effects on systemic immunity. There is some evidence to suggest that maternal emotional stress might reduce immune defences in the neonate through the transfer of maternal corticosteroids via breast milk [106]. The data from this study showing an inverse correlation between cortisol levels in maternal milk and SIgA levels in term infants were not conclusive, as these mothers of preterm infants also had positive correlations of anger (on their psychological profiles) with SIgA levels in their breast-milk. The endogenous production of stress hormones around the time of birth might also result in temporary immunosuppression in the neonate.

Exposure during pregnancy to toxic chemicals that cross the placenta can result in developmental disorders causing immune suppression [107]. Maternal ethanol consumption impairs in utero development of immunity and postnatally modulates lymphocyte proliferation

responses [108]. Maternal smoking is associated with impaired gastrointestinal function [109], and lower mucosal IgA levels [110], which might also reduce the passive protection provided to breast-fed neonates. This could account, in part, for the increased respiratory morbidity and atopy in infants of mothers who smoke [111,112]. Each of these toxic substances will affect the development of effective mucosal immunity in children and might determine many of the clinical outcomes in later life.

### 8.5. Other influences

Interactions occur between the mucosal immune system and a range of other exogenous and endogenous factors in adults, including climate, pollution, sleep deprivation, obesity, psychological and physiological stressors have been reported [8,56,113–115]. Whether these factors modify immune profiles in neonates is unknown; however, respiratory infections early in life can cause significant immune dysregulation and induce IgA-deficiency [61]. Similarly, lower levels of salivary IgA following chronic physiological and psychological stressors have also been associated with increased incidences of upper respiratory illness [116,117].

## 9. Relationship of mucosal immune development to SIDS

One of the plausible hypotheses to explain SIDS is that a common bacterial toxin can trigger the events leading to death [118]. As no single pathogen or commensal bacteria have been identified as the cause of SIDS, it is probable that in “at-risk” infants overwhelming inflammatory processes, as a result of antigenic stimulation, are a significant contributing factor [87,119,120]. Investigations of mucosal immunity in SIDS have supported this hypothesis.

The earliest study of mucosal immunity by Ogra et al. [121] reported that SC was absent or reduced in the bronchopulmonary epithelium of SIDS infants. The implication being that the SIDS infants could have impaired secretory antibody responses to mucosally presented antigens. Subsequent research has consistently shown the reverse and that overstimulation of the mucosal immune system occurs in the respiratory and gastrointestinal tracts of SIDS infants [87,122–124]. Postmortem findings have revealed significant mucosal immune stimulation in the salivary glands [125], tonsils [126], bronchial tracts and the duodenum [124] of many SIDS infants compared to infants who died of accidental deaths. A second common feature of these studies has been the association with mild respiratory tract infections prior to death.

During the 1–6 months age range during which most SIDS death occur, the infant’s mucosal immune system

is undergoing rapid maturation in response to significant mucosal antigenic challenge from non-pathogenic as well as potentially pathogenic organisms and other protein antigens (food and allergens). In developed countries, there are also early challenge from vaccines. This rapid maturation process is particularly characterised by a rapid polyclonal expansion of the mucosal effector network. At the peak age of SIDS of 8–12 weeks, the mucosal antibody levels are often declining (Fig. 1), particularly in non-breast-fed infants (Fig. 2). There could be interactions with other known risk factors for SIDS during this period: the prone sleeping position; changes in night time cortisol levels associated with establishment of circadian rhythm; and exposure to cigarette smoke. It is possible during this period for an inappropriate uncontrolled inflammatory immune response to occur to an otherwise harmless antigen. The proinflammatory cytokine responses observed in SIDS infants [127,128] are most likely important links between the hyperimmune response, apnea and death. During this peak age of risk of SIDS, many infants are also being weaned and might lose the protective effect of passive antibody and the regulatory influence that breast feeding exerts on the mucosal immune response.

In a prospective study of the development of mucosal immunity in healthy children, an unexpected death of an infant occurred at 10 weeks of age and was classified as SIDS [87]. The infant had a normal ontogeny pattern until a mild upper respiratory tract infection was reported at 3½ weeks. The early levels of salivary IgA and IgM had been normal for age, but following the cessation of breast-feeding and the respiratory infection, the antibody levels increased to approximately 5–10 times the age-related medians just prior to death. This indicated an uncontrolled mucosal immune response. A recent study of infants admitted to hospital with acute life threatening events (ALTE), suspected of being “near-miss SIDS” [129], also showed an association between prior mild upper respiratory infections and the induction of hyperimmune salivary antibody responses indicative of mucosal immune dysfunction. Genetically influenced control of the pro-inflammatory responses [130] induced by normally non-lethal infections might explain the why some infants are at-risk of SIDS and why there are ethnic differences in SIDS incidences throughout the world [119].

## 10. Conclusions

Anatomically, the human mucosal immune system is fully developed by 200 days gestation, but in the absence of an intrauterine infection, the development of effective mucosal immunity occurs in the postnatal period. At birth all the essential components of the mucosal immune system are present and have the potential to

Table 1  
Prenatal and postnatal factors influencing the development of the mucosal immune system in infancy

Factors influencing mucosal immunity in infancy
<i>Prenatal factors</i>
<ul style="list-style-type: none"> <li>• Genetic factors, particularly cytokine gene polymorphisms</li> <li>• Maternal nutritional status</li> <li>• Intrauterine infections</li> <li>• Toxic chemical exposure (alcohol, products of smoking cigarettes and illicit drugs)</li> </ul>
<i>Perinatal factors</i>
<ul style="list-style-type: none"> <li>• Hormonal influences at birth (immunosuppressive)</li> <li>• Infant feeding practices, particularly non-breast fed infants</li> <li>• Timing of mucosal membrane closure (exclusion of antigenic stimulation)</li> <li>• Bacterial colonisation, particularly gut flora in response to feeding practices</li> <li>• Up-regulation of T-cell regulatory cytokines in response to antigenic stimuli</li> </ul>
<i>Postnatal factors</i>
<ul style="list-style-type: none"> <li>• Level of protective antibodies in maternal serum and breast-milk</li> <li>• Immunisation</li> <li>• Exposure to new infections (family members, particularly older siblings; day-care or child-minding centers; hospitalisation; commencing school)</li> <li>• Exposure to cigarette smoke</li> <li>• Inherited or induction of IgA-deficiency states</li> <li>• Diet and nutritional status of infant, (adequate protein, essential vitamins and minerals)</li> <li>• Exposure to physiological and psychological stressors</li> </ul>

respond rapidly to antigenic stimulation. In conjunction with innate immune factors and initial passive protective maternal antibodies, the mucosal immune system develops rapidly after birth to provide effective immune protection.

The ontogeny patterns indicate the first appearance of immunocytes at mucosal surfaces and secretory antibodies between 2 and 4 weeks. Mucosal immunity is usually established by 3 months of age, but maturation markers indicate that the age of maturation varies between individuals and the period from 1 to 6 months is critical to this process. This period of maturation of effective immune protection at mucosal surfaces coincides with the period in which the majority of deaths classified as SIDS occur.

After birth the ontogeny of mucosal immunity is influenced by a number of factors (Table 1) including: the initial suppressive effect of maternal antibodies on the infant's immune development; neonatal feeding practices; establishment of commensal bacteria; maternal and infant nutritional status; vaccinations and natural exposure to infections. Maternal factors have significant influences on the development of mucosal immunity both in utero (toxic chemical exposures, nutrition, infections) and postnatally (immunosuppressive passive antibodies, nutrition, smoke exposure).

The mucosal immune responses and inflammatory markers detected in SIDS infants indicate that uncontrolled hyper-immune inflammatory responses are a common finding and are often associated with prior respiratory tract infections. Immune dysregulation at the time of maturation and aberrant genetic control of

the pro-inflammatory immune responses are potential contributing factors that lead to death in at-risk infants.

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Our Ref: SG: 10332  
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8 March 2019

Professor Robert Clancy  
[REDACTED]

**By Email:** [REDACTED]

Dear Professor Clancy,

**Re: Kathleen Megan Folbigg**

We advise that we act on behalf of Ms Kathleen Megan Folbigg in relation to the Inquiry ordered into her convictions by the NSW Governor on 22 August 2018.

### Report

We have been instructed to seek your expert opinion report in relation to the cause of death of Caleb Gibson Folbigg, Patrick Allen Folbigg, Sarah Kathleen Folbigg and Laura Elizabeth Folbigg. I draw your attention to the following Court Rules, which have been provided to you:

1. UCPR 31.23 Code of Conduct;
2. UCPR 31.27 Experts' reports; and
3. UCPR Schedule 7 – Expert witness code of conduct.

In order for the report to be of value in the current proceedings, your report must state:-

1. Your name and address.
2. That you comply with Regulation 31.27,
3. That as an expert, you have specialised knowledge based upon your training, study or experience set out in the report,

4. Sets out the opinion that you hold as an expert, and which is wholly or substantially based upon that specialised knowledge, and
5. Set out your reasons for your opinion, and to the extent that you have relied on any scientific study or other literature, refer to that literature by footnote or bibliography.
6. Attach a copy of this letter, the letter of instruction and its attachments to your report;
7. Complete and attach the Certificate - Expert Report;
8. Complete and attach the Expert Certificate, s 177 Evidence Act

### Attached Documents

We provide you the following documents:

1. Autopsy reports for Caleb, Patrick, Sarah and Laura Folbigg;
2. Report of Professor Duflou dated 13 February 2019;
3. Report of Professor Horne undated;
4. Report of Professor Hutchinson dated 17 April 2003;
5. Report of Dr Drucker dated 18 February 2003;
6. Handwritten notes from Allison Colley;
7. Relevant scientific literature.

### Questions

We refer to our conference with you today, 8 March 2019. It was during this conference that you agreed to prepare a report regarding mucosal immunology. We thank you in advance.

We undertake to be responsible for your professional fee.

Would you please address your tax invoice as follows:

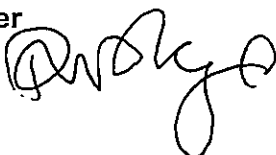
Kathleen Megan Folbigg  
CO/ Cardillo Gray Partners  
PO Box 409  
Newcastle NSW 2300

Should you have any questions or wish to discuss this matter please do not hesitate to contact us on (02) 4910 0677.

Yours Faithfully

**CARDILLO GRAY PARTNERS**

Stuart Gray  
Partner

per   
Enc:

## **Supplementary Report for the Inquiry into the Convictions of Kathleen Megan Folbigg**

1. My name is Professor Robert Llewellyn Clancy AM, BSc (Med) Hons, MB BS (Hons), PhD DSc, FRACP, FRCP(A), FRCP(C).
2. This report is further and addition to my report of 13 March 2019.
3. I have now been provided, on 15 March 2019, with microbiology reports for Sarah and Laura Folbigg. I had not seen these reports previously. I have read the expert code of conduct and have prepared this report in accordance with the code.
4. The microbiology reports that were provided to me are attached to this supplementary report. This more detailed information was not previously provided or known to me. It is very important to the formation of my opinion.
5. As outlined in my CV and DSc to my previous report, I was the Foundation Head of Clinical Immunology at Royal Prince Alfred Hospital. My 50 years of research has specifically focused on the relationship between infection at mucosal surfaces (especially the lung) and the immune response and protection mechanisms directed against those infections. I know and have worked with Doctors Benn and Mcleod, described in the documents as being part of the Department of Microbiology at Royal Prince Alfred Hospital, where the microbiological tests have been done. I have found both of these doctors to be competent and skilled professionals. In my studies and clinical practice, I have become familiar with the testing of autopsy samples, and the processes that are employed to preserve tissue for the purpose of microbiological testing. I have also had dealings with Professor Hilton, Senior Pathologist at Glebe mortuary, and the microbiology department at Royal Prince Alfred Hospital.

### **Review of Microbiology: Sarah**

6. A tissue biopsy of the lung in deceased persons is for the purpose of analysing the tissue and detection of infection in the small airways.
7. The bacteria of the type found in Sarah's lung tissue are commonly present in children that die unexpectedly and without any readily identifiable alternate cause of death. This is not unique, but rather characteristic of any disorder where the protective immune mechanisms in the airways are compromised. For example, individuals exposed to smoke (which is a noted risk factor for SIDS and ALTE).



8. A confident diagnosis of SIDS can be made as the cause of death in Sarah on clinical grounds. The microbiological report in my opinion adds confidence to this diagnosis by the finding of both streptococcal and staphylococcal species in the lung cultures. The pure growth of coliforms in the spleen, and absence of streptococcal and staphylococcal species in the colon cultures, argues against post-mortem contamination from the gut for the particular bacteria of streptococcal and staphylococcal species. While airway contamination from cardio-respiratory resuscitation is a possibility, I do not consider that to be a likely cause of the bacteria found here in the lung biopsy. Subjects with SIDS have defective immunity and airway clearance of bacteria, that would have descended from upper airway in life. Thus, in SIDS there is disordered transition of effective immunity in the airways which allows colonisation by bacteria to occur. This upheaval of the mucosal immune system in SIDS (well documented in the studies from my department, in the world's only prospective study of SIDS) is often triggered by an intercurrent virus infection. This severely disturbed immune status allows the bacteria to colonise, what would otherwise be a sterile environment. There is extensive precedent for this occurring in other disorders in humans, for example, smokers.
9. More recent studies suggest these bacteria can contribute to death through the production of toxins. While I believe this sequence of events occurred in Sarah Folbigg, bacterial detection at post-mortem in the lower airways is not essential for the diagnosis of SIDS.
10. Specific comments: (i) The samples taken at post-mortem of bacterial culture results would reflect the infection status at the time of post-mortem (as sterile tubes are used and samples kept at 4 degrees Celsius).
11. Specific comments: (ii) it appears the microbiologist was surprised and uncertain of the significance of the findings of streptococcal and staphylococcal in the lung tissue (perhaps not so surprised by the coliforms which may reflect gut contamination). This supports a pre-mortem presence of streptococcal and staphylococcal bacteria in the lower airways, having descended from the upper airways due to the defective immune status of the child. For clarification I note that the additional data on the microbiology report requested two days after the post-mortem, as the microbiologist attempted to clarify the findings he/she had noted. These subsequent cultures of the spleen grew coliforms suggesting contamination of that organ at this later time by gut bacteria.

## **Review of Microbiology: Laura**

12. I note that the lung and spleen were examined in Laura. I have been provided with documents relating to these test results.
13. I note that coliforms were found in the spleen, together with profuse alpha haemolytic streptococcus and moderate growth of staphylococcus which was eventually identified as aureus.
14. The microbiological reports are very different to those in her sister Sarah. (i) only coliforms (gut bacteria) were found in the lungs (in profuse growth). There were no streptococcus and staphylococcus, as found in Sarah. (ii) I agree with the microbiological report, that post-mortem contamination is likely to account for the coliforms present. (iii) The presence or absence of bacteria in the airways of Laura of whatever description is irrelevant to her diagnosis of myocarditis.

## **Conclusion**

15. The material that has been supplied to me does not cause me to alter my opinion as expressed in my report of 13 March 2019. However, having examined these original documents, I am of the opinion more likely than not, that bacteria (i.e. streptococcus and staphylococcus), identified in the cultures taken from Sarah's lung are not the result of contamination or from cardio-respiratory resuscitation. Rather, they represent colonisation of the lower airways by bacteria at a critical time of mucosal immune paresis, as has been documented in subjects with SIDS and the related condition of ALTE. Although not required for SIDS to occur, the presence of streptococcus and staphylococcus species in cultures of lung tissue add an important mechanism that could contribute to respiratory arrest, through the production of toxins as has recently been documented. Thus, the presence of these bacteria acquired prior to death due to a unique abnormality of impaired airways clearance reinforces the probability of SIDS as the cause of death in Sarah.
16. Analysis of the microbiology report in Laura contrasts with that of Sarah. Here there is a dominant finding of gut-derived bacteria in the spleen and lungs with a conclusion by the attending microbiologist that this likely represents post-mortem contamination. A conclusion with which I concur. In Laura, the cause of death in my view is myocarditis, and the microbiological findings neither support nor refute that diagnosis, which is based firmly on histological examination.

Robert Clancy.

Professor Robert Clancy

17 March 2019

# ROYAL PRINCE ALFRED HOSPITAL

NSW INS FORENSIC MEDICINE  
PO BOX 90  
GLEBE NSW 2037

Requested by: HILTON

Patient No: (2247)0045824

Name: FOLBIGG, SARAH  
Sex/Age: FEMALE UNKNOWN UNK

Location: NSW FORENSIC MED

## DEPARTMENT OF MICROBIOLOGY

Drs Benn, Kappagoda & Macleod Enquiries: 516 8278

### Virus

VIRUS: CULTURE

MB-93-53691

COLLECTED: 31AUG93

SOURCE: LUNG

RECEIVED: 31AUG93 1458

FINAL REPORT

NO VIRUS ISOLATED AFTER 10 DAYS. NO FURTHER REPORT WILL  
BE ISSUED UNLESS GROWTH OCCURS IN THE NEXT 2 WEEKS.

### Miscellaneous

TISSUE/BIOPSY CULTURE

MB-93-53691

COLLECTED: 31AUG93

SOURCE: LUNG

RECEIVED: 31AUG93 1458

FINAL REPORT

PROFUSE COLIFORM

PROFUSE STREPTOCOCCUS, ALPHA HAEMOLYTIC

SCANTY STAPHYLOCOCCUS AUREUS

TISSUE/BIOPSY CULTURE

MB-93-54258

COLLECTED: 02SEP93

SOURCE: SPLEEN

RECEIVED: 02SEP93 1109

FINAL REPORT

MODERATE COLIFORMS OF 3 COLONIAL TYPES

TISSUE/BIOPSY CULTURE

MB-93-54259

COLLECTED: 02SEP93

SOURCE: TISSUE

RECEIVED: 02SEP93 1109

LARGE INTESTINE

FINAL REPORT

MODERATE COLIFORMS OF 3 COLONIAL TYPES

MODERATE ENTEROCOCCUS FAECALIS

MODERATE DIPHTHEROIDS

TISSUE/BIOPSY CULTURE

MB-93-54260

COLLECTED: 02SEP93

SOURCE: TISSUE

RECEIVED: 02SEP93 1109

SMALL INTESTINE

FINAL REPORT

PROFUSE COLIFORMS OF 2 COLONIAL TYPES

# ROYAL PRINCE ALFRED HOSPITAL

NSW INS FORENSIC MEDICINE  
PO BOX 90  
GLEBE NSW 2037

Requested by: HILTON

Patient No: (2247)0045824

Name: FOLBIGG, SARAH  
Sex/Age: FEMALE UNKNOWN UNK

Location: NSW FORENSIC MED

## DEPARTMENT OF MICROBIOLOGY

Drs Benn, Kappagoda & Macleod. Enquiries: 516 8278

### Virus

VIRUS CULTURE MB-93-53691 COLLECTED: 31AUG93  
SOURCE: LUNG RECEIVED: 31AUG93 1458

FINAL REPORT

NO VIRUS ISOLATED AFTER 10 DAYS. NO FURTHER REPORT WILL  
BE ISSUED UNLESS GROWTH OCCURS IN THE NEXT 2 WEEKS.

### Miscellaneous

TISSUE/BIOPSY CULTURE MB-93-53691 COLLECTED: 31AUG93  
SOURCE: LUNG RECEIVED: 31AUG93 1458

FINAL REPORT

PROFUSE COLIFORM  
X PROFUSE STREPTOCOCCUS, ALPHA HAEMOLYTIC  
SCANTY STAPHYLOCOCCUS AUREUS

TISSUE/BIOPSY CULTURE MB-93-54258 COLLECTED: 02SEP93  
SOURCE: SPLEEN RECEIVED: 02SEP93 1109

FINAL REPORT

MODERATE COLIFORMS OF 3 COLONIAL TYPES

TISSUE/BIOPSY CULTURE MB-93-54259 COLLECTED: 02SEP93  
SOURCE: TISSUE RECEIVED: 02SEP93 1109  
LARGE INTESTINE

FINAL REPORT

MODERATE COLIFORMS OF 3 COLONIAL TYPES  
MODERATE ENTEROCOCCUS FAECALIS  
MODERATE DIPHTHEROIDS

TISSUE/BIOPSY CULTURE MB-93-54260 COLLECTED: 02SEP93  
SOURCE: TISSUE RECEIVED: 02SEP93 1109  
SMALL INTESTINE

FINAL REPORT

PROFUSE COLIFORMS OF 2 COLONIAL TYPES

# CENTRAL SYDNEY LABORATORY SERVICE

NSW INS FORENSIC MEDICINE  
PO BOX 90  
GLEBE NSW 2037

Requested by: CALA, A

Patient No: (2247)0999322

Name: FOLBIGG, LAURA  
Sex/Age: FEMALE 01/01/98 14 MOS

Location: NSW FORENSIC MED

## DEPARTMENT OF MICROBIOLOGY

Dr R Benn, Dr C MacLeod. Enquiries: 9515 8278

### Fluids

CSF CULTURE

MB-99-014698

COLLECTED: 02MAR99

SOURCE: CEREBROSPINAL FLUID

RECEIVED: 02MAR99 0904

PRELIMINARY

No growth overnight.  
Further incubation in progress.

### Miscellaneous

TISSUE/BIOPSY CULTURE

MB-99-014696

COLLECTED: 02MAR99

SOURCE: SPLEEN

RECEIVED: 02MAR99 0904

PRELIMINARY

Moderate coliforms of 2 colonial types  
Profuse alpha haemolytic Streptococcus  
Moderate Staphylococcus epidermidis (presumptive)  
Further incubation in progress.

Printed: 03MAR99 1327

\*\*\* End of Report \*\*\*

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Ref No. MB-99-014696

# CENTRAL SYDNEY LABORATORY SERVICE

NSW INS FORENSIC MEDICINE  
PO BOX 90  
GLEBE NSW 2037

Requested by: CALA, A

Patient No: (2247)0999322

Name: FOLBIGG, LAURA  
Sex/Age: FEMALE 01/01/98 14 MOS

Location: NSW FORENSIC MED

## DEPARTMENT OF MICROBIOLOGY

Dr R Benn, Dr C MacLeod. Enquiries: 9515 8278

### Fluids

CSF CULTURE MB-99-014698 COLLECTED: 02MAR99  
SOURCE: CEREBROSPINAL FLUID RECEIVED: 02MAR99 0904

PRELIMINARY

No growth overnight.  
Further incubation in progress.

### Faeces

STOOL CULTURE MB-99-014699 COLLECTED: 02MAR99  
SOURCE: RECTAL SWAB RECEIVED: 02MAR99 0918

AMENDED REPORT

Profuse normal enteric flora isolated.  
No Salmonella/Shigella/Campylobacter isolated.

### Miscellaneous

TISSUE/BIOPSY CULTURE MB-99-014695 COLLECTED: 02MAR99  
SOURCE: LUNG RECEIVED: 02MAR99 0904

PRELIMINARY

Profuse Post mortem contaminants.  
Further incubation in progress.

TISSUE/BIOPSY CULTURE MB-99-014696 COLLECTED: 02MAR99  
SOURCE: SPLEEN RECEIVED: 02MAR99 0904

PRELIMINARY

Moderate coliforms of 2 colonial types  
Profuse alpha haemolytic Streptococcus of 2 colonial types  
Moderate Staphylococcus aureus (presumptive)  
Identification and susceptibilities proceeding.

Printed: 04MAR99 1326

\*\*\* End of Report \*\*\*

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# CENTRAL SYDNEY LABORATORY SERVICE

NSW INS FORENSIC MEDICINE  
PO BOX 90  
GLEBE NSW 2037

Requested by: CALA, A

Patient No: (2247)0999322

Name: FOLBIGG, LAURA  
Sex/Age: FEMALE 01/01/98 14 MOS

Location: NSW FORENSIC MED

## DEPARTMENT OF MICROBIOLOGY

Dr R Benn, Dr C MacLeod. Enquiries: 9515 8278

### Fluids

CSF CULTURE

MB-99-014698

COLLECTED: 02MAR99

SOURCE: CEREBROSPINAL FLUID

RECEIVED: 02MAR99 0904

FINAL REPORT

No growth

### Faeces

STOOL CULTURE

MB-99-014699

COLLECTED: 02MAR99

SOURCE: RECTAL SWAB

RECEIVED: 02MAR99 0918

AMENDED REPORT

Profuse normal enteric flora isolated.  
No Salmonella/Shigella/Campylobacter isolated.

### Miscellaneous

TISSUE/BIOPSY CULTURE

MB-99-014695

COLLECTED: 02MAR99

SOURCE: LUNG

RECEIVED: 02MAR99 0904

FINAL REPORT

Profuse Post mortem contaminants.  
Profuse coliform

Printed: 09MAR99 1327

Continued ...

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# CENTRAL SYDNEY LABORATORY SERVICE

NSW INS FORENSIC MEDICINE  
PO BOX 90  
GLEBE NSW 2037

Requested by: CALA, A

Patient No: (2247)0999322

Name: FOLBIGG, LAURA  
Sex/Age: FEMALE 01/01/98 14 MOS

Location: NSW FORENSIC MED

## DEPARTMENT OF MICROBIOLOGY

Dr R Benn, Dr C MacLeod. Enquiries: 9515 8278

### Miscellaneous

TISSUE/BIOPSY CULTURE  
SOURCE: SPLEEN

MB-99-014696

COLLECTED: 02MAR99  
RECEIVED: 02MAR99 0904

#### FINAL REPORT

Moderate coliforms of 2 colonial types  
Profuse alpha haemolytic Streptococcus of 2 colonial types  
Moderate Staphylococcus aureus

#### SUSCEPTIBILITIES

##### S. aureus

	<u>MIC</u>	<u>INTERP</u>
Penicillin	<=0.125	S
Flucloxacillin	<=4	S
Cefazolin	<=4	S
Eryth/Clinda	<=1	S

Printed: 09MAR99 1327

\*\*\* End of Report \*\*\*

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