AIRWAY DIMENSIONS IN THE SUDDEN INFANT DEATH SYNDROME

BY

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DEDICATION

This work is dedicated to my wife Joanna, without whose love and encouragement this undertaking would not have happened.

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PREFACE

This thesis represents work which forms part of ongoing studies into infant mortality due to SIDS. The first two results chapters (Chapter4 and Chapter 5) have been published in the American Journal of Respiratory and Critical Care Medicine (AJRCCM). Chapter 4 is In Press (1999) and Chapter 5 AJRCCM 1998, 158:802-806. Chapter 6 is currently under review by the American Journal of Respiratory and Critical Care Medicine. As such I have decided to include these chapters as they were prepared for submission to the journal. This has resulted in a degree of repetition particularly with regard to methodology. However I feel that presenting the work in this way would maintain continuity and flow.

ABSTRACT

Sudden Infant Death Syndrome (SIDS) is the major cause of death in infants during the first year of life. SIDS describes the sudden unexpected death of an apparently well infant. A SIDS diagnosis is one of exclusion, that is, death cannot be explained by history, circumstance or a specific pathological condition. The underlying pathophysiological mechanism resulting in death in SIDS is unknown. Although a number of abnormalities in several organ systems have been associated with SIDS, no specific diagnostic pathology has been identified. Epidemiological data suggests a range of environmental and physiological conditions are associated with an increased risk of death from SIDS. These include frothy fluid in the airways, nose and mouth, intrathoracic petechial haemorrhage, delayed dendritic spine maturation and delayed myelination of regions of the central nervous system and vagus nerves. The peak incidence of SIDS occurs between 6 to 11 weeks of life with 90% of SIDS deaths occurring by 6 months of age. Death is usually uncommon in the first 1 to 2 weeks of life. Ethnicity, low birth weight, short gestation, socioeconomic background, excessive clothing and bedding, reduced amount of breast feeding, a prone sleeping position, co-sleeping, a winter peak, maternal use of narcotics and maternal cigarette smoking have all been associated with an increased risk of SIDS. Among the risk factors, an association between maternal smoking and an increased risk of SIDS has been clearly identified. A number of studies have demonstrated that *in-utero* cigarette smoke exposure is related to abnormalities in lung function in newborn infants. However, despite these findings the actual mechanisms resulting in abnormal post-natal lung function following *in-utero* cigarette smoke exposure is unknown. Small airway

closure has been hypothesized as a potential mechanism of death in SIDS and may be related to airway pathology. This thesis examines the hypothesis that small airway closure could be due to altered lung/airway structure in infants who die of SIDS in response to maternal cigarette smoke exposure.

We obtained lung tissue from infants dying with and without SIDS which had been obtained at autopsy from the Victorian Department of Forensic Medicine. Cases were identified from a previous epidemiological study conducted by the Victorian Sudden Infant Death Research Foundation from 1991 to 1993. In this study mothers whose infants had died from SIDS and were asked to indicate whether they smoked before becoming pregnant and separately during the first, second, and third trimester of pregnancy. Mothers were also asked whether they smoked between the birth and death of their child. For all questions, smoking was rated on a 5 point scale with 0) reflecting no smoking, 1) less than 10 cigarettes a day, 2) 10-20 cigarettes a day, 3) 20-30 cigarettes a day and 4 a smoking habit of greater than 30 cigarettes a day.

Morphometric analysis was performed on tissue obtained from 57 infants who died from SIDS and compared with 21 age-matched infants who had died from causes other than SIDS. To increase the number of control cases, 8 cases from a study in Western Australia where SIDS was not the cause of death were included. Sections of tissue had been obtained in a standardised way as part of a protocol for the pathological examination of all (including SIDS) deaths. Identical methodologies were used in both studies to measure airway dimensions. Transverse sections of airway stained with haematoxylin and eosin

were examined using a video-linked microscope linked to an on-line image analysis system. Airway wall dimensions included measurements of the inner and outer airway wall thickness and the areas occupied by smooth muscle, mucous glands and cartilage within the airway wall. The number of alveolar attachments to each airway were also measured. In addition, a prospective study of airway dimensions and alveolar attachment points was conducted in neonatal guinea pigs with and without *in-utero* cigarette smoke exposure.

Airways from infants who died from SIDS showed a significantly higher proportion of airway smooth muscle than control airways when corrected for age and sex ($p<0.01$). There was no significant difference between the groups for wall thickness, epithelial thickness or areas of mucous glands or cartilage. Increased airway smooth muscle in infants who die from SIDS might lead to excessive airway narrowing raising the possibility that the cause of death in this condition might be related to abnormal airway function.

In a separate analysis, we examined airway dimensions in 19 children who died from SIDS whose mothers smoked more than 20 cigarettes a day both prenatally and post-natally and compared these data with those from 19 infants who died from SIDS and had non smoking mothers. Inner airway wall thickness was greater in the larger airways of those infants whose mothers had smoked more than 20 cigarettes a day. These findings suggest that infants passively exposed to high levels of cigarette smoke develop significant structural changes in their airways. Increased airway wall thickness may contribute to exaggerated airway narrowing and may help explain the observed abnormalities in neonatal lung

function which have been described in infants of smoking mothers. We also found that the number of alveolar attachment points connected to the airway adventitia was decreased in SIDS cases whose mothers smoked compared to those who did not. If a decreased number of alveolar attachments leads to a reduced effect of lung elastic recoil on the airway then this raises the possibility that this may also contribute to altered airway function in these infants.

To further test this hypothesis we used an animal model of *in-utero* smoke exposure to examine the effects of *in-utero* smoke exposure on post-natal lung function and airway and lung morphology in a group of neonatal guinea pigs 21 days after delivery. Pregnant guinea pigs were exposed to cigarette smoke from day 28 to term (day 68 of gestation). Following delivery newborn animals did not receive any smoke exposure. Airway wall thickness, smooth muscle area and the number of points where the alveoli attached to the airway adventitia were measured. Airway responsiveness was increased ($p<0.05$) 6 fold and the mean number of alveolar attachment points per mm of the outer perimeter of the airway was decreased ($p<0.05$) (mean 19.4 ± SE 0.41 v 21.6 ± 0.81) in animals exposed to cigarette smoke *in-utero* compared with non-exposed animals. Although not statistically significant, both the inner and outer airway wall and the smooth muscle area were greater in exposed animals compared with non-exposed animals. These findings suggest that the increased airway responsiveness observed in post-natal animals, subsequent to *in-utero* cigarette smoke exposure, may be the result of decreased alveolar attachment points to the airways combined with subtle changes in airway wall dimensions.

It is concluded that structural changes in the airway, which may mechanically increase airway responsiveness, occur in the small airways in infants dying of SIDS. The structural changes include increased airway smooth muscle and an increase in the thickness of the inner airway wall in infants who are exposed to maternal smoking. Whether these changes are confined to small airways only or are also seen in larger airways is not known. No evidence of exaggerated smooth muscle shortening was seen in the SIDS cases however whether this is an effect of the post mortem process or not is not known. These findings also emphasise the dangers of passive cigarette smoke exposure to infants and highlight the importance of anti-smoking measures aimed at reducing the changes in airway and lung structure that have been observed in these cases.

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CHAPTER 1 -INTRODUCTION

THE SUDDEN INFANT DEATH SYNDROME (SIDS)

The sudden death of infants has been documented throughout history and was previously known as "overlay". The reference to the overlaying of an infant goes as far back as the Old Testament. It was not until the 18th century that the medical fraternity started to recognize the condition of the sudden unexpected and unexplained death of an infant. Sudden Infant Death Syndrome (SIDS) has no specific pathological characteristics that can be used as a means of a positive diagnosis. SIDS is the major cause of death in infants during the first year of life National Institute of Child Health and Human Development (1990). Epidemiological data has shown associations with specific maternal and social practices and an increased risk of SIDS. However, how these practices result in death is still unknown.

Autopsy studies have observed a number of pathophysiological differences in some infants who have died from SIDS. However the underlying pathological cause of death remains uncertain. Abnormalities have been identified in several organ systems including the nervous and respiratory systems, and in the pulmonary circulation of some infants dying of SIDS. The direct relationship between these changes and the actual mechanism of death remains unclear.

The tragedy of an infant's sudden unexpected death can be dramatic for the family and all community members who are affected by the death. SIDS occurs in all socioeconomic groups and the infants dying of SIDS appear to be healthy prior to death. The eradication

of this most puzzling mystery of medical science has been paramount for scientific researchers for many years. Whilst the numbers of SIDS deaths have fallen in recent years the mechanism of death is still unexplained. While the avoidance of some risk factors for SIDS may reduce the incidence of SIDS, no specific risk factor has been shown to be directly related to the cause of death. As we discuss various risk factors associated with SIDS, it is however, important to understand SIDS is not a result of abuse or neglect.

THE MODERN DEFINITION

The modern definition was agreed in the United States at the Second International Conference in 1969 on sudden death in infants and the term "*Sudden Infant Death Syndrome*" (SIDS) was adopted. The definition is "The sudden death of an infant, that was unexplained by history and in which a thorough postmortem examination failed to demonstrate an adequate cause of death". This definition is still widely accepted. In 1989 NICHD issued a statement suggesting that the SIDS diagnosis be limited to infants under one year of age as approximately 98% of sudden infant death cases occurred in infants under the age of one year and the inclusion of infants above this age was likely to represent a different group. The panel also recommended an investigation of the death scene as a means of excluding environmental factors and any suspicious circumstances (1).

As there are no categorical features of SIDS that may be utilized as criteria for a positive diagnosis the process of exclusion remains the only means possible of a positive diagnosis. Unfortunately, this can vary between investigator, time and place. The comparison of data is limited as a result of this procedure.

INCIDENCE

There were 27 cases of SIDS confirmed in Victoria in 1997 and in 1998, forty three cases in 1996 and 40 cases in 1995 compared with the 137 cases in 1989 at the onset of the "Reduce the risk" campaign. These figures highlight a marked decline of nearly 80% in SIDS deaths across this time period (2). SIDS deaths represent 0. 44% of every 1000 deliveries or 1 SIDS death in every 2270 live births in the state of Victoria in 1997.

AIRWAY STRUCTURE AND SIDS

Changes in pulmonary function, thought to be due to airway inflammation and structural changes as a result of smoking are well documented (3-5). Changes in airway function may be acute in response to damage to airway components and cells, stimulation of smooth muscle shortening or increased mucus secretion. On the other hand, changes in airway function may be the result of chronic changes such as remodelling of the airway wall, hypertrophy of airway components, and collagen deposition within the airway, all of which might lead to altered airway function. Airway inflammation may be a response to inhaled environmental pathogens or viral infections which are readily identified or result from endogenous stimulation of the immune system by unknown factors. To what extent these pathways are acting in the lungs of SIDS victims and whether they alter airway structure, function and maturation is largely unknown.

There are few studies which have examined lung and/or airway pathology in SIDS. Fewer still have looked at the relationship between maternal smoking, airway structure and SIDS.

Morphometric assessment of the lungs allows us to quantify the pathology of the airways and provides us with a reasonable interpretation of airway structure. It also provides us with important information on the nature of the airway at the time of death.

Cellular hypertrophy and hyperplasia, inflammatory infiltrates, airway wall remodelling and destruction of the lung parenchyma are pathological changes that are seen in various disease states. Fibrosis of the airway wall and damage to the lung parenchyma leads to loss of elastic recoil and is commonly associated with smoking. Hypertrophy and hyperplasia of the airway smooth muscle may lead to increased airway responsiveness to non-specific bronchoconstricting stimuli as is seen in asthma. Inflammatory cell infiltration and activation in the airway wall can result in the release of inflammatory mediators which cause increased vascular permeability and airway oedema. Oedema fluid may alter epithelial permeability thereby limiting the epithelium's ability to act as an effective barrier to inhaled pathogens.

Modelling the effects of changes in airway dimensions have shown that a small increase in the thickness of the inner airway wall can lead to a dramatic increase in airway resistance when smooth muscle shortens (6) while thickening of the outer airway wall might reduce the distending forces of the lung parenchyma and decrease the loads opposing smooth muscle when it shortens (7) .

Haque *et al* (8) examined 25 cases of SIDS and 18 controls in their study of airway morphometry in SIDS. They examined over 1,000 airways, predominantly membranous

bronchioles from SIDS cases and found a significantly increased index of airway wall thickness compared with control cases.

Martinez (9) has postulated that small airway closure could be associated with SIDS. The delayed development of parenchyma and its attachment to the airway wall in the postnatal period relative to the fully mature bronchial tree has been suggested as a possible cause of small airway occlusion in SIDS. Delayed development may be an explanation for the findings of Doershuk *et al* (10) who demonstrated that during infancy peripheral airway conductance falls after birth, is lowest between 31 to 60 days of age and then gradually increases. Tepper (11) similarly found, using pulmonary function tests, that the maximal respiratory flow at functional residual capacity (VmaxFRC) fell from birth to 1 month of age before improving again. Stocks and Godfrey (12) found that premature infants had reduced airway conductance at birth, which continued to fall in the first month after birth. They suggested that postnatal lung development was similar in premature infants compared with babies born at full-term. McFawn and Mitchell (13) observed a significant change in bronchial distensibility in the early postnatal development in pigs. Tepper (11) found that female infants had higher flow rates when corrected for lung size in the first year of life compared with males. This may be related to the association with male gender and the SIDS age peak of 6 to 11 weeks.

Ninety percent of SIDS cases occur by 6 months of age, a period in which reduced airway conductance is seen in infants. To what extent this results from altered airway function during this age period is unclear.

MATERNAL SMOKING AND LUNG FUNCTION

Maternal cigarette smoking is known to be a significant health risk to infants (14). Infants exposed to passive cigarette smoking in the first year of life have an increased incidence of lower respiratory tract infections (15, 16). *In-utero* cigarette smoke exposure has been shown to produce abnormalities in lung function in newborn infants. While *in-utero* smoke exposure is a unique form of passive smoke exposure in that there is no direct exposure of the foetal lung to cigarette smoke, there is still evidence for altered lung function in these infants. Tager *et al* (17) showed that infants born to mothers who smoked during pregnancy had approximately 10% reduced expiratory flow parameters when compared to infants whose mothers did not smoke during pregnancy. Young *et al* (18) showed that infants whose mothers smoked during pregnancy had increased airway responsiveness to inhaled histamine 4 weeks after delivery. In epidemiological studies, exclusion of the effects of any post-natal smoke exposure is difficult, however the use of statistical methods, in which these confounding variables are adjusted for, suggests that increased responsiveness in these infants is primarily associated with *in-utero* cigarette smoke exposure (19). Despite these conclusions the actual mechanisms resulting in abnormal post-natal lung function following *in-utero* cigarette smoke exposure are unknown. Saetta *et al* (20) have previously shown that number of alveolar attachment points to the surrounding airway adventitia are reduced in active smokers and that this reduction is associated with a reduction in lung elastic recoil. Several studies (19, 21-23) have reported reduced respiratory function at birth in infants whose mothers smoked during pregnancy and propose altered lung/airway development *in-utero* as a likely mechanism although no

attempts were made to test these hypotheses.

SMOKING AND LUNG DISEASE

The harmful effects of cigarette smoking have been well documented. The functional characteristics of chronic obstructive pulmonary disease (COPD) caused by cigarette smoking are air-flow limitation, reduced lung elastic recoil and increased airway hyperresponsiveness. Functional abnormalities seen in COPD may be a result of pathological changes in the structure of the airways and lung. These changes are characterised by; (1) airway remodelling (ie. inflammation and fibrosis of the airway wall and mucus gland proliferation), (2) destruction of the lung parenchyma and a reduced amount of elastic fibres. The destruction of alveolar walls observed in patients with moderate to severe emphysema associated with cigarette smoking is associated with airflow obstruction, increased airway hyperresponsiveness and reduced elastic recoil of the lung. Saetta *et al* (20) have previously shown a reduction in the number of alveolar attachment points, an increase in the distance between attachments, and an increase in the percentage of abnormal attachments in cigarette smokers compared with nonsmokers. This reduction was related with the score for airway inflammation and with reduced elastic recoil pressure in smokers. Airway responsiveness was not assessed in this study. Willems *et al* (24) have shown a causal relationship between the airway disease process and parenchymal destruction. Inflammatory processes have been implicated in the destruction of the lung parenchyma. It has been shown that inflammatory cells are capable of releasing elastolytic enzymes which can cause parenchymal destruction (emphysema). Eidelman *et al* (25) has reported hypercellularity of the lung parenchyma in cigarette smokers. Petty *et al* (26) have

also shown that in patients with mild emphysema, elastic recoil pressure is reduced but that this is not associated with air-flow limitation.

It is apparent from the literature that there are a number of potential mechanisms by which environmental factors might interact in the first few months of life to increase the risk of death. These factors include socio-economic factors, cigarette smoke exposure, viral respiratory infection and those related to the age of the infants, such as:

(1) Immaturity / development of central nervous system.

The central nervous system continues to develop post-natally and reduced arousal mechanisms or respiratory control mechanisms may result in inappropriate or absent responses to harmful stimuli. Instability of respiratory control may result in intermittent and prolonged apneas, either spontaneously or in response to noxious stimuli.

(2) Immaturity / development of the immune system.

The immune system develops intra-natally and post-natally and abnormalities of development may be responsible for allergies or inappropriate responses to antigens. This is a relatively new area of research with little data related to SIDS infants.

(3) Immaturity / development of the lung.

Underdevelopment of the lung may predispose the infant to excessive airway narrowing or inflammation in response to respiratory infection. Exposure to cigarette smoke *in-utero* or in the early post-natal period may contribute to abnormal airway structure and function.

Increased loads on the respiratory system, possibly associated with poor ventilatory control, may predispose infants to apnea or asphyxia.

The overall aim of this thesis is to examine differences in airway structure in infants dying from SIDS and from other causes and to examine the relationship between airway structure and cigarette smoke exposure in cases of SIDS. Finally, using an animal model, the effect of *in-utero* cigarette smoke exposure on airway structure post-natally was examined.

AIMS

The specific aims of this study were;

1) To examine the small airways structure of SIDS and compare them with controls.
2) To examine the effects of maternal smoking on airway structure in SIDS.
3) To test the effects of *in-utero* smoke exposure on airway hyperresponsiveness and airway structure in an animal model.

HYPOTHESES

The hypotheses of this study are;

- Altered small airway structure is present in infants dying of SIDS.
- Altered small airway structure is present in infants dying of SIDS, as a result of exposure to maternal cigarette smoke.

- Abnormalities of the small airways in SIDS may produce exaggerated airway contraction.
- Altered post-natal lung function and airway hyperresponsiveness observed following in- utero cigarette smoke exposure are due to altered lung/airway structure in a small animal model.

CHAPTER 2 - LITERATURE REVIEW

EPIDEMIOLOGY OF SIDS

The epidemiological study of SIDS is difficult because the majority of the observed risk factors are interrelated. The majority of studies that have been undertaken have been retrospective studies, not hypothesis based and so may have recall bias effects. Confounding this also is the dramatic fall in the rate of SIDS since the late 1980s.

The "Reduce The Risks" (RTR) campaign highlighted specific risk factors associated with SIDS and has led to a significant reduction in the rate of SIDS death. The campaign also encouraged breast-feeding as a means of reducing risks.

A possible shift in the classification of SIDS over time may have caused a reduction in the overall numbers of SIDS cases reported (1, 27). An overall fall in sudden postnatal deaths in South Australia in this period suggests that a diagnostic shift is not responsible for the reduction in the rates of SIDS deaths (28).

A possible result of the RTR campaign is that the epidemiological characteristics of the remaining SIDS cases may have changed. Sawaguchi *et al* (29) reported a statistically significant increase in SIDS death in Japan and Hong Kong in contrast to western countries. Post-neonatal mortality in infants between the ages of 1-5 months fell, suggesting that the increased rates of SIDS deaths could be due to a change in diagnostic characteristics.

The decrease in SIDS deaths may have led to changes in the epidemiological characteristics

of the remaining SIDS deaths. Nevertheless the relevance of work compiled before the introduction of RTR has demonstrated a number of key associations which have led to the dramatic decline in SIDS numbers.

Many factors have been associated with SIDS. Some of these factors were first reported in the last century in Dundee, Scotland (30). The poor social conditions of the time were reflective of what we now regard as high-risk practices. Cases reported as being accidental overlaying by the parents were frequent, as bed sharing was common place at that time (30). As with most modern studies, a winter and age peak was evident and is still a consistent characteristic of SIDS. Other associations and risk factors relating to the high prevalence of SIDS include a higher male to female ratio, an association with premature birth, low birth weight, low socioeconomic status, prone sleeping and parental cigarette smoking.

PREMATURITY AND LOW BIRTH WEIGHT

Many authors have reported an association between prematurity, low birth weight, and SIDS (31-38). This association is independent of the length of gestation, suggesting a sub-optimal *in-utero* environment may be important in the development of the foetus (31, 35, 36). Taylor and Sanderson (37) reported that this association disappeared when SIDS deaths were compared with post-neonatal deaths in the first year of life. However, maternal smoking remained independently associated with SIDS and may be the primary cause of the association with low birth-weight and prematurity.

BREAST FEEDING

It has been postulated that breast-feeding has a protective role against SIDS. Mitchell *et al.* (39) found that after adjusting for other risk factors, a significant association between breast-feeding and reduced risk of SIDS existed. Golding (40) was unable to reproduce Mitchell's findings and concluded that other previous studies had failed to adjust for other confounding factors (eg. maternal smoking and viral respiratory infections). Breast feeding also reduces the risk of RSV infections (41). Brooke and Gibson (36), after adjusting for maternal smoking, concluded that maternal smoking was a more important issue than the protective effects of breast-feeding.

GENDER

A male to female ratio of approximately 1.6:1.0 has been reported in the literature, compared with non-SIDS post-natal deaths, which affect the sexes equally. The small disparity of male to female births -1.05:1.0- does not account for this excess in male to female deaths (33, 36).

AGE AT DEATH

Age at death is the most consistent factor in SIDS and is consistent internationally and is independent of other factors such as ethnicity, birth weight, gender, seasons and maternal age (33, 39, 42-53). SIDS peaks at 6 to 11 weeks and 90% of cases occur by 6 months however death is uncommon in the first 1 to 2 weeks of life (53, 54).

FAMILY SIZE AND BIRTH ORDER

An increase in maternal parity has been reported by many authors to increase the rate of SIDS (32, 45, 46, 55-60). Increased parity has an effect on infant mortality and is increased with young mothers (30). Spiers *et al.* (61) reported that larger families have a greater risk of SIDS and within the family the first born child is at greatest risk while the risk diminishes relative to the first born. Spiers concluded that the risk of SIDS was related to sibship not maternal parity, and may reflect the negative correlation between socioeconomic status and family size.

BEDDING

Kemp *et al* (62) examined whether bedding used by infants who are at risk of SIDS differed in physical properties, which may favour rebreathing of exhaled gases. They concluded that sociodemographic factors dictated use of bedding that had physical properties which may favour rebreathing.

TEMPERATURE IN BED

Bed temperature, excess clothing and bedding have been identified as independent risk factors for SIDS and may be implicated in the death of the older infants (63, 64). Overheating may be also associated with external temperature, which has been demonstrated to be a significant risk factor for SIDS. Infants are at greatest risk of SIDS during warmer minimum temperatures or when small hourly variation in temperature is recorded (65). Fleming *et al* (66) later suggested that overheating may be associated with poorer thermoregulation and increase in the body mass to surface area ratio of the older

infants.

CO-SLEEPING

A case-control study in New Zealand examined the association of co-sleeping and SIDS (67). SIDS infants were significantly more likely to co-sleep and have a smoking mother. Conversely the risk of SIDS was significantly reduced if the infant slept alone in a cot in the same room as the parents, given the parents did not smoke.

PRONE SLEEPING

The association between prone sleeping and SIDS has been well documented (68). SIDS is relatively rare in countries where prone sleeping is uncommon (69). The reduction in the prevalence of prone sleeping has made a significant reduction in the incidence of SIDS (63, 67, 70-74). Scragg and Mitchell (75) suggest that a further substantial decrease in SIDS death could be achieved if infants were placed on their backs to sleep. It is likely that the increase of infant back sleeping contributed to the fall in the number of SIDS cases, however, the reduction of other risk factors associated with SIDS will have also contributed significantly.

ETHNICITY

A number of epidemiological studies have examined the relationship between ethnic groups and the incidence of SIDS. These studies suggest that the difference in the rates of SIDS reflect the prevalence of risk factors rather than any ethnic predisposition. Bulterys (76) examined the rate of SIDS in native north Americans from different demographic regions

and determined that the difference in the rates of SIDS was possibly due to the variation in the socioeconomic status and prenatal care. After adjusting for these various factors, maternal smoking was the only significant factor between the different regions. The rate of SIDS in the Caucasian community in the southwestern region was the same as in the indigenous community from the same region. This suggests that environmental factors rather than ethnicity may explain the relatively high rates in the same racial groups. Kraus *et al.* (31) demonstrated that, after adjusting for socioeconomic status and maternal education, the previously higher rates of SIDS deaths among black infants disappeared.

WINTER PEAK

In some centres, a winter peak has been observed in deaths occurring in infants over two months of age ((32, 53, 65, 69, 77-79). However this has not been observed in Scandinavian studies (77). A winter peak may reflect an increase in viral respiratory infection in the general community in this time period however this relationship has not been consistently demonstrated (66). Deacon (80) demonstrated that in the northern region of North America, there was an increased incidence of SIDS compared with southern regions. Similarly, incidence of death in mid summer in South Australia was 0.7 per 1,000 live births while in Tasmania the incidence was 6.3 per 1,000 live births in mid winter (77). Schluter *et al.* (65) confirmed the seasonality of SIDS in a retrospective epidemiological study in New Zealand from 1968 to 1989. They also observed that on days that showed little change in hourly temperature and on days with warmer minimum temperatures there was a significantly increased rate of SIDS.

SOCIOECONOMIC STATUS

Many authors have reported an association with various indexes of low socioeconomic status and SIDS. Ford and Nelson (81) suggests that the observed reduction in SIDS deaths since the late 1980's have occurred in the middle to upper income groups and that low income groups are three times more at risk of SIDS than middle to upper income groups. The suggestion that maternal smoking may confound this association is supported by Taylor and Sanderson (37) who compared factors associated with post-neonatal death in the first year of life with factors associated with death from SIDS. After adjusting for low level of education and maternal smoking, maternal smoking continued to be significantly more common amongst SIDS, leading the authors to conclude that maternal smoking is the only factor independently associated with an increased risk of SIDS.

MATERNAL NARCOTIC USE

An association between maternal narcotic use and SIDS has been shown in the literature (82, 83). Whilst maternal narcotic use increases the risk of SIDS it would be difficult to assess other confounding factors such as low socioeconomic status, maternal care and maternal smoking. This association has not been demonstrated independently of these other factors.

ALLERGY AND THE RISK OF SIDS

It has been postulated that a link between allergy and SIDS may exist (84-86). However this has not always been a consistent finding. A study conducted in New Zealand examined rates of eczema in a nationwide case-control study covering 78% of all births from 1987-

90. The authors concluded that infants with parental diagnosed eczema had a low risk of SIDS (81). Other studies have examined tryptase levels and allergen-specific IgE in SIDS with varying findings (87, 84). Mast cell degranulation and elevated concentrations of tryptase in blood serum has been reported in some cases of SIDS (86).

MATERNAL CIGARETTE SMOKING

Since 1966 maternal cigarette smoking has been recognized as a statistically significant risk factor associated with the increase risk of SIDS (88). In later years it has emerged as one of the most important risk factors. Cooke (38) examined the link between maternal cigarette smoking and low birth-weight in a case control study of 104 SIDS infants and 206 controls. Using a birth-weight ratio (BWR), head circumference ratio (OFCR) and a growth retardation ratio (GRR = OFCR / BWR). Cooke found no significant differences between SIDS and controls. However, classifying the infants into those exposed to cigarette smoke and those not, they found a significant growth retardation in the smoke exposed infants. The non-smoke exposed SIDS cases as a group were not growth retarded, but had a lower gestation period to birth. The author concluded that the risk associated with growth retardation and SIDS may be accounted for by maternal cigarette smoking.

Taylor and Sanderson (37) concluded that maternal smoking, when adjusted for low levels of maternal education, was the only independent risk factor associated with SIDS. Since Steele and Langworth's (88) article other authors have identified maternal smoking as a risk factor for SIDS. Lewak (89) and McGlashan (32) observed that smoking tended to be more common amongst low socioeconomic groups but neither author adjusted for this

factor. Other researchers, who have tried to adjust confounding factors such as socioeconomic status, birth weight, age and race by selecting appropriate controls, similarly found that mothers of victims of SIDS's were more likely to smoke (54, 67, 79, 90, 91).

A dose-response relationship between the amount smoked and the risk of SIDS has implicated a biologically causal role (88, 67, 79, 90). The contribution of *in-utero* and postnatal cigarette smoke exposure is hard to examine because women who smoke during pregnancy will tend to smoke after birth (92). Mitchell reported that infants of parents who smoked, but not in the house were still at a statistically significantly increased risk of SIDS. This suggests that the increased risk of SIDS may be primarily due to the effect of *in-utero* cigarette smoke exposure. Haglund *et al.* (90) reported that the risk of SIDS associated with maternal smoking did not vary with season. They suggested that infants were more likely to be exposed to passive cigarette smoke in winter suggesting that the effect of smoking is *in-utero* rather than postnatal. A study by DiFranza and Lew (93), who pooled published risk ratios and performed a meta-analysis to determine population attributable risk, estimated that in the United States alone tobacco products were responsible for 1,200 to 2,200 cases of SIDS each year.

RSV BRONCHIOLITIS AND SIDS

Parallels between SIDS and RSV bronchiolitis have been documented. The similarity between the age distribution and the age at which the most hospital admissions for RSV occur and SIDS death has been demonstrated by Parrot *et al* (94). Beal (69) observed a common "high incidence years" and a winter peak. Similarly with SIDS, RSV has a higher

male to female ratio, ethnic and sociodemographic distribution (94, 95). There is also evidence that parental smoking is associated with an increased incidence of lower respiratory tract infections in infants (96). Mc Connochie and Roghman (97) demonstrated that infants from a smoking environment were 4 times more likely to develop bronchiolitis than a smoke free infant. Taylor and Wadsworth (98) demonstrated an association between pre-natal smoking and an increased frequency of hospitalisation for bronchiolitis.

THE POSTMORTEM EXAMINATION

The definition of SIDS is one of exclusion and cannot be explained by history, environmental factors or by any inherent lethal pathology. If the death can be attributed to a specific condition, the death should not be classified as a SIDS death. Postmortem examinations have identified a number of pathophysiological differences in several organ systems in some infants who have died of SIDS. These include the nervous and respiratory systems, and the pulmonary circulation. The direct relationship between these differences and the actual underlying pathological cause of death remains uncertain.

ENVIRONMENTAL EXAMINATION

The Coronial investigators examine the bedroom environment and conduct interviews with parents to identify the possible cause of death. Once these factors are excluded as a possible cause of death a post mortem examination is conducted to identify any physiological abnormality or trauma that may explain death.

EXTERNAL EXAMINATION

Berry (99) reported the presence of frothy fluid in the mouth, nose and the airways in 50% of SIDS cases. Beckwith (100) reported up to 80% of cases with the presence of frothy fluid. Berry also reported cyanosis of the nailbeds and of the lips, post-mortem hypostatic staining of the anterior surface, (indicating a face down position at death) and clothing damp with sweat in some SIDS cases. While SIDS babies tend to be below average weight, they are usually well nourished.

INTERNAL EXAMINATION

In the state of Victoria the Victorian Institute of Forensic pathology reviews cases of sudden unexplained death in infants. An experienced paediatric pathologist performs a post mortem examination in all cases. Sections of several organ systems are taken, fixed in formalin and embedded in paraffin wax for sectioning.

PULMONARY PATHOLOGY IN SIDS

Intrathoracic petechial haemorrhage has been observed in many cases of SIDS and was one of the first pathological findings of SIDS (30). Beckwith (101) postulated that negative intrathoracic pressure generated by obstructed inspiration may be involved in the observed petchiae in SIDS. Intrathoracic petchiae has been observed in animal models by tracheal occlusion (102). Cambell and Read concluded that the intrathoracic petechial haemorrhage observed in rabbits was an effect of vigorous respiratory effort and was not seen in the animals that were sacrificed by an induced quiet apnea. Visceral petechiae in SIDS are considered to be a non-specific manifestation of SIDS rather than a direct suggestion of any

role of upper airway obstruction. Further, the paper by Berry et al. (99) showed that while intrathoracic petechiae are found in a high proportion of SIDS cases, subconjunctival petechiae are not, whereas in cases of strangulation (a true upper airway obstruction) subconjunctival petechiae are common and intrathoracic petechiae still uncommon suggesting that the petechiae seen in SIDS are not simple markers of upper airway obstruction. However intrathoracic petechiae still remains a non-specific pathological observation in SIDS.

Pulmonary lavage samples from SIDS cases suggest that plasma may be transudated from the airway wall into the lumen at the time of death (103). This may interfere with surfactant function (104). Martinez (9) postulated that this frothy fluid might be produced during a fatal episode of small airway occlusion. He suggests that a liquid meniscus is formed across the collapsed airway and is unable to be cleared due to different opposing surface tensions. A large pressure difference is required to move a stable meniscus distally (105). . Gas trapped between the menisci may be aggravated by a pressure difference across the films, providing a gradient for gaseous diffusion into the trapped space (106). As the lung inflates more menisci may be produced, and as they move distally they would involve more and more airways. This may result in frothy fluid in the mouth and nose in SIDS victims.

Baxendine *et al* (107) found an increase in eosinophil numbers in the lungs of SIDS infants while Howat *et al* (108) found a T lymphocyte-mediated pulmonary inflammatory response present in the parenchyma and in the peribronchial area. Williams (109) reported the

presence of lymphoid inflammatory infiltrates in around 70% of cases and their rarity in control infants who die of trauma. Oedema is also reported to be present in the lungs of some SIDS victims (99). Berry (99) suggested that interstitial oedema is a result of viral respiratory infection, which is present in approximately 20% of SIDS victims (110).

NEUROLOGICAL PATHOLOGY IN SIDS

A wide range of changes in the central and peripheral nervous systems have been reported in SIDS cases. These findings are mainly suggestive of delayed maturation and possible previous hypoxic episodes. Filliano *et al* (111) believed that the hypoplasia of the arcuate nucleus observed was an indicator of delayed maturation.

Findings suggestive of previous hypoxic episodes in the nervous system of SIDS infants include microglial activation and elevated neurotransmitters in the carotid body brain stem, astrogliosis and periventricular and subcortical leukomalacia (112-115).

PATHOLOGICAL MARKERS OF HYPOXIC EPISODES

Naeye (116) first reported changes due to previous hypoxic episodes in the small pulmonary arteries. He reported 1.6 times as much muscle in the small pulmonary arteries of infants diagnosed as SIDS. Approximately 60% of SIDS infants have an increase in the thickness of small pulmonary artery (117). Other studies have confirmed increased muscle in the small pulmonary arteries (118-120) however this has been contradicted (121). Naeye's findings of increased smooth muscle within small pulmonary arteries remains controversial.

Naeye *et al* (79) also reported an abnormally heavy right ventricle in many of the SIDS victims and that weight was directly proportional to the mass of muscle in the small pulmonary arteries. However, Valdes-Dapena (120) was unable to find a significant difference.

Hypoxanthine levels in plasma, urine and CSF are believed to increase during hypoxia. Rognum *et al* (122) found elevated hypoxanthine levels in the vitreous humour of 32 SIDS infants compared with 8 infants dying of trauma and 7 neonates ($p<0.01$).

STRUCTURAL DEVELOPMENT OF THE LUNGS AND AIRWAYS

Knowledge of airway pathology has been gained largely from autopsy studies and in animal models. *In-utero* airway development in the human lung occurs between the 10th and 14th week of gestation. Sixty five to seventy five percent of the bronchial branching has occurred at this point and is completed by the 16th week. Cilia are noted at the 13th week and type I type and II cells can be distinguished in the epithelium from the 16th and 26th weeks. From around the 26th week to birth the alveolar sacs develop and continue to proliferate (123). Sparrow *et al* (124) speculated that the bronchomotor tone (spontaneous contraction) of the airway observed in foetal pigs was providing movement of lung fluid essential for continued growth and development of the lung.

TRACHEOBRONCHIAL TREE

The tracheobronchial tree in the human lung is a series of branching airways. Each series

of airway size or generation becomes progressively narrower, shorter, and greater in number as they progress deeper into the lung. The generations are essentially broken up into 3 groups, 1) cartilaginous airways, 2) noncartilaginous airways and 3) sites of gas exchange. A slow and gradual simplification of the histologic structures of the airways takes place towards the terminal bronchioles.

• Trachea	Cartilaginous airways
• Main stem bronchi	Cartilaginous airways
• Lobar bronchi	Cartilaginous airways
• Segmental bronchi	Cartilaginous airways
• Subsegmental bronchi	Cartilaginous airways
• Bronchioles	Non cartilaginous airways (Membranous airways)
• Respiratory bronchioles	Sites of gas exchange
• Terminal bronchioles	Sites of gas exchange
• Alveolar ducts	Sites of gas exchange
• Alveolar sacs	Sites of gas exchange
• Alveoli	Sites of gas exchange

STRUCTURE AND FUNCTION

The airway wall can be divided into a number of compartmental areas which contain a

number of structural components (125). The inner airway wall comprises the epithelial layer, the basement membrane, the vascular capillary bed, the sub-basement membrane connective tissue matrix, elastic fibre bundles and smooth muscle. The outer airway wall comprises cartilage, mucous glands (in the cartilaginous airways), bronchial blood vessels and loose connective tissue.

The role of the cartilaginous bronchi and membranous bronchi is to conduct air from out side of the body to the sites of gas exchange. Cartilage plates help maintain the patency of the central airways. The epithelium consists of a number of cells including basal cells, columnar ciliated cells and goblet cells, sitting on the basement membrane which is normally not visible under the light microscope. Longitudinal elastic bundles are prominent below the basement membrane. Discrete bundles of smooth muscle lie below or between the cartilage plates but do not insert into them. The submucosal mucus glands lie outside the smooth muscle, between the cartilage plates or below them. Their collecting ducts cross the muscle layer and open onto the mucosal surface where their epithelium becomes continuous with that of the airway lumen.

The bronchioles do not have cartilage, or glands. The smooth muscle bundles are arranged spirally and more completely surround the bronchi. Patency is maintained by the stiffness of the airway wall and the elastic recoil of the surrounding lung which attaches directly to the adventitial surface of the airway. The epithelium has become cuboid with a decreased number of goblet cells. Cilia are still present however become sparse in the terminal bronchioles. In the distal airways the inner airway wall has become thin in appearance,

consisting mainly of elastic fibres which are interlaced with the smooth muscle cells. The smooth muscle, although reduced in absolute amount, occupies relatively more of the distal inner airway wall. The outer airway wall is also thinner than the central airways, extending outwards and continuing with the rest of the structure of the pulmonary parenchyma.

The respiratory bronchioles have well developed smooth muscle and elastic fibres. The respiratory and terminal bronchioles are at a point of transition between the conducting and respiratory portions of the respiratory system. Gas exchange increasingly occurs and the mechanical properties of the airways approximate that of the lung parenchyma as greater number of alveoli occupy the airway wall.

The alveolar ducts appear discontinuous in shape with numerous branching to alveolar sacs and alveoli. The alveoli are small pockets open on one side and the wall consists of a thin double epithelial partition that contains capillaries. This honeycomb like structure consisting of elastic and collagen structural elements determines the mechanical properties of the lung. It links the pleural surface to the airways via the forces of interdependence (126)

THE AIRWAY EPITHELIUM

The airway epithelium forms a continuous layer from the larynx to the terminal bronchioles and contains the first cells to come into contact with the external environment (see Fig.1). The epithelial layer has a number of roles in the lung. The mucociliary layer constantly clears inhaled particles and micro-organisms from the airways. Mucus is produced from

the goblet cells and submucosal glands. The epithelial and basal cells act as a tight mesh and a physical barrier to inhaled toxins and organisms. The airway epithelial cells also have been reported to play a role in the regulatory control of smooth muscle stimulation and inflammation in the airway (127-129). Epithelial damage, desquamation and metaplasia can occur in response to a variety of clinical conditions in the airway. These processes may lead to airway hyperresponsiveness and contribute to toxin, viral and bacterial permeability in to the airway wall and, in turn, to the blood stream (130-133). The airway epithelium also contains a network of nerves, which may act to modulate the bronchoconstrictor response to inhaled stimuli.

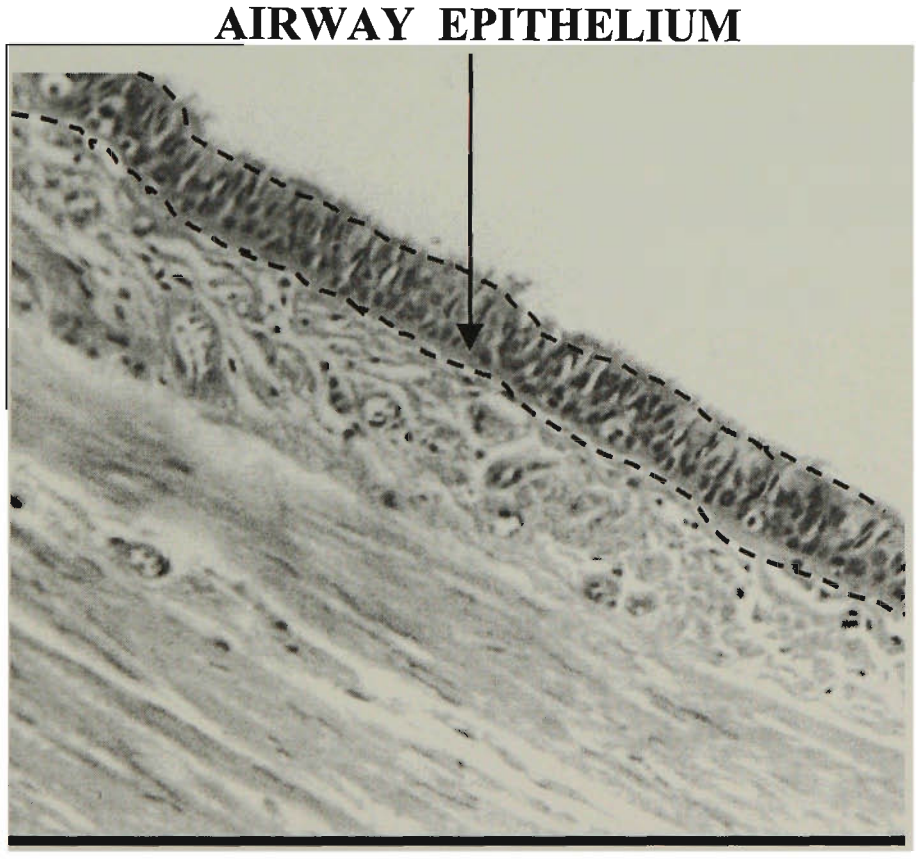

Figure 1 Photomicrograph of the airway epithelium and the basement membrane running directly under the epithelium.

THE BASEMENT MEMBRANE

The basement membrane of the airway is an elastic structure located in the inner airway wall, directly beneath the epithelium (see Fig.2). Lambert (134) examined the mechanical properties of the basement membrane and its potential to be load bearing. Increased mechanical stiffness in the airway created by the basement membrane would reduce the deformability of the airway wall when smooth muscle shortens. Lambert concluded that the ability of the basement membrane to resist deformation was a function of the number of folds that occurred. The buckling pressure required varied as the square of the number of folds. The greater the number of folds that occur in the basement membrane as the smooth muscle shortens, the greater the resistance to lumenal narrowing. A reduced number of folds may contribute to excessive narrowing. In asthma, a thickened basement membrane is a uniform pathological finding (135-139). However it does not correlate with the duration of clinical disease or severity (138). The increased thickness has been attributed to the deposition of collagens and fibronectin by activated fibroblasts beneath the basement membrane (139).

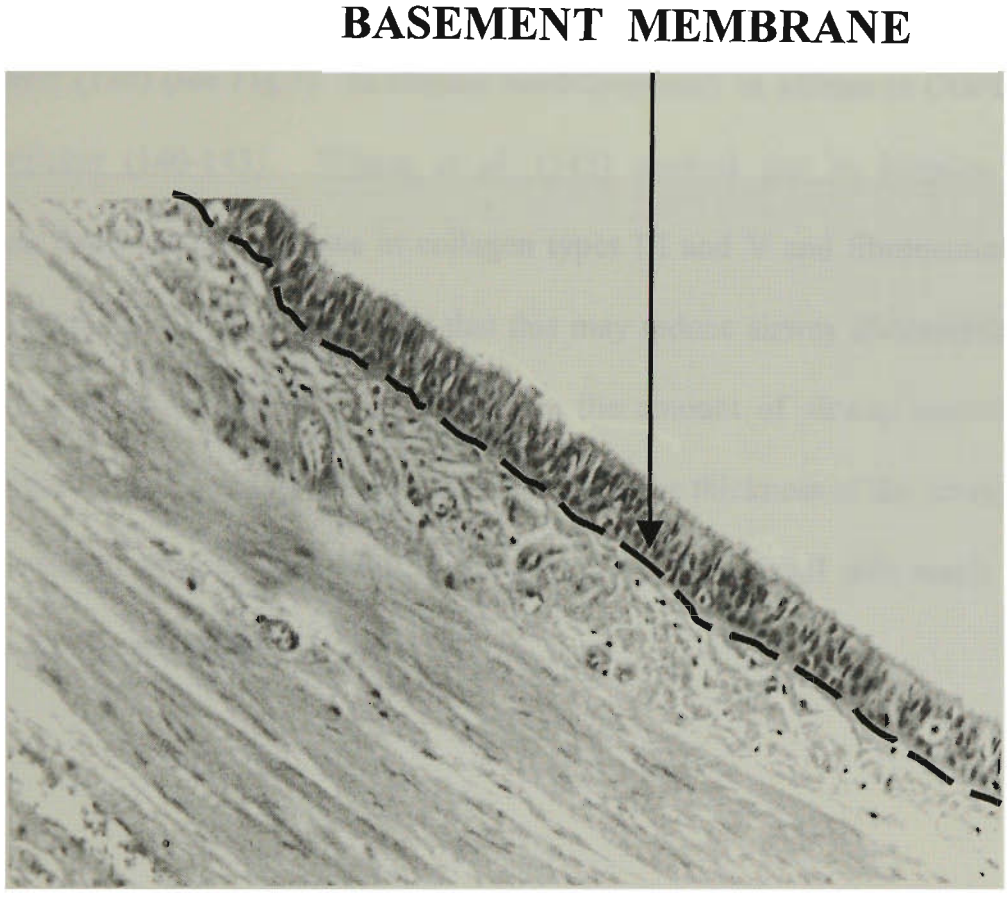

Figure 2 Photomicrograph of the airway basement membrane located under the epithelial layer.

THE INNER AIRWAY WALL

The inner airway wall is characterised by the epithelial layer, the vascular capillary bed, the basement membrane, the sub-basement membrane connective tissue matrix and the smooth muscle layer (125) (see Fig.3). In clinical conditions such as asthma or COPD the airway wall is thicker (140-143). Wilson *et al.* (145) showed that in biopsies taken from asthmatics, there was an increase in collagen types III and V and fibronectin beneath the basement membrane. They postulated that this may reduce airway distensibility (144). A number of studies have shown an increase in the amount of airway smooth muscle in asthmatic patients (140, 141). This will also increase the thickness of the airway wall. As a result of smooth muscle shortening, a thicker inner airway wall will result in a greater change in airway resistance (6).

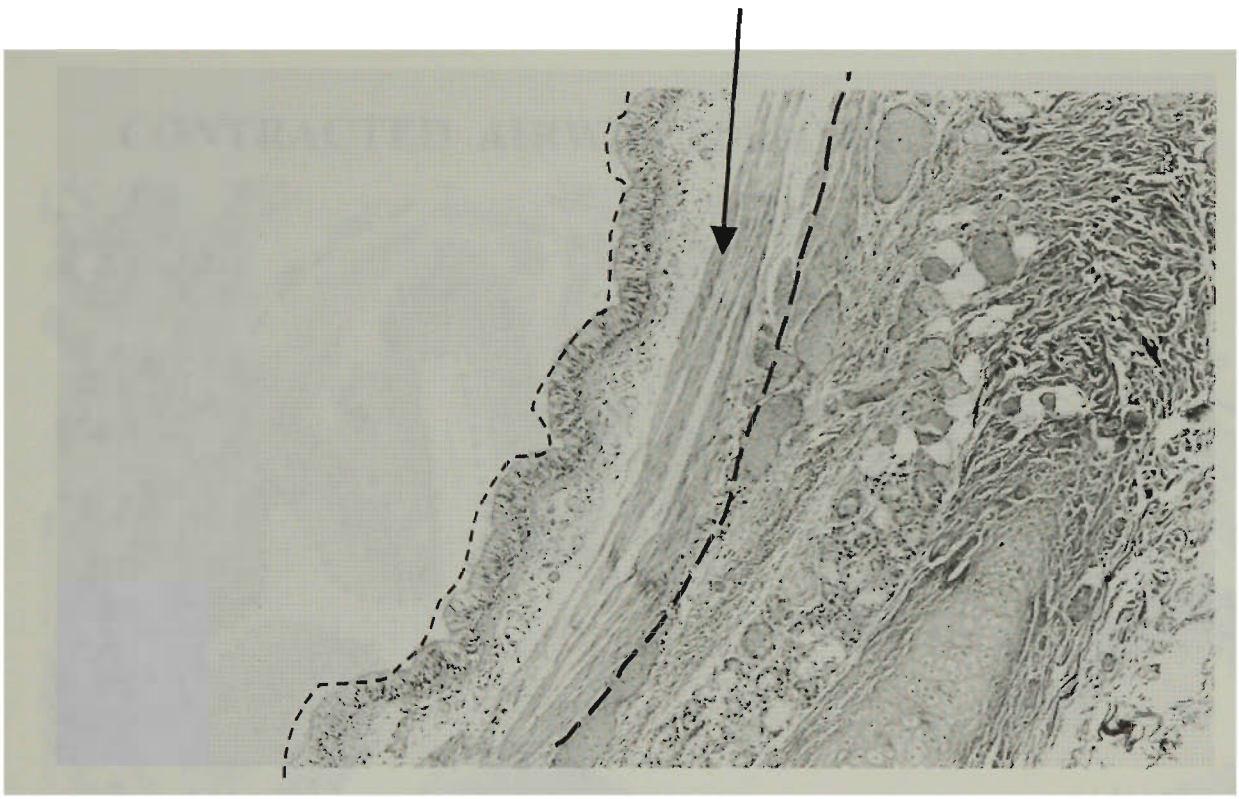

Figure 3 Photomicrograph of the inner wall area characterised by the epithelial layer, the vascular capillary bed, the basement membrane, the sub-basement membrane connective tissue matrix and the smooth muscle layer.

AIRWAY SMOOTH MUSCLE

The airway smooth muscle is under the control of the vagus nerve and the sympathetic nervous system. When stimulated to contract by a variety of stimuli, the airway smooth muscle will produce a decrease in the airway diameter and thus airflow (see Fig.4).

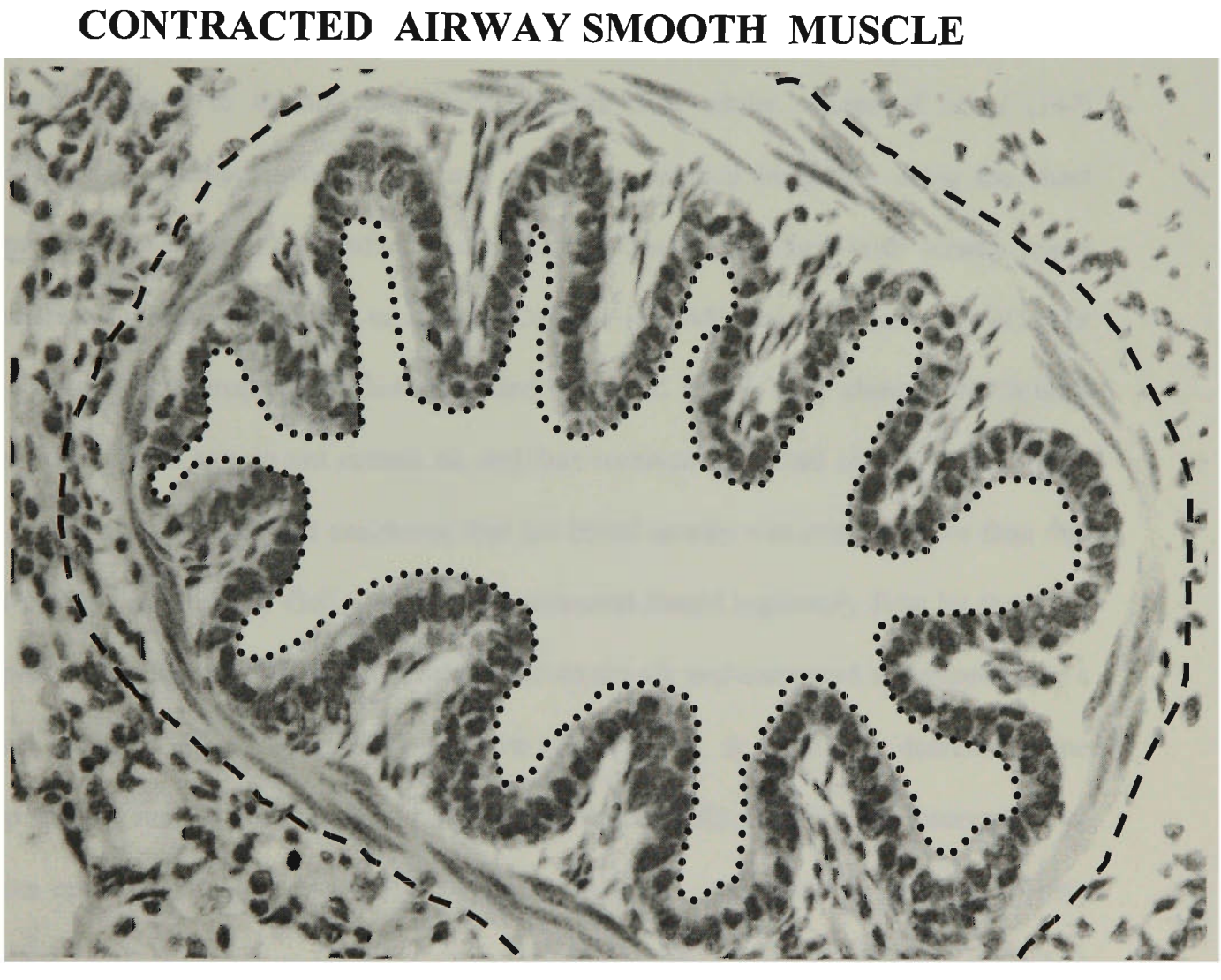

Figure 4 Photomicrograph of a contracted airway. The lumenal area is decreased as the smooth muscle shortens around the airway.

Tepper (11) demonstrated increased bronchial smooth muscle reactivity in healthy infants. A 40% drop in the VmaxFRC of sleeping infants was measured after the administration of doubling doses of methacholine (mean dose of 1.2 mg/ml). Infants then inhaled bronchodilator metaproterenol and 10 minutes later the VmaxFRC had returned to normal. The mean dose to obtain a 40% drop in adults is around 48 mg/ml, approximately 40 times the dose per unit lung volume required in infants (146). Tepper concluded that infants may be more sensitive to inhaled bronchoconstrictors than adults. Lesouef *et al* (147) investigated the responsiveness of infant airways to inhaled histamine using the chest compression technique. All infants responded to doses associated with airway hyper responsiveness in older children or adults. Collis *et al* (148) looked at entrainment of air that occurred when inspiratory flow exceeded nebulised flow. They showed that infants under 6 months of age do not entrain air and thus received undiluted aerosol. This called into question studies that had concluded that the infant airway was more reactive than that adult airway. A study by Geller *et al* (149) measured forced expiratory flow by the chest compression technique after administration of cold dry air and compared the responses to a control group of infants who did not receive cold dry air. A significant decrease in the group mean VmaxFRC in the infants who received the cold dry air was observed. The authors concluded that a non-specific airway reactivity may exist from early infancy. Stephens (150) demonstrated that in vitro airway smooth muscle is able to contract to around 20 % of its starting length against a zero load. This degree of shortening would be expected to cause airway closure in-vivo. Since airway narrowing is limited in-vivo in normal subjects (151) it is suggested that powerful opposing forces maintain airway patency (6, 152). It has been postulated that increase smooth muscle is associated with the

excessive airway narrowing that is characteristic of changes in pulmonary function in diseases such as asthma. Hypertrophy and hyperplasia of airway smooth muscle has been reported in autopsy studies of patients dying of asthma (153, 154). In vitro and in vivo comparisons have supported this hypothesis. Schellenberg and Foster (155) and De Jongste *et al* (156) found that some airway smooth muscle from asthmatic patients were able to generate 2 to 3 fold greater tension than smooth muscle from non asthmatic controls. An increase in airway smooth muscle is not only necessarily specific to asthma. A number of studies examining the small bronchioles in smokers have also found the area of smooth muscle is increased compared with non-obstructed control patients (3, 143). Some of these studies have been confounded by the failure to standardise for the total amount of smooth muscle in each preparation.

James *et al.* (157) found that the internal perimeter of the airway lumen defined by the lumenal border of the airway epithelium was independent of smooth muscle tone and lung volume, and could by used as a marker of airway size. This technique, similar to the technique used to correct for smooth muscle shortening in the pulmonary arteries (158-160), has enabled quantitative assessment of the amount of muscle shortening present in an airway (see Fig.4).

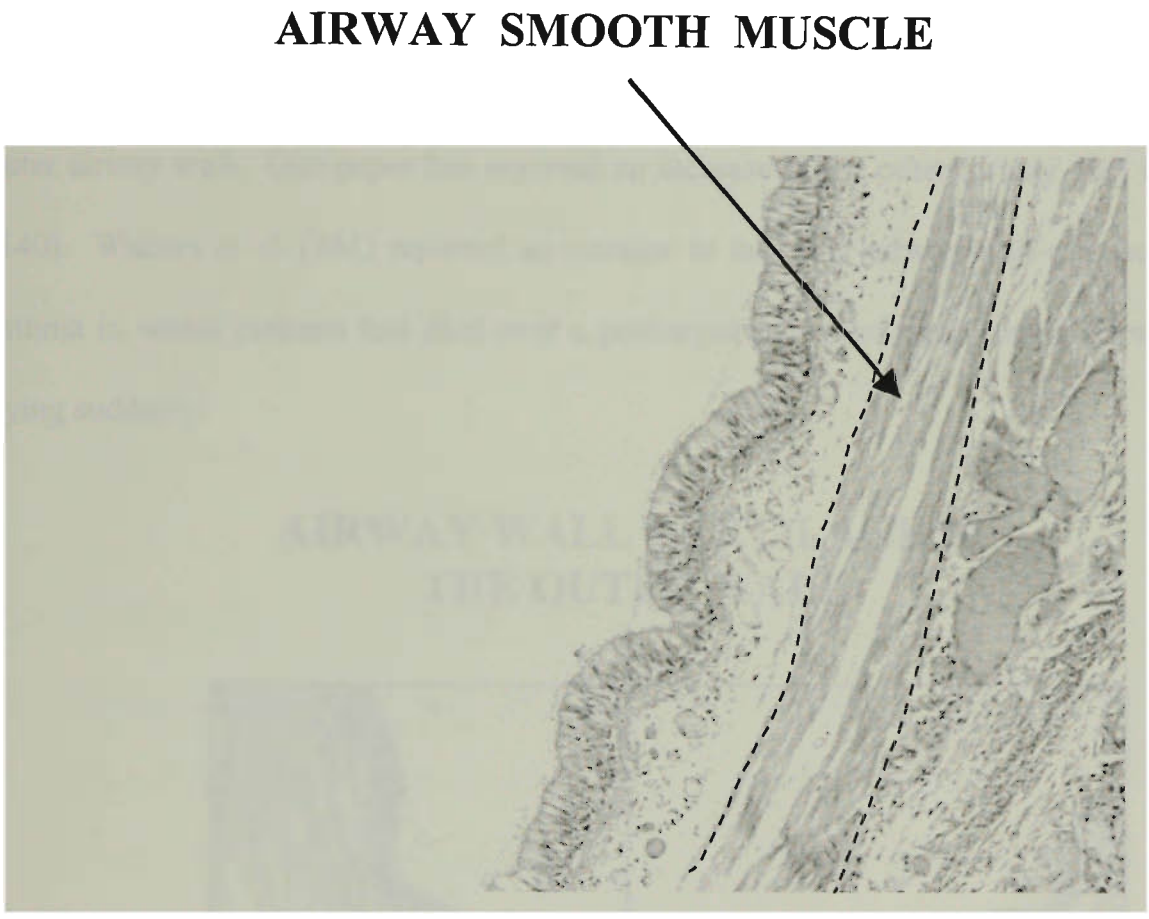

Figure 5 Photomicrograph of airway smooth muscle bundles in the airway wall. The smooth muscle bundles are arranged spirally and completely surround the smaller airways.

THE OUTER AIRWAY WALL

The outer airway wall extends from the outer border of the smooth muscle to the outer perimeter of the airway wall (see Fig.6). The presence of cartilage and mucus glands is restricted to the larger airways. Large and small blood vessels are contained within the outer airway wall. One paper has reported an increase in the outer airway wall in asthma (140). Walters *et al.* (161) reported an increase in the outer airway wall of cases of fatal asthma in which patients had died over a prolonged period of time compared with those dying suddenly.

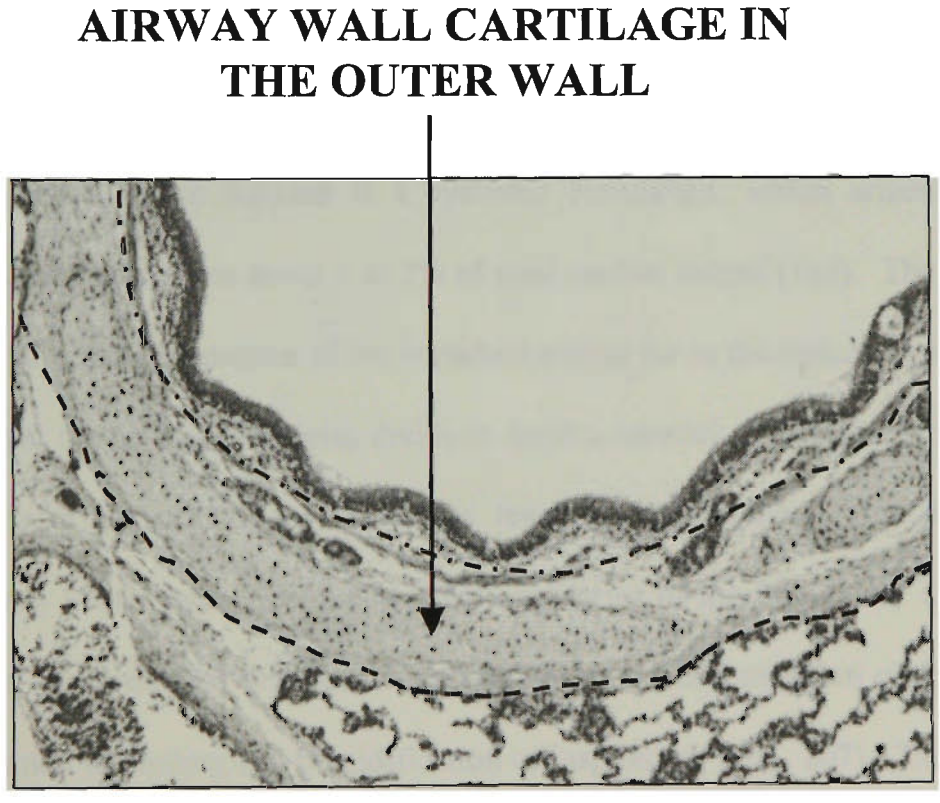

Figure 6 Photomicrograph highlights the outer airway wall, from the outer border of smooth muscle to the outer perimeter of the airway wall. The cartilage plates are easily seen.

THE AIRWAY WALL CARTILAGE

The role of cartilage in the airway wall is to maintain the structural integrity and patency of the upper airways (see Fig.6). It has been hypothesised that deficiency of airway wall cartilage may contribute to flow limitation observed in obstructive airways disease (162). The lack of cartilage may alter airway compliance and increase airway wall collapsibility and therefore lead to increased flow limitation due to dynamic compression of the airways. Rees *et al* (163) observed in foetal growth retarded lambs, a reduction in the area of tracheal cartilage. This may lead to an increase in airway responsiveness (6). Softening of the cartilage has been shown to result in increased airway resistance (164).

BRONCHIAL BLOOD VESSELS

The bronchial circulation in humans is a systemic circulation, which arises from the descending aorta and comprises about 1 to 2% of total cardiac output (165). The bronchial arteries follow the branching pattern of the bronchial tree as far as the terminal bronchioles. In the airway wall the bronchial arteries divide to form a network of capillaries which run subepithelially from the central airways to the level of the peripheral bronchioles (see Fig.7), where they anastomose with the alveolar capillaries of the pulmonary circulation. The functions of the bronchial circulation are thought to include nutrition of the bronchi (166), the regulation of heating and humidification of inspired air (165, 167). The transport of mediators from one part of the airway to another and clearance of substances inhaled into the lung are also the function of the bronchial circulation. It has been postulated that vascular dilation and engorgement may contribute to airway obstruction by increasing the thickness of the airway mucosa and reducing the airway lumen and exaggerating the effects

of airway smooth muscle shortening on lumenal narrowing (6, 168), although this seems unlikely based on calculated maximal dimensions of subepithelial vessels (169).

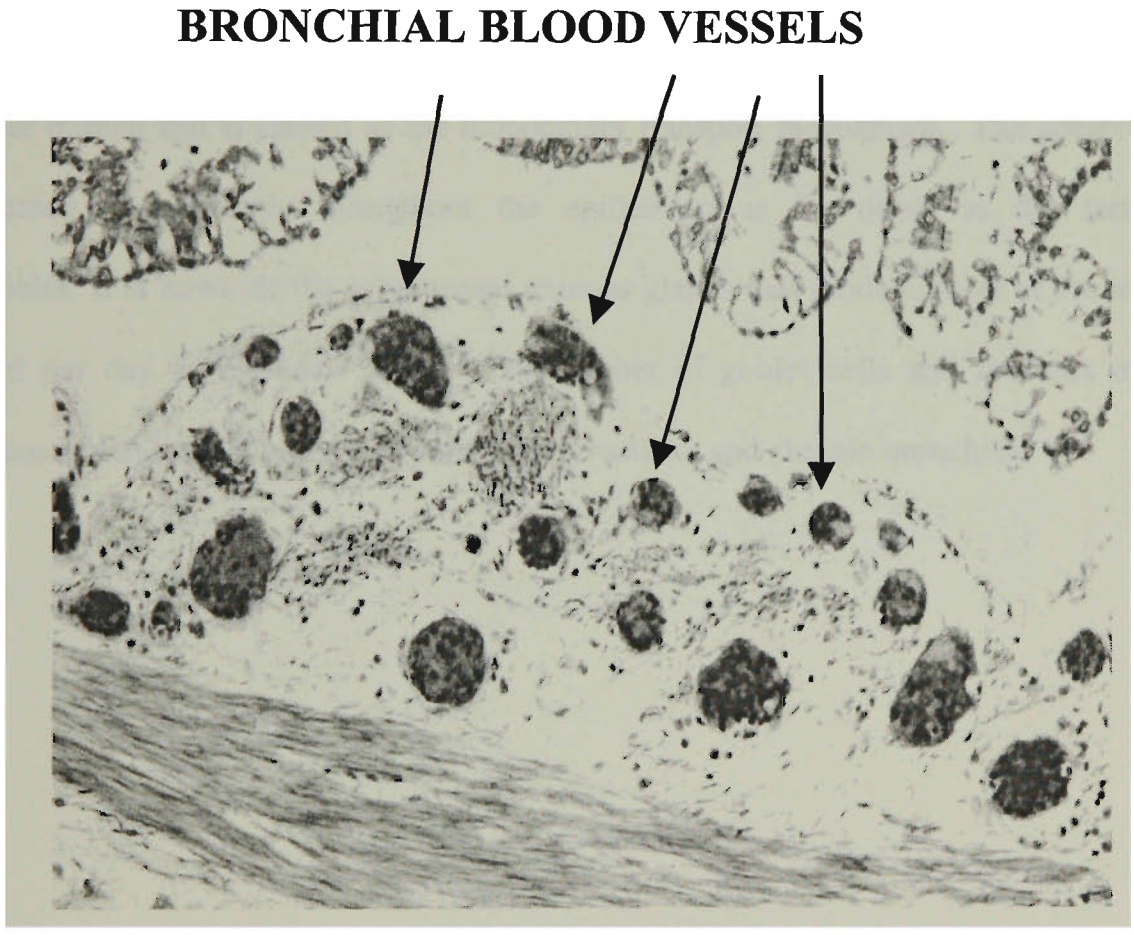

Figure 7 Photomicrograph of the vascular capillary bed in the inner airway wall.

THE SUBMUCOSAL MUCOUS GLANDS AND GOBLET CELLS

The submucosal mucous glands are located in the cartilaginous airways and are particularly numerous in the medium size bronchi (see Fig. 8). Mucous excretion into the airways is a means of clearing inhaled debris. The mucous blanket, covering the epithelial lining consists of two layers, the sol layer and the gel layer. The mucous blanket is constantly moved in a wave-like fashion by the cilia towards the larynx at an estimated average rate of 2 cm per minute and is known as the mucociliary transport mechanism. The goblet cells are located intermittently throughout the epithelium as far down as the terminal bronchioles. It is however the submucosal mucous glands that produce most of the mucus (100 ml per day in the adult lung). The number of goblet cells and the area of the submucosal glands are increased in patients with asthma and chronic bronchitis.

AIRWAY MUCUS GLAND AND DUCT

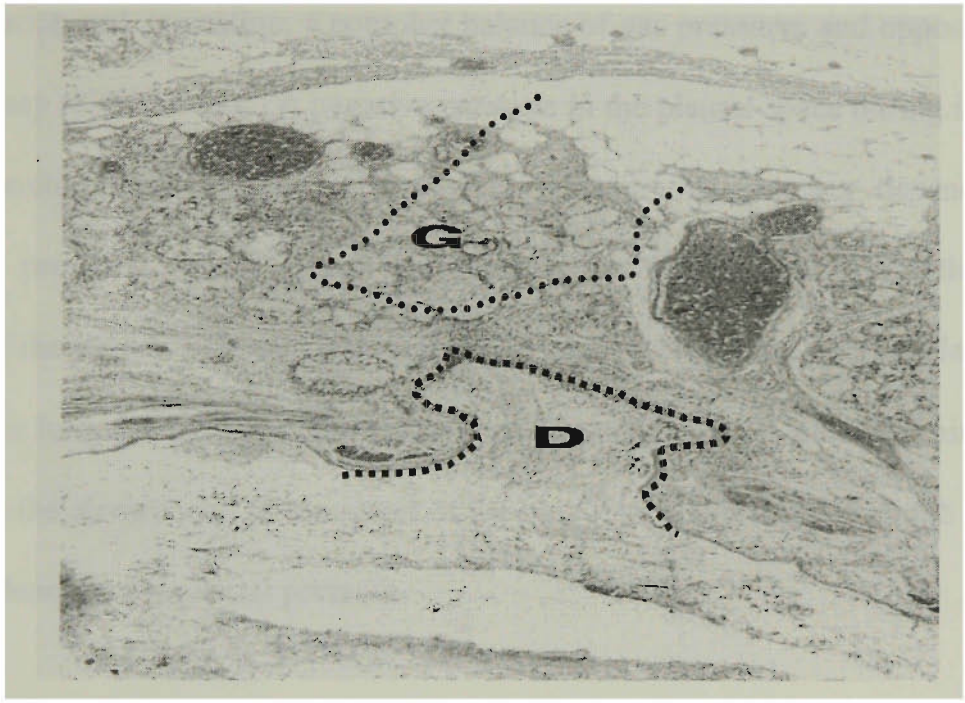

Figure 8 Photomicrograph of the airway mucous glands (G) and mucous duct (D). The role of the mucous glands is to secrete mucus into the airways as a means of clearing any inhaled debris.

THE AIRWAY WALL ATTACHMENTS

The airway wall attachments play an important role in airway patency and maintenance of airflow by exerting an opposing load to airway smooth muscle shortening (see Fig. 9). During inspiration and expiration, a complex balance of gas pressures and opposing forces maintain patency of the airway. A negative pressure in the pleural space during inspiration exerts a distending force on the airways. During expiration, patency depends on the opposing gas pressures in the alveoli surrounding the airway and within the airway, compliance of the airway wall which is related to smooth muscle tone and the elastic recoil pressure of the lung (126, 170). The parenchymal attachments on the outer airway wall perimeter link the airway wall to the recoil of the lung parenchyma and, through the forces of interdependence to the pleural pressure.

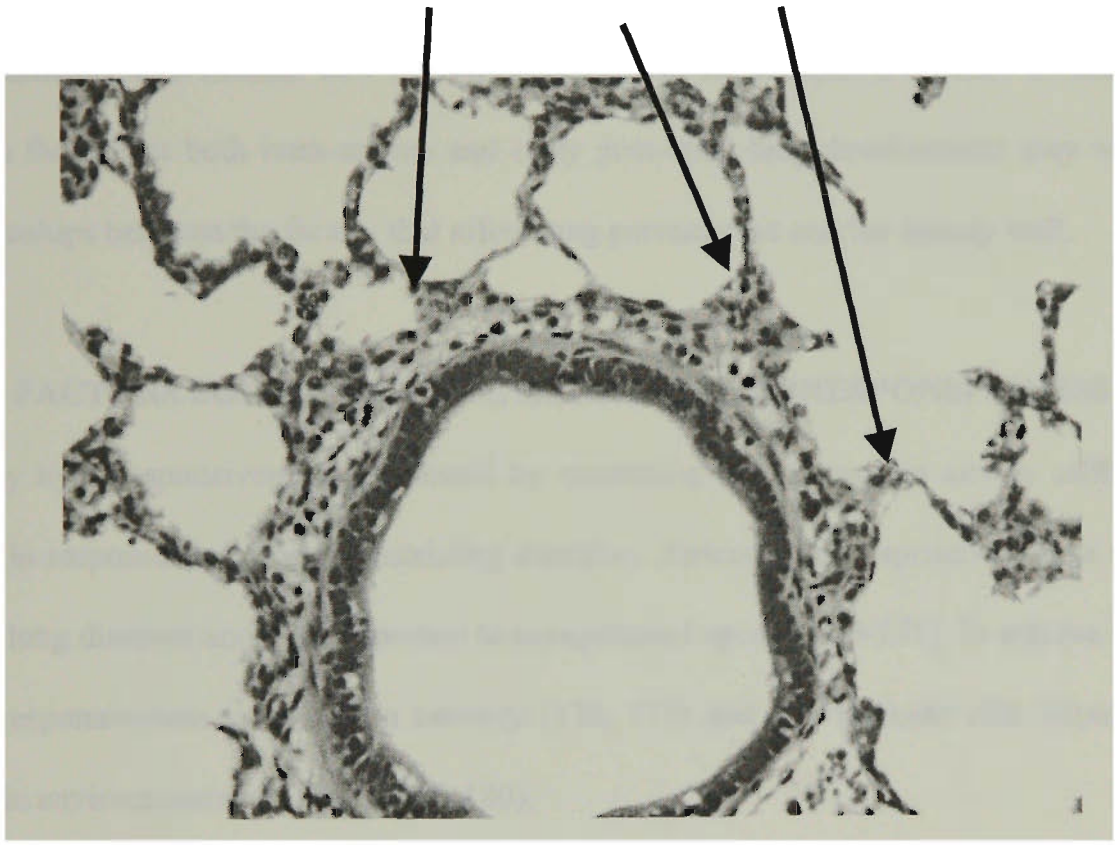

Figure 9 Photomicrograph of the airway wall attachments. The role of airway attachments play an important role in airway patency and maintains air flow by exerting an opposing load to airway smooth muscle shortening.

At birth the airways are fully mature in their structure and branching pattern and they increase approximately 2 fold in length in the first 2 months (171). In contrast the parenchymal support network develops mainly during the postnatal period. Thurlbeck (172) examined morphometrically the lungs from 36 boys and 20 girls and estimated that approximately 200 million new alveoli were formed in the first 6 months of life. The factors that affect both intra-uterine and early post-natal lung development may alter the relationships between the factors that affect lung parenchyma and the airway wall.

RISK FACTORS FOR DEVELOPING AIRWAY HYPERRESPONSIVENESS

Airway hyperresponsiveness is assessed by measuring the changes in airway caliber that occur in response to bronchoconstricting stimulus. Airway hyperresponsiveness is seen in many lung diseases and after exposure to occupational agents (173-175). In asthma, airway hyperresponsiveness is related to severity (176, 177) and can increase after exposure to specific environmental allergens (178-180).

VIRAL INFECTION

Mucus secretion, inflammation of the airway walls and lymphocyte infiltration is common in respiratory viral infection. Inflammation and mucus secretion can contribute to airflow limitation. Mier-Jedrzejowicz *et al* (181) observed abnormalities in respiratory muscle function in subjects with upper respiratory tract infection. Viral infections in asthmatic patients and in normal subjects can enhance airway responsiveness to degrees, especially in children (182, 183). A significant correlation between viral infection in SIDS and viral isolation in the same time period suggests that respiratory viruses may be associated with

SIDS (184).

CIGARETTE SMOKING

The harmful effects of cigarette smoking are well known (185). Airflow limitation, airway hyperresponsiveness and mucus gland hypersecretion are all associated with smoking. Airway wall remodelling and destruction of the alveoli (emphysema) and inflammatory cell infiltration may contribute to making the lung more responsive to inhaled stimuli.

VARIABILITY OF MORPHOMETRIC MEASUREMENTS

A number of studies have examined the reproducibility of measurements of airway dimensions. The study of Carroll *et al* (186) showed that for airway wall dimensions, the inter and intra observer variations expressed as the coefficient of variation were less than 5 % for most measurements and was similar when comparing airways between or within subjects. Their data showed that measurements of airway dimensions defined as perimeters and areas were less variable than measurements of smooth muscle, gland and cartilage area. The coefficient of variation for both intra and interobserver error was greater in small airway than large airways. Sullivan *et al* (187) measured basement membrane thickness and inflammatory cells numbers in specimens obtained at bronchial biopsy and found that approximately 70-100 measurements of the basement membrane thickness will give a precision of 10% for the measurement. Using the variation of measurements from control cases, Carroll *et al* showed that it was necessary to compare 10-15 cases in each group to detect a difference of 100% in airway dimensions in cases of asthma while a 50% difference between groups would require up to 50 cases in each group.

CHAPTER 3 – METHODS

ETHICS APPROVAL

All work conducted in this study was approved by the Royal Children's Hospital Ethics committee (REF. 94064C) and also by the Victorian Institute of Forensic Medicine Ethics committee (access number: 36/94, human ethics number 119/94).

SUBJECTS

In the state of Victoria, the Victorian Institute of Forensic Medicine (VIFM) reviews cases of sudden unexplained death in infants. The SIDS cases used were part of an epidemiological study conducted by the Victorian Sudden Infant Death Research Foundation from 1991 to 1993, where an autopsy had been performed and where information had been stored on the SIDS VIFM autopsy database. A post-mortem examination is performed in all cases by an experienced paediatric pathologist and a diagnosis of SIDS is only made if the post mortem examination and circumstances surrounding death do not suggest an alternative cause. Routinely, sections of several organ systems are taken and stored in paraffin in the event that further review of the case is required. There were 83 SIDS cases with maternal smoking data available: 48 males and 35 females with a mean (± SD) age 5.7 ± 4.3 months.

Age-matched control cases were obtained with permission from the VIFM by accessing the records of infant deaths reviewed by the Institute from 1991. Cases were selected where there was no previous pulmonary pathology and sections were obtained from archived lung tissue samples. Cases included were sudden death (motor vehicle accidents or homicide) in

previously healthy children: 9 male, 4 female, with a mean (± SD) age 7.6 ± 4.7 months. A further 8 control cases with similar causes of death were included from a study in Western Australia: 4 male and 4 females with a mean (± SD) age 6.5 ± 5.8 months. Sections of tissue had been obtained in a standardised way as part of a protocol for the pathological examination of all infant (including SIDS) deaths. Measurements of airway dimensions in all cases were performed by the author (JE) using identical techniques.

MATERNAL SMOKE EXPOSURE HISTORY

In 1991 the Victorian Sudden Infant Death Research Foundation commenced a 3 year study into the Epidemiology of SIDS in the State of Victoria, Australia. The investigators used a variety of sources including ambulance officers, paediatric emergency nursing staff, SIDS grief counsellors and family members, to identify families who had lost a child from SIDS. The investigators then approached the families and invited them to participate in a formal interview where a detailed questionnaire covering various epidemiological factors associated with SIDS was administered by a trained interviewer. The questionnaire included specific questions related to maternal smoking. Mothers were asked to indicate whether they smoked before becoming pregnant and separately during the first, second, and third trimester of pregnancy. Mothers were also asked whether they smoked between the birth and death of their child. For all questions, smoking was rated on a 5 point scale.

- no smoking = 0
- < 10 cigarettes a day = 1
- 10-20 cigarettes a day = 2

- 20-30 cigarettes a day = 3
- > 30 cigarettes a day = 4

The investigators were granted access, with permission from the SIDSF, to the case numbers of these infants and the data relating to maternal smoking. Nineteen cases of SIDS were identified as high smoke exposure, where maternal smoking was > 20 cigarettes a day pre and post-natally: 12 males and 7 females with a mean (± SD) age of 4.9 ± 2.9 months. A further 19 cases were also categorised as no smoke exposure where no maternal smoking took place either before, during or after the pregnancy: 12 males and 7 females with a mean (± SD) age of 5 ± 3.8 months.

POSTMORTEM TISSUE

Using a standard post mortem examination protocol at the VIFM, sections of tissue were obtained from several organ systems including the lungs. Parenchymal tissue was sectioned at random from several sites in lung. Sections of lung tissue were fixed by immersion in 10% buffered formalin solution for a minimum of 24 hours. Tissue sections were then put though a series of graded alcohols and embedded in paraffin wax ready for sectioning and staining.

SECTIONING OF LUNG TISSUE

Paraffin blocks were trimmed until the whole face of the tissue surface was exposed and section were cut at 5 microns using a 'Biocut 20:30' microtome. The tissue sections were then mounted on glass slides, stained with haematoxylin and eosin (H&E) and covered with

a glass coverslip. A further 8 control infants were included from Western Australia in which the same procedure for fixation, embedding, sectioning and staining had been followed.

BLINDING OF THE TISSUE SAMPLES

The VIFM case number was written on the mounted slides. A separate investigator then placed a sticky label over the case number and issued the slide with a study number. The identity of the slides therefore was not made known to the measurer until all airways had been measured.

AIRWAY SELECTION CRITERIA

Airways were selected for measurement using the following criteria.

Cases in which significant pulmonary damage was likely to be a feature of the cause of death such as pneumonia, congenital heart disease, or history of premature birth, were excluded. From the 90 SIDS cases who had maternal smoking data, 33 had no airways suitable for morphometric analysis.

1) Morphometric measurements were performed on all airways that were cut in cross section, or near cross section.
2) Airways with a short to long axis ratio of less than 0.6 were excluded to avoid errors arising from tangential sectioning.
3) Airways which showed >50% epithelial detachment were excluded.

4) Airways that had signs of branching were excluded.

AIRWAY MEASUREMENTS

Four perimeters and areas were measured (see Fig.10);

1) The internal perimeter (Pi) and area (Ai), defined by the luminal border of the epithelia,
2) The perimeter (Pbm) and area (Abm), defined by the outer border of the basement membrane,
3) The perimeter (Pmo) and area (Amo) defined by the outer border of the smooth muscle,
4) The total perimeter (Po), and area (Ao), defined by the outer edge of the adventitia surrounding the airway.

In addition, the area of airway smooth muscle was measured directly with a digitiser using an image analysis system. The numbers of airway attachment points were counted directly (see below).

AIRWAY WALL AREAS

The compartmental areas of the airway wall (125) were calculated from the concentric measurements of the airway layers. The areas measured were the epithelial area (WAepi), inner wall area (WAi), outer wall area (WAo), and total wall area (WAt).

The areas were calculated as follows;

- the epithelial wall area (WAepi = Abm - Ai),
- the inner wall area (WAi = Amo- Ai)
- the outer wall area (WAo = Ao - A mo)
- the total wall area (WAt = Ao - Ai)

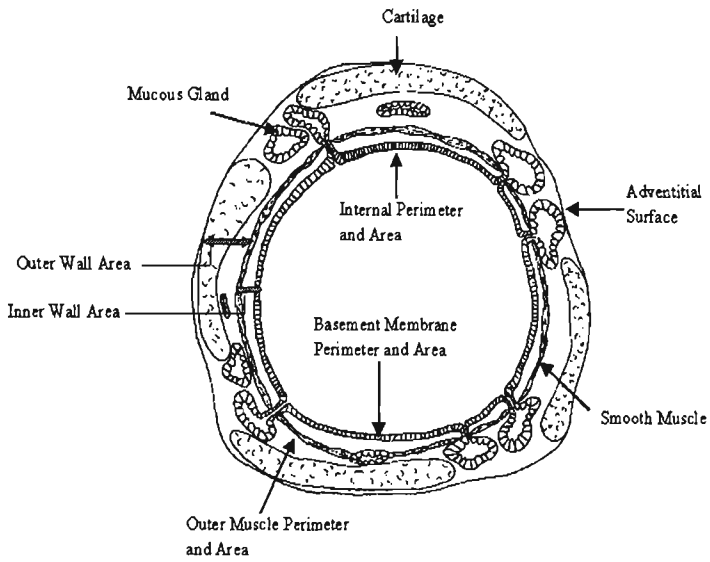

Figure 10 Schematic representation of an airway showing dimensions measured in this study. The airway wall is divided into inner and outer areas by the outer perimeter of the airway smooth muscle. Standard nomenclature as described by Bai *et al* (1994) has been employed.

AIRWAY SIZE AND PERCENT OF SMOOTH MUSCLE SHORTENING

The length of the basement membrane perimeter (Pbm) was used as a marker of airway size since it is independent of lung volume or smooth muscle contraction (157). The area of smooth muscle was standardised for airway size by dividing it by the Pbm.

The percent of smooth muscle shortening (PMS) present in each airway was calculated as follows. The "relaxed" lumen area (Abmr) is the area of a circle with a circumference equal to the measured Pbm. $(Abmr) = (Pbm^2) / 4\pi$. By adding the measured area of wall which changes little during muscle contraction (157) between the basement membrane and the outer muscle border (Amo - Abm) to Abmr, the relaxed outer area (Amor) is obtained. The perimeter (Pmor) of this area is the length of the 'relaxed' smooth muscle. By comparing the measured 'muscle length' (Pmo) to the relaxed muscle length (Pmor), the amount of muscle shortening that is present can be calculated.

$PMS = (Pmor - Pmo)/Pmor \times 100$, where $Pmor = \sqrt{}(4\pi \times Amor)$.

While PMS reflects the degree to which the airway smooth muscle has shortened in the airway wall, this may be influenced in a number of ways, such as the length of time from death to the fasciation of the tissue, or whether the lung was inflated or submerged in fixative

ALVEOLAR ATTACHMENTS POINTS

The adventitial surface of the airway wall is attached to the surrounding parenchyma at a

number of points by tissue resembling alveolar walls. These attachment points are contiguous with the outer airway wall adventitial connective tissue. Attachment points were counted in all airways. The number of attachment points were expressed as the number per mm of external airway perimeter (Po) to obtain the mean distance between each attachment point or alternatively the mean number of points per mm of outer airway wall perimeter.

IMAGE ANALYSIS SYSTEM

Slides were examined using a video-linked microscope Leica Laborlux D, (Leica, Germany) with the image being projected onto the monitor screen of a 486 DX computer. Images were assessed using the colour image analysis program Quantimet 500+ (Leica Cambridge Ltd, Cambridge, England).

REPRODUCIBILITY OF MEASUREMENTS

The reproducibility of airway measurements in the present study was examined by estimating the intraobserver error. The coefficient of variation of mean i.e.(SD/mean x 100) was calculated from measurements of airway dimensions made on 10 different airways measured on separate occasions. The coefficient of variation for airway measurements ranged from 0. 44% to 3. 7% for all airway dimensions with an overall mean value of 2% ± 0. 7%. Measurements were made blinded to the clinical histories of all cases and were made by the author unless otherwise stated.

SAMPLE SIZE CALCULATIONS

To calculate the number of cases needed to detect differences between the means of two subject groups we used the formula of Dobson (1984) (188); $n = 2S2 / \Delta 2 \times f(a,\beta)$, where n = sample size, S = standard deviation of the measurement, Δ = difference to be detected between the means of the two groups, a = significance (eg. 0. 05), and β = power of the test (eg 0. 8). A value for $f(a,\beta)$ of 7.85 was used for all calculations using $a = 0.05$ and $\beta = 0.8$. The standard deviation from 3 measurements per airway made in control cases was used as the value for "S". We calculated sample size using the variation in measurements (ie.SD) 10 from airways < 4 mm of the basement membrane perimeter. Using this formula we calculated that we would need to examine between 10-20 cases in each group depending on the measurement being undertaken.

DATA ANALYSIS

The data were analysed in a variety of ways to address various factors. To assess the effects of SIDS, age and sex on airway dimensions, a weighted least squares model was applied to the data. In brief, airway wall areas and airway smooth muscle area in relation to airway size were linearised by plotting the square root of area against the Pbm for each of the airway wall compartments. Using linear regression analysis, the data for each case were plotted and the slope and intercept, together with their standard errors (SE) were calculated (189). The slopes and intercepts were then regressed, adjusting for the effects of cases group (SIDS or no SIDS), sex (male or female) and age (months). The latter was designed to adjust for the effects of airway growth when comparing airway dimensions. A probability of < 5 % was considered significant.

The two groups of smoke exposed SIDS cases were well matched for age. Thus, to compare similar sized airways from different subjects, airways were divided into 3 arbitrary size groups using the Pbm, < 1mm, 1-2mm, and >2-4mm. Airway size groups were chosen to minimise the effects of growth, and to enable comparisons to other studies (8, 190). All measurements of area were normalised for airway size by dividing by the basement membrane perimeter (157). For each variable, the mean value for each case was calculated. The differences between the means of size groups were tested using a one-way analysis of variance (ANOVA).

ANIMAL MODEL

In-utero model of smoke exposure

Nine pregnant Cam Hartley guinea pigs were obtained from a local breeding facility (Monash University breeding facilities, Clayton, Victoria, Australia) at between 15 and 22 days post conception. Smoke exposure commenced at day 27 post conception and occurred for 15 minutes a day for 4 days each week. Smoke exposure commenced at day 27 to minimise the risk of failed implantation and/or spontaneous abortion due to the acute effects of cigarette smoke inhalation. The guinea pigs were placed in a 16 litre perspex chamber developed for this project. A cigarette was connected to the access port in the wall of the chamber. A bi-directional syringe pump was also connected to the chamber to enable cigarette smoke to be drawn in. Following the lighting of the cigarette, smoke was drawn into the syringe by the pump and the smoke was then returned into the chamber through a further porthole. An oxygen monitor continuously monitored the oxygen levels

in the exposure chamber and supplemental oxygen was bled into the chamber, as the combustion process continued, to maintain normoxic conditions. At the end of the 15 minute exposure animals were removed and returned to their standard cages. In order to limit any effects related to the stress of handling, control animals were placed in an identical exposure chamber at the same frequency but received no smoke exposure.

Serum cotinine measurements

Blood samples were drawn from each pregnant animal under light halothane anaesthesia twice a week for measurement of serum cotinine. Blood samples were centrifuged and the serum removed. Analysis of serum cotinine was performed using a cotinine assay kit (DPC nicotine metabolite assay, Bio-Mediq DPC Pty Ltd. California USA). Blood samples were drawn immediately prior to the first smoke exposure of each week and 24 hours following the last smoke exposure each week. To control for any possible effect of the halothane anaesthesia on foetal development, control non-smoke exposed animals were also anaesthetised and bled at an identical frequency.

Neonatal Animals

Pregnant animals were allowed to proceed to normal delivery, usually at 68 days gestation. To limit any effects of smoke metabolites which may cross through breast milk new born infants were removed from mothers as soon as possible and placed with lactating animals who had not received smoke exposure during pregnancy. New born animals received no smoke exposure post-natally and were studied at day 27 of post-natal life.

Assessment of airway responsiveness

Animals were anaesthetised with 0.3 ml ketamine and xylazine by intraperitoneal injection. Under supplemental halothane anaesthesia a tracheostomy was performed and the animals were then placed in a pressure sensitive plethysmograph, designed for the guinea pig and ventilated with a Harvard small animal ventilator (model 608, Harvard Apparatus, South Natick, MA) delivering a tidal volume of 3 ml at a frequency of 60 breaths per minute. Animals were paralysed with succinylcholine (0.5mg/kg im) immediately after attachment to the ventilator. Volume signals were obtained from a pressure-sensitive transducer (MP 45-2 ± 2 cm H_2O, Validyne, Northridge, CA) which measured changes in box pressure. The plethysmograph was calibrated with a 10ml syringe and a 3 ml volume change resulted in a 0.3 cm H_2O pressure change within the box. The pressure response was linear to a volume change of 20ml. The volume signal was electronically differentiated to determine flow. Transpulmonary pressure was determined using a differential pressure transducer (model 267BC: Hewlett Packard, Waltham, MA) by comparing pressure at the tracheal opening with oesophageal pressure measured from a saline filled PE-90 tube in the distal oesophagus. The pressure transducer was calibrated using a water-filled manometer. The response was linear over the range ± 50 cm H_2O. The volume, flow and pressure signals were recorded (General Scanning, model RS4-5P recorder) and pulmonary resistance (R_L) was calculated at 50% of tidal volume from these signals using the method of Von Neergard and Wirz (191). After stable baseline R_L values were present, guinea pigs were given isotonic saline aerosol followed by increasing doses of aerosolized acetylcholine solution (Ach), and the changes in R_L were measured.

Acetylcholine chloride (Sigma Chemical) was dissolved in normal saline to produce a stock solution of 50 mg/ml. Serial dilutions were made to produce solutions of 15, 5, 1.5, and 0.5 mg/ml. Aerosol was generated by placing 5 ml of saline or Ach solution into a Hudson nebuliser (Hudson Oxygen Therapy Sales Co., Temecula, CA) driven by compressed air at 7 l/min and connected by a T piece to the attachment tube for the tracheostomy. The ventilator was disconnected from the circuit during the administration of the Ach. Six 3 ml tidal breaths of nebulised Ach were delivered for each concentration of Ach. Following the administration of each dose, the ventilator was immediately reconnected to the circuit. The computer analyses the first 20 breaths following recommencement of ventilation. Peak responses following Ach challenges generally occur within the first 10 breaths after reconnection to the ventilator. The average of the three highest R_L values at 50% tidal volume was taken as the response to each Ach concentration. The next dose was delivered after R_L had returned to baseline.

SPECIMEN PREPARATION

At the completion of the assessment of airway responsiveness the animal was sacrificed and removed from the plethysmograph. The anterior chest wall was then removed and the trachea cannulated though the tracheostomy site. The lungs were then inflated over 60 minutes with 10% buffered formalin at 20 cm H_2O. Following inflation the lungs and heart were removed from the chest cavity *en bloc* and immersed in formalin. Multiple tissue blocks were cut in a saggital plane through the perihilar regions of both lungs and embedded in paraffin for histological processing. Three-micron sections were cut and stained with haematoxylin and eosin for morphometric analysis. Slides were examined

using a video-linked microscope Leica Laborlux D, (Leica, Germany) with the image being projected onto the monitor screen of a 486 DX computer. Images were assessed using the colour image analysis program Quantimet 500+ (Leica Cambridge Ltd, Cambridge, England.

DATA ANALYSIS

When comparing data from the animal model there were so few large cartilaginous airways available for examination, only airways with a Pbm of less than 4mm were analysed. These airways were predominantly membranous and small intraparenchymal cartilaginous airways. The results were expressed as the mean ± standard error (SE). Differences between the two groups were tested using an unpaired t-test. A probability of less than 5 % was considered significant. When the frequency distribution of internal airway and outer airway perimeters was compared there was no difference between the groups suggesting that *in-utero* smoke exposure does not result in a systematic change in airway calibre. The means and standard errors also support this data, for both of these measurements which were virtually identical for the two groups. Thus we feel confident that the methods used to normalise the data in this study are valid. Similar methods have been used previously ((6, 141, 157) and the matching of airways by order or generation is not possible when lung tissue is collected and processed in this way.

CHAPTER 4 - INCREASED AIRWAY SMOOTH MUSCLE IN SUDDEN INFANT DEATH SYNDROME

ABSTRACT

The underlying patho physiological mechanism behind death in the Sudden Infant Death Syndrome (SIDS) is uncertain. Although infants dying from SIDS frequently have a post mortem examination performed, no specific diagnostic pathology in any organ system has been identified. Previous theories relating to the cause of death in SIDS have included increased lower airway closure. We examined the airway morphometry of 57 infants who died from SIDS and compared these findings to those obtained from 21 age-matched infants who had died from non SIDS causes. Airway wall dimensions, epithelial thickness and the area of smooth muscle within the airway wall were measured. Airways from infants who died from SIDS showed a significantly higher proportion of airway smooth muscle than control airways when corrected for age and sex ($p<0.01$). There was no significant difference between the groups for wall thickness or epithelial thickness. Increased airway smooth muscle in infants who have died from SIDS may contribute to excessive airway narrowing raising the possibility that the cause of death in this condition is related to abnormalities in lower airway function.

INTRODUCTION

The underlying pathophysiological cause of the sudden infant death syndrome (SIDS) remains uncertain. Several pathologic abnormalities have been identified in several organ systems including the nervous and respiratory systems, and the pulmonary circulation in some infants dying of SIDS however the direct relationship between these changes and the actual mechanism of death remains unclear (119, 192-198). Previous studies, which have examined the airways of infants who died from SIDS have failed to identify a consistent pulmonary pathological feature. Baxendine *et al* (107) found an increase in eosinophil numbers in the lungs of SIDS infants while Howat *et al* (108) found a T lymphocyte-mediated pulmonary inflammatory response present in the parenchyma and in the peribronchial area.

Small airway closure has been suggested as a pathophysiological abnormality in SIDS (9). We postulated that exaggerated small airway closure could be due to increased airway smooth muscle mass. We therefore examined the structure of small airways in infants who died from SIDS with particular attention to the amount of airway smooth muscle and compared these findings with findings from age matched controls.

METHODS

In the state of Victoria cases of sudden unexplained death in infants are reviewed by the Victorian Institute of Forensic pathology. A post mortem examination is performed in all cases by an experienced pediatric pathologist and a diagnosis of SIDS is only made if the post mortem examination and circumstances surrounding death do not suggest an

alternative cause. Routinely, sections of several organ systems are taken and stored in paraffin in the event that further review of the case is required.

The investigators of this present report obtained permission from the Victorian Institute of Forensic Pathology to examine stored lung blocks from infants who had died from SIDS. A total of 90 cases of SIDS were made available. From each lung block one 5μ section was taken and stained with haematoxylin and eosin. Slides were examined using a video-linked microscope Leica Laborlux D, (Leica, Germany) with the image being projected onto the monitor screen of a 486 DX computer. Images were assessed using the color image analysis program Quantimet 500+ (Leica Cambridge Ltd, Cambridge, England). Airways were subjected to standard airway morphometric analysis, as described below.

Control cases

Permission was obtained from the Victorian Institute of Forensic Pathology to access the records of infant deaths reviewed by the Institute from 1991. In age matched cases where no previous pulmonary pathology was likely, sections were obtained from the stored lung blocks as described in the previous section. Cases in which significant pulmonary damage was likely to be a feature of the cause of death such as pneumonia, congenital heart disease, or history of premature birth, were excluded. Cases included were those in which previously healthy children had died suddenly e.g. as a result of motor vehicle accidents or homicide. A further 8 control cases with similar causes of death were included from a study in Western Australia by three of the investigators (AJ, NC, JE). Sections of tissue had been obtained in a standardised way as part of a protocol for the pathological examination

of all (including SIDS) deaths. Measurements of airway dimensions in these cases were performed by the same investigator (JE) using identical techniques.

Airway morphometry

Morphometric analysis was performed on all airways that were cut in cross section, or near cross section. To avoid errors arising from tangential sectioning, airways with a short to long axis ratio of less than 0.6 were excluded from subsequent analysis (125). Four perimeters and areas were measured; 1) the internal perimeter (Pi) and area (Ai), defined by the luminal border of the epithelial, 2) the perimeter (Pbm) and area (Abm), defined by the outer border of the basement membrane, 3) the perimeter (Pmo) and area (Amo) defined by the outer border of the smooth muscle, and 4) the total perimeter (Po), and area (Ao), defined by the outer edge of the adventitia surrounding the airway (Fig 10). In addition, the area of airway smooth muscle was traced.

Airways which showed >50% epithelial detachment or branching were excluded, however where smaller sections of epithelium were missing, the border was interpolated between two intact areas (125)

Calculations

The areas defined by the perimeters were used to calculate wall areas ie, the epithelial wall area (Waepi = A bm - Ai), the inner wall area (Wai = Amo- Ai) the outer wall area (Wao = Ao - Amo) and the total wall area (Wat = Ao - Ai) as has been previously outlined by Bai *et al* . (125). Because the airway wall areas, and proportion of smooth muscle within the

airway wall, are a function of airway size, the areas were divided by the basement membrane perimeter (125, 157). The airway distribution between groups is shown in Fig.11.

DATA ANALYSIS

To assess the effects of SIDS, age and sex on airway dimensions, a weighted least squares model was applied to the data. In brief, airway wall areas and airway smooth muscle area data were linearised by plotting the square root of area against the Pbm for each of the airway wall compartments. Using linear regression analysis, the data for each case were plotted and the slope and intercept, together with their standard errors (SE) calculated (189).

The individual slopes and intercepts are weighted by calculating ($1/SE^2$). The slopes and intercepts were then regressed, adjusting for the effects of exposure (SIDS or no SIDS), sex (male or female) and age (months). This is designed to adjust for the effects of airway growth when comparing airway dimensions. Where data was normally distributed, a student's t-test was used to compare the mean data for airway dimensions and numbers of attachment points in the different airway size groups. A probability of < 5 % is considered significant.

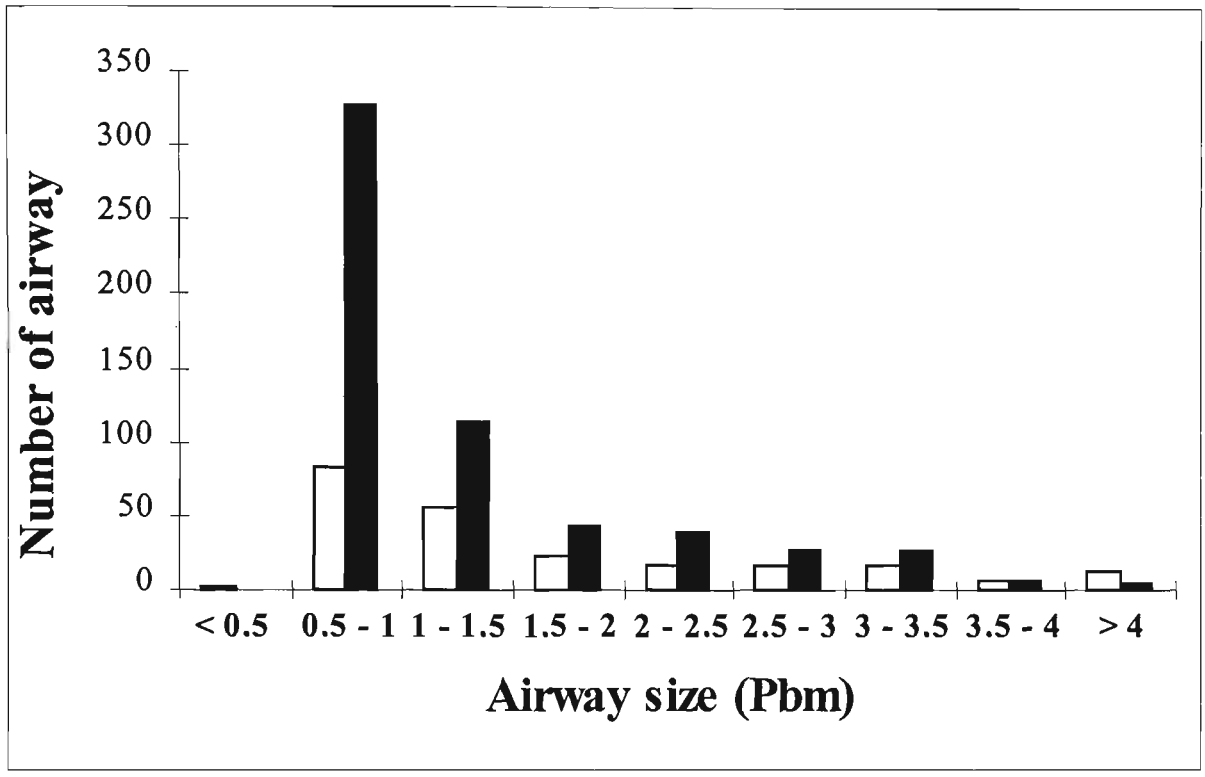

Figure 11 Airway size distribution of patients studied. SIDS cases are represented as solid bars and clear bars represent control cases. The airways are sized by the basement membrane perimeter which has been shown to be independent of muscle contraction and lung volume (157).

Observer Error

Intraobserver error was assessed by calculating the coefficient of variation for measurements of airway dimensions made on 10 different airway measurements on separate occasions (186). All measurements were made by the one observer who was blinded to the case classification.

RESULTS

Results were obtained from 57 infants who had died from SIDS: 36 males and 21 females with a mean (± SD) age 4.8 ± 3.1 months and 21 control infants: 13 males and 8 females with a mean (± SD) age 7.2 ± 5 months. (Fig 12) In a further 33 cases of SIDS no airways suitable for morphometric analysis were seen on the obtained section. All control infants had had a post mortem examination performed by a paediatric pathologist and in all cases the cause of the sudden death was not associated with evidence of significant lung pathology. The causes of death are outlined in Table 1. There was no significant difference in the age or sex distribution between the control group from Victoria: 9 males and 4 females with a mean (± SD) age 7.6 ± 4.7 months and Western Australia: 4 males and 4 females with a mean (± SD) age 6.5 ± 5.8 months, nor between the group of SIDS cases included in this study and those where morphometric assessment was not possible. There was no significant difference in any of the measured morphometric parameters between the two control groups and their results are thus pooled for comparison with the SIDS cases.

TABLE 1.

CAUSE OF DEATH IN CONTROL CASES

Case	Age (mths)	Sex	Cause of death
1	7	Female	Meningitis
2	1	Male	Meningitis
3	4	Male	Disseminated sepsis
4	1	Female	Septicaemia
5	3	Male	Septicaemia
6	18.5	Female	Skull fracture(Motor vehicle accident)
7	11	Female	Head injury
8	18	Female	Skull fracture
9	7	Female	Asphyxiation
10	5	Female	Hydranencephaly
11	4	Male	Asphyxiation
12	0.3	Male	Blood loss (Post operative)
13	3.1	Male	Head injury
14	7	Male	Head injury
15	4.6	Male	Haemorrhagic cerebral infarction
16	13	Male	Asphyxiation
17	8.5	Male	Asphyxiation
18	9	Male	Drowning
19	7	Female	Blood loss (Motor vehicle accident)
20	7.1	Male	Carbon monoxide poisoning
21	11.2	Female	Head injury

Table 1 Cause of death in the 21 infants used in this study as controls cases. The cause of death was determined following examination of the circumstances surrounding death by investigators from the coroner's office as well as a post mortem examination performed by an experienced paediatric pathologist.

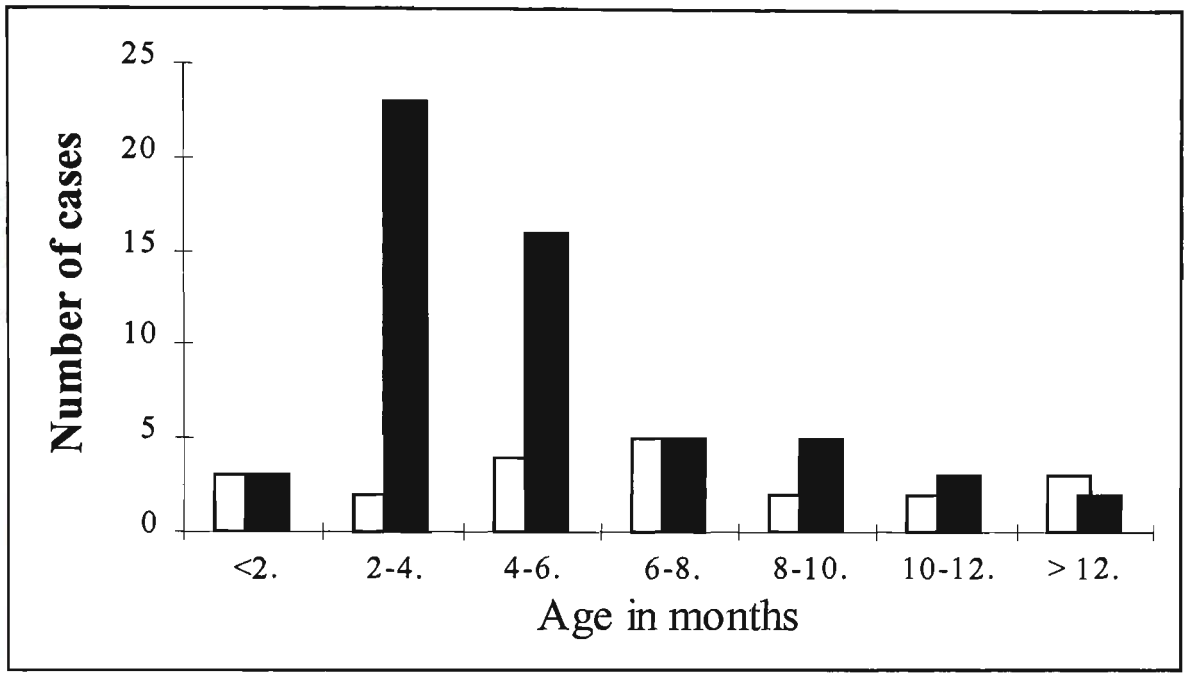

Figure 12 Age distribution of patients studied. Age is expressed in months for both SIDS cases (solid bars) and clear bars (control cases). In the SIDS group one patient was over 12 months of age (17 months), in the control group 3 patients were over 12 months of age (17 months and two 18 month old infants).

Airway morphometry

The coefficient of variation for airway measurements ranged from 0.44% to 3.7% with a overall mean value of 2% ± 0.7%.

Five hundred and eighty seven airways from infants dying from SIDS were examined and compared with 227 airways from control infants. The intercepts of regression lines were similar between case groups. There was a significant effect of SIDS and sex on the slope of the airway smooth muscle area plot. Cases of SIDS had greater smooth muscle area for any given airway size (Fig 13) and males had a steeper slope than females. There was no difference between SIDS and control cases for inner airway wall area, outer airway wall area or epithelial thickness, however, there was an independent effect of sex on the slopes for the inner and outer wall area with males having a steeper slope for inner wall area and females a steeper slope for outer wall area. This may be due to the effect of the larger airway smooth muscle area in the male infants.

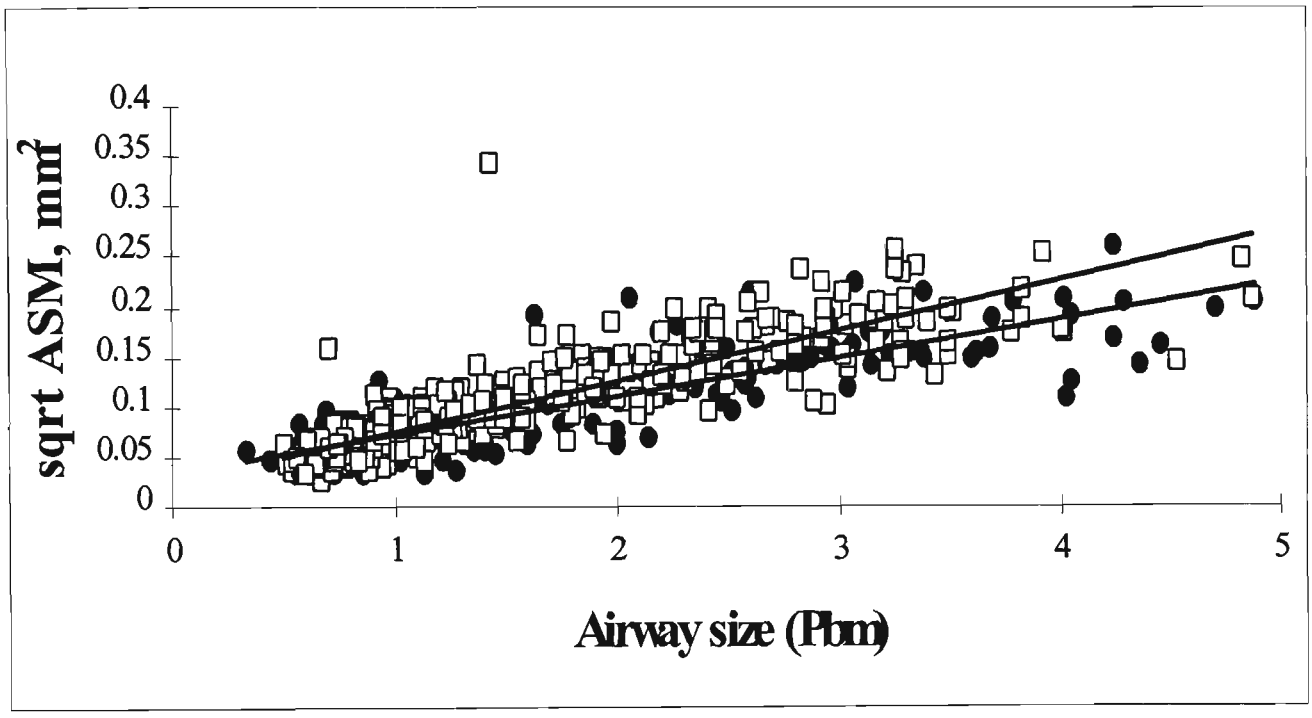

Figure 13 Plot of the square root of area of airway smooth muscle against basement membrane perimeter (Pbm) from 57 infants who died from SIDS (□) and 21 age matched control infants who died suddenly from other causes (●). There is a significantly increased amount of airway smooth muscle in the SIDS group compared to the control group ($p < 0.01$).

DISCUSSION

This study shows that airways obtained from infants who died from SIDS have a larger proportion of smooth muscle in their airways than age and sex matched infants dying from other causes. The Sudden Infant Death Syndrome describes the unexpected and unexplained death of an apparently previously well infant (30). The diagnosis is one of exclusion and is made after post mortem examination and a detailed assessment of the environment in which death occurred. While many post mortem examinations have been performed on infants whose death has subsequently been attributed to SIDS there is no uniform pathological feature which aids in the diagnosis of this condition. The post mortem examination in cases of SIDS is, in many ways, to exclude other causes of death such as undiagnosed infections eg. meningitis or pneumonia. By examining tissue obtained at post mortem from infants dying from SIDS many investigators have tried to identify the pathophysiological mechanism or mechanisms underlying the cause of death. Most interest has centered on two organ systems, the neurological (192-195) and respiratory systems (196, 197). Investigators have postulated abnormalities of the neural centres responsible for the control of breathing or have examined pulmonary tissue for evidence of altered structure or function. Haque *et al* (8) examined 25 cases of SIDS and 18 controls in their study on airway morphometry in SIDS. They examined over 1,000 airways, predominantly membranous bronchioles from SIDS cases and found a significantly increased index of airway wall thickness compared with control cases. Smooth muscle content of the airway wall was not measured. In the present study the thickness of the airway wall, and the area occupied by airway smooth muscle were measured directly. We did not find any significant difference in airway wall thickness between SIDS cases and controls, contrasting our

results with those of Haque. We have previously examined the airways from 38 infants who died from SIDS and shown that in the 19 infants who were exposed to high levels of cigarette smoke (maternal smoking >20 cigarettes a day) both *in-utero* and post-nataly, there was a significant increase in the inner airway wall thickness compared with the 19 infants who had died from SIDS whose mothers did not smoke either during pregnancy or post-nataly (199). In this study we also examined the amount of smooth muscle in the airways and found that there was no difference between smoke exposed and non smoke exposed groups of infants who died from SIDS. In the study by Haque, no allowance was made for the degree of cigarette smoke exposure in infants in the SIDS group. It is likely given our recent findings that the finding of increased inner airway wall thickness in the SIDS group as a whole in Haque's study reflects the number of smoke exposed infants in their study group. Given that we previously have not shown any difference in airway smooth muscle mass within a group of SIDS infants when infants exposed to high levels of smoke are compared with those infants not exposed to any maternal smoke, the findings of this present study of increased airway smooth muscle in lungs from infants who died from SIDS compared to controls would suggest that this difference is not a smoke related effect and may indeed reflect a SIDS effect (199).

In this present study we were given access to stored lung blocks from the coroners court. We therefore had no control over the site of sampling of the lung and further no control over the degree of lung inflation, which occurred prior to tissue sampling. These limitations prevent us from using the alternative form of tissue morphometry, stereology, as pioneered by Cruz -Orive, Gundersen and Weibe and more recently reviewed by Bolender (200-202).

This technique permits assessment of epithelial and smooth muscle volume providing that full lung inflation to TLC is achieved before lung sectioning commences. From this starting point isotropic uniform random sections can be obtained by vertical sectioning of the lung thus ensuring that all orientations and positions of airways are sampled. Point counting using a cycloid grid placed over the sections permits a very detailed and accurate assessment of epithelial and smooth muscle volume. This detailed and precise method of measurement may well provide a more accurate way of measuring airway smooth muscle mass than that employed by us in this present study and ideally our results will need to be confirmed by future studies using this technique. In reality however the ability of researchers to ensure full lung inflation at a coronial post mortem is not only difficult to achieve on a pure practicality basis but in this era is further limited by both ethical and legal constraints. The ability of researchers to obtain any tissue from a post mortem on an infant dying from SIDS is in many areas, including our own state of Victoria now very tightly restricted by legal directives. In this climate the use of the more detailed methods of stereology, while desirable, are for most cases unachievable.

The present study has shown that airway smooth muscle is increased in infants who die from SIDS. What effect could this increase in airway smooth muscle produce? For a given airway size an increase in the degree of airway smooth muscle may predispose to excessive airway narrowing due to increased force development during stimulation (6). Increased activation of smooth muscle, independent of any actual increase in airway smooth muscle content might predispose to small airway closure. Martinez *et al* (9) have postulated that small airway closure could be associated with SIDS and our present findings suggest that in

some cases this mechanism may be an important factor in the cause of death. While this study has shown an increase in airway smooth muscle mass within the airway wall of infants who die from SIDS it is not possible to draw conclusions from this data regarding the level of maximal smooth muscle contraction. To answer these questions it would be necessary to obtain fresh tissue for *in vitro* studies as well as submitting resected tissue to a maximal contractile stimulus before sectioning to allow for analysis of maximal contractile ability (157).

What is the underlying mechanism for this observed increase in smooth muscle? This present study is unable to answer whether the observed increase in airway smooth muscle is a primary effect which directly produces the pathophysiological abnormalities resulting in sudden death which leads to SIDS or whether the alterations in airway smooth muscle are secondary to other factors which may cause death in SIDS through other pathophysiological mechanisms. While unrecognised genetic factors may be responsible for producing this increased amount of airway smooth muscle, environmental factors, such as *in-utero* cigarette smoke exposure, a known risk factor for SIDS, may be responsible. *In utero* cigarette smoke exposure is known to produce abnormalities in lung development which may be evident as alterations in lung function in the neonatal period (17, 18, 203). While these findings would tend to support Martinez's theory of small airway closure in SIDS, animal studies which have shown anatomical abnormalities in lung development in offspring from animals exposed to cigarette smoke during pregnancy, have reported abnormalities in alveolar and parenchymal elastic tissue development but have not documented alterations in airway smooth muscle content (204). Clearly as SIDS may also

occur in situations where infants were not exposed to any degree of maternal smoking other factors which may influence airway smooth muscle development are likely to be important.

It is further possible that the observed increase in airway smooth muscle in the SIDS lungs in this group is reflecting pathological mechanisms occurring elsewhere. Chronic upper airway obstruction has frequently been postulated to be an important mechanism in the pathogenesis of SIDS (116). While Naeye has previously described an increase in smooth muscle content in pulmonary arteries in infants dying from SIDS - a finding also seen in other forms of chronic upper airway obstruction such as obstructive sleep apnoea (OSA), there has never been a description from any other cause of chronic upper airway obstruction (including OSA) of increased airway smooth muscle. In the absence of any precedence from other conditions, as well as the difficulty in explaining such a relationship on physiological principles, it is hard to postulate that the observed increase in airway smooth muscle seen in this study could be secondary to chronic upper airway obstruction.

In summary this study has shown that infants who die from the Sudden Infant Death Syndrome have a higher proportion of airway smooth muscle in their small airways than age matched infants who die suddenly from causes not associated with underlying cardiorespiratory pathology. The increase in smooth muscle may contribute to excessive airway narrowing which along with other factors, such as immature ventilatory control mechanisms, may result in sudden death.

CHAPTER 5 - MATERNAL CIGARETTE SMOKING IS ASSOCIATEDWITH INCREASED INNER AIRWAY WALL THICKNESS AND DECREASED AIRWAY ATTACHMENT POINTS IN CHILDREN WHO DIE FROM SUDDEN INFANT DEATH SYNDROME

ABSTRACT

The harmful effects of passive cigarette smoke exposure to infants include an increased frequency of asthma exacerbations, lower respiratory viral infections, and the Sudden Infant Death Syndrome (SIDS). Because of a difficulty in obtaining airway tissue from infants, little information is available on the effects of passive cigarette smoke exposure on the structure of the infant airway wall. We examined airway dimensions in 19 children who died from SIDS whose mothers smoked more than 20 cigarettes a day pre and post-nataly, and compared this data with that from 19 infants who died from SIDS and had non smoking mothers. Total, inner and outer wall area were calculated for each airway and expressed in terms of the basement membrane perimeter (Pbm). The number of alveolar attachment points was counted for each airway and expressed as the mean number per mm of the outer airway perimeter (Po). Inner airway wall thickness was greater in the larger airways from those infants whose mothers had smoked more than 20 cigarettes compared with non-smoke exposed infants while the mean number of attachment points was decreased in all airways in all smoke exposed infants. These findings suggest that infants exposed to a high level of passive cigarette smoke develop significant structural changes in their airways. Both increased airway wall thickness and loss of alveolar attachments may

contribute to exaggerated airway narrowing and may help explain the previously observed abnormalities in neonatal lung function which have been described in infants of smoking mothers.

INTRODUCTION

Passive cigarette smoking is known to be a significant health risk (205). Infants exposed to passive cigarette smoking in the first year of life have an increased incidence of lower respiratory tract infections (15, 16). A large proportion of infants exposed to maternal passive cigarette smoke will also have been exposed to *in-utero* cigarette smoke exposure. Infants whose mothers have smoked during pregnancy have been shown to reduce lung function in the neonatal period (17, 18, 203). Despite this evidence there is often strong debate in the public domain about whether passive cigarette smoking in infants is in fact harmful. While direct anatomical studies examining the airways of adult smokers have documented structural changes associated with this insult (3), there is no anatomical information on the effect of passive cigarette smoking on the infant airway. Alveolar destruction in association with a reduction in lung elastic recoil and increased airflow obstruction is a common finding in symptomatic adults who smoke cigarettes (20).

The Sudden Infant Death Syndrome (SIDS) describes the unexpected and unexplained death of an apparently well infant (30). The diagnosis is one of exclusion and is made after a post-mortem examination and detailed assessment of the environment in which death occurred. Most epidemiological studies have linked maternal smoking with an increased risk of SIDS (30, 32, 39, 90, 93, 206, 207). As part of a larger study into lung structure in SIDS we elected to examine whether airways from infants, who had died from SIDS and who had been exposed to high levels of maternal smoking were structurally different to airways from infants who had died from SIDS and who had not been exposed to maternal smoking. We postulated that infants who had been exposed to high levels of maternal

smoking would show damage to their airways or lungs as a result of the passive cigarette smoke, independent of any changes associated with SIDS.

METHODS

In 1991 the Victorian Sudden Infant Death Research Foundation commenced a 3 year study into the epidemiology of SIDS in the State of Victoria, Australia. Families who had lost a child from SIDS were identified by the investigators using a variety of sources including ambulance officers, paediatric emergency nursing staff, SIDS grief counsellors and family members. The investigators then approached the families and invited them to participate in a formal interview where a detailed questionnaire covering various epidemiological factors associated with SIDS was conducted by a trained interviewer. The questionnaire included specific questions relating to maternal smoking. Mothers were asked to indicate whether they smoked before becoming pregnant and separately during the first, second, and third trimester of pregnancy. Mothers were also asked whether they smoked between the birth and death of their child. For all questions, smoking was rated on a 5 point scale with 0) reflecting no smoking, 1) less than 10 cigarettes a day, 2) 10-20 cigarettes a day, 3) 20-30 cigarettes a day and 4 a smoking habit of greater than 30 cigarettes a day.

As part of a larger study into lung structure in SIDS the chief investigators of this present report were granted access to the raw smoking data from these questionnaires. The investigators were also granted access to the stored lung blocks from all of the infants who died from SIDS in the state of Victoria over the period 1991-1993. This allowed interpretation of lung histology in the light of the degree of smoke exposure of the infant.

Tissue preparation

All cases of SIDS involved in this study had had a post-mortem examination performed by an experienced paediatric pathologist at the Victorian Institute of Forensic Medicine (VIFM). Permission was obtained from the Institute's ethics committee to access the stored lung tissue from those post-mortems performed during 1991-93. From each lung block one 5 micron section was taken and stained with haematoxylin and eosin. To preserve the block for possible future use by the VIFM the investigators were limited to one section from each block. Slides were examined using a video-linked microscope Leica Laborlux D, (Leica, Germany) with the image being projected onto the monitor screen of a 486 DX computer. Images were assessed using the colour image analysis program Quantimet 500+ (Leica Cambridge Ltd, Cambridge, England). Airways were subjected to standard airway morphometric analysis, as described below.

Airway morphometry

On all airways cut in transverse section, (defined as an even thickness of epithelium and an even thickness from the basement membrane to the outer smooth muscle layer) the following perimeters were measured; the inner airway wall perimeter (Pi), defined by the luminal surface of the epithelial border, the perimeter of the outer border of the basement membrane, (Pbm), the outer muscle perimeter (Pmo) defined by the outer border of the smooth muscle, and the outer airway wall perimeter (Po), defined by the outer edge of the adventitia surrounding the airway (Fig.10). The areas of airway smooth muscle, mucous gland and cartilage within the airway wall were also measured. Airways which showed

>50% epithelial detachment or branching were excluded, however where smaller sections of epithelium were missing, the border was interpolated between two intact areas.

The number of alveolar attachment points to the adventitial surface were counted for each airway and expressed as the number of attachment points per millimetre of the outer airway wall perimeter (Po) which is defined by the surrounding airway adventitia. Where a large pulmonary vessel was adjacent to the airway and no alveolar attachments were possible, the length of the outer perimeter where the airway and vessel were adjacent was subtracted from the total outer perimeter to give the total length over which alveolar attachments could be counted.

Calculations

Using the measured perimeters, the image analysis program calculated the areas defined by each of these perimeters. The epithelial wall area (WAe), the inner wall area (WAi) the outer wall area (WAo) and the total wall area (WAt). Because the wall areas and proportion of smooth muscle, mucous glands and cartilage within the airway wall are influenced by the size, the areas were expressed in terms of the traced Pbm. Airways were divided into three size groups on the basis of the measured Pbm, <1mm, 1-2mm and 2-4 mm.

The percent of smooth muscle shortening (PMS) is present in each airway was calculated as follows. The "relaxed" lumen area (Abmr) is the area of a circle with a circumference equal to the measured Pbm. $(Abmr) = (Pbm^2) / 4\pi$. By adding the measured area of wall

which changes little during muscle contraction (157) between the basement membrane and the outer muscle border (Amo - Abm) to Abmr, the relaxed outer area (Amor) is obtained. The perimeter (Pmor) of this area is the length of the 'relaxed' smooth muscle. By comparing the measured 'muscle length' (Pmo) to the relaxed muscle length (Pmor), the amount of muscle shortening that is present can be calculated.

PMS = (Pmor - Po)/Pmor x 100, where Pmor = $\sqrt{}$ ($4\pi \times$ Amor).

Data analysis

To compare similar size airways from different subjects, airways were divided into 3 arbitrary size groups using the Pbm, < 1mm, 1-2mm, and 2-4mm. Airway size groups were chosen to minimise the effects of growth, and to enable comparisons to other studies (8, 190). All airway measurements of area were normalised for airway size by dividing by the basement membrane perimeter (157). For each variable, the mean value for each case was calculated. The differences between the means of size groups were tested using a one-way analysis of variance (ANOVA). A probability value of less than 5% was considered to be significant.

Intra observer error was expressed as the coefficient of variation, and was calculated for measurements made on 10 airways 10 times as previously described by Carroll *et al* (140). All measurements were made by the one observer who was blinded to the case classification.

RESULTS

A total of 83 cases of SIDS with completed questionnaire smoking data and lung blocks were available to the investigators. There were 48 males and 35 females with a mean (± SD) age 5.7 ± 4.3 months. The time to interview was mean (± SD) 31 ± 19 days from the death of the infant. In 38 cases there was a consistent level of smoking through all 4 time periods assessed in the questionnaire. Nineteen mothers did not smoke either before, at any stage during, or after pregnancy (no smoke group). A further 19 mothers smoked more than 20 cigarettes a day in all these time periods (high smoke group).

In the remaining 45 cases infants had been exposed to variable levels of cigarette smoke both during and following pregnancy with a mean (± SD) age 6.5 ± 5.4 months. This was not significantly different from the 38 infants whose morphometry is reported in this study. As the variability in smoke exposure made the assessment of any relationship between observed histological changes and smoking history impossible this present report will concentrate on those two groups where smoking level was constant throughout all time periods.

The age at death of the infants in the high exposure group was mean (± SD) 4.9 ± 2.9 months and 5 ± 3.8 months in the non smoke exposure group. In each group there were 12 males and 7 females.

Airway morphometry

Two hundred and twenty eight airways from the 19 infants in high smoke group were compared to 158 airways from the 19 infants in the no smoke group. (Table 2) There was

no detectable difference in the airways from the two groups when viewed under simple light microscopy. Inner wall area expressed with relationship to Pbm was significantly greater in the high smoke group in airways in the Pbm 2-4 mm group. (Table 3) The epithelial thickness expressed in relationship to Pbm was also significantly increased in this airway size group in the high smoke exposure group compared to the no smoke exposure group. The increased inner wall thickness was independent of the increase in the epithelial thickness. Total and outer wall areas were not significantly different in the larger airway size group.

TABLE 2

AIRWAYS NUMBER AND SIZE

	< 1mm	**1 - 2mm**	**2 - 4mm**
High smoke group	132 (58%)	49 (22%)	47 (20%)
No smoke group	77 (49%)	58 (37%)	23 (14%)

Table 2 Number of airways of differing sizes measured. The no smoke group refers to infants who died from SIDS and whose mothers did not smoke either during or following pregnancy and high smoke group refers to those infants whose mothers smoked over 20 cigarettes a day both during and following pregnancy.

TABLE 3.

COMPARTMENTAL WALL DIMENSIONS

	No Smoke	High Smoke	p value
WAi/Pbm	0.055±0.008	0.07±0.013	p<0.05*
Wao/Pbm	0.12±0.062	0.15±0.067	p>0.05
WAt/Pbm	0.17±0.067	0.22±0.075	p>0.05
Epi/Pbm	0.02±0.003	0.03±0.007	p<0.01**
Muscle/Pbm	0.01±0.003	0.01±0.003	p>0.05
% muscle shortening	12.2±7.91	15.5±6.03	p>0.05

Table 3. Morphometric data from airways with a basement membrane perimeter between 2-4 mm of infants who died from SIDS and whose mothers did not smoke either during or following pregnancy (no smoke) compared with airways from infants whose mothers smoked over 20 cigarettes a day both during and following pregnancy (high smoke). Results are expressed as mean ±standard deviation.

TABLE 4.

COMPARTMENTAL WALL DIMENSIONS IN AIRWAYS <1mm and 1-2mm.

TABLE 4A.	No Smoke	High Smoke	p value
WAi/Pbm	0.028±0.008	0.028±0.008	p>0.05
Wao/Pbm	0.028±0.012	0.29±0.015	p>0.05
WAt/Pbm	0.056±0.018	0.057±0.02	p>0.05
Epi/Pbm	0.014±0.004	0.014±0.004	p>0.05
Muscle/Pbm	0.005±0.002	0.004±0.002	p>0.05
% muscle shortening	3.9±3.7	3.4±4.5	p>0.05

TABLE 4B.	No Smoke	High Smoke	p value
WAi/Pbm	0.04±0.012	0.043±0.014	p>0.05
Wao/Pbm	0.055±0.036	0.052±0.032	p>0.05
WAt/Pbm	0.095±0.044	0.095±0.043	p>0.05
Epi/Pbm	0.019±0.005	0.02±0.006	p>0.05
Muscle/Pbm	0.006±0.003	0.007±0.003	p>0.05
% muscle shortening	11.3±11.5	5.6±5.8	p>0.05

Table 4 Morphometric data from airways with a basement membrane perimeter between <1mm (Table A) and 1-2mm (Table B) of infants who died from SIDS and whose mothers did not smoke either during or following pregnancy (no smoke) compared to airways from infants whose mothers smoked over 20 cigarettes a day both during and following pregnancy (high smoke). Results are expressed as mean ±standard deviation.

There was no significant difference between the high and no smoke groups in the 2 smaller airways size groups (Pbm <1 mm and Pbm 1-2 mm) for epithelial, inner, outer and total wall thickness or the area of smooth muscle.

Airway smooth muscle

There was no significant difference in the amount of measured smooth muscle within the two groups in all airway size groups. The percentage of airway smooth muscle shortening showed marked variability both between airways in particularly and between cases, ranging from 0% to 40% throughout all cases. There was no significant difference in the degree of airway smooth muscle shortening between the high, and no exposure groups in any airway size. There was no significant difference in the amount of mucous gland or cartilage content of the airways between the two groups in any airway size.

Airway Attachment Points

Compared with non-smoke exposed infants the mean number of alveolar attachment points was reduced ($P<0.05$) in SIDS cases (14 ± 1 v 11 ± 0.5) in small membranous airways and (13 ± 1 v 10 ± 0.5) in larger membranous airways. These findings were regardless of whether the passive smoke exposure was categorised as high or low and independent of the stage of the pregnancy when smoke exposure occurred.

The coefficient of variation of the observations was $2\% \pm 0.7\%$.

TABLE 5

MATERNAL SMOKE EXPOSURE

Case	Sex	AGE/M	weight	Height	birth weight	Gest/wks	1t	2t	3t	post >
1	M	7	7.3	66	3.52	38	0	0	0	0
2	M	3	6.2	61	4.352	40	0	0	0	0
3	M	2	6.1	58	4.069	40	0	0	0	0
4	F	6	8	64	3.435	39	0	0	0	0
5	F	5	6.1	63	2.715	40	0	0	0	0
6	F	2.4	5	60	3.004	41	0	0	0	0
7	F	3.2	7.7	63.4	3.65	38	0	0	0	0
8	F	2.5	5.1	60	3.557	40	0	0	0	0
9	M	6			3.999	40	0	0	0	1
10	M	13	9	74	1.193	31	0	0	0	4
11	F	2			2.93	38	0	0	0	1
12	F	5	7.4	55	3.986	42	0	0	0	1
13	M	10			3.999	40	2	2	0	0
14	M	2			3.549	40	2	0	2	0
15	M	5.5	8	65	4.14	40	1	1	1	0
16	F	5.5	7	61	3.885	40	2	1	1	0
17	M	2.9			3.117	41	1	2	2	2
18	M	2.7			2.947	38	1	2	2	1
19	M	2.6			2.313	28	0	2	3	2
20	M	3			3.298	38	0	1	1	2
21	M	4.3			3.53	38	2	2	0	1
22	M	5.6			2.99	38	2	2	1	2
23	M	2.6			2.037	42	2	3	4	1
24	F	2.5			3.772	43	2	2	2	1
25	F	4.3			3.145	39	1	2	2	2
26	F	8	9	69	3.943	40	1	1	0	1
27	M	2.3	5.4	62	3.65	40	4	4	4	4
28	M	4	5.4	61	2.833	37	4	2	4	4
29	M	4	7	66			4	4	4	3
30	F	9	6	64	2.369	40	4	4	4	3
31	F	1.2	3.8	51	2.947	39	4	4	4	4
32	F	7.6	8	66	2.525	41	4	4	4	4

Table 5 Data relating to the 32 cases of SIDS death used in this study. The diagnosis of SIDS was determined following examination of the circumstances surrounding death by investigators from the coroners office as well as a post mortem examination performed by an experienced paediatric pathologist.

DISCUSSION

The present study has documented changes in airway wall dimensions in infants who have been exposed to consistently high levels of maternal smoking when compared to infants who have died from the same cause but had no exposure to maternal cigarette smoke and loss of alveolar attachments in infants exposed to cigarette smoke regardless of the level of exposure. We believe this is the first reported study documenting anatomical abnormalities in the infant airway associated with passive cigarette smoking.

All lung blocks used in this present study were obtained from infants who had died from the Sudden Infant Death Syndrome. This diagnosis was made following a careful post-mortem examination and detailed assessment of the circumstances surrounding death in order to exclude other causes of death. No diagnostic pulmonary pathology has been described in the Sudden Infant Death Syndrome, specifically no previous studies of lung structure in SIDS utilising direct measurements of airway thickness have documented increased airway wall thickness as a feature, and we are therefore confident that the findings of this present study reflect more on the degree of smoke exposure of the infants than the subsequent death from SIDS (8, 107, 108).

The infants from the high smoke exposure group had mothers who smoked more than 20 cigarettes a day both during pregnancy and post-nataly. *In-utero* smoke exposure represents a different insult to the growing airways than post-natal smoke exposure, as *in-utero* smoke exposure is not associated with direct contact between inhaled smoke and the airway. Post-natal smoke exposure is a true inhalational insult with the infant directly inhaling side-

stream cigarette smoke. Although increased inner airway wall thickness in infants of smoking mothers was found in this present study we are unable to determine from our findings whether this alteration in airway morphometry is due to an *in-utero* smoke exposure effect or a post-natal smoke exposure effect. It is unclear by what mechanism the indirect effect of *in-utero* smoke exposure might result in a thickened inner airway wall but it is tempting to speculate that inflammatory changes similar to those seen in adults may be responsible. If this were the case then it suggests that the placental blood barrier may not be sufficient to protect infants in-utero from some environmental pathogens (eg. cigarette smoke). Alternatively direct irritation of the inner airway wall by passive smoke exposure post-natally might have a more direct effect on the lung and could explain the finding of our present study. Nevertheless, an increase in the inner airway wall, whatever the mechanism, can amplify the effects of smooth muscle shortening thereby resulting in altered lung function.

It is interesting that the number of alveolar attachment points was reduced even in SIDS cases in which the cigarette smoke exposure had been relatively low. A larger cohort of cases with more clearly defined exposure patterns would be needed to determine whether there is a dose-response relationship between smoke exposure and reduced attachment points. We did not assess the degree of alveolar damage in this study and cannot be sure whether our findings reflect immature lung development or a reduced number of attachments due to direct damage associated with passive smoke exposure. We propose to measure airway and lung inflammation in addition to measuring the mean linear intercept to estimate the mean number of alveoli in these infants. This would help in determining the

likely mechanism to explain our findings. Clearly though, if reduced numbers of alveolar attachments result in decreased lung elastic recoil, then one of the major loads opposing airway smooth muscle shortening would be reduced allowing greater muscle shortening for the same stimulus. Whether or not excessive airway narrowing or altered airway smooth muscle shortening is important in the pathogenesis of SIDS remains unknown.

The exact mechanism by which maternal smoking increases the risk of Sudden Infant Death Syndrome is as yet undetermined. Some investigators have suggested that direct toxic effect on lung growth may occur secondary to *in-utero* smoke exposure and that the altered lung growth may predispose infants to impaired lung function post-nataly and subsequently an increased risk of SIDS. Maternal smoking has also been identified to be associated with alterations in development of areas of the nervous system associated with respiration. Abnormalities in these areas may then produce secondary abnormalities in either *in-utero* or post-natal lung development and/or function which may predispose to SIDS. The findings of this present study showing increased inner airway wall thickness in infants whose mothers smoked during pregnancy is unable to differentiate between a direct effect of the cigarette smoke exposure on lung development or whether smoke exposure produced abnormalities in other systems such as the nervous system.

Several epidemiological studies have documented abnormalities in pulmonary function in infants in the first month of life born to smoking mothers (17, 18, 203). Most investigators have considered that these changes are a result of the detrimental effects of *in-utero* smoke exposure. Hogg *et al* (208) have previously shown that the small airways of young

children (defined by them as those less than 2mm in internal diameter) are responsible for a large part of the total airway resistance. Alterations to the airway wall structure, particularly increased inner airway wall thickness in airways this size could then have major effects on airway physiology. This may indeed explain the observed alterations in increased airway responsiveness that have been described in infants with a history of exposure to maternal cigarette smoke (18).

Maternal smoking has been shown to be associated with an increased incidence of the Sudden Infant Death Syndrome (32, 37, 39, 90, 93, 206, 207). Martinez has postulated that the patho-physiology of SIDS may be related to small airway closure (9). Our findings that the inner airway wall is thickened and the number of alveolar attachments is reduced in membranous airways lends plausibility to this hypothesis. Clearly while this postulated mechanism may explain the course of death in infants who died from SIDS and who were 0exposed to high levels of maternal smoking, they do not explain the cause of death in infants whose mothers did not smoke. This may simply reflect the spectrum of pathophysiology of death in infants who die from SIDS (30).

Exposure to passive cigarette smoke in the infant has also been shown to be associated with an increased frequency of lower respiratory tract infections (15, 16). While a relationship between SIDS and lower airway infection has been postulated, no direct pathological evidence from cases involved in this present study identified any evidence of lower airway infection. Lower airway infection by itself would not explain the observed increased inner airway wall thickness or loss of alveolar attachments because infection derived

inflammatory processes would be expected to produce transmural increases in wall thickness rather than be isolated to the inner airway wall only.

We conclude that passive cigarette smoking produces significant alterations in the structure of the developing infant airways and lungs which may have significant physiological sequela and may be related to the cause of death in SIDS in infants with a history of maternal smoking. These findings also emphasis the dangers of passive cigarette smoke exposure to infants and highlight the importance of anti-smoking measures aimed at reducing this risk.

CHAPTER 6 - INCREASED AIRWAY REACTIVITY AND DECREASED AIRWAY ATTACHMENT POINTS FOLLOWING *IN-UTERO* SMOKE EXPOSURE IN THE GUINEA PIG

ABSTRACT

Maternal smoking during pregnancy has been shown to result in abnormalities in lung function in newborn infants including reduced expiratory flow and increased airway responsiveness to inhaled agonists. The mechanism by which this occurs remains unclear. Using a guinea pig model of *in-utero* smoke exposure we measured airway responsiveness and lung morphology in a group of neonatal guinea pigs 21 days after delivery. Pregnant guinea pigs were exposed to cigarette smoke from day 28 to term (day 68 of gestation). Following delivery newborn animals did not receive any smoke exposure. Airway wall thickness, smooth muscle area and the number of points where the alveoli attached to the airway adventitia were measured. Airway responsiveness was increased ($p<0.05$) 6 fold and the mean number of alveolar attachment points per mm of the outer perimeter of the airway was decreased ($p<0.05$) (mean 19.4 ± SE 0.41 v 21.6 ± 0.81) in animals exposed to cigarette smoke *in-utero* compared with non-exposed animals. Although not statistically significant, both the inner and outer airway wall and the smooth muscle area were greater in exposed animals compared with non-exposed animals. These findings suggest that the increased airway responsiveness observed in post-natal animals, subsequent to *in-utero* cigarette smoke exposure, may be the result of decreased alveolar attachment points to the airways and changes in airway dimensions.

INTRODUCTION

In-utero cigarette smoke exposure has been shown to produce abnormalities in lung function in newborn infants. Tager *et al* (17) showed that infants born to mothers who smoked during pregnancy had approximately 10% reduced expiratory flow parameters when compared to infants whose mothers did not smoke during pregnancy. Young *et al* (18) showed that infants whose mothers smoked during pregnancy had increased airway responsiveness to inhaled histamine 4 weeks after delivery. In epidemiological studies, exclusion of the effects of any post-natal smoke exposure is difficult, however the use of statistical methods, in which these confounding variables are adjusted for, suggests that increased responsiveness in these infants is primarily associated with *in-utero* cigarette smoke exposure (19). Despite these conclusions the actual mechanisms resulting in abnormal post-natal lung function following *in-utero* cigarette smoke exposure are unknown. Saetta *et al* (20) have previously shown that number of alveolar attachment points to the surrounding airway adventitia are reduced in active smokers and that this reduction is associated with a reduction in lung elastic recoil. Several studies (19, 21, 22) and (23) have reported reduced respiratory function at birth in infants whose mothers smoked during pregnancy and propose altered lung/airway development *in-utero* as a likely mechanism although no attempts were made to test these hypotheses. Therefore, we hypothesised that alterations in post-natal lung function observed following *in-utero* cigarette smoke exposure are due to altered lung/airway structure. The aims of this study were to examine the effects of *in-utero* smoke exposure on post-natal lung function, specifically airway responsiveness, and lung (alveolar attachments) and airway (airway dimensions) structure using a small animal model of *in-utero* smoke exposure.

METHODS

In-utero model of smoke exposure

Nine pregnant Cam Hartley guinea pigs were obtained from a local breeding facility (Monash University breeding facilities, Clayton, Victoria, Australia) at between 15 and 22 days post conception. Smoke exposure commenced at day 27 post conception and occurred for 15 minutes a day for 4 days each week. Smoke exposure commenced at day 27 to minimise the risk of failed implantation and/or spontaneous abortion due to the acute effects of cigarette smoke inhalation. The guinea pigs were placed in a 16 litre perspex chamber developed for this project. A cigarette was connected to the access port in the wall of the chamber. A bi-directional syringe pump was also connected to the chamber to enable cigarette smoke to be drawn in. Following the lighting of the cigarette, smoke was drawn into the syringe by the pump and the smoke was then returned into the chamber through a further porthole. An oxygen monitor continuously monitored the oxygen levels in the exposure chamber and supplemental oxygen was bled into the chamber, as the combustion process continued, to maintain normoxic conditions. At the end of the 15 minute exposure animals were removed and returned to their standard cages. In order to limit any effects related to the stress of handling, control animals were placed in an identical exposure chamber at the same frequency but received no smoke exposure.

Serum cotinine measurements

Blood samples were drawn from each pregnant animal under light halothane anaesthesia twice a week for measurement of serum cotinine. Blood samples were centrifuged and the

serum removed. Analysis of serum cotinine was performed using a cotinine assay kit (DPC nicotine metabolite assay, Bio-Mediq DPC Pty Ltd. California USA). Blood samples were drawn immediately prior to the first smoke exposure of each week and 24 hours following the last smoke exposure each week. To control for any possible effect of the halothane anaesthesia on foetal development, control non-smoke exposed animals were also anaesthetised and bled at an identical frequency.

Neonatal Animals

Pregnant animals were allowed to proceed to normal delivery, usually at 68 days gestation. To limit any effects of smoke metabolites which may cross through breast milk new born infants were removed from mothers as soon as possible and placed with lactating animals who had not received smoke exposure during pregnancy. New born animals received no smoke exposure post-natally and were studied at day 27 of post-natal life.

Assessment of airway responsiveness

Animals were anaesthetised with 0.3 ml ketamine and xylazine by intraperitoneal injection. Under supplemental halothane anaesthesia a tracheostomy was performed and the animals were then placed in a pressure sensitive plethysmograph, designed for the guinea pig and ventilated with a Harvard small animal ventilator (model 608, Harvard Apparatus, South Natick, MA) delivering a tidal volume of 3 ml at a frequency of 60 breaths per minute. Animals were paralysed with succinylcholine (0.5mg/kg im) immediately after attachment to the ventilator. Volume signals were obtained from a pressure-sensitive transducer (MP 45-2 $\pm$ 2 cm H_2O, Validyne, Northridge, CA) which measured changes in box pressure. The

plethysmograph was calibrated with a 10ml syringe and a 3 ml volume change resulted in a 0.3 cm H_2O pressure change within the box. The pressure response was linear to a volume change of 20ml. The volume signal was electronically differentiated to determine flow. Transpulmonary pressure was determined using a differential pressure transducer (model 267BC: Hewlett Packard, Waltham, MA) by comparing pressure at the tracheal opening with oesophageal pressure measured from a saline filled PE-90 tube in the distal oesophagus. The pressure transducer was calibrated using a water-filled manometer. The response was linear over the range $\pm$ 50 cm H_2O. The volume, flow and pressure signals were recorded (General Scanning, model RS4-5P recorder) and pulmonary resistance (R_L) was calculated at 50% of tidal volume from these signals using the method of Von Neergard and Wirz (191). After stable baseline R_L values were present, guinea pigs were given isotonic saline aerosol followed by increasing doses of aerosolized acetylcholine solution (Ach), and the changes in R_L were measured.

Acetylcholine chloride (Sigma Chemical) was dissolved in normal saline to produce a stock solution of 50 mg/ml. Serial dilutions were made to produce solutions of 15, 5, 1.5, and 0.5 mg/ml. Aerosol was generated by placing 5 ml of saline or Ach solution into a Hudson nebuliser (Hudson Oxygen Therapy Sales Co., Temecula, CA) driven by compressed air at 7 1/min and connected by a T piece to the attachment tube for the tracheostomy. The ventilator was disconnected from the circuit during the administration of the Ach. Six 3 ml tidal breaths of nebulised Ach were delivered for each concentration of Ach. Following the administration of each dose, the ventilator was immediately reconnected to the circuit. The computer analyses the first 20 breaths following recommencement of ventilation. Peak

responses following Ach challenges generally occur within the first 10 breaths after reconnection to the ventilator. The average of the three highest R_L values at 50% tidal volume was taken as the response to each Ach concentration. The next dose was delivered after R_L had returned to baseline.

Specimen preparation

At the completion of the assessment of airway responsiveness the animal was sacrificed and removed from the plethysmograph. The anterior chest wall was then removed and the trachea cannulated though the tracheostomy site. The lungs were then inflated over 60 minutes with 10% buffered formalin at 20 cm H_2O. Following inflation the lungs and heart were removed from the chest cavity *en bloc* and immersed in formalin. Multiple tissue blocks were cut in a saggital plane through the perihilar regions of both lungs and embedded in paraffin for histological processing. Three micron sections were cut and stained with haematoxylin and eosin for morphometric analysis. Slides were examined using a video-linked microscope Leica Laborlux D, (Leica, Germany) with the image being projected onto the monitor screen of a 486 DX computer. Images were assessed using the colour image analysis program Quantimet 500+ (Leica Cambridge Ltd, Cambridge, England.

Airway morphometry

On all airways cut in transverse section, (defined as an even thickness of epithelium and an even thickness from the basement membrane to the inner smooth muscle layer and a min/max diameter ratio1:3) the following areas (A) and perimeters (P) were measured; the

internal area and perimeter (Ai and Pi), defined by the luminal surface of the epithelial border, the basement membrane area and perimeter (Abm and Pbm), defined by the basement membrane; the outer muscle area and perimeter (Amo and Pmo), defined by the outer border of the airway smooth muscle, and the outer airway wall area and perimeter (Ao and Po), defined by the outer edge of the adventitia surrounding the airway (125) (Fig10). The area of airway smooth muscle in the airway wall was measured by direct tracing. Airways in which more than 50% of the surface epithelium was missing or which were cut at a bifurcation were not measured. In airways with more than 50% of the epithelium intact, the lumenal border of the epithelium was interpolated between two intact areas. The number of alveolar attachment points to the adventitial surface were counted for each airway and expressed as the number of attachment points per millimeter of the outer airway wall perimeter which is defined by the surrounding airway adventitia. Where a large pulmonary vessel was adjacent to the airway and no alveolar attachments were possible, the length of the outer perimeter where the airway and vessel were adjacent was subtracted from the total outer perimeter to give the total length over which alveolar attachments could be counted.

Calculations

The inner wall area (WAi) was calculated by subtracting Ai from Amo. The outer wall area (WAo) was calculated by subtracting Amo from Ao. The area of smooth muscle in the airway wall was normalised by dividing the measured area by the basement membrane perimeter which has been shown to be independent of muscle contraction and lung volume (157).

Data analysis

The basement membrane perimeter (Pbm) was used to define airway size. As there were so few large cartilaginous airways available for examination only airways with a Pbm of less than 4mm were analysed. These airways were predominantly membranous and small intraparenchymal cartilaginous airways. The results were expressed as the mean ± standard error (SE). Differences between the two groups were tested using an unpaired t-test. A probability of < 5 % was considered significant.

Intra observer error was expressed as the coefficient of variation, and was calculated for measurements made on 6 airways 6 times, as previously described by Carroll *et al* (186). All measurements were made by the one observer who was blinded to the case classification.

The study was reviewed by and approved by the Royal Children's Hospital Animal Experimentation Ethics committee.

RESULTS

Results were obtained from 17 animals (7 males and 10 female) born to 6 guinea pigs exposed to cigarette smoke during pregnancy. These results were compared to findings from 8 animals (5 males and 3 female) born to 3 guinea pigs not exposed to cigarette smoke during pregnancy. I*n-utero* smoke exposed animals had a significantly lower weight at birth compared with non-exposed animals (269g ±42g v 309g± 35g) ($p<0.05$), however the *in-*

utero smoke exposed animals displayed accelerated post-natal growth. By day 27 when airway responsiveness was measured there was no significant difference between the two groups in mean weight ($p=0.11$).

Smoke exposure during pregnancy

Serum cotinine levels during pregnancy in the smoke exposed group showed significant variation through the week following the four day exposure pattern. Serum cotinine levels peaked at day 4 of exposure and had reached non-recordable levels by the commencement of the following weeks exposures. Peak serum cotinine levels ranged from 22 ±12 ng/ml to 76 ± 12 ng/ml at day 35 of gestation while all animals reached non-recordable levels by the end of each week. No recordable cotinine levels were obtained from control animals.

Airway Responsiveness

There was no difference in baseline transpulmonary resistance between the smoke-exposed animals and controls. There was increased airway responsiveness ($p<0.05$) to inhaled acetylcholine at higher doses in the animals who had received smoke exposure during pregnancy compared to control non-smoke exposed animals. (Fig 14)

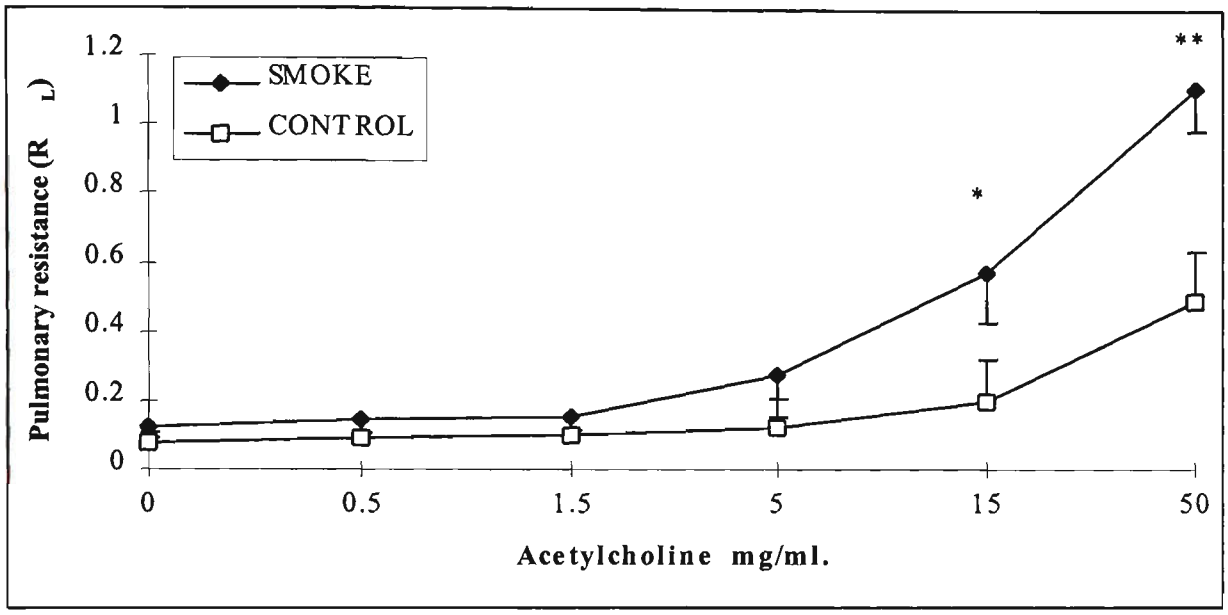

Figure 14 Pulmonary resistance values following administration of 6 breaths of increasing concentrations of nebulised acetylcholine to 27 day old guinea pigs who were exposed to cigarette smoke *in-utero* (n=17) or non smoke exposed controls (n=8). *p<0.05 ** P<0.01.

Airway Morphometry

A mean number of 8 ± 2 airways were measured from each animal. The basement membrane perimeter (Pbm) was the same (1.32mm ± 0.11mm v 1.35mm ± 0.08mm) in the non-exposed and exposed animals respectively showing that similar sized airways were compared. The outer airway perimeter (Po) adjusted for any lengths where adjacent pulmonary vessels prevented alveolar attachments was also similar in the two groups (0.94mm ± 0.06mm v 1.0mm ± 0.05mm) in the non-exposed and exposed animals respectively validating the expression of attachment points in this way. In the non-exposed and exposed animals the inner airway wall thickness was (0.032mm ± 0.01mm v 0.033mm ± 0.02mm), the outer airway wall thickness was (0.029mm ± 0.047mm v 0.039mm ± 0.07mm) and the area of airway smooth muscle was (0.016mm ± 0.01mm v 0.018mm ± 0.017mm) respectively. Compared with non-exposed animals, the inner and outer airway wall and the muscle area were increased up to 25 % in the smoke-exposed animals although these differences were not significant.

The mean number of alveolar attachment points was decreased ($p=0.008$) in the smoke-exposed animals (19.4 ± 0.4 /mm) compared with non-exposed animals (21.6 ± 0.8 /mm). There was no difference between the two groups for the amount of smooth muscle shortening observed. The coefficient of variation for airway measurements ranged from 0.68% to 3.2% with a mean value of 1.8% ± 0.5%.

DISCUSSION

In this study, *in-utero* exposure to cigarette smoke during pregnancy, in the absence of

post-natal cigarette smoke exposure, resulted in increased airway responsiveness to inhaled acetylcholine at day 27 of life in the neonatal guinea pig. This increase in airway responsiveness was associated with a significant decrease in the number of alveolar attachment points in membranous and small cartilaginous airways. The inner airway wall area and the airway smooth muscle area were increased by 25% and the outer airway wall was increased by 30% in the smoke-exposed animals compared with the non-exposed animals.

When interpreting the findings in this study, one needs to carefully consider a number of technical and methodological factors. Firstly, we are confident that the level of smoke exposure to pregnant animals in this study was similar to that seen in studies of maternal smoking. To this extent, The pregnant animals exposed to cigarette smoke during this study had peak serum cotinine levels in the range of 50-80 ng/ml. This level approximates the levels seen in humans who smoke between 5 and 10 cigarettes a day (205). The decrease in serum cotinine levels to zero over the 3 days each week in which animals were not exposed to cigarette smoke suggests that the half-life of cotinine in the guinea pig is similar to that seen in adults which is between 12 -18 hours. We believe therefore that the degree of foetal smoke exposure produced in this study is similar to the degree of exposure experienced by human foetuses whose mothers smoke to a moderate degree.

Secondly, for valid comparisons of morphological data between different animals it is important that airways of similar size and dimensions are analysed. When the frequency distribution of internal airway and outer airway perimeters was compared there was no difference between the groups suggesting that *in-utero* smoke exposure does not result in a

systematic change in airway calibre. This data is also supported by the means and standard errors for both of these measurements which were virtually identical for the two groups. Thus we feel confident that the methods used to normalise the data in this study are valid. Similar methods have been used previously (6, 141) and the matching of airways by order or generation is not possible when lung tissue is collected and processed in this way.

There are a number of studies (19, 21-23) in which lung function has been measured in infants close to birth whose mothers smoked during pregnancy. These studies report reduced respiratory function at birth in such infants and propose altered lung/airway development *in-utero* as a likely cause although no attempts were made to test these hypotheses. Several previous animal models have documented abnormalities in lung development following smoke exposure during pregnancy. Our animal study examined the effect of smoke exposure during pregnancy on post-natal lung function and on post-natal lung structure several weeks after birth in an attempt to elucidate possible mechanisms for this altered lung function. We examined the animals at day 27 to allow smoke exposed animals a catch up period so thy would be of similar size and weight as this can effect lung function. Collins *et al* (204) studied rats and found that in pregnant animals exposed to cigarette smoke from day 5 to term at day 21, their offspring were not only smaller but had reduced lung elastic tissue, and increased size but reduced number of saccules (foetal alveoli). They did not examine the effects of these structural changes on post-natal lung function nor did they examine the effect of post-natal lung growth in a smoke free environment.

Changes in lung and/or airway structure as a result of *in-utero* cigarette smoke exposure might give rise to increased responsiveness to inhaled smooth muscle agonists in a number of ways. In-vivo, when airway smooth muscle is stimulated, it must overcome a number of forces which act to oppose muscle shortening. One of the major forces resisting smooth muscle shortening in-vivo is the load imparted on the muscle due to the elastic recoil pressure of the lung parenchyma (6). This resistive load imparted on the muscle is translated to the airway wall via alveolar attachments to the airway. Thus, any reduction in the number of alveolar attachments to the airway may reduce the loads opposing smooth muscle shortening and allow increased shortening for the same degree of stimulus. Similarly, an increase in the thickness of the airway wall outside the smooth muscle layer will have the same effect and reduce the effect of lung elastic recoil pressure when smooth muscle shortens. On the other hand, an increase in the amount of smooth muscle may enable the muscle to generate more force when stimulated to shorten while an increase in the thickness of the airway wall inside the smooth muscle layer will result in greater lumenal narrowing for the same degree of smooth muscle shortening. Therefore, it is plausible that the net effect of the modest increases in airway wall thickness and smooth muscle area seen in this study, coupled to the significant reduction in the number of alveolar attachments, might result in airway hyperresponsiveness such as that observed in the smoke-exposed animals.

Saetta *et al* (20) have previously shown a reduction in the number of alveolar attachment points, an increase in the distance between attachments, and an increase in the percentage

of abnormal attachments in cigarette smokers compared with nonsmokers. This reduction was associated with the score for airway inflammation and with reduced elastic recoil pressure in smokers. Airway responsiveness was not assessed in the study of Saetta *et al* Petty *et al* (26) have also shown that in patients with mild emphysema (ie. destruction and loss of the alveolar walls) that elastic recoil pressure is reduced but that this is not associated with air-flow limitation. The destruction of alveolar walls observed in patients with moderate to severe emphysema associated with cigarette smoking is associated with airflow obstruction and increased airway hyperresponsiveness. These studies have generally been performed in adults while our study examined the effects of passive cigarette smoke exposure *in-utero* in a developing lung system. We did not examine airway or alveolar inflammation in this study so whether the decreased number of alveolar attachment points in smoke-exposed animals in this study was the result of cellular infiltration and destruction of existing alveoli or abnormal growth *in-utero* is unknown. Unlike the study by Saetta *et al* we made no attempt to assess the degree of damage to existing alveolar attachments. Structural changes to the alveoli such as a reduction in elastic fibers or collagen deposition (fibrosis) of alveolar walls although not measured in this study might also alter lung function or response to inhaled agonists.

We did not observe any difference between the smoke-exposed and non-exposed animals in the amount of smooth muscle shortening observed despite a significant increased response to inhaled acetylcholine at the higher doses in the smoke-exposed animals. We can think of two possible explanations for this observation. Firstly, the lungs were inflated to a pressure of $20cmH_20$ with fixative prior to histological examination. This distending pressure may

reduce the amount of muscle shortening observed following fixation and may have been preserved if the lungs had been fixed in immersion without inflation. Carroll *et al* (140) compared smooth muscle shortening in cases of fatal asthma with nonasthmatic control cases dying of nonrespiratory causes in which the lungs were inflated to a pressure of 25cm H_20 and found no difference between the groups. We made a conscious decision to inflate the lungs in this study to increase our ability to clearly delineate alveolar attachments, which are more accurately measured in the inflated lung. A second possible explanation for our findings is that the relatively modest increase in the inner airway wall thickness in the smoke-exposed group resulted in exaggerated lumenal narrowing at high doses of acetylcholine. This is supported by studies which have modelled the effects of airway wall thickening in patients with asthma. Wiggs *et al* (209) showed that the most pronounced effects of increased thickness of the inner airway wall and lumenal narrowing are seen at higher degrees of muscle shortening. This might explain why the changes were seen at the highest doses where presumably the smooth muscle is highly stimulated.

Young *et al* (18) examined post-natal lung function in 63 infants at 4.5 weeks of age and found a strong association between increased airway reactivity and exposure to cigarette smoke *in-utero*. Other investigators have also documented abnormalities in neonatal respiratory function which are associated with maternal smoking during pregnancy (17, 19). Similarly, in the present study we observed increased airway responsiveness to acetylcholine at day 27 post-natally in guinea pigs whose mothers had been directly exposed to cigarette smoke during pregnancy but in the absence of post-natal smoke exposure. This suggests that the mechanisms resulting in increased airway responsiveness

may develop *in-utero* in response to passive smoke exposure. To what extent the findings in guinea pigs can be extrapolated to humans is not clear however we feel confident that the findings from the present study provide important new information about possible mechanisms to explain the deleterious effect of *in-utero* smoke exposure on post-natal lung function.

In-utero smoke exposure could be considered as a distinct form of "passive" cigarette smoking in that the foetus is not directly exposed to cigarette smoke and thus any effects on foetal development are a result of indirect exposure to cigarette smoke. A variety of hypotheses to explain such changes have been postulated including factors such as alterations in placental blood flow, altered cortisol levels and changes in foetal breathing patterns, all of which may influence foetal lung development (210, 211).

In conclusion neonatal guinea pigs born to animals exposed to cigarette smoke during pregnancy show increased airway responsiveness at day 27 of post-natal life. Examination of lung structure at this stage show a reduced number of alveolar attachment points and modest changes in airway dimensions suggesting that the observed increase in airway responsiveness may be related to altered airway and lung structure secondary to *in-utero* cigarette smoke exposure.

CHAPTER 7 - SUMMARY AND DISCUSSION

The cause of death in SIDS remains unknown despite extensive research into possible pathological mechanisms which might lead to death. The risk factors for SIDS have been identified through extensive epidemiological studies and paternal cigarette smoking, particularly maternal cigarette smoking, has been reported as a significant risk factor. Much of the pathological data acquired from postmortem examinations of SIDS deaths suggests that hypoxia, possibly associated with apnea, may be a contributing factor in these cases. Because altered lung function and increased airway hyperresponsiveness has been shown in infants and has been related to maternal cigarette smoking, we hypothesised that changes in airway structure which could give rise to altered airway function may be present in babies dying of SIDS. To test this hypothesis we retrospectively examined lung tissue from SIDS cases in which an autopsy had been carried out by the Victorian Department of Forensic Medicine during the period from 1991 to 1993. In these cases, detailed information had been obtained from parents regarding environmental factors and smoking histories of the parents. Tissue was also collected from infants in whom the cause of death was known (non-SIDS) and these cases were used as controls. We employed standardised morphometric techniques in an attempt to identify any changes in airway or lung structure which might cause or contribute to death.

Because tissue was collected retrospectively and only parenchymal blocks of tissue were available, we were confined to examining predominantly smaller airways because there are generally few well preserved large cartilaginous airways cut in cross section when tissue is

collected in this way. Tissue sections which had been fixed in formalin and embedded in paraffin were stained with haematoxylin and eosin for morphological assessment. On all airways cut in transverse section we measured airway dimensions including airway wall thickness and the amount of smooth muscle. We also counted the number of alveolar attachments to the airway adventitia and calculated the percent smooth muscle shortening present in each airway. Because our initial data suggested that some of the morphological changes seen in SIDS cases were related to maternal smoking, we decided to carry out a controlled study in guinea pigs examining the effects of *in-utero* cigarette smoke exposure on airway responsiveness and airway and lung structure.

In our initial studies we compared airway dimensions in all SIDS cases, regardless of maternal smoking histories, with control cases. Compared with control cases, we found that the area of smooth muscle in membranous airways was significantly greater in SIDS cases.

The thickness of the inner airway wall, the outer airway wall and the percent smooth muscle shortening was similar in the two groups. An increase in the amount of airway smooth muscle might lead to excessive airway narrowing if increased muscle results in increased force when the muscle is stimulated (6). Because we only had a complete maternal smoking history in the cases of SIDS, we decided to analyse only the cases of SIDS to see whether there was any effect of maternal cigarette smoking on the airway dimensions we had measured. Because destruction of lung parenchyma caused by cigarette smoking has been shown to effect lung function in adults (20) we also counted the number of alveolar attachments to the airway adventitia in smoke exposed and non-smoke exposed

SIDS cases. Analysis in this way showed that both the thickness of the epithelium and the thickness of the inner airway wall (between the basement membrane and the outer border of the airway smooth muscle) were increased and the number of alveolar attachment points was decreased in SIDS cases where there was a history of maternal smoking compared with SIDS cases without cigarette smoke exposure. An increase in the thickness of the inner airway wall will amplify the effects of smooth muscle shortening on lumenal narrowing while a decrease in the number of alveolar attachment points might reduce the force of the lung elastic acting on the airway wall recoil and hence the load against which smooth muscle has to contract when shortening. Both of these factors could give rise to excessive airway narrowing when airway smooth muscle is stimulated.

To further test this, we used an animal model of *in-utero* cigarette smoke exposure to examine airway responsiveness to inhaled acetylcholine and airway dimensions and structure in neonatal animals whose mothers had been exposed to moderate levels of cigarette smoke. In this study we showed that airway responsiveness to inhaled acetylcholine was significantly increased in animals that had been exposed to cigarette smoke *in-utero* compared with those who had not. The number of alveolar attachment points was significantly reduced in the smoke-exposed animals compared with the non-smoke exposed animals. Airway wall thickness and the amount of smooth muscle were all greater but not significantly so in the smoke exposed animals. This suggested that the airway hyperresponsiveness observed in the smoke-exposed animals was associated with a reduction in alveolar attachment points, possibly combined with subtle changes in airway structure.

Before discussing possible mechanisms or implications of these findings it is important to consider a number of methodological issues which may have influenced our findings. Firstly, the diagnosis of SIDS is one of exclusion and cannot be explained by history, environmental factors or pathology. Minor inflammatory changes are found in the respiratory system of some SIDS cases (107, 108), however the distinction between an associated finding and a cause of death is difficult to make. Because SIDS may not represent a distinct pathological entity, there may be a number of different mechanisms which lead to death in these infants. Therefore specific findings which are related to SIDS deaths may not be generalisable to all infants dying of SIDS. Indeed our data suggests that infants who die of SIDS who have been exposed to cigarette smoke might represent a distinct subgroup of SIDS cases in which the risk of death is increased compared with those who are not exposed to cigarette smoke while on the other hand, our findings might be incidental and not related to the cause of death at all.

Tissue samples from SIDS and control cases were collected at autopsy using a standard protocol. Blocks of parenchymal tissue were obtained at random from different sites in the lung. Sampling was not stratified to collect both large and small airways. Consequently, the majority of airways that were suitable for examination in this study (ie. present in sufficient numbers and cut in transverse section) were smaller membranous airways. Large cartilaginous airways suitable for examination were only seen occasionally and were therefore excluded from analysis. This means that we cannot extrapolate our findings in small airways to the large airways. In order to increase the statistical power of the study,

airway dimensions from a further 8 control infants who were part of a Western Australian study into infant airway dimensions were included in our data set. This additional tissue had been collected and measured using identical techniques to those used in this study. Therefore we are confident that pooling of the data from these two separate groups did not confound our data set. The only difference in the protocols between the 2 studies was that whole lungs were fixed in inflation in the Western Australian study while tissue sections from the Victorian study were fixed in immersion without inflation. We do not feel that this was a problem because airway wall dimensions have been shown to be independent of lung inflation and smooth muscle tone (157, 212). The control cases obtained from Western Australian study were similar to the control cases that were collected in the Victorian cases. The age and airway size distributions were similar in the two groups (see Fig.15 and Fig.16) as was the amount of airway smooth muscle (Fig.17).

Where possible, we attempted to match our control cases with SIDS cases for age and sex to minimise variations in our measurements that may relate to these factors. The number of control cases we were able to collect was less than the number of SIDS cases which reflects the low number of non-SIDS deaths in the first 12 month of life. The mean age of our control cases was greater than our SIDS cases, however there was no independent effect of age on airway dimensions or number of attachment points when a weighted least squares regression analysis was applied to the data. It could be argued that our control cases are not "normal" by virtue of the fact that the infants had died. We cannot be sure to what extent the cause of death in our control group might influence our measurements of airway and

lung structure. There was no history of respiratory disease in our control cases and with the exception of four cases in whom asphyxiation was the cause of death, the causes of death were non-respiratory.

We were only able to analyze the effect of cigarette smoke exposure on airway and lung structure in the SIDS cases because the study was conducted retrospectively and questionnaire data regarding parental smoking was not available for our control cases. This may influence our findings in a number of ways. Firstly, we cannot be sure to what extent cigarette smoke exposure influenced airway and lung structure in the control group. Our data showed that the thickness of the inner airway wall and the epithelium was increased in SIDS cases in whom the mothers smoked. While there was no independent effect of cigarette smoke exposure on the airway smooth muscle area in the SIDS cases we cannot exclude the possibility that cigarette smoke exposure may have influenced the findings in our first study in which SIDS cases were found to have more smooth muscle than control cases. In adults, an increase in the amount of smooth muscle in membranous airways has been previously shown in active cigarette smokers (3) and we know that 50 out of 83 SIDS cases had mothers who smoked up until the time of death.

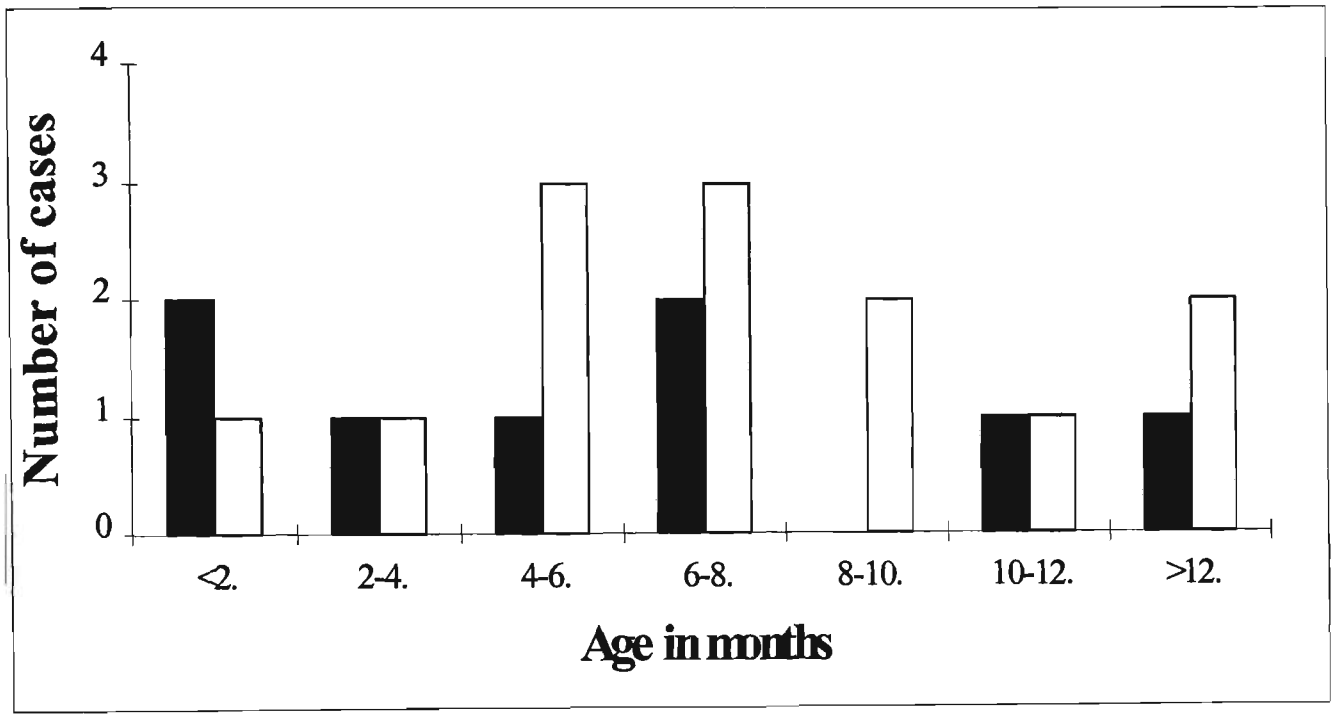

Figure 15 Age distribution of the two control groups show no marked difference in age. Age is expressed in months for both Western Australian. cases (solid bars) and Victorian cases (clear bars).

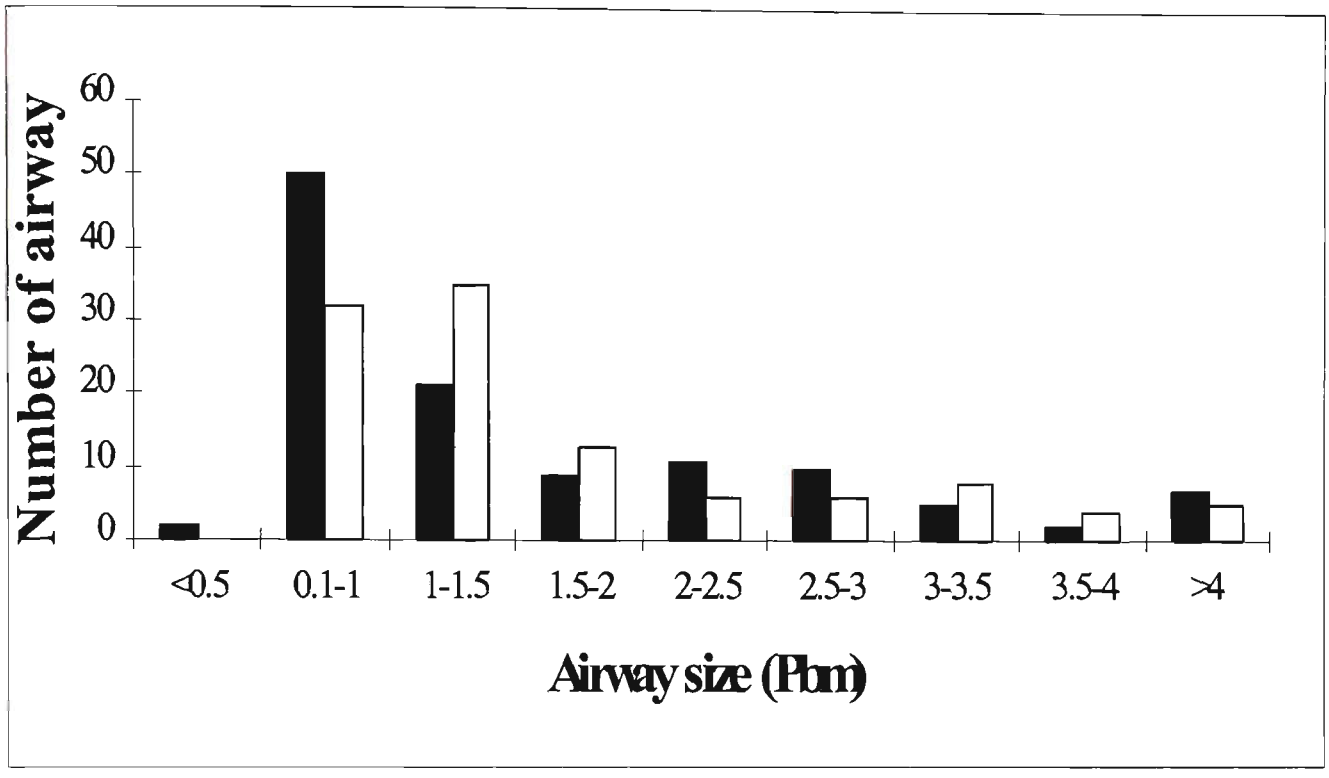

Figure 16 Airway size distribution of the two control groups show no significant difference in airway size. Western Australian cases are expressed as solid bars and clear bars represent Victorian. The airways are sized by the basement membrane perimeter which has been shown to be independent of muscle contraction and lung volume (157).

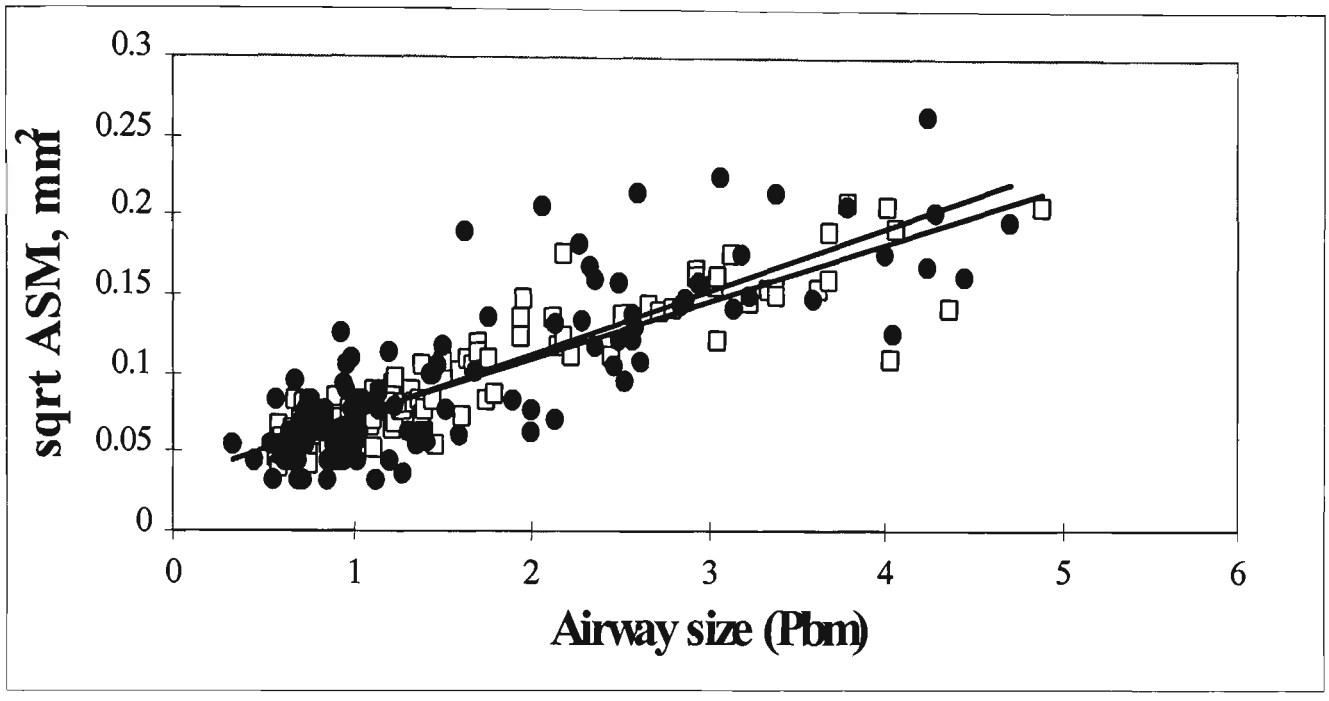

Figure 17 Plot of the square root of area of airway smooth muscle against basement membrane perimeter (Pbm) from 8 control infants who died in Western Australia (□) and 13 age matched control infants from Victoria (●). There is no significance difference in the amount of airway smooth muscle in either group.

The methods used to measure airway wall dimensions in this study have been used in a number of previous studies of airway dimensions (140-142) and utilise the standard nomenclature for quantifying subdivisions of the bronchial wall as proposed by Bai *et al* (125). These methods have been used to assess airway wall dimensions in a variety of diseases in humans and in animals models. The coefficient of variation for the range of measurements made in this study was very low (<10%) so that our techniques are sensitive enough to detect subtle differences between groups. We decided to use direct planimetry and image analysis to make our measurements of airway wall dimensions. Other methods of measuring such as point counting and mean linear intercept have been shown to produce similar results when comparing airway dimensions. Estimating the volume proportions of different tissue types within the airway wall using a variety of different stereological techniques has been the subject of numerous reviews (200-202). Many of these techniques are used to obtain absolute volume fractions of tissue types and require the volume of the whole organ, in this case the lung, to be measured initially. Stereology permits assessment of epithelial and smooth muscle volume providing that full lung inflation to TLC is achieved before lung sectioning commences. From this starting point isotropic uniform random sections can be obtained by vertical sectioning of the lung thus ensuring that all orientations and positions of airways are sampled. Point counting using a cycloid grid placed over the section permits a very detailed and accurate assessment of absolute epithelial and smooth muscle volume to be estimated. This detailed and precise method of measurement may well provide a more accurate way of measuring absolute airway smooth muscle volume than that employed in the present study and ideally our results will need to be confirmed by future studies using this technique. These stereological methods requiring

whole lung sections are limited when making comparisons of airway dimensions between different groups and do not allow for the variable effects of smooth muscle shortening or variations in measurements due to airway size. The latter may depend on generation or order number and on growth factors which may to some extent independently affect airways and parenchyma. In addition the relative proportions of smooth muscle, epithelium and glands to total airway wall volume varies down the bronchial tree as do the functional effects of these varying proportions. Despite potential sampling bias using the methods we have used in this study, we still feel that comparison between groups, using airways of similar sizes, will yield useful information regarding the likely functional effects of changes in airway structure on changes in lung function. We feel that the robustness of these methods is supported by;

- The similarity between findings from different laboratories when comparing airway dimensions between control and disease groups.
- The similarity of results using different sampling methods eg. transverse sections or bronchial biopsy.
- The ability to detect small differences in airway dimensions in relatively small groups.
- The direct relationship demonstrated between severity of changes in airway structure and clinical severity of disease.

Maternal smoking histories were obtained as part of a three year epidemiological study conducted in 1991 by the Victorian Sudden Infant Death Research Foundation. Mothers were asked to indicate whether they smoked before becoming pregnant and separately during the first, second, and third trimester of pregnancy or whether they smoked between

the birth and death of their child. For all questions, smoking was rated on a 5 point scale. Considering the delicate nature of the questionnaires it is possible that there was a degree of under reporting with regard to the amount of cigarette smoking by the mothers of infants who died of SIDS. If under reporting of cigarette smoking was the case then it would only serve to strengthen the differences we showed between those infants exposed to cigarette smoke and those who were not.

The fact that there was no difference in the amount of airway smooth muscle within the SIDS group between infants exposed to high levels of cigarette smoke and infants not exposed to cigarette smoke suggests that the changes are not due to maternal smoking and may therefore be related to the causation of SIDS itself. Control infants who died from sepsis or asphyxia would have died acutely without any evidence on history or examination of an underlying predisposition such as immune deficiency or upper airways obstruction. In the absence of these findings we feel it is very unlikely that smooth muscle bulk would be increased in a septic illness of only a short period. Further, if such an event had occurred this would actually strengthen the significance of our findings as it would serve to over-represent the "normal" level of airway smooth muscle in the control group.

Physiologically it is very difficult to explain how chronic upper airway obstruction would produce an increase in airway smooth muscle. Certainly there is no precedence from other causes of chronic upper airway obstruction such as adult OSA where even major changes in pulmonary artery smooth muscle content occur (accepted by most as being due to hypoxia rather than the actual obstruction itself) but are not associated with any alteration in airway

smooth muscle content.

The cause of the decreased number of alveolar attachment points in smoke-exposed cases of SIDS or animals exposed to cigarette smoke *in-utero* in this study is unknown. Possibilities include, cellular infiltration and destruction of existing alveoli or abnormal growth *in-utero* is unknown. We did not examine airway or alveolar inflammation in this study. In patients with emphysema, infiltration of the lung parenchyma with neutrophils and increased levels of elastase in bronchoalveolar lavage samples are well documented (25, 213, 214). Elastase is a proteolytic enzyme capable of damaging elastic fibres (215) within the lung parenchyma and is thought to be the major cause of alveolar damage in cigarette smoke-induced emphysema. Although not measured in the current studies, further examination of both airway and parenchymal inflammation will be useful to determine the potential mechanisms for reduced number of alveolar attachment points in cigarette smoke exposed infants and animals. It is interesting that the birth weight of cigarette smoke exposed animals was significantly lower compared with non-smoke exposed animals. Whether this lower birth weight reflects retarded lung development such as a decreased number of alveoli requires further clarification.

Cigarette smoke induced inflammation may also result in the release of inflammatory mediators capable of inducing acute changes in airway structure such as accumulation of oedema fluid, resulting in a thickening of the airway wall or changes in epithelial permeability which might allow greater access of an inhaled agonist to the site of action. Both of these factors might lead to increased airway responsiveness to inhaled

bronchoconstricting agonists.

The findings that alveolar attachment points were reduced in animals following *in-utero* cigarette smoke exposure suggests that cigarette smoke exposure is the most likely mechanism by which this difference occurs. We did not count the number of attachment points in our control cases because we did not have paternal smoking histories for these cases. Therefore while cigarette smoke exposure is the most likely mechanism for this, we cannot exclude the possibility that retarded lung growth in the SIDS cases per se, if this in fact occurs at all, also contributes to this finding.

Whatever the mechanisms, the work in this thesis confirms the presence of altered airway structure in cases of SIDS as previously reported by Haque *et al* (8). Our findings differ from those previously published in that we were able to examine the effect of maternal cigarette smoking on airway and lung structure in our SIDS cases and we were able to measure the amount of smooth muscle within the airway wall and show that it was increased in cases of SIDS. In fact the measurements of inner airway wall thickness reported in the study by Haque *et al* included the area occupied by smooth muscle within their measurement thus the changes in the index of airway wall thickness observed in membranous bronchioles in their study may be due, at least in part, to increases in the amount of airway smooth muscle.

The present study has shown that airway smooth muscle is increased in infants who die from SIDS. What effect could this increase in airway smooth muscle produce? For any

given airway, an increase in the amount of airway smooth muscle may result in excessive airway narrowing if increased muscle volume results in increased force development when the muscle is stimulated (6). Increased stimulation of smooth muscle, independent of any actual increase in airway smooth muscle content might also result in increased shortening. To our knowledge, in-vitro experiments examining the contractile responses of airway smooth muscle preparations from SIDS cases have not been reported. Preliminary data suggests that the in vitro responsiveness of smooth muscle may be markedly reduced in SIDS (Prof. H. Mitchell, Dept. Physiology U.W.A.; Personal communication). Increased airway responsiveness to smooth muscle agonists has been shown to be related to viral infection, cigarette smoke exposure and airway inflammation, however their effects in the cases in the present study is unknown. As discussed below we did not observe differences in the amount of smooth muscle shortening between SIDS and control cases.

If small airway closure is an important mechanism in SIDS cases then one might expect to see increased muscle shortening in the small airways. Indeed, the percent of smooth muscle shortening was calculated in SIDS cases and control cases and was not different. It may be that the extent of smooth muscle shortening is not preserved in autopsy specimens and the time course between death, postmortem examination and tissue collection might variably alter the amount of muscle shortening observed in these cases. Martinez *et al* (9) have postulated that small airway closure could be associated with SIDS and our present findings suggest that in some cases, the potential for excessive airway narrowing caused by increased airway wall thickness and an increased amount of airway smooth muscle make this hypothesis feasible.

What is the underlying mechanism for this observed increase in smooth muscle? It is not clear from our study whether the observed increase in airway smooth muscle in SIDS cases is a primary effect which directly results in the pathophysiological abnormalities resulting in sudden death in SIDS or whether the alterations in airway smooth muscle are secondary to other factors which may cause death in SIDS through other pathophysiological mechanisms. Host factors such as initial airway structure warrant consideration as well as potential pathological mechanisms. Genetic factors alone might influence the amount of airway smooth muscle, while environmental factors such as *in-utero* cigarette smoke exposure, viral infections or inflammatory processes may interact with this change increasing the risk of death in SIDS. *In utero* cigarette smoke exposure is known to result in altered lung function in neonatal infants (17, 18, 203) and abnormalities in lung development and/or structure are postulated as the most likely mechanisms responsible for these observations. Animal studies examining the offspring from animals exposed to cigarette smoke during pregnancy, have reported abnormalities in alveolar and parenchymal elastic tissue development but have not documented alterations in airway smooth muscle content (204). Clearly as SIDS may also occur in situations where infants were not exposed to any degree of maternal smoking, other factors which may influence airway smooth muscle development are likely to be important.

It is also possible that the observed increase in airway smooth muscle in the SIDS cases reflect pathological mechanisms occurring elsewhere. Chronic upper airway obstruction has frequently been suggested to be an important mechanism in the pathogenesis of SIDS

(116). While Naeye has previously described an increase in smooth muscle content in pulmonary arteries in infants dying from SIDS, a finding also seen in other forms of chronic upper airway obstruction such as obstructive sleep apnoea (OSA), increased airway smooth muscle in such cases has not been previously described in other cases in which chronic upper airway obstruction (including OSA) is apparent.

Interstitial oedema and inflammatory infiltrates have been reported in some SIDS lungs and are suggested to be a result of viral respiratory infection. It is likely that persistent airway inflammation results in structural changes such as increased airway wall thickness and increased smooth muscle similar to that seen in patients with asthma. Whether these structural changes occur over years or could take place within the relatively brief postnatal period (months) in which most SIDS deaths occur is unknown.

The epidemiological study of SIDS is difficult because the majority of the observed risk factors are interrelated. The majority of studies that have been undertaken have been retrospective studies, not hypothesis based and are likely to have recall bias effects. Confounding this also is the dramatic fall in the rate of SIDS since the inception of the RTR campaign. This decrease may have led to changes in the epidemiological characteristics of the remaining SIDS cases. Nevertheless the relevance of work compiled before the introduction of RTR has demonstrated a number of key associations which have led to the dramatic decline in SIDS numbers.

In conclusion, this study has shown that infants who die from the Sudden Infant Death

Syndrome have a higher proportion of airway smooth muscle in their small airways than age matched infants who die suddenly from causes not associated with underlying cardiorespiratory pathology. The increase in smooth muscle may contribute to excessive airway narrowing which along with other factors, such as immature ventilatory control mechanisms, may result in sudden infant death. In cases of SIDS whose mothers smoked >20 cigarettes a day during pregnancy and in the postnatal period to death, the inner airway wall in the small airways was significantly increased and the number of alveolar attachment points was decreased when compared with infants who have died from SIDS but whose mothers did not smoke during the pregnancy or in the postnatal period. We conclude that passive cigarette smoking produces significant alterations in the structure of the developing infant airway which may have significant physiological consequences and may be related to the cause of death in SIDS in infants with a history of maternal smoking. These findings also emphasise the dangers of passive cigarette smoke exposure to infants and highlight the importance of anti-smoking measures aimed at reducing the likelihood of damage to the airways in this way.

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