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An evaluation of mucus glycoproteins in the larynges of victims of sudden infant death syndrome

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Abstract

Larynges from 24 victims of Sudden Infant Death Syndrome (S.I.D.S.) and 10 controls, with ages ranging from two days to 24 weeks in the controls and from two to 116 weeks in the S.I.D.S. victims, were transversely, step-serially sectioned and then stained to show acid, neutral and mixed mucus glycoproteins.

The proportion of sulphated mucus glycoprotein and sialylated mucus glycoprotein to total acid mucus glycoprotein was determined by the use of various staining techniques and a comparison was made for each type between S.I.D.S. and controls. The differences were significant with a mean of 27 per cent in the controls compared to 59 per cent in the S.I.D.S. for sulphomucin (difference 32 per cent; standard error of difference 6 per cent; $p<0.01$) and 73 per cent in the controls compared to 41 per cent in the S.I.D.S. larynges (difference 32 per cent; standard error of difference six per cent; $p<0.01$) for sialomucin. The results suggest that sulphated mucus glycoprotein is secreted in excess in some victims of Sudden Infant Death Syndrome.

Key words: Sudden infant death; Larynx; Mucins

Introduction

Sudden Infant Death Syndrome is the most common cause of death in infancy, comprising approximately 50 per cent of all deaths after the first week of birth, although it is relatively uncommon in relation to the number of live births each year, 0.15–0.35 per cent for all countries with variations e.g. 0.05 per cent in Sweden. Although S.I.D.S. can occur at any time, around two-thirds of cases occur between noon and midnight and more often during the colder months. Also, in general more boys than girls die of S.I.D.S. It occurs more commonly amongst babies who are small for their gestational age i.e. infants weighing between 2000 and 2500 g at 37 weeks (Peterson, 1989). Various investigators have found that S.I.D.S. is uncommon under one week of age, peaks at 12 weeks and gradually declines until the 36th week (Wedgewood and Benditt, 1963). Mucus hypersection is recognized as a contributory factor in morbidity and mortality in chronic airway diseases (Jany and Basbaum, 1991) as it can become a source of disability when the type of mucus glycoprotein is altered and its function is no longer protective.

Airway mucins consist of water, salts, protein and high molecular weight glycoconjugates. It is the mucus glycoprotein content which gives mucus its characteristic viscoelastic properties which enable the cilia to export it (Sheehan *et al.*, 1991). Respiratory mucins are glycoproteins possessing a wide variety of different peptides or apomucins with multiple carbohydrate chains (mucins are 70–80 per cent CHO) attached to the peptide by O-glycosidic linkages. Mucins appear as linear threads from 200 to 1000 nm in length (Lamblin *et al.*, 1991).

Various types of mucus glycoproteins (mucins) have been identified of which the major types are sulphated, carboxylated and neutral. The neutral mucins are those which do not possess an acidic radical. Sulphated mucins may be either strongly or weakly sulphated and carboxylated mucins may be either sialidase-resistant or sialidase-labile sialomucins. When sulphated glycoprotein is the predominant acid type in an airway surface the sialylated type is normally sialidase resistant. More than one type of mucin may be found within a cell (Lamb, 1969). These are found in combinations and are referred to as mixed mucins. For the purpose of this study, acidic mucins were divided into sulphated and carboxylated and their relative proportions determined as well as their relationship to neutral mucins.

In the human bronchial submucosal gland it has been found that the proportions of acidic groups on epithelial glycoproteins may vary in disease. For example, the proportion of cells secreting sulphated mucins increases (Lamb, 1969) in cases of chronic bronchitis. Also, it is known that there is an increase

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in acid mucins with a corresponding decrease in neutral mucins in bronchial disease e.g. in the bronchial submucosal glands of pigs with enzootic pneumonia (Jones and Reid, 1978).

The submucosal glands of the larynx and trachea are similar to bronchial glands (Spicer *et al.*, 1983) and are of the compound tubuloacinar type with a mucus-secreting duct epithelium. The alveoli or acini of the duct secrete mucus and may have serous crescents. The ducts open onto the surface which consists of respiratory type mucosa (which is columnar and ciliated). Laryngeal submucosal glands are specifically defined according to their anatomical sites (Nassar and Bridger, 1971). The supraglottic and subglottic regions of the larynx are rich in submucosal glands, particularly in the ventricular region. There are very few of these glands in the glottic region, posterior surfaces of the epiglottis and aryepiglottic folds where squamous epithelium lines the free margin of the vocal folds. Stimulation of secretion by the submucosal glands is by cholinergic and adrenergic agonists (Sheehan *et al.*, 1991).

The cells of the tracheobronchial submucosal glands of the foetus all produce heavily sulphated acid mucin and no sialic acid-containing mucin (Lamb and Reid, 1972). At birth there is a gradual decline in the number of glands producing secretions which react strongly with staining techniques for sulphated mucins and a gradual increase in those reacting with stains which are highly selective for sialomucins. By the age of three years the pattern seen in normal older children and adults has been established.

Conversely, in the foetal larynx, mainly sialomucins are present in the submucosal glands (author's observation). Laryngeal glands in various sites from two foetuses, of 22 and 24 weeks gestation, were stained with both alcian blue–periodic acid Schiff and aldehyde fuchsin–alcian blue and it was observed that those in the epiglottis of both larynges were sialic acid-containing only. In the vestibular folds there were a few very weakly sulphated mucin-containing cells with sialomucin or mixed mucins contained within the ducts of some of these immature glands. The vocal folds contained sialomucins only as did the submucosal gland cells at the cricoid–trachea junction while a few sulphomucins were present in the anterior tracheal submucosal glands.

There is no general failure in foetal life to produce sialomucins as they have been found in the foetal submandibular glands (Lamb and Reid, 1972).

There is an increase in the amount of mucus secreted by the acini of the vestibular folds of larynges of some S.I.D.S. victims in comparison with those of control specimens and it is believed that excess mucus in the larynx may cause a reflex laryngeal spasm (Fink and Beckwith, 1980) similar to the type of apnoea which has been postulated as a contributing factor to S.I.D.S. (Downing and Lee, 1975). A recent investigation (Harrison, 1991b) showed a reduction in the subglottic airway in the larynges of S.I.D.S. victims, due to submucosal gland hyperplasia which is suggestive of hypersection. In chronic bronchitis there is hyperplasia in the submucosal glands with a corresponding increase in the ratio of thickness of gland to that of the bronchial wall. There is an increase in sulphated mucin-secreting cells in the airway surface (Lamb and Reid, 1968). Similar changes are seen in cystic fibrosis (Reid and de Haller, 1967; Jones and Reid, 1978). Mucin with a sulphate radical only has been found to be of low viscosity and was thought to offer the best protection in airways as it could reduce the danger of blockage (Reid, 1977). Therefore mucus glycoprotein with a high sulphate concentration is not believed to be a contributory factor in the viscous secretion of certain diseases e.g. chronic bronchitis. It is important to identify the relative proportions of various types of mucins present and to determine the type secreted in excess by the larynges of S.I.D.S. victims.

Materials and methods

Whole larynges with their tracheas, which were formalin fixed before receipt, of 24 S.I.D.S. babies aged between two and 116 weeks and 10 control specimens from babies aged two days to 24 weeks, were radiographed (exposure time five minutes, tube voltage 115 KVP) to check for presence of calcium. After any necessary calcium removal from the hyoid bone by 10 per cent formic acid and further radiography to determine the end-point of decalcification, the specimens had excess external muscle removed. Initially, a few specimens were embedded in low viscosity nitrocellulose and wax, a double-embedding technique which provides adequate support for whole organs, but it was found that background staining with aldehyde fuchsin was too

TABLE I

PLOTS TO SHOW THE RELATIVE PROPORTIONS OF ACID, NEUTRAL AND MIXED MUCUS GLYCOPROTEINS IN S.I.D.S. AND CONTROL LARYNGES

S.I.D.S. acid	Control acid	S.I.D.S. neutral
0	0	0 0000011222233334578 8
1	1	1 013
2	2	2 1
3 9	3	3
4 6799	4 48	4
5 0001225699	5 3566899	5
6 0033569	6 2	6
7 24	7	7
8	8	8
9	9	9
10	10	10
Control neutral	**S.I.D.S. mixed**	**Control mixed**
0 0122333	0	0
1 337	1 36	1
2	2 028	2 8
3	3 3448	3 468
4	4 00112467788	4 011139
5	5 123	5
6	6 1	6
7	7	7
8	8	8
9	9	9
10	10	10

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TABLE II
(a) THE RELATIVE AMOUNTS OF SULPHOMUCIN IN S.I.D.S. AND CONTROLS

S.I.D.S.	Controls
0	0 6
1	1 366
2	2 279
3 89	3 48
4 03379	4
5 125888	5
6 024578	6 5
7 5569	7
8	8
9 2	9
10	10

TABLE II
(b) THE RELATIVE AMOUNTS OF SIALOMUCIN IN S.I.D.S. AND CONTROLS

S.I.D.S.	Controls
0 7	0
1	1
2 1345	2
3 134689	3 5
4 222579	4
5 0277	5
6 011	6 25
7	7 027
8	8 347
9	9 4
10	10

heavy. Subsequent specimens were embedded using 'Fibrowax'.

Transverse 5 μm sections were cut step-serially, taken from the epiglottis, aryepiglottic folds and vestibular folds of the supraglottis, the vocal fold area of the glottis, the cricoid region of the subglottis and also from the first tracheal ring.

Initially sections were stained with haematoxylin and eosin, using Ehrlich's haematoxylin as it is selective for acid mucins demonstrating the general structure of cartilage and mucous glands. For each mucin technique employed a known positive stain control, i.e. sections of human adult trachea, was stained concurrently with the infant larynx or trachea sections. Confirmatory techniques were also employed as mucin stains can be capricious. Sections were next stained by the pH 2.5 alcian blue–periodic acid Schiff technique (A.B.P.A.S.) for acid, neutral and mixed mucins. This was confirmed using the Hale's dialysed iron–P.A.S. method. For the Hale's method a negative control section, which was a duplicate of one of the sections being stained, was taken through the method simultaneously with the omission of the dialysed iron step, as false positive results can occur due to any haemosiderin present.

To determine the relative amounts of sulphated and carboxylated mucins present the aldehyde fuchsin–alcian blue method was employed. An attempt was made initially to confirm the results given by this technique by the use of the combined high diamine–alcian blue technique. As this method is both time consuming and hazardous (known carcinogens present), confirmation for the aldehyde fuchsin-alcian blue staining was repeated using the aluminium sulphate–neutral red method and the high diamine method was abandoned. The aluminium sulphate method demonstrates sulphated mucins only.

Quantitation

On completion of staining a Clay-Adams point counter was used to count firstly acid, mixed and neutral mucin secretions (Table I) and secondly sulphated and carboyxlated mucin secretions i.e. sulphomucins and sialomucins respectively (Table II). All specimens were counted 'blind' i.e. it was not known whether they were S.I.D.S. or control.

Six sections for each stain technique employed were counted: three from the supraglottis, one from the glottis, one from the subglottis and one from the trachea: 1200 cells per larynx were counted in all and the percentage of glandular area secreting each type of mucin was calculated as an average over the six areas for each larynx.

For the A.B.–P.A.S. method the results of the amounts of different types of mucin secretions present were confirmed with the dialysed iron–P.A.S. method and an average between the two

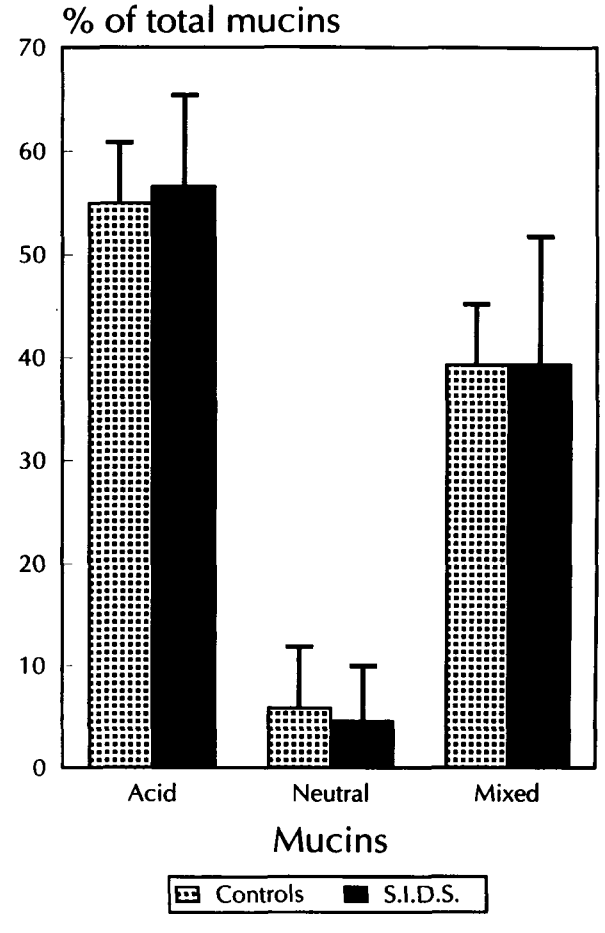

FIG. 1

The percentage of acid, neutral and mixed mucins found in control and S.I.D.S. larynges is expressed as the mean ± SD. There were no significant differences between control and S.I.D.S. ($p>0.05$).

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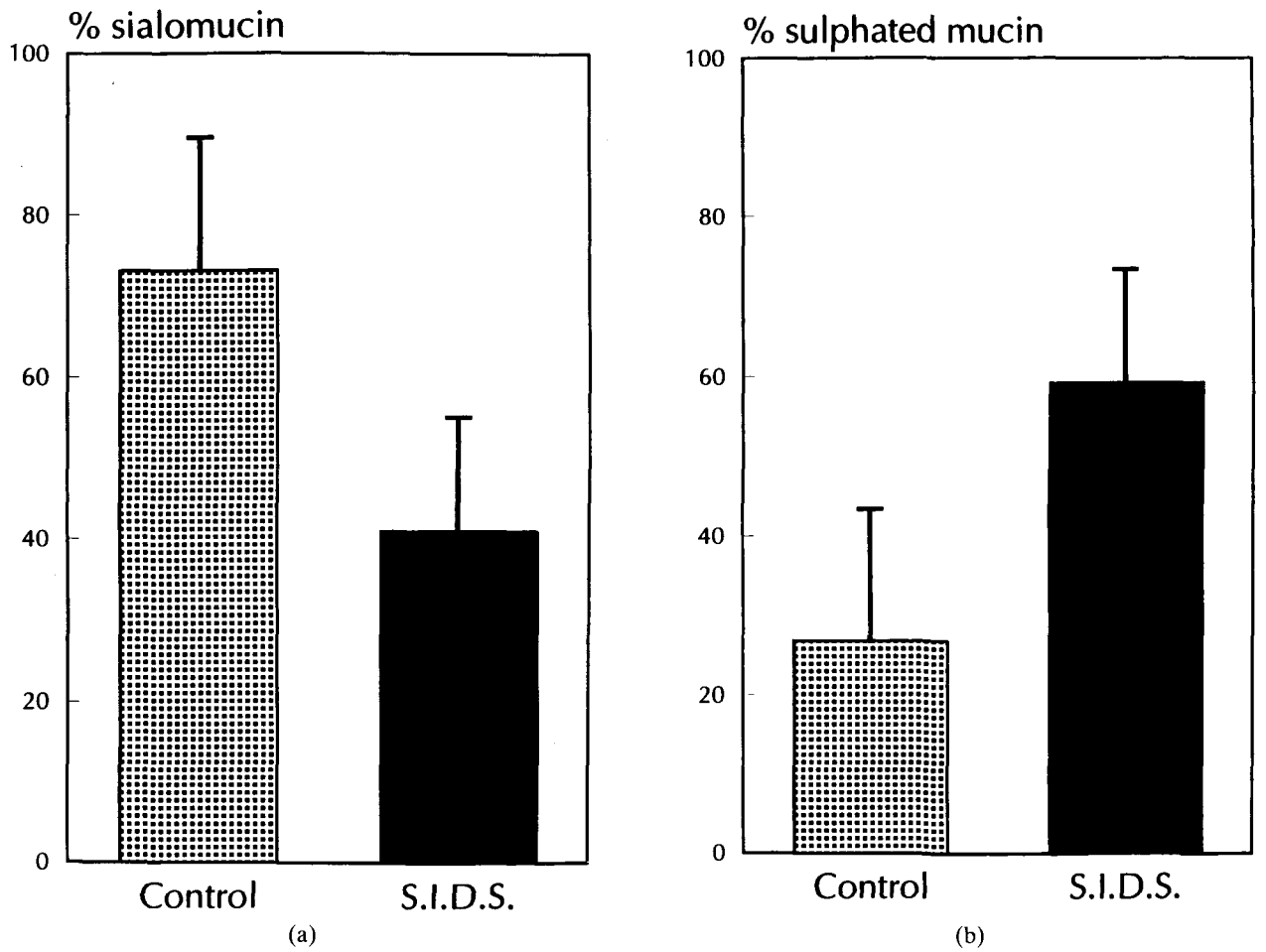

FIG. 2

Larynges of S.I.D.S. infants show (a) a significant ($p<0.05$) decrease in the percentage of cells expressing sialomucins and (b) a significant increase ($p<0.01$) in the percentge of cells expressing sulphated mucins.

was taken. To determine whether or not there were any between site differences the result of the mean for each area for each mucin type was tabulated.

All weakly and strongly stained sulphated mucins were counted together. A comparison of the relative amounts of mucin secretions was made between S.I.D.S. and control specimens, taking into consideration the age of the infant and the area from which the section was taken. A final result could then be calculated by taking the mean of both sulphated and sialic acid mucins contained in S.I.D.S. and control larynges. A Mann-Whitney U test was used to compare the non-parametric data of these two groups.

Results

The percentage of acid, neutral and mixed mucus glycoproteins were similar in both S.I.D.S. and control larynges (Figure 1). The Mann-Whitney

TABLE III

BETWEEN SITE DIFFERENCES OF ALL MUCUS GLYCOPROTEINS FOR S.I.D.S. AND CONTROL LARYNGES

Mucin type	Mean percentage per six laryngeal areas						± SD
S.I.D.S.	1	2	3	4	5	6	
Acid	54	55	60.5	57.5	56	56	2.28
Neutral	4	3	4	3.5	4	6.5	1.21
Mixed	42	42	35.5	39	40	37.5	2.56
Sulphated	60	61	61.5	57.5	60	56.5	1.98
Sialylated	40	39	38.5	42.5	40	43.5	1.98
Controls							
Acid	54	51	54	60	56	54.5	2.97
Neutral	6.5	4.5	4.5	5	5.5	9	1.72
Mixed	39.5	44.5	41.5	35	38.5	36.5	3.43
Sulphated	29	29	28.5	27	26.5	21	3.04
Sialylated	71	71	71.5	73.5	73	79	3.04

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TABLE IV

(a) AVERAGE PERCENTAGE ACID, NEUTRAL AND MIXED MUCINS PER LARYNX SHOWING NO CONSISTENT CHANGES WITH AGE

Case No.	Age (weeks)	Sex	Weight P.M. (g)	Controls			S.I.D.S		
				Acid	Neutral	Mixed	Acid	Neutral	Mixed
880	2 days	M	3780	58	1	41			
902	2	M	3450				59	1	40
879	4	F	3208	59	0	41			
912	4	M	4050				59	0	41
921	4	M	5476				60	2	38
987	7	M	3760	55	17	28			
903	8	M	5850				63.5	2	34.5
919	8	M	4309				52	8	40
920	8	M	4630				49	2.5	48.5
942	8	F	4804				69	3	28
878	12	M	4075				46.5	1	52.5
881	12	F	4800	56	3.5	40.5			
882	12	M	3430	59	3	38			
900	12	M	4510				47	0	53
904	12	F	4370				63.5	2	34.5
940	12	M	5542				50	3	47
986	12	F	4170	56	2.5	41.5			
877	14	M	N.K.				39	0	61
901	14	M	5975				52	0	48
988	14	M	4170	44	13	43			
913	16	M	7170				66	21	13
911	20	M	6640				50	3	47
918	20	F	5750				74	10	16
922	20	M	5840				72	8	20
943	20	M	7020				56.5	1.5	42
989	20	M	4840	48	3	49			
923	24	M	4961	53	13	34			
985	24	M	7800	62	2	36			
914	36	M	3600				65	13	22
915	36	M	6850				60	7	33
917	40	F	9940				50	4	46
941	44	M	5428				55.5	3	41.5
916	52	F	8770				51	5	44
944	116	F	4280				49	0	51
Mean				55	6	39	57	4	39
± SD				5.44	6.07	5.7	8.87	5.12	12.3

test confirmed that there was no significant difference between these two groups. The amounts of total acid mucins were for the controls 55 per cent and for the S.I.D.S. 57 per cent (difference two per cent; ± standard error of difference 2.43 per cent; $p>0.05$). For neutral mucins the amounts were for the controls 6 per cent and for the S.I.D.S. 4 per cent (difference 2 per cent; ± standard error of difference 2.18 per cent; $p>0.05$) and for mixed mucins for the controls 39 per cent and for S.I.D.S. 39 per cent (± standard error of difference 3.09 per cent; $p>0.05$).

There was, however, a significant difference between S.I.D.S. and controls in the relative proportions of sialomucin (Figure 2a) and sulphomucin (Figure 2b) present. The percentage of sulphated mucin to total acid mucin was for the controls 39 per cent and for the S.I.D.S. 59 per cent (difference 32 per cent; ± standard error of difference six per cent; $p<0.01$). The sialomucin content of the controls was 73 per cent and for the S.I.D.S. 41 per cent (difference 32 per cent; ± standard error of difference six per cent; $p<0.01$.

There were no consistent changes in relation to the laryngeotracheal area (Table III) in either S.I.D.S. or controls for acid, neutral, mixed, sulphated or sialylated mucins, neither were there any consistent changes with age in any of these groups (Tables IVa and b).

There was a predominance of males in both the S.I.D.S. and the control specimens but with a higher ratio in the S.I.D.S. (3:1) than in the controls (7:3).

Discussion

The present investigation showed an overall increase in the presence of sulphated mucus glycoproteins in the S.I.D.S. laryngeal submucosal glands with a reduction in sialomucin. A variation in the proportion of mucins may be due to the activity of glycosyl transferases (Berry *et al.*, 1992).

Evidence of infection and inflammation has been noted in the respiratory tract of S.I.D.S. victims yet these inflammatory changes have not been so severe as to have been considered the cause of death (Williams, 1980).

There have been numerous reports that parents of S.I.D.S. victims have noticed symptoms indicative of a mild respiratory infection in the infant approximately two weeks before death. Studies have shown that in around 25 per cent of cases there is microbiological and or histological evidence of respiratory virus infection (Scott *et al.*, 1978) compared with 18 per cent of controls (Williams *et*

TABLE IV

(b) AVERAGE PERCENTAGE PER LARYNX OF MUCUS GLYCOPROTEIN TYPE, SHOWING NO CONSISTENT CHANGES WITH AGE

Case no.	Test	Age (weeks)	Sialomucin	Sulphomucin
880	Control	2 days	83.5	16.5
902	S.I.D.S.	2	60	40
879	Control	4	70.5	29.5
912	S.I.D.S.	4	52.5	47.5
921	S.I.D.S.	4	34.5	65.5
987	Control	7	62	38
903	S.I.D.S.	8	57	43
919	S.I.D.S.	8	25	75
920	S.I.D.S.	8	21	79
942	S.I.D.S.	8	33	67
878	S.I.D.S.	12	49	51
881	Control	12	77.5	22.5
882	Control	12	94	6
900	S.I.D.S.	12	45	55
904	S.I.D.S.	12	61.5	38.5
940	S.I.D.S.	12	23.5	76.5
986	Control	12	87	13
877	S.I.D.S.	14	42	58
901	S.I.D.S.	14	50.5	49.5
988	Control	14	72.5	27.5
913	S.I.D.S.	16	7.5	92.5
911	S.I.D.S.	20	61	39
918	S.I.D.S.	20	36	64
922	S.I.D.S.	20	39.5	60.5
943	S.I.D.S.	20	42	58
989	Control	20	84	16
923	Control	24	35	65
985	Control	24	65.5	34.5
914	S.I.D.S.	36	31.5	68.5
915	S.I.D.S.	36	42	58
917	S.I.D.S.	40	57	43
941	S.I.D.S.	44	24.5	75.5
916	S.I.D.S.	52	38	62
944	S.I.D.S.	116	47.5	52.5

al., 1984). Upper respiratory tract infection was also reported in 45 per cent of infants preceding near-miss episodes (Jeffery *et al.*, 1983).

Certain bacteria and other microorganisms have an affinity for particular airway mucins which facilitates their clearance. *Pseudomonas aeruginosa*, for example, has an affinity for neutral and sialylated mucus glycoproteins but none for those which are sulphated (Lamblin *et al.*, 1991). Viscoelastic properties are related to sialomucin which is more easily cleared (Berry *et al.*, 1992), therefore it is beneficial for the predominant mucus glycoprotein to be sialylated. Conversely, in certain disease states, even a minor infection or irritation could be detrimental to a baby as the increase in thickness of the laryngeal submucosal glands and the corresponding decrease in the size of the lumen (Harrison, 1991a) in conjunction with hypersection and an increase in sulphated mucin which has a low clearance rate could possibly lead to failure to clear secretions and therefore to apnoea.

It has been recognized for some time that irritants such as certain gases, drugs, infectious agents e.g. bacteria and viruses, can cause an alteration of respiratory tract submucosal glands, producing hypertrophy and hyperplasia, leading to basal rate hypersecretion of mucus glycoprotein (Lamb and Reid, 1968; Jones *et al.*, 1972; Jefferey and Reid, 1977; Sheehan *et al.*, 1991). Tobacco smoke is an irritant that induces similar results and continuing exposure produces mainly acid glycoprotein. The cell type is modified and the intracellular quantity is increased. Stimulus by noxious substances often increases sulphated mucin at the expense of sialylated or neutral glucoproteins (Jones and Reid, 1978). The induced hypertrophy by tobacco smoke of the mucous tubules, partly due to an increase in the size of individual mucous cells, has been noted both in laryngeal and tracheal glands as well as an increase in the size of the collecting ducts (Coles *et al.*, 1979). Mitotic rate and epithelial thickness increase also after exposure to marijuana smoke (Hayashi *et al.*, 1980) and to sulphur dioxide (Lamb and Reid, 1968), the gland size increasing proportionately to the time of exposure to sulphur dioxide with a shift to sulphomucin from sialomucin.

The cot death rate has decreased by 75 per cent recently (Radio news broadcast, August, 1993). This may be due to an increased awareness of the dangers of exposing infants to atmospheric pollutants such as tobacco smoke and traffic exhaust fumes, as well as a better education in other factors such as sleeping position and overheating.

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