Supplementary Report for the Inquiry into the Convictions of Kathleen Megan Folbigg
Relating to Caleb Folbigg

1. The only abnormal finding for Caleb appears to be the histology of the lungs, "congested and in places show incomplete aeration, in other sections their alveoli contain extravasated red blood cells and a small amount of eosinophilic exudate. These are proteins that pick up the eosin stain and a common finding in post mortem lungs and often seen in samples from children diagnosed with SUDI (Prof. J. A. Morris, personal communication).

2. The source of Caleb’s eosinophilic exudate was not investigated. No microbiology examination was carried out on samples from the child. This is important as a variety of infectious agents identified in SIDS/SUDI can elicit inflammatory responses. These include a variety of viruses, bacteria, Chlamydia trachomatis [Lundemose et al., 1990], and Pneumocystis carinii [Vargas et al., 1999] Pneumocystis jerovici (Prof. P.N. Goldwater, personal communication).

3. There was also no assessment of lung immunoglobulins which also found to be raised in SIDS/SUDI [Forsyth et al., 1989].

References


Professor Cecelia Caroline Blackwell

13 March 2019
Lung immunoglobulins in the sudden infant death syndrome

Kevin D Forsyth, Sandy C Weeks, Lin Koh, John Skinner, John Bradley

Abstract

The incidence of the sudden infant death syndrome parallels that of respiratory tract infections in the paediatric community. On the basis that the aetiology of the sudden infant death syndrome may lie in an unusual response to a trivial intercurrent respiratory tract infection a necropsy study was carried out investigating pulmonary immunoglobulins in 16 victims of the syndrome and a series of infants (controls) who had died of non-pulmonary causes. Compared with the controls victims of the sudden infant death syndrome had grossly raised concentrations of IgG, IgM, and to a less extent IgA in lung lavage samples. In addition, pulmonary interstitial and terminal airway cells expressing these immunoglobulins were identified far more often in victims of the syndrome and a series of infants who died of both respiratory viral infections and sudden infant death syndrome. Immunoglobulins were identified in at least 97% of lung samples from victims of the sudden infant death syndrome. Immunoglobulins were identified in 82% of the controls. Immunoglobulins were found to persist up to five minutes after death and were not detected in samples obtained prenatally.

In conclusion the reduction in cognitive performance combined with the adverse changes in mood show less than desirable conditions for personal wellbeing and the practice of medicine. Superimposed night duty rosters may be a stress adversely affecting the welfare of both the doctor and the patient.


(Received 7 November 1988)

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months, range 1-7). Using the same procedure we also collected lung lavage samples from eight controls (mean age 4-8 months, range 2-10) and lung tissue from four controls (mean age 5-3 months, range 3-9). The controls were previously well children who had died of acute, non-pulmonary causes. They came to necropsy because they had congenital abnormalities or had died as a result of an accident. Accident victims with pulmonary contusion were excluded. In all cases specimens were collected 12 to 24 hours after the presumed time of death. The results of cellular and virological studies have been reported.6

**Lung lavage fluid immunoglobulin concentrations**—Immunoglobulins IgG, IgA, and IgM were measured by a sensitive competitive inhibition enzyme immunoassay. Briefly, polystyrene tubes were used as the solid phase and coated with the appropriate pure immunoglobulin (rabbit antihuman IgG, IgA, or IgM; DAKO, Denmark). The lavage samples were diluted 50% and added to the solid phase together with purified antihuman IgG, IgA, or IgM coupled to horseradish peroxidase. 5-Aminosalicylic acid was used as the enzyme substrate and the absorbance measured at 474 nm. In this assay, as the amount of solid phase immunoglobulin is limited, the amount of enzyme bound is inversely proportional to the concentration of immunoglobulin in the test sample. With this assay IgG is detectable down to 2-5 µg/l, IgA down to 4-2 µg/l, and IgM down to 7-2 µg/l. There is undetectable cross reactivity among the immunoglobulin classes with the exception of purified IgM, which cross reacted at 1-9% with the IgG enzyme immunoassay. For standardisation all results were expressed as µg immunoglobulin/mg total protein in the lavage sample. Total protein in mg/ml was measured by the Lowry method.1

**Immunostaining for lung immunoglobulins**—Lung tissue collected at necropsy was stored at −70°C. Sections were cut on a cryostat and mounted on glass slides. Endogenous peroxidase was blocked with 1-6% hydrogen peroxide in absolute methanol for 20 minutes. Fc receptors were blocked with normal horse serum diluted 1/50 in isotonic saline for 20 minutes. Antihuman IgG (HB-60) and antihuman IgM (HB-57) were obtained from American Type Culture Collection, Maryland, and used as neat supernatant. The IgA monoclonal antibody (H-11) is reactive with human IgA secretory component. It was supplied as ascites and diluted 1/50 for use. The antibodies were applied for two hours, followed by the secondary antibody (horse antimouse IgG-biotin conjugate) for 30 minutes. Avidin-Biotin complex (Vector Laboratories) was applied for one hour and the complex developed with 0-05% diaminobenzidine hydrochloride (Sigma Chemicals) and 0-01% hydrogen peroxide for 10 minutes. Nuclei were counterstained with Mayers haematoxylin for 30 seconds. All incubation steps were performed at room temperature, with the sections washed three times in TRIS hydrochloric acid buffer 0-05 mol/l pH 7-6 between each step. Positive cells on immunohistological staining were identified by two observers through a double headed Olympus microscope with ×25 objective. On average 20 fields were studied per specimen. Results were expressed as the total number of positive cells per field divided by the number of fields studied and multiplied by 100.

![FIG 1.](image)

**FIG 1**—Pulmonary lavage fluid concentrations of IgG (expressed as µg/mg total protein) in eight controls and 16 victims of sudden infant death syndrome. Roughly half of lavage fluid protein in sudden infant death syndrome group was IgG

**Statistics—Results** are expressed as the mean and one standard deviation (1SD). As the immunoglobulin results did not conform to a normal distribution, the Mann-Whitney U test was used to compare values between the sudden infant death syndrome and control groups.

**Results**

**Lung lavage samples**—IgG was present in the lavage samples of all subjects, with remarkably high values in the sudden infant death syndrome group: just over half the total protein in the lavage samples was IgG (fig 1). The mean IgG concentration in the sudden infant death syndrome group was 578 (216) µg/mg total protein and in the controls 93 (22) µg/mg (p<0.005). IgA and IgM concentrations were similar and quantitatively much lower than the IgG concentrations. The mean IgA concentration in the lavage fluid in the sudden infant death syndrome group was 7 (2) µg/mg total protein and in the controls 3 (3) µg/mg (p<0.005) (fig 2). The mean IgM concentration in the sudden infant death syndrome group was 8 (3) µg/mg total protein and in the controls 0-5 (0-4) µg/mg (p<0.005) (fig 2). Protein concentrations in the lavage samples were not significantly different between the two groups.
Lung immunohistory.—Positive cellular staining for immunoglobulins was evident in the terminal airways and alveolar interstitium and was extensive in victims of the sudden infant death syndrome (fig 3). The mean numbers of IgG-positive cells on immunohistological staining expressed per field per cent were 151 (140) in the sudden infant death syndrome group and 26 (16) in the controls (p<0.005). For the IgA secretory component there were 119 (76) positive cells in the sudden infant death syndrome group and 29 (33) in the controls (p<0.05). Similarly, for IgM there were 176 (78) positive cells in the sudden infant death syndrome group and 53 (14) in the controls (p<0.005). At the cellular level the staining pattern was diffuse, not granular, and distributed throughout the cytoplasm, not solely membrane staining. In general positive cells were diffuse throughout the section, though in a few sections in the sudden infant death syndrome group there were clumps of IgA or IgG-positive cells around terminal airways. The staining suggested that they were plasma cells.

Discussion

In this paper we have shown by two separate methods (analysis of pulmonary lavage fluid and pulmonary immunohistological staining) that there is a difference in the amounts of immunoglobulin present in the lungs of children who have died of the sudden infant death syndrome compared with controls. The concentration of IgG in lavage fluid was high and the number of pulmonary interstitial and terminal airway plasma cells that expressed IgG was increased in the sudden infant death syndrome. In addition, in comparison with the controls over half the lavage fluid protein was IgG, there was an increase in lavage fluid and cellular IgM, and there was a smaller but significant increase in the IgA secretoiy component. These differences were apparent whether the lavage fluid immunoglobulin concentration was expressed as μg immunoglobulin/ml lavage fluid or mg immunoglobulin/mg total protein or mg immunoglobulin/g lavage fluid (data not shown).

In a recent study by our group of respiratory tract immunoglobulins from ventilated neonates IgG was found to be the predominant immunoglobulin.\(^1\) In this series victims of the sudden infant death syndrome were on average one month younger than the controls. Data on lower respiratory tract immunoglobulin concentrations in normal children are not available. Concentrations in the serum and lower respiratory tract of intubated neonates, however, suggest a decline in IgG values with increasing age and a slow increase in IgA and IgM.\(^2\) These differences are not significant over one month.

Previous work also by our group on lung lavage cell phenotype in the sudden infant death syndrome has shown a difference between victims of the syndrome and controls.\(^2\) The relevance of this difference (lack of CD14A reactivity in the sudden infant death syndrome group) is uncertain but it is compatible with cellular activation (N Hogg (London), personal communication).

There has been little work on pulmonary immune mechanisms in the sudden infant death syndrome. A study by Ogra \textit{et al} in 1975 using a qualitative (radial gel diffusion) rather than quantitative method showed IgG and IgM to be present in lavage fluid in the sudden infant death syndrome, but secretory component to be absent in pulmonary tissue.\(^3\) Ackermann \textit{et al}, in agreement with our findings, detected significantly increased IgG expression in pulmonary tissue from victims of the syndrome with a fluorescein labelled sheep antihuman IgG antibody.\(^4\)

Our studies suggest that there is altered immunological function in the sudden infant death syndrome. There is alteration of cellular phenotypes and a gross increase in IgG and IgM concentrations in lung lavage fluid. The relevance of these changes is not clear. Possibly an unrecognised respiratory pathogen is stimulating high immunoglobulin concentrations in the respiratory tract. More probably, however, a dysregulation of immunoglobulin production stimulated by a trivial infection might serve to "trigger" the attack in an infant at risk by exposing antigens or modulating the immune response.\(^5\) It has been postulated, for example, that the sudden infant death syndrome may be a modified form of local anaphylaxis occurring in the lungs, possibly mediated by IgG; infection might produce or expose the appropriate antigen for this type of reaction in susceptible children.

Though extravasation of IgG might be due to an anaphylactoid type of reaction, this could not account for the numbers of plasma cells seen in tissues where they are rarely if ever present. This finding points to local production of immunoglobulin. The evidence, both epidemiological and from this study, suggests that the respiratory tract is the prime target organ in the sudden infant death syndrome. Further investigation of pulmonary immunological responses is required.

This work was supported by the Sudden Infant Death Research Foundation of South Australia. We are grateful to A Hohmann, of Flinders Medical Centre, for the gift of IgG monoclonal antibody (H-11).


Serious eye injuries due to war games

J F Acheson, M F P Griffiths, R J Cooling

"War games" are an outdoor activity combining recreation, military style manoeuvres, and fantasy. Combatants in the games use gelatin pellets filled with dyes, at each other from powerful air guns. War games' weaponry developed from paint guns used by forestry workers to mark trees for felling. These guns are not classified as firearms in the United Kingdom, in contrast to rounds detonated by percussion. Gas cartridge weapons are not classified as firearms in the United States.3:4 Again, goggles were being worn but did not prevent hyphaema, vitreous haemorrhage, and a retinal tear.1

Goggles were supplied to all our six patients; a lens from one pair, however, dropped out spontaneously, and in three cases the goggles were removed for extra comfort and visibility before injury. In the remaining two patients who were wearing goggles both injuries resulted from the plastic pellet dislocating the lens from the frame and driving it on to the eye. The toughened protective glasses and rubber rimmed industrial goggles were inadequate, and single piece polycarbonate eye protectors such as those recommended for racket sports seem more suitable.

The injuries reported were of a blunt, non-penetrating type normally associated with fairly low kinetic energies further dissipated by goggles, when worn. Permanent visual loss, however, may result from these games as the weapons have sufficient muzzle velocities to cause penetration of the eye. Moreover, though the retained subconjunctival dye in case 4 did not seem to be toxic, blunt ocular injury carries considerable morbidity. Emergency admission to hospital was required in three of our six cases to treat raised intracranial pressure and to minimise the risk of rebleeding hyphaema. All six patients required prolonged follow up to identify and treat delayed complications such as secondary glaucoma, contusion catarract, recurrent corneal erosion syndrome, retinal pigment epithelial degeneration, and retinal detachment.

We gratefully acknowledge the help of Mr D Pryor, Metropolitan police laboratory, firearms section.

Details of six cases of ocular injury due to war games

<table>
<thead>
<tr>
<th>Case No</th>
<th>Age (years)</th>
<th>Wearing goggles</th>
<th>Emergency admission to hospital</th>
<th>Initial visual acuity (months later)</th>
<th>Final visual acuity (months later)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>No</td>
<td>Yes</td>
<td>Hand movement</td>
<td>6/12</td>
</tr>
<tr>
<td>2</td>
<td>35</td>
<td>Yes</td>
<td>Yes</td>
<td>Central abrasion, hyphaema, conjunctival laceration, corneal abrasion</td>
<td>6/12 (Six months; after extraction of cataract and insertion of aphakic contact lens)</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>No</td>
<td>Yes</td>
<td>Hyphaema, central abrasion, corneal abrasion</td>
<td>6/6 (Two months)</td>
</tr>
<tr>
<td>4</td>
<td>58</td>
<td>Yes</td>
<td>Yes</td>
<td>Conjunctival laceration, corneal abrasion, corneal erosion of retina</td>
<td>6/24 (Three months)</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>No</td>
<td>No</td>
<td>Hyphaema, transient raised intraocular pressure, vitreous haemorrhage, retinal break (replaced by cryotherapy)</td>
<td>6/3 (Three months)</td>
</tr>
<tr>
<td>6</td>
<td>19</td>
<td>No</td>
<td>No</td>
<td>Hyphaema, drainage angle recession</td>
<td>6/5 (One month)</td>
</tr>
</tbody>
</table>

Original works

Chlamydia and sudden infant death syndrome.
A study of 166 SIDS and 30 control cases

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Received March 28, 1990 / Received in revised version May 23, 1990

Summary. Chlamydia inclusions could be demonstrated by an immunofluorescence assay in formalin-fixed lung sections in 32 of 166 cases (19.4%) of Sudden Infant Death Syndrome (SIDS) and in the lungs of only 1 of 30 infants with a known cause of death (3.3%). The difference is statistically significant (P = 0.04). Chlamydia trachomatis is an agent of pneumonia in 1-4 month-old infants who have acquired the disease from an infected cervix during birth, but other chlamydia species are also capable of causing pneumonia. The lung sections of the 32 chlamydia positive SIDS cases did not show typical histological signs of pneumonia. Even though chlamydia inclusions were detected in the lungs of 32 SIDS cases a causal relation between chlamydia infection and SIDS could not be demonstrated.

Key words: Sudden Infant Death Syndrome – Chlamydia – Bacteria – Diagnosis – Immunofluorescence

Introduction

In Western countries about half of all postneonatal deaths are classified as Sudden Infant Death Syndrome (SIDS). The cause of SIDS is still unknown in spite of an increased public awareness and intensified research and its prevalence is unchanged [1]. In most of the SIDS cases the autopsy shows no pathological changes, but some cases have minor signs of infections in the respiratory organs insufficient to explain the cause of death. A number of the infants have had history of coughs and other symptoms of respiratory infection preceding death [2] but no causal relation between infectious agents and SIDS has until now been demonstrated.

Chlamydia trachomatis is among the most common agents of lower respiratory infections during the first three months of infancy [3, 4], but no studies of the relation between this microorganism and SIDS have ever been performed. In this paper a possible connection between chlamydia and SIDS was studied by determining the occurrence of chlamydia inclusions in the lungs of these infants.

The genus Chlamydia consists of three species, Chlamydia trachomatis, Chlamydia psittaci and Chlamydia pneumoniae. C. trachomatis is a human pathogen causing ocular genital infections and pneumonia in 3-4 month-old infants [5]. C. psittaci is mainly an animal pathogen, but in man it produces ornithosis which occurs as a systemic infection with a severe pneumonia [5]. C. psittaci has also been associated with abortion in women [6, 7].
C. pneumoniae causes respiratory infections including pneumonia [8]. Chlamydia is an obligate intracellular bacteria with a biphasic life cycle. The characteristic chlamydia morphology is represented by the inclusion, which is the metabolically active part of chlamydia. We have previously described a method to detect chlamydia inclusions in postmortal formalin-fixed tissue with an immunofluorescence assay using two genus-specific monoclonal antibodies [9]. In this study the frequency of chlamydia inclusions in the lungs of 166 SIDS cases and a comparable control group of 30 infants which died of known causes is described and discussed.

Materials and methods

SIDS cases. A prospective study of all cases of sudden infant death was carried out in Denmark in 1987 and 1988. According to Danish law all cases of SIDS are submitted to a postmortal medico-legal investigation and in most cases a medico-legal autopsy is performed. The autopsy rate was 95% in the years 1987 and 1988. Two hundred and seven infants between the age of 1 week and 1 year died suddenly in 1987 and 1988. The cause of death was explained by autopsy in 24 cases. Eleven cases were not autopsied due to prohibition by the parents. From the 172 autopsied infants with no known cause of death, 166 cases were selected and were classified as SIDS according to the definition "The sudden death of any infant or young child which is unexpected by history and in which a thorough post mortem examination fails to reveal an adequate cause of death" [10]. One hundred and twenty-six cases had no pathological changes, but 5 infants with histologically minor signs of viral pneumonia insufficient to explain death and 35 infants with cellular inflammatory changes considered insignificant for the death, were included in the 166 SIDS cases selected.

The age interval of the selected SIDS group in this study was between 28 days and 1 year. The age distribution of the SIDS cases was from 1 to 10 months, with 2 month-old infants making up 28% of the total and 86% aged between 1 and 4 months.

Control cases. Thirty control cases aged between 28 days and 1 year were found in the files of the Danish Institutes of Forensic Medicine from January 1 1985 and December 31 1988. Only infants with a known cause of death were selected and included 7 cases of previous undiagnosed congenital heart disease, 2 cases of bacterial meningitis, 4 cases of cerebral disorders (2

![Fig. 1a Two chlamydia inclusions in the lung of one of the chlamydia positive SIDS cases. Original magnification x 500. Bar represents 10 µm. b The left upper inclusion from a magnified x 1500. The reticulate bodies are located in the cytoplasm around the nucleus of the host cell](image-url)
thrombosis and 2 incarcerations), 5 major respiratory infections (with verified bacteriology), 1 abdominal haemorrhagic infarction, 8 accidents (domestic or traffic) and 3 homicides.

The age distribution of the infants in the control group was from 1 to 12 months, 66% were from 1-4 months old and 4-month-old infants making up 20% of the total.

**Immunofluorescence method.** Formalin-fixed lung sections from the SIDS and control cases were retrospectively examined for chlamydia inclusions as described by Lundemose et al. [9]. Two monoclonal antibodies 12.1 and 15.1 against the genus-specific chlamydia lipopolysaccharide (LPS) epitope were used [11]. These antibodies react only with the chlamydia-specific LPS epitope and no cross reactions occur. Intracellular inclusions or LPS accumulated in the membrane of the infected host cell were visualized with immunofluorescence after reaction with fluorescein-conjugated anti-mouse antibody (IgG). Problems with background staining were avoided by using trypsin, rabbit serum and Evans-blue. Trypsin removes the non-specific antibody binding Fc-receptors in the tissue by digestion and the rabbit serum minimizes the non-specific antibody adsorption by competition with the specific antibody. Evans-blue was used as counterstain.

The positive immunofluorescence diagnosis in this study was exclusively based on the bright apple-green fluorescence and the characteristic morphology of the chlamydia inclusion as described earlier [9]. In all the chlamydia positive cases distinct and sharply demarcated inclusions were visualized (Fig.1). The case was only considered positive if inclusions were seen in at least 2 of the 4 lung sections. LPS accumulated in the membrane of the infected host cells was sometimes visualized as a bright applegreen ting of the cell/epithelium, also called the alveolar lining.

**Demonstration of chlamydia inclusions in the lung tissue by immunofluorescence.** Four tissue sections, two from the central and two from peripheral parts of both lungs were examined for each case. The tissue sections were examined in the form of a blind trial and were all examined by the same individual. The results were reproducible in 100% of the cases when re-examined 1 day later (75% were re-examined). The positive controls were lung sections from a mouse artificially infected with *C. trachomatis* serovar L2. The negative controls were lung sections from a 42-year-old man who had died from suffocation with no histological signs of pneumonia.

**Statistical analysis.** Fisher's exact test was used for statistical analysis of the results observed in this study.

**Results**

Thirty-two of the 166 SIDS cases (19.4%) and one of the control cases (3.3%) had typical chlamydia inclusions in the lungs. The data was subjected to Fisher's exact analysis, and associations with *P* values below 0.05 were regarded as significant (*P* = 0.04).

All the chlamydia positive cases had 10 or more inclusions in each positive lung section, 10 cases had more than 25 inclusions and 3 cases more than 100 inclusions. The inclusions were usually seen in clusters and always in the peripheral parts of the lungs located in relation to the alveoli. In the 13 heavily (25 inclusions or more) infected cases accumulation of LPS in the membrane of the infected host cell (membrane/alveolar lining) was detected. The alveolar lining was nearly always located in the central parts of the lung.

Chlamydia positive cases were aged from 1-7 months, the first 4 months making up 84% with a peak at 1-2 months. The histological examination of the lung sections from the chlamydia positive cases did not show any signs of typical chlamydia pneumonia with inflammatory exudate in the alveoli and lymphocytic infiltration in the bronchial submucosa. There were no specific reports of symptoms of tachypnea, staccato cough and inspiratory stridor, although 6 of the 32 chlamydia positive infants were reported to have had colds and coughs immediately preceding death. One of the 3 chlamydia positive cases with more than 100 inclusions per positive lung section and 1 of the chlamydia positive cases with more than 25 inclusions per lung section had minor signs of viral pneumonia insufficient to explain death. In addition, 9 of the chlamydia positive infants with more than 25 inclusions per lung section showed non-specific lymphocytic infiltration in the lungs. None of these histological pictures were serious enough to explain the cause of death. The remaining 21 of the chlamydia positive cases showed no evidence of infectious diseases either in the medical history or in the histological examination. Forty of the 166 cases in the total SIDS group had inflammatory cellular changes in the lungs insufficient to explain death. Colds and coughs were reported in 43 of the 166 cases.

The single chlamydia positive infant in the control group was a 8 1/2 month-old female with Down's Syndrome. The girl had a severe congenital heart malformation, which was considered the cause of death. The histological examination of the lungs showed signs of bronchopneumonia. The immunofluorescence examination showed more than 10 chlamydia inclusions per positive lung section. Fourteen of the 30 cases in the control group had non-specific cellular infiltrations or slight focal bronchopneumonia in the lungs as a secondary finding to the primary cause of death.

**Discussion**

The occurrence of chlamydia inclusions in the lungs of a SIDS group has been examined and compared with a control group. Due to the high overall autopsy rate in cases of infant deaths in Denmark, the SIDS group as well as the control group was well-defined. A simple and easily performed immunofluorescence technique has been used which is applicable on formalin-fixed tissue sections [9]. It was found that 32 of the 166 SIDS cases examined were positive for chlamydia inclusions. Only 1 child was found positive in the control group. The difference between the SIDS group and the control group was statistically significant (*P* = 0.04). The *P* value was relatively higher than expected due to the low number of cases in the control group. However, we were not able to allocate more than 30 infants to the control group and could only have increased the number by expanding the age and time interval or by including material from ordinary pathological autopsies.

The immunofluorescence assay was selected as the diagnostic tool, since cultivation from autopsy material is difficult because of postmortal loss of viability of the chlamydia. Genus-specific monoclonal antibodies against LPS were selected instead of the species-specific monoclonal antibodies against the Major Outer Membrane Protein, because the non-protein origin of LPS allows...
treatment with the background-reducing trypsin which is very important for the assay [9]. Furthermore LPS is in contrast to proteins, resistant to proteolysis and to post-mortem autolysis [9]. The membrane lining observed in some sections is probably caused by LPS incorporated into the plasma membrane of the infected host cell during infection [12]. Birkeland et al. [13] have shown that chlamydial elementary bodies treated with monoclonal LPS antibodies liberate LPS from the surface into the surroundings. Thus an alternative explanation to the formation of the membrane lining could be that LPS is liberated from the infected cell during infection. The membrane lining seen in the sections from the central part of the lungs could thus represent free LPS removed by the alveolar macrophages.

Conjunctivitis and nasopharyngeal infections in infants of C. trachomatis infected mothers has been observed and it has been reported that this may result in pneumonia [14]. The disease is usually mild and does not require hospitalization, but it reportedly accounts for approximately one third of the cases of pneumonia in hospitalized infants from 1-6 months of age and may occasionally be life-threatening [15]. Impairment of lung function may persist for a longer period than after a viral pneumonia [16]. The chlamydia positive SIDS cases in our study were aged between 1-7 months, 86% between 1-4 months in which C. trachomatis pneumonia usually occurs. The age distribution of the SIDS group and the control group was almost identical.

Although C. trachomatis is the classic agent of chlamydia pneumonia in young infants, the 2 other chlamydial species may be responsible for some of the inclusions detected in the lung sections of the chlamydia positive cases. The applied immunofluorescence assay does not permit distinction between the 3 chlamydia species. As C. pneumoniae is a new chlamydia species capable of producing lower respiratory diseases transmitted by droplet infection, it might be considered a possible source of at least some of the inclusions observed in the lungs of the SIDS cases. C. psittaci in humans is associated with severe pneumonia, but has also been reported to produce acute placentitis and abortion in women, where both the mother and the fetus have been infected [17]. Therefore C. psittaci is difficult to exclude as a possible agent of some of the inclusions found in the lungs of the chlamydia positive cases.

In Denmark pregnant women are not routinely screened for genital chlamydia infections and gynaecological and obstetric data on the mothers, including venereal status, preceding the births of the infants included in this study are therefore not available. Several authors [for ref. see 15] have shown that approximately 7%-12% of cervices are infected with C. trachomatis before delivery. Approximately 2 out of 3 infants who are exposed to C. trachomatis acquire the infection, and pneumonia will develop in about 10%-20% [18]. Using these figures, the expected frequency of C. trachomatis pneumonia in this study should have been about 1% to 2%. In the present study we found 3.3% positive for chlamydia in the control group. The difference between the expected and the actual chlamydia frequency in the control group could either be caused by the presence of chlamydia inclusions from the other 2 chlamydia species or simply be due to the small number (30) in our control group. The high frequency of chlamydia in the SIDS cases could theoretically be regarded as a normal finding, explained by a 100% transmission rate from the infected mother to the infant, but since a similar frequency was not seen in the control group, the occurrence of chlamydia in the SIDS cases must be regarded as significant.

An association between chlamydia and SIDS has been noted before but was not further examined [19]. The chlamydia organism has been well studied and it seems odd that chlamydia infection in SIDS infants has been overlooked before. However, cultivation of chlamydia from autopsy material is, as already mentioned, difficult and a direct antigen detecting method applicable on postmortem formalin-fixed tissue, has not been described until recently [9].

We did not find any histologically verified signs of typical chlamydia pneumonia in the 32 chlamydia positive SIDS cases or in the 1 chlamydia positive control case. Nor did we find any cellular reactions in the lungs of these infants adequate to explain the cause of death. Twenty-one of the positive infants did not have any signs of pulmonary inflammation and 11 infants with more than 25 inclusions per lung section had only non-specific lymphocytic infiltration or minor histological signs of viral pneumonia. Based on this observation it could be speculated whether some infants who may be incapable of producing an adequate response to a microorganism could die of a chlamydia infection, without significant histological signs of inflammation.

Evidence of respiratory inflammation has been found in a considerable proportion of SIDS cases [20, 21]. Some investigators have sought the explanation in a specific microorganism. Püschel et al. [22] examined the parotid gland and/or the submandibular gland for Cytomegalovirus (CMV) in 255 SIDS cases by means of HE staining, immunohistochemical analysis, in situ hybridization and electron microscopy. Typical cytomegalic inclusions were recognized in 10% of the cases. A localized CMV infection of the salivary glands does not adequately explain the sudden death of these infants, but the investigators emphasize that cytomegaly may influence the immunological status of the organism. Telford et al. [23] found significantly higher rates of streptococcal and enterobacterial carriage in the nasopharyngeal flora in a SIDS group than in a matched living control group, indicating a disordered nasopharyngeal flora in SIDS cases. However, these studies do not deal with a control group of infants at the same age and dying in the same period as the SIDS group examined.

It is difficult to establish a causal relationship between certain microorganisms and SIDS. However, in support of a causal relation between chlamydia infection and SIDS, we have found a disseminated chlamydia infection in a SIDS infant who died in 1989. Chlamydia inclusions were found in the lungs, heart, liver and prostate gland, with an inflammatory response only in the prostate gland.

We have not examined all the organs from the SIDS cases in this study, but are in the future planning to ex-
amine the degree of dissemination of the chlamydia in­
fection in the 32 SIDS cases with chlamydia inclusions in
the lungs. Whether a disseminated infection with chla­
mydia or other microorganisms is the cause of death in
some SIDS cases as a result of an inadequate response to
the infection needs further investigation.

Acknowledgements. This work was supported by The Danish Re­
search Academy. We are indebted to Jytte Jacobsen, Susanne An­
dersen and Lis Nielsen for technical assistance.

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tissue. APMIS 97: 68–74
Association of Primary *Pneumocystis carinii* Infection and Sudden Infant Death Syndrome

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To delineate clinical and histological features of the first *Pneumocystis carinii* infection affecting the immunocompetent host, *P. carinii*-specific histological stains were performed on autopsy lung specimens from 534 consecutive pediatric patients (those with AIDS and malignancies were excluded) in Santiago, Chile. *P. carinii* clusters were found in 4 (25%) of 16 infants who died of no apparent cause at arrival to the emergency department, and in 10 (2.9%) of 342 infants who died of multiple conditions at the hospital (*P* = .002, Fisher's exact test). This prompted us to analyze additional series of infants with sudden infant death syndrome (SIDS). In 161 additional SIDS cases, 47 (35.1%) of 134 infants from Chile and 4 (14.8%) of 27 infants from Oxford, United Kingdom, were found to have *P. carinii* clusters in the lungs. The quantity of *P. carinii* cysts was small compared with the numbers seen in immunocompromised hosts with *P. carinii* pneumonitis. This study provides histological evidence that primary *P. carinii* infection is associated with SIDS.

There is well-documented serological evidence that up to 94% of normal immunocompetent children have detectable antibody to *Pneumocystis carinii* by 30 months to 4 years of age [1, 2], which indicates that primary *P. carinii* infection is one of the most common infections in humans and that exposure occurs early in life. Experimental evidence shows that the host response pattern associated with primary *P. carinii* infection in otherwise healthy animals is milder [3-5] than that associated with the usually massive infection seen in an immunocompromised host. Data for humans are scant: *P. carinii* in low numbers or mild focal pneumonitis has rarely been reported as an autopsy finding for presumably immunocompetent children, and then the infection has been judged to be latent, incidental organisms [6]. In contrast, there are abundant autopsy reports of interstitial plasma cell pneumonia in certain groups of debilitated, undernourished, or premature infants, and reports of *P. carinii* pneumonia in infants and children with primary and secondary immunodeficiency syndromes [7, 8].

Cross-sectional studies of children's lungs at autopsy might provide histological support for the assumption, based on serology, that primary *P. carinii* infection is a common occurrence in small children. Furthermore, a correlate with diagnosis before death might provide insight to the clinical presentation of primary infection in immunocompromised infants and children. Because specific stains are needed to identify *P. carinii* in tissue samples, we undertook a prospective search for *P. carinii* by studying autopsy lung specimens from 534 consecutive children (those with AIDS and malignancies were excluded) that were obtained over a 6-year period at 2 major pediatric hospitals in Santiago, Chile. *P. carinii* was detected more frequently in infants who were dead on arrival at the emergency department and had an autopsy diagnosis compatible with sudden infant death syndrome (SIDS) than in children who died of multiple conditions at the hospital. These findings prompted us to expand our study to include an additional 134 infants in Chile who died at home and had a postautopsy diagnosis of SIDS, and 27 infants in Oxford, United Kingdom, who died of SIDS.

Methods

**Lung specimens.** A total of 695 autopsy lung samples were studied from infants and children in 4 different series. Series 1

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Received 26 February 1999; revised 6 August 1999.

This work was presented in part at the 36th annual meeting of the Infectious Diseases Society of America held on 12-15 November 1998 in Denver, Colorado.

Financial support: This work was supported in part by Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT research grant 1960940), Santiago, Chile, and by the St. Jude International Outreach Program and American Lebanese Syrian Associated Charities, St. Jude Children's Research Hospital, Memphis, Tennessee.

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comprised 534 consecutive pediatric patients (those with malignancies and AIDS were excluded) who were autopsied between January 1990 and December 1996 at the Department of Pathology, Luis Calvo Mackenna Children's Hospital, and the Department of Pathology, Roberto del Rio Children's Hospital, in Santiago. Series 2 comprised 94 infants with SIDS who were autopsied at the Legal Medicine Institute of Chile during 1996 and 1997; series 3, 40 infants with SIDS who were autopsied at the Department of Pathology, Exequiel Gonzalez Cortés Children's Hospital in Santiago between 1990 and 1993. Series 4 comprised 27 infants with SIDS who were autopsied at the Department of Pathology, John Radcliffe Hospital, University of Oxford, Oxford, from 1996 to 1998.

Formalin-fixed paraffin-embedded lung specimens were provided by pathologists from each institution. Age, circumstances of death, and postautopsy diagnosis were recorded when available. SIDS was diagnosed if there was no recognized premortem disease, no significant microscopic or macroscopic pathological findings, and toxicology studies were negative.

Control subjects. All 342 infants who were aged between 5 days and 12 months at the time of death at the hospital were identified from the original 534 infants in series 1. These were selected as control subjects for the purpose of statistical comparison with the age-matched infants who died suddenly at home. Newborns aged <5 days were excluded.

Processing of lung specimens and stains. Lung tissue specimens were sectioned (5 µm) and stained with Grocott-Gomori methenamine-silver nitrate and hematoxylin-eosin stains. Slides from series 1 were examined by investigators blind to the diagnosis of SIDS, and slides from series 2 and 3 were examined by investigators aware of the diagnosis of SIDS. Slides in cases from Oxford were examined by investigators blind to the diagnosis of SIDS who also analyzed slides for possibly immunocompromised or immunocompromised patients with unknown P. carinii status. Specimens were examined by 2 different investigators (S.L.V. and J.C.W., C.P., or P.M.) in all cases. Discordant results were discussed, and cases were labeled as positive only if typical P. carinii cysts in clusters of 3 or more organisms were seen by both investigators. A third investigator reviewed positive cases (W.H. for Chilean samples, and S.G. for Oxford samples). Positive slides were subsequently stained with monoclonal antibody 3F6 (Dako Diagnostics, Carpinteria, CA), which recognizes an 82-kDa protein present in the cyst wall that is not altered by formalin or paraffin; all positive cases were confirmed by both methods (figure 1).

Clinicopathologic correlation. To gain insight into the clinical history and to better describe the histopathologic pattern of primary P. carinii infection in these children, the criteria described by Price and Hughes [9] for children with malignancies were retrospectively applied to P. carinii-positive patients with SIDS. Briefly, this scale of lung involvement with clinical correlation considers 2 asymptomatic stages and 1 symptomatic stage of P. carinii infection. Asymptomatic stages were described as isolated cysts with no parenchymal reaction of the lung (stage 1) or desquamation of organisms into the alveolar lumen with an increasing number of P. carinii and minimal or no inflammatory response in alveolar septa (stage 2). The symptomatic stage was defined as a host response consisting of alveolar desquamation and lymphocytic and plasma cell alveolar infiltrates (stage 3). Stage 3 was found by these investigators to correlate with clinical symptoms and radiographic signs of P. carinii pneumonitis in children with different types of cancer.

Statistical analysis. To compare the incidence of P. carinii-positive and -negative lung samples among infants who died at home of SIDS with the incidence among those who died at the hospital of multiple conditions, we used Fisher's exact test (using Epi-Info version 6; Centers for Disease Control and Prevention, Atlanta, GA). Control subjects were compared with infants with SIDS from series 1 and also with infants from series 2 and 3 combined. P < .05 was considered statistically significant.

Results

Series 1. Of 534 lung tissue specimens from consecutive pediatric patients that were blind to investigators with respect to age and diagnosis, 16 (3%) were found to be positive for P. carinii clusters. Primary autopsy diagnoses for these children were as follows: bronchopneumonia, 5 children; SIDS, 4; bronchitis, 1; generalized lipidosis, 1; and no diagnosis available, 1. The following underlying diseases suggestive of an immune defect were present in 4 patients who also had bronchopneumonia as a secondary diagnosis: severe combined immunodeficiency syndrome, congenital medullary aplasia, mucocutaneous candidiasis, and fulminant hepatitis. Age distribution and primary autopsy diagnoses for these patients are shown in table 1. P. carinii was detected in 4 (25%) of 16 infants who were dead at arrival to the emergency department and had a postautopsy diagnosis of SIDS compared with 10 (2.9%) of 342 infants who were aged between 5 days and 1 year and died of multiple conditions at the hospital (P = .002, Fisher's exact test). On the basis of this observation, we elected to examine a larger number of infants with a postautopsy diagnosis of SIDS.

Series 2–4 and control subjects. We examined an additional 134 infants with a primary autopsy diagnosis of SIDS who were autopsied at different hospitals in Santiago (series 2 and 3) and 27 infants who died of SIDS and were autopsied at a hospital in Oxford (series 4) (table 2). Ages of these infants ranged from 20 to 575 days (mean, 95 days; median, 60 days). Ages of control subjects ranged from 5 to 365 days (mean, 88 days; median, 60 days); control subjects were matched according to the age criterion for the diagnosis of SIDS.

Ten (2.9%) of 342 controls had P. carinii clusters compared with 47 (35.1%) of 134 Chilean infants with an autopsy diagnosis of SIDS in series 2 and 3 (P = .000001, Fisher's exact test). Four (14.8%) of the 27 infants who died of SIDS in Oxford were found to have P. carinii clusters by histological analysis (table 2).

Lung reaction, extent of P. carinii infection, and retrospective correlation with clinical manifestations before death. Of 35 P. carinii-positive patients with SIDS, 13 were not evaluable because the specimens had extensive postmortem autolysis. The clinicopathologic correlation criteria developed by Price and Hughes [9] were applied to 42 evaluable cases. Twelve and 25
Figure 1. A. Pneumocystis carinii clusters in lung tissue specimen from a 2-month-old infant diagnosed with sudden infant death syndrome (SIDS) (Grocott-Gomori methenamine-silver nitrate stain; original mag, ×330; bar, 10 µm). B. Smaller magnification (×60) of A, illustrating that number of P. carinii clusters in patients with SIDS is few. C. Immunohistochemical analysis with monoclonal antibody 3F6 (Dako Diagnostics, Carpinteria, CA) of lung tissue specimen from a 4-month-old infant diagnosed with SIDS that reveals P. carinii cluster filling an alveolus (original mag, ×330; bar, 10 µm). Grocott-Gomori methenamine-silver nitrate stain is generally considered standard for detection of P. carinii in tissue specimens. It correlates well with immunohistochemical analysis with monoclonal antibody 3F6. P. carinii was detected by both techniques in all cases considered positive.

Discussion

This study provides histological evidence of mild infection by P. carinii in presumably normal immunocompetent infants, a finding in agreement with serological evidence that most normal children are exposed to P. carinii at an early age [1, 2]. Mild, naturally occurring P. carinii infection has previously been observed in other mammals shortly after weaning: rabbits [3, 4] and piglets [5]. The young age of the patients and the characteristically mild histological pattern encountered suggest that our findings correspond to primary infection rather than to reactivated or secondary infection which has been histologically well described for the immunocompromised host [7–12].

This study also suggests an association between primary P. carinii infection and SIDS (table 2). A small number of reports of cases of mild, focal P. carinii pneumonitis in infants with SIDS in Germany, the United States, and Chile in the 1950s [6, 10–12] provide further support of this association. In this study, P. carinii was also found in a relatively high proportion of patients with SIDS in Santiago and Oxford. Some innate flaws in the study must be considered. In series 2 and 3, slides were examined by investigators aware of the diagnosis of SIDS. To further assess the statistical significance found in series 1, the proportion of P. carinii-positive cases in series 2 and 3 was compared with that of P. carinii-positive control subjects (table 2); however, deaths in control subjects were not sudden, and
cases were not matched with respect to underlying health status, prior drug therapies, and awareness of the diagnosis by investigators examining slides. The samples from Oxford (series 4) demonstrated that these findings were not a local phenomenon restricted to Chile; and so have much wider implications.

Small numbers of P. carinii organisms found in infants with SIDS do not necessarily represent the onset of primary P. carinii infection; these organisms could correspond to a phase of P. carinii clearance that may take up to 1 year [13]. Therefore, a well-defined control group is needed in further studies to document the relevance of a potential pathogenic role of P. carinii in a proportion of SIDS cases.

A possible explanation for the pathogenic role of P. carinii in some infants with SIDS is that it reduces the level of pulmonary surfactant. This hypothesis is supported by evidence in both experimental models and studies of humans that showed that P. carinii infection leads to a decrease in the level of pulmonary surfactant [14-16]. Furthermore, a decreased surfactant level has been recently shown in an animal model to be directly related to P. carinii growth and occurs at early stages of P. carinii development [14]. Relevant to this study, infants with a postautopsy diagnosis of SIDS also have been consistently found to have decreased levels of surfactant [17-19]. In 1985, Talbert and Southall [20] first hypothesized that a decrease in surfactant level may be a mechanism that triggers sudden death in infants when it occurs at a critical stage of their lung development. Our documentation of P. carinii in a relatively high proportion of SIDS cases suggests that P. carinii might play a role, alone or as an accompanying pathogen, in the decreased level of pulmonary surfactant that is documented in SIDS cases. Whether other pathogens that possess activity against phospholipase A2 or other mechanisms could be implicated needs to be determined [18].

Most (37 [88.1%] of 42) infants with SIDS who were positive for P. carinii and were evaluated by the clinicopathologic correlate developed by Price and Hughes [9] were categorized in stage I or 2, thus providing postmortem evidence that primary infection was asymptomatic in most SIDS cases. In agreement with this evaluation, the load of P. carinii organisms in SIDS cases was mild, and clusters were difficult to find histologically.
Therefore, the terminal event of SIDS cannot be explained by these findings under the current understanding of *P. carinii* disease.

Alternatively, because *P. carinii* is largely a pathogen of the immunocompromised host, finding *P. carinii* more frequently in infants with SIDS might suggest that it marks the presence of an underlying immune defect in SIDS, just as *P. carinii* has served as a marker for HIV infection [21–25].

Previous reports indicate that *P. carinii* can present as pneumonia in immunocompetent infants aged <3 months [26] and also suggest that *P. carinii* pneumonia might be associated with apnea [27, 28]. In our study, 5 (7.3%) of 68 immunocompetent infants aged from 5 days to 1 year in series 1 had bronchopneumonia as a primary autopsy diagnosis. This proportion, which agrees with findings in other studies [26, 27, 29, 30], suggests that *P. carinii* infection should be included in the differential diagnosis of bronchopneumonia in presumably immunocompetent infants. Whether a clinically identifiable pattern is present in mild forms of primary infection occurring in infants who spontaneously recover is not known.

The data provide histological evidence of primary infection by *P. carinii* in apparently immunocompetent infants and children. They show that *P. carinii* infection is more common in infants aged <1 year who die in the hospital setting and that this infection can be asymptomatic. The high prevalence of *P. carinii* infection in infants with SIDS warrants further investigation.

References
