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Immunocytologic Characterization Using Monoclonal Antibodies of Lung Lavage Cell Phenotype in Infants Who Have Died from Sudden Infant Death Syndrome

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ABSTRACT. The phenotype of cells obtained from pulmonary lavage in infants who have died from sudden infant death syndrome were examined and compared with cells from control subjects. The only striking difference between the groups was a lack of reactivity of lavage cells with antibody of the CD14A cluster in the sudden infant death syndrome subjects, while antibodies of the CD14B cluster reacted strongly with cells from both the sudden infant death syndrome group and controls. Major histocompatibility complex class II antigens were expressed on approximately 95% of lavage cells, with DQ expressed more frequently than DR or DP. The majority of the lavage cells was macrophages, yet they reacted with CD3 (OKT3) and CD8 (OKT8) antibodies. No interleukin 2 was found in the lavage fluid. (*Pediatr Res* 23: 187–190, 1988)

Abbreviations

SIDS, sudden infant death syndrome
DAB, diaminobenzidine hydrochloride
IL-2, interleukin 2
HLA, human leukocyte antigen

The first report of SIDS in South Australia was in 1837, when it was considered to be due to overlying (1), as was also reported in the days of King Solomon (2). The current incidence of SIDS in South Australia is 1.83/1000 live births (3), similar to that reported in upstate New York [1.43/1000 live births (4)], but lower than that reported in Scotland [2.7/1000 live births (5)]. Epidemiologically, SIDS is more common in colder weather, and correlates well with an increase in respiratory tract infections, particularly admission rates to the hospital for bronchiolitis (3). A highly significant correlation was found between respiratory viral isolation in the general pediatric population and the incidence of SIDS (6). If SIDS were due simply to overwhelming respiratory viral disease, there ought to be readily discernible features of extensive pneumonitis in the lungs at autopsy. However, this is not the case (7); the pressure volume characteristics of the lung in SIDS as studied at autopsy is no different to age-matched controls (8). An unusual response to a relatively common viral pathogen is feasible epidemiologically. Concurrent respiratory viral infection may serve as a "trigger" mechanism in the infant at risk (9). It has been postulated that SIDS may be due to a modified anaphylaxis occurring in the lung, perhaps IgG mediated (10). If there is abnormal immune regulation within the lung, triggered perhaps by a concurrent respiratory viral infection, an alteration in the relative proportion of lung cell types might be seen. This would be reflected in the cell phenotypes defined by surface markers, or the release of inflammatory mediators such as interleukin.

Our aim herein was to harvest lung cells from children who have died from SIDS, and using monoclonal antibodies, assess the phenotype of the cells and compare them with cells from controls. In addition, lavage fluid was assayed for the presence of IL-2.

PATIENTS

All children suspected of dying from SIDS in South Australia have autopsies performed by direction of the State Coroner, at the Adelaide Children's Hospital. During 1986, specimens were collected from 16 of these autopsies (mean age 3.75 months, range 1–7). In addition, specimens were collected at autopsy from four controls (mean age 15.5 months, range 1–32) in the same manner. Specimens were collected from all autopsies 12 to 24 h after the presumed time of death. The control subjects were previously well children who had died from acute, nonpulmonary causes.

METHODS

Lung lavage. The lungs were removed intact at autopsy. An endotracheal tube was passed through the trachea and 500 ml of normal saline at 37° C introduced into the lung in 50-ml aliquots. The lungs were aspirated between each aliquot. The resultant lavage fluid was pooled and centrifuged at 400 × *g* for 10 min, the supernatant removed, and the cells harvested.

Cells. The cells were resuspended in RPMI 1640 enriched with 10% fetal calf serum and gently rocked at 37° C for 1 h. The cells were again harvested by centrifugation at 400 × *g* for 10 min, counted on a hemocytometer, and viability determined by trypan blue exclusion. Because the lavage was performed from the level of the distal trachea rather than a subsegmental bronchus, the resultant contamination of the lavage by mucus was removed by Ficoll-Hypaque separation. The cells were centrifuged at 700 × *g* for 15 min over a layer of Ficoll-Hypaque (specific gravity 1.077) and the cells concentrated at the interface between the Ficoll-Hypaque and tissue culture medium were collected. Cells were also collected from the cell pellet for CD15 (neutrophil) analysis. Cell types obtained from the lavage were

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identified on morphologic criteria with Geimsa stain and by the presence of acid phosphatase.

Immunostaining. These cells were centrifuged onto acid-alcohol cleaned glass slides in a cytocentrifuge, and air dried. All incubation steps were performed at room temperature. Endogenous peroxidase was blocked with 1.6% hydrogen peroxide in absolute methanol for 20 min. The slides were washed three times in tris/HCl buffer, 0.05 M, pH 7.6 between each step. Fc receptors were blocked with normal horse serum diluted 1/50 in normal saline for 20 min. The monoclonal antibodies were applied for 2–12 h, followed by the secondary antibody (horse anti-mouse IgG/biotin conjugate) for 30 minutes. Avidin-Biotin complex (Vector Laboratories) was applied for 1 h, and the complex developed with 0.05% DAB (Sigma Chemicals, St. Louis, MO) and 0.01% hydrogen peroxide for 10 min. Nuclei were counterstained with Mayers progressive hematoxylin for 30 sec.

After air drying, the immunostained cell preparations were mounted in methylacrylate slide mounting medium. Five hundred cells were counted for each preparation, and cells were scored as positive or negative according to their staining pattern on microscopy. Results were expressed as percent positive.

Monoclonal antibodies. The monoclonal antibodies used in the study are listed in Table 1. To ensure that the positive results obtained with OKT3 and OKT8 (IgG2a antibodies) was not mediated by Fc binding, an irrelevant antibody of the same isotype was also used (FMC 21), directed against an antigen of *Toxoplasma gondii.*

Virology. On all tracheal aspirates, immunofluorescence was performed using antisera to parainfluenzae 1 and 3, influenzae A and B, adenovirus, and respiratory syncytial virus (Wellcome Diagnostics). In addition, viral culture was performed using three cell lines (rhesus monkey kidney, Hep 2, and MRC 5).

IL-2 assay. Lung lavage supernatants were concentrated 10-fold by ultrafiltration using a filter with a 5000 nominal molecular weight cut off. The CTLL-2 mouse cell line (IL-2 dependent) was grown in McCoy's medium, suplemented with 2 mercaptoethanol, and IL-2 containing supernatant. This IL-2 dependent cell line dies within 24 h in the absence of IL-2 in the culture media. The CTLL-2 cells were washed three times with IL-2-free media to remove IL-2 before use in the assay. The cells were suspended at a concentration of 5×10^4 cells/ml, and 100 μl of this suspension was added to each well of a 96-well flat bottom microtiter plate (Linbro). One hundred μl of either test sample or IL-2 standard at doubling dilutions were added to each well. All dilutions were assayed in triplicate. A negative control consisting of 100 μl of media was also included, in triplicate, for each assay. Plates were cultured for 24 h in a humidified 37° C incubator in an atmosphere of 5% carbon dioxide. After 20 h incubation, 0.42 μCi of tritiated thymidine was added to each well, and the plates incubated for another 4 h. Plates were harvested using a Skatron cell harvester, filters were dried, scintillation fluid was added, and radioactivity was counted in a beta counter. Absolute amounts of IL-2 were calculated using a Probit analysis (24) with 1 U of IL-2 being defined as that dilution of test sample which produced 50% of maximum tritiated thymidine uptake by the CTLL-2 line.

Table 1. *Monoclonal antibody characteristics**

Antibody	Class	Specificity	Reference
X63	IgG1	Negative control	11
FMC 16	IgG2a	MHC† class I	12
FMC 17	IgG2b	Monocyte/macrophage CD14B	13
FMC 32	IgG1	Monocyte/macrophage CD14A	13
FMC 33	IgG1	Monocyte/macrophage CD14A	13
FMC 10	IgM	Granulocytes CD15	14
FMC 21	IgG2a	Toxoplasma antigens	15
FMC 4	IgG1	MHC class II DR, DP, and DQ	16
FMC 15	IgG2b	MHC class II DR	17
TU 22	IgG2	MHC class II DQ	18
B7/21	IgG1	MHC class II DP	19
OKT3	IgG2a	Pan T CD3	20
OKT4	IgG2b	T helper CD4	21
OKT8	IgG2a	T cytotoxic/suppressor CD8	22
OKT9	IgG1	Transferrin receptor CD45	23

* A rabbit polyclonal antiserum, SMIG, was used to detect B cells.
† Major histocompatibility complex.

RESULTS

Pulmonary tissue histology. No abnormalities of pulmonary tissue in any of the subjects were found on light microscopy.

Virology. Immunofluorescence and culture on all four control subjects was negative for the viruses tested. In the SIDS group, three of 16 subjects were positive; one was positive for parainfluenzae type 1, one was positive for both coxsackie B4 and cytomegalovirus, and one was positive for respiratory syncytial virus.

Cells. Mean cell numbers obtained from the lavage in SIDS subjects was 295×10^4 (range $100–520 \times 10^4$). This was not significantly different in the control subjects: 270×10^4 (range $210–345 \times 10^4$). The cell types were not significantly different between the two groups (Table 2). Cell viability based on trypan blue exclusion ranged from 20–80%. Although the membrane immunostaining was better preserved in cell populations with high viabilities, it was still very satisfactory in those with low viabilities. There was more cytoplasmic staining (as opposed to membrane staining) in the lower viability cells, but results were the same as in the better preserved cells.

Monoclonal antibodies. Cell preparations incubated with the negative control X63, were uniformly unstained, while the positive control FMC 16 gave positive staining on all cells (Table 3). Preparations incubated with monoclonal antibodies of the monocyte-macrophage lineage gave variable staining patterns. FMC 17 (CD14B) gave strongly positive staining in both the SIDS group and the controls. No infant from the SIDS group had expression of the antigen recognized by FMC 33 (CD14A), but all control infants had some degree of this antigen expression. For FMC 32 (CD14A), again all control infants had some degree of antigen expression, but only two SIDS subjects were positive, and then at very low levels (1 and 3% of cells were positive). This difference in FMC 32 and 33 expression between the SIDS group and controls was statistically significant ($p < 0.001$).

Class II antigens were expressed in 90% of the cells. FMC 15 (DR) antigen was expressed slightly less often than FMC 4 (DR, DP, and DQ), as was B7/21 (DP) compared with Tu22 (DQ) (Table 3). This difference was statistically significant ($p < 0.001$).

Many of the cells gave positive staining when incubated with OKT3 and OKT8 antibodies, but were less frequently positive with OKT4. All subjects in both groups had some degree of OKT3 antigen expression. Only two subjects in the SIDS group had OKT4 antigen expression, while all expressed the OKT8 antigen, generally on a similar percentage of cells expressing OKT3 antigen. In the control subjects, the pattern was similar;

Table 2. *Lavage cell population characteristics (mean and range)*

	SIDS ($n = 16$)	Controls ($n = 4$)
Cell no. ($\times 10^4$)	295 (100–520)	270 (210–345)
Viability (%)	46 (20–82)	59 (48–76)
Macrophages (%)	78.9 (60–93)	86.6 (85–88)
Bronchial epithelial cells (%)	17.2 (4–36)	10.6 (8–14)
Lymphocytes (%)	2.4 (0–7)	2 (1–3)
Neutrophils (%)	1.5 (0–15)	0.7 (0–2)

Table 3. *Cellular antigen expression (mean and range)*

Antibody	SIDS ($n = 16$)	Controls ($n = 4$)
X63	0 (0)	0 (0)
FMC 16	100 (100)	100 (100)
FMC 17	83.6 (44–95)	87.7 (86–90)
FMC 32	0.3 (0–3)	9.2 (6–14)
FMC 33	0 (0)	25 (10–55)
FMC 4	93 (71–99)	95 (90–96)
FMC 15	82 (58–92)	84 (65–95)
TU 22	97 (95–100)	99 (98–100)
B7/21	85 (81–93)	87 (84–92)
OKT3	73.3 (25–98)	63.7 (30–83)
OKT4	6.8 (0–90)	10 (0–20)
OKT8	54 (5–95)	55 (0–80)
OKT9	78.2 (58–95)	71.5 (55–85)
SMIG	0.4 (0–2)	0.7 (0–2)
FMC 10	3 (0–16)	2 (1–5)

there was no statistical difference in expression of the antigens recognized by OKT3, T4, or T8 antibodies between the two groups. Morphologically and histochemically by the presence of acid phosphatase, the cells positive with OKT3 and T8 antibodies were identified as macrophages. Approximately 75% of cells were positive with OKT9 (CD45) antibody.

The antigens recognized by FMC 10 (CD15) and SMIG were very seldom expressed, the one exception being a SIDS subject who had 15% of lavage cells positive for the FMC 10 antibody. This patient had both cytomegalovirus and coxsackie B4 isolated from the lavage fluid.

IL-2. No evidence of IL-2 was found in concentrated specimens from both SIDS subjects and controls.

DISCUSSION

Clearly the comparisons between the SIDS subjects and controls are limited by the small number of controls (4). This study has, however, given useful insight into lung cell phenotypes in the child, and in addition revealed major differences in cell phenotype in SIDS cases.

Studies in the adult (25) using monoclonal antibodies of the monocyte/macrophage lineage, found FMC 17, FMC 32, and FMC 33 antigens positive on alveolar macrophages. We have found a different pattern of reactivity in the SIDS group, with FMC 32 and 33 negative. However, these antigens were expressed in the controls. This statistically significant difference ($p < 0.001$) in expression between the SIDS group and controls is of considerable interest. The function of the antigen recognized by FMC 32 and 33 is unknown. FMC 32 and 33 belong to CD group CD14A, while FMC 17 belongs to CD group CD14B (26). These closely related antibodies are against 2 molecules coded for by the same gene, of mol. wt. 50,000 to 55,000 (26). The nature of the difference between the two clusters is not understood. However, it is clear that CD14A and B are not always coexpressed, both from the workshop cluster analysis (26) and from individual studies (25). The CD14A and B antibodies may be recognizing different epitopes on the same molecule (26).

Class II antigen expression in normal adult pulmonary macrophages has been reported to vary between 21% (27) and 90% (28). We have found 94% FMC 4 (class II, DR, DP, and DQ)-positive cells in the lavage. As DP (B7/21) and DR (FMC 15) antigens were expressed on approximately 80% of cells, it is the DQ component of the FMC 4 antigen that is being expressed most frequently. This is confirmed by finding 97% expression of TU22 (DQ) antigen on the cells. The difference in expression between TU22 and both B7/21 and FMC 15 is highly significant ($p < 0.001$). Gonwa and Stobo (29) have reported that all human peripheral blood monocytes express DR, but only 50% express DQ. Navarrete et al. (30) have reported that blood monocytes express DR throughout all stages of differentiation, whereas only a subset of mature monocytes have DQ antigens. More recently Gonwa *et al.* (32) have demonstarted that freshly isolated blood monocytes had 91% DR expression, 32% DQ expression, and 15% DP expression. However, incubation with interferon-γ for 96 h induced the expression of all three antigens on all monocytes, suggesting that interferon-γ regulates class II antigen expressionon the peripheral blood monocyte. On blood monocytes in the human, Gonwa *et al.* (32) found that antigen presentation correlated with the expression of HLA-DQ. Alveolar macrophages are thought to express approximately five times more class II antigens on their surface than blood monocytes (33). It would be of interest to know if interferon-γ is involved in local maturation of monocytes into class II-positive alveolar macrophages. Our findings in the pediatric population are similar therefore to previous work in adults.

Monoclonal antibodies of the OKT3, T4, and T8 series are often regarded as recognizing antigens restricted to lymphocytes. However, we have found that many lavage cells express these antigens, and they are morphologically and histochemically typical of alveolar macrophages. In particular, the most common pattern of expression is for 55–75% of the cells to express OKT3 and OKT8 antigens, indicating cytotoxic/suppressor phenotype when present on lymphocytes. To ensure that expression of OKT3 and OKT8 antigens was not due to Fc binding, we also used an irrelevant monoclonal antibody FMC 21 (toxoplasma antigen) which is of the same isotype (IgG2a); this was uniformly negative. While these results demonstrate unequivocally that pulmonary macrophages react specifically with OKT3 and OKT8, we have not demonstrated that the molecules responsible are the same as those on T cells, or that the macrophages synthesize the molecules (as opposed to absorbing them). The observation that cells of the monocyte/macrophage lineage can express "T lymphocyte" antigens has also been noted by Bradley and Skinner (personal communication), and by Pilkington *et al.* (34), who found that a human macrophage cell line expressed OKT3 and OKT4 antigens. He also found that OKT4 stained subcapsular and medullary macrophages and dendritic cells of lymph nodes. Other workers (35, 36) have also shown the presence of OKT4 antigen on human peripheral blood monocytes and macrophage cell lines. Stewart *et al.* (37) have in addition demonstrated that the T4 antigen on human lymphocytes and monocyte/macrophage cell lines are structurally identical. However, our findings are somewhat different in that we have found pulmonary alveolar macrophages to be expressing predominantly the cytotoxic/suppressor (T8) phenotype and only rarely the helper (T4) phenotype.

The significance of OKT8 (CD8) antibody expression by pulmonary macrophages is unknown. Holt (38) has argued that alveolar macrophages in the normal lung down-regulate immune processes through T cell suppression, thus avoiding potential lung responses to irrelevant air-borne antigens. In a study of alveolar macrophages from asthmatics, Aubas *et al.* (39) found decreased suppressor cell activity of these cells, which could result in increased immunogenicity of inhaled antigens and subsequent stimulation of the inflammatory cascade by release of mediators giving rise to clinical symptoms. As mentioned above, the alveolar macrophage is strongly positive for class II antigens, yet these cells are poor antigen-presenting cells in T cell proliferative assays, perhaps [as reasoned by Lyons *et al.* (40)] because of poor macrophage T cell binding. Although alveolar macrophages have high DQ expression, suggesting they should function well as antigen-presenting cells, Lipscomb *et al.* (41) found that despite high class II antigen expression on alveolar macrophages of both DP and DQ, these cells functioned poorly as antigen-presenting and argue that their main role is in phagocytosis and restimulation of already activated T cells. If alveolar macrophage suppression is important in maintaining avoidance of immune-mediated pulmonary damage, it is of considerable interest that we have demonstrated virtual universal expression of the antigen recognized by the OKT8 antigen on the surface of

the alveolar macrophage. Perhaps the poor antigen-presenting function and suppression of alveolar macrophages is related in some way to their OKT8 antigen expression.

If the alveolar macrophages were initiating the immune cascade as antigen-presenting cells, we might expect measurable levels of IL-2. The fact that IL-2 was undetectable, suggests that in this pediatric population there is little immune amplification occurring.

Blood monocytes are transferrin receptor antigen (OKT9) negative, although the receptor has previously been reported to be present on approximately 86% of adult alveolar macrophages (42). Our finding of 75% of cells expressing the transferrin receptor in this pediatric population is therefore very similar to the adult. As iron is needed for cells to function and proliferate, transferrin receptors are generally present on cells capable of proliferation and suggests that these cells are capable of a proliferative response.

There were few FMC 10 (granulocyte)-positive cells or SMIG (B cells) present in bronchoalveolar lavage. The one exception was one of the SIDS subjects who had 10% of lavage cells as granulocytes. This subject also had two viruses cultured from the lavage-coxsackie B4 and cytomegalovirus. It is possible that this infant was not a "classical" SIDS death, but died from respiratory tract viral infection. However, there were no symptoms of respiratory tract infection before death, and lung histology was normal.

In summary, we have described the phenotype of lung lavage cells from children in the first 2 yr of life. The fact that antigens of the CD14A group are expressed less frequently in the SIDS group requires further investigation. Macrophages frequently express cytotoxic/suppressor "lymphocyte" antigens; the functional significance of this finding is unclear, and again further studies would be of value. There is high class II expression, in particular DQ. No IL-2 was found in the lavage fluid. There is no convincing evidence that in SIDS there is abnormal immune regulation within the lung.

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