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Bound Immunoglobulin and Foreign Antigen in Lungs of Sudden Infant Death Syndrome Victims

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Lung sections from 33 infants who died suddenly and unexpectedly and were diagnosed by medical examiners as sudden infant death syndrome (SIDS) gave evidence of bound immunoglobulin G (IgG) when examined by direct fluorescent antibody technique. Ten tissues from appropriate control infants were negative. Specimens containing IgG exhibited no IgA or IgE, but three contained IgM. Sixty-one percent of lung sections with IgG contained either K or λ antigens; the remainder contained both. The indirect fluorescent antibody technique gave similar results. Blood sera of some individuals in the study which were tested all contained both K and λ antigens. Fluorescent-labeled immunoglobulin from one SIDS victim stained 7 of 17 SIDS lung sections tested, including his own. Labeled immunoglobulin from three mothers of SIDS victims exhibited differential selectivity in reaction with antigen in lungs of a group of 18 SIDS infants. They did not react with 10 control infant tissues. Various labeled adult sera, cord sera, and serum from an apneic child did not react with the various lungs of SIDS victims in the study.

Recently we reported that lung sections from 22 sudden infant death syndrome (SIDS) victims gave evidence of bound immunoglobulin G (IgG) when examined by the direct fluorescent antibody technique (FAT). IgG was not found in postmortem tissue from traumatic deaths (6). This report confirms and extends previous findings. Additional, recent SIDS and control cases from different medical examiners in various geographic areas are included. Lungs with bound IgG are examined for K- and λ-chain antigens. The immunoglobulin content and presence of K and λ antigens in blood sera are reported for SIDS victims, their mothers, normal infants, and parents.

Bound IgG in a lung of a SIDS victim implies the presence of foreign antigen there and antibody to the antigen in the infant's blood serum. Since immunoglobulin G (IgG) is in lungs of very young victims (under 4 weeks) and IgM is absent, sera from these infants likely contain maternal antibody to the SIDS antigen in their lung. If this is so, serum taken from a mother, close upon the death of a young SIDS victim, should contain antibody to the infant lung antigen. Therefore, serum from the victim and the mother should react in vitro with lung tissue of the infant, as well as that of other victims with immunologically related antigen. The study of such interactions could approach an understanding of the diversity of foreign antigens in various SIDS lungs.

This rationale prompted the present investigation in which the reactivities of fluorescent-labeled immunoglobulins from selected human sera were determined with lung sections of SIDS victims and control cases.

MATERIALS AND METHODS

Lung specimens. Human lung specimens were from 33 infants who died suddenly and unexpectedly and whose cause of death was diagnosed by medical examiners as SIDS (Table 1). Specimens were received from Toledo, Ohio (Alfred Golden) and the following Michigan counties: Lapeer (Leon R. Boruch), Jackson (J. H. Ahronheim), Washtenaw (R. Hendrix) and Wayne (C. Raven).

Controls. Control lung tissues were from 10 infants who had died of various traumatic injuries or accidents as indicated in Table 2.

Tissue sections. Tissue sections were cut (3 μm) from Formalin-fixed, paraffin-embedded or buffered Formalin-fixed, paraplast-embedded lung tissue and used for histopathology and fluorescent antibody studies. This type of material and procedure has been used effectively in demonstration by immunofluorescence of both immunoglobulins and viral antigens in association with tissues (7-9).

Processing and cutting of tissues were done at St. Vincent's Hospital (Toledo, Ohio), Wayne County's Medical Examiner's facilities (Detroit, Mich.) and by J. H. Ahronheim (Jackson, Mich.).

Paraffin tissue sections for fluorescent antibody staining were processed before staining by first dipping the slides containing the paraffin tissue sections in xylene (analytical grade) and then placing them in the following reagents for the prescribed time and sequence: xylene (5 min), absolute ethyl alcohol (5 min), 95% ethyl alcohol (5 min), and 70% ethyl alcohol (5 min). After the 70% ethyl alcohol treatment, the slides were washed in two changes of phosphate-buffered saline (pH 7.8) for 10 min each.

Sera. Human sera were obtained from blood of four mothers who had recently experienced sudden infant deaths and one mother of an apneic child. Serum from the apneic child, cord sera from three apparently normal infants, and postmortem serum from one SIDS victim were also collected.

Anti-human immunoglobulins. Anti-human immunoglobulins were obtained from various commercial sources. Unlabeled sheep anti-human IgG-specific antiserum (nonreactive with IgM and IgA by gel diffusion or by direct fluorescent test on monoclonal bone marrow) was obtained from Burroughs Wellcome Co., Research Triangle Park, N.C. Unlabeled goat anti-human IgG specific for γ, or $F(ab)_2$ antigens and horse anti-human λ or K light-chain antigens (specific for IgG bound and free antigens) were from Hyland Laboratories Inc., Costa Mesa, Calif. Unlabeled goat anti-human IgM, IgA, and IgE were purchased from Antibodies Inc., Davis, Calif. Unlabeled goat anti-human Ig (IgM, IgA, and IgG) was obtained from Cappel Laboratories, Inc. Cochranville, Pa.

Sources of fluorescein isothiocynate (FITC) prelabeled sera were as follows: sheep anti-human IgG and rabbit anti-horse (Burroughs Wellcome Co.) and IgG fraction of rabbit anti-goat (Cappel Laboratories, Inc.).

Labeling of specific antisera and human sera was done with the IgG fractions of the sera obtained by ammonium sulfate fractionation (3). A portion of each serum was retained unlabeled for later use in a blocking test of the specificity of the direct fluorescent antibody reaction and also for indirect fluorescent antibody tests. The number of precipitations and the concentration of ammonium sulfate were dependent on the animal serum (sheep, goat, or human). After fractionation, sera were labeled with FITC using the dialysis method (3). Unattached FITC was removed by dialysis, and the fluorescein-protein (F/P) molar ratio of each was determined (5). F/P ratios of sera from Antibodies Inc., and Burroughs-Wellcome Co. were 2.0:3.0, while those from Hyland Laboratories Inc. were 1.5:2.25. Antibody concentrations of conjugated antisera were determined by a standard gel diffusion precipitation test. Immunoglobulins in human sera were determined by radial immunodiffusion plates (Hyland Laboratories Inc.). Conjugates were used at a dilution showing definite precipitation. The specificity of each serum was checked by immunoelectrophoresis with purified IgG, IgA, and IgM purchased from Cappel Laboratories Inc. and against pooled human serum. Each conjugate was then tested against positive and negative control tissue for its staining qualities.

Fluorescent staining of lung sections. (i) Direct staining. Direct staining was accomplished by allowing FITC-labeled antiserum to react with deparaffinized tissue sections for 45 min in a moist chamber at room temperature, washing in phosphate-buffered saline (pH 7.6) for 10 min, and mounting under no. 0 cover slips with buffered glycerine, pH 7.6.

(ii) Blocking reactions. Blocking reactions were carried out by the one-step method to test for the specificity of the direct staining. Labeled antiserum was mixed with aliquots of the same serum which had not been labeled and applied to specimens as described for the direct staining procedure. Direct staining was carried out at the same time by using serial sections from the same block of tissue.

(iii) Indirect staining. Indirect staining was used on serial sections of some specimens in order to verify the results obtained by the direct staining procedure. Serial tissue sections were allowed to react with appropriate dilutions of unlabeled specific immune serum or serum from an unvaccinated animal of the same species. After 45 min of incubation, the tissue sections were rinsed in phosphate-buffered saline and washed in phosphate-buffered saline for 10 min. Next they were incubated for 45 min with the appropriate species-specific FITC-labeled antiserum. After washing in phosphate-buffered saline for 10 min, the slices were mounted under cover slips as described above.

Examination of specimens was made with a Zeiss fluorescent microscope using a BG-12 exciter filter and OG-4 and BG-4 barrier filters. Pictures were taken with a KP-490 exciter filter and either Polaroid ASA 3000-speed film or Ektachrome 400.

Judging criteria. Criteria for judging adequacy of fluorescent staining areas were that the tissue be flat within the observed field, not folded or wrinkled, the fluorescence be within the tissue, not at the edge of the tissue, and all positive fluorescing tissues show a 3 to 4 reactivity.

RESULTS AND DISCUSSION

Infant lung sections stained with labeled specific anti-human immunoglobulins using the direct immunofluorescent method. Thirty-three lung specimens from SIDS victims were negative when stained with fluorescent-labeled (goat) anti-human IgA or IgE. Three were positive with (goat) anti-human IgM and 33 were positive with sheep anti-human IgG. With the direct fluorescent antibody method and specific sera (sheep) directed to human K or λ light-chain antigens, 8 specimens displayed only λ antigens, 10 only K, and 12 contained both K and λ (Table 1). Fluorescent-positive fields were observed with various frequency, depending on the specimen. However, in all cases a single randomly selected tissue section was sufficient to demonstrate bound IgG. Differences in the character of the staining with the antisera against IgG (γ specific), K and λ antigens were not discernible. Generally, areas of fluorescence were in the basement membrane of bronchi, bronchioles, and alveolar ducts and in intact or desquamated bronchial and alveolar epithelium,

alveolar septa, alveolar capillary endothelium, and interstitium.

Lung specimens from 10 infants of age and sex similar to SIDS victims who died of various known injuries (chiefly mechanical) were used as control specimens (Table 2). These did not show evidence of bound IgG when tested under the same conditions as the SIDS victims using the direct immunofluorescent methods with specific fluorescein-labeled sheep anti-human IgG.

Blocking of direct fluorescent staining with unlabeled specific antisera. To verify the specificity of direct immunofluorescent staining, the staining of some specimens was blocked by use of unlabeled antiserum. This is illustrated in the micrographs of Fig. 1. The tissue is from case 76-211, a 7-month-old white female brought to the hospital and maintained in a Bird respirator until shortly before autopsy. Initially the oxygen concentration was 40%, then it was lowered to 30%, and then to 26%. The oxygen concentration stayed at 26%, was raised to 60% on one occasion, promptly dropped to 50%, and then again was raised to 60% when the brain scan became flat and oxygen was discontinued. Tissue preservation was good, as indicated by hematoxilin-eosin-stained sections. The diagnosis was SIDS and acute interstitial pneumononitis. One section was stained with fluorescein-labeled (goat) γ-specific anti-human IgG (dilution 1:4) plus the same unlabeled immune serum (1:2) (Fig. 1B). Each photograph was taken with a 30-s exposure. Staining by the fluorescein-labeled immunoglobulin was blocked entirely by the unlabeled serum. Similar staining results were obtained with $F(ab)_2$-specific anti-human IgG (goat) sera which were inhibited by that unlabeled antibody (the whole molecule).

Detection of K and λ antigens using the indirect immunofluorescent technique.

TABLE 1. *Immunofluorescence of sections of SIDS lungs stained with specific labeled anti-human immunoglobulins*[a]

SIDS tissue	Age (months)	Sex/ race	Fluorescent-labeled antiserum			
			IgG	IgM	K	λ
77-184	0.75	M/W	+	−	ND	ND
76-29	0.75	M/W	+	−	+	+
62-713	1	F/W	+	−	−	+
61-125	1	F/W	+	−	−	+
A77-150	1	M/W	+	ND	−	+
78-4718	1.4	M/W	+	+	+	+
A77-136	1.5	F/W	+	ND	+	+
A77-143	1.5	M/W	+	ND	+	+
62-458	2	M/B	+	−	+	−
62-375	2	M/B	+	−	+	+
77-148	2	M/W	+	−	+	−
AA-2-77[b]	2	F/NA	+	−	+	+
LP-3-77[b]	NA	NA	+	−	+	+
LP-4-77[b]	2	F/NA	+	+	+	−
76-38	2.25	NA	+	−	+	+
62-439	3	M/W	+	−	+	+
62-353	3	M/W	+	−	+	−
A77-126	3	F/W	+	ND	−	+
62-679	4	F/W	+	−	+	−
62-832B	4	F/B	+	−	−	+
A77-144	4	N/W	+	ND	+	−
A78-1	4	M/W	+	ND	+	−
A77-50	6	M/W	+	ND	−	−
62-531	7	M/B	+	−	+	+
76-211	7	F/W	+	−	+	+
62-809B	8	F/B	+	−	+	−
A78-20	8	F/W	+	ND	−	+
78-470-70	8.5	M/W	+	ND	−	−
62-755	9	F/W	+	−	−	+
62-916	9	NA	+	−	+	+
76-17	NA	NA	+	−	+	−
A77-54	9	M/W	ND	ND	−	+
AA-1-77[b]	NA	F/B	+	+	+	−

[a] ND, Not done; NA, not available.

[b] These tissues were snap-frozen and then fixed and embedded in paraffin.

TABLE 2. *Reaction of control infant lung sections to fluorescein-labeled anti-human IgG and human sera*

Specimen	Age months	Sex/race	Clinical history	Reaction to fluorescent-labeled serum			
				IgG[a]	30[b]	11[b]	10[b]
62-477	3	F/B	Bathtub burns, 90%	—[d]	ND[c]	—	—
62-333	3	M/W	Inhaled a marble	—	ND	ND	ND
65-168	3	M/W	Asphyxia, plastic bag	—	—	—	—
65-643	4.5	M/W	Multiple bruises	—	—	—	—
62-376	5	M/W	Fell from crib	—	ND	ND	ND
LA-7-78	5	M/B	Hydrocephalus	—	—	—	ND
62-405	5	M/W	Asphyxia by pillow	—	—	—	—
65-1183	9	F/B	Asphyxia by toy balloon	—	—	—	—
65-1511	9	M/B	Possible skull fracture	—	—	—	—
65-208	10	F/W	Child abuse	—	—	—	—

[a] Sheep anti-human IgG.

[b] Labeled immunoglobulin from mothers of three SIDS victims.

[c] ND, Not done.

[d] —, Negative reaction.

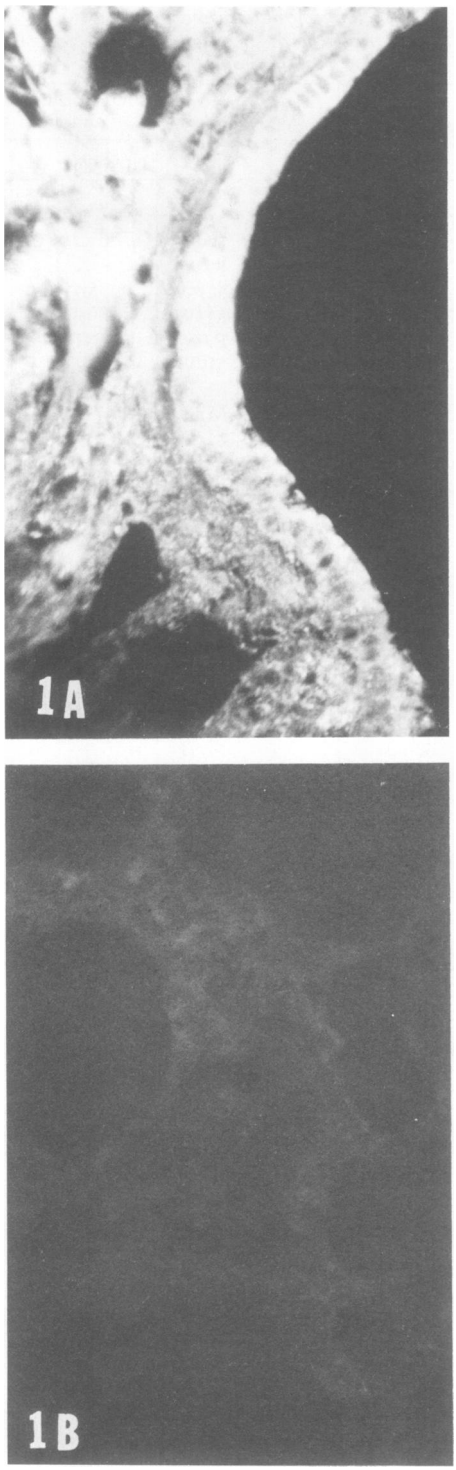


FIG. 1. *(A) Direct immunofluorescent staining of specimen 76-211 with FITC conjugated goat γ-specific anti-human IgG showing fluorescence localized in the epithelial lining and wall of a bronchus, as well as in the surrounding interstitium. (B) Specimen 76-211 stained as in (A), but blocked with unlabeled specific serum showing no fluorescence in alveoli nor interstitium. ×250; exposure 30 s for both figures.*

Specimen 76-211 could be stained with either horse-specific anti-human K or λ serum by using the direct immunofluorescent technique (Table 1). Those reactions were further verified by use of the indirect method. Four serial tissue sections were allowed to react with either saline, horse serum anti-λ-specific or anti-K-specific serum and then stained with FITC-labeled rabbit anti-horse serum. In Fig. 2A and B, staining is apparent in bronchus and alveolus when λ or K serum was used, respectively, but no staining occurred with the controls. Similar results were obtained with the indirect method using unlabeled goat anti-human IgG [λ or $F(ab)_2$ specific] with labeled rabbit anti-goat serum.

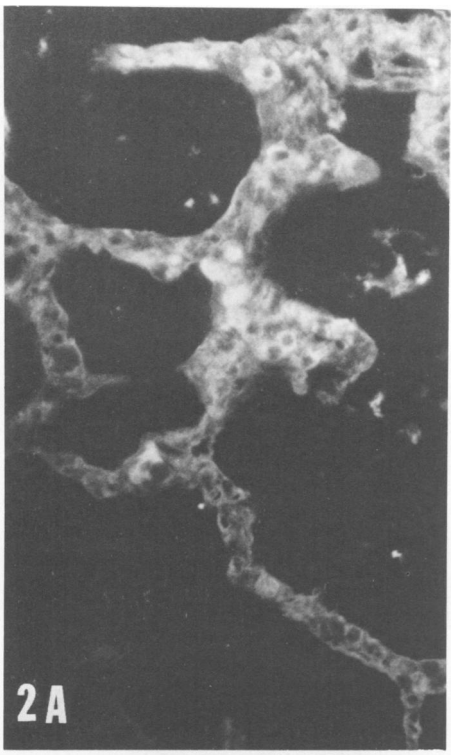
Immunoglobulin content of selected human sera. Because of the structural specificity of immunoglobulin found bound in SIDS lungs, immunoglobulins from blood sera of selected individuals were examined. Included were sera from one SIDS victim, an apneic child (regarded by some as a high-risk infant), two mothers of recently deceased infants, cord sera of three apparently normal newborns, and postmortem sera from a control infant case of hydrocephalus. All sera contained a mixture of K- and λ-chain antigens, and the contents of IgG, IgM, and IgA were in normal ranges (Table 3).

Staining of SIDS lung sections with FITC-labeled human sera by using the direct immunofluorescent technique. The immunoglobulin fractions of the following blood sera were FITC labeled: Four mothers (numbers 10, 11, 29, and 30) and two fathers of SIDS victims, one SIDS victim (postmortem serum), the mother of an apneic child and the child itself, cord sera of three apparently healthy newborns, and two unmarried adult women.

The labeled serum from the SIDS victim reacted in vitro with lung sections from the victim. The serum also stained lung sections from several other victims. Labeled sera from four mothers reacted with various SIDS lung sections (Table 4). However, all other sera failed to react with the exception of a reaction of one lung section with the serum of the mother of the apneic child.

Labeled sera from three of the mothers (numbers 10, 11, and 30) failed to react with any of the control tissues listed in Table 2 by using the direct method.

Some combinations of tissue and sera were not done because of limited material.



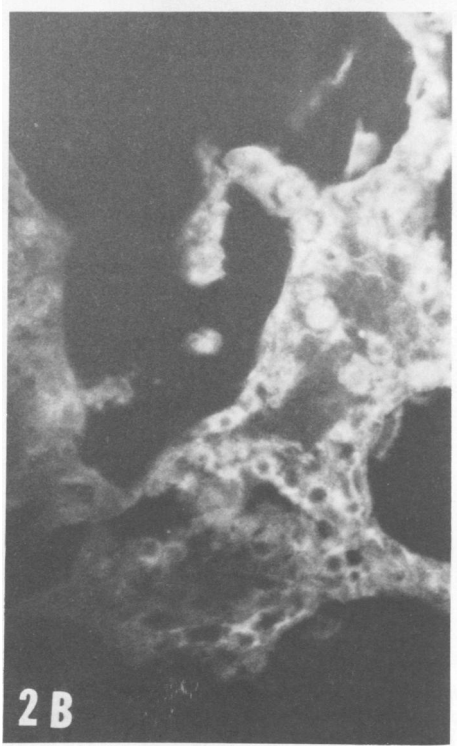


FIG. 2. *Indirect immunofluorescent staining of specimen 76-211 with horse-specific anti-human λ (A) and anti-human K (B) sera. Fluorescent staining is in alveolar cells, duct epithelium, and interstitium. ×250.*

TABLE 3. *Immunoglobulin content of selected human sera*

Human serum[a] specimen	Anti-human serum component				
	λ	K	IgG[b]	IgM[b]	IgA[b]
11	+	+	1,250	57	115
30	+	+	980	28	135
2	+	+	310	88	32.5
13	+	+	1,350	4	
14	+	+	1,440	11	
15	+	+	1,060	4.2	
19	+	+	31	5.6	29
20	+	+	830	1.5	145
21	+	+	23		

[a] Specimens 11 and 30 are from mothers of SIDS victims; 2 is a postmortem serum of an SIDS victim; 13, 14, and 15 are individual cord sera of newborns; 19 is a postmortem serum of a hydrocephalic infant; 20 and 21 are from an apneic child and his mother, respectively.

[b] Immunoglobulins were determined by radial immunodiffusion, expressed in mg/dliter.

TABLE 4. *Immunofluorescence of sections of SIDS lungs stained with various fluorescein-labeled human immunoglobulins*

Lung tissue[a]	Human serum[b]						
	11	29	30	2	21	20	13
AA-1-77	+	−	+	+	−	−	−
AA-2-77	+	ND	+	+	−	−	−
L-3-77	+	ND	+	+	−	−	−
L-4-77	+	ND	+	+	+	−	−
76-211	−	−	+	+	−	−	ND
76-38	+	−	+	−	ND	ND	−
76-17	+	−	+	−	−	−	−
76-29	+	+	+	−	ND	ND	−
A78-1	+	+	+	ND	ND	ND	ND
A77-136	−	+	−	+			
A77-50	−	+	−	−			
A77-143	+	+	+	ND			
A78-20	−	+	+	ND			
76-126	+	+	−	+			
A77-144	−	+	+	+			
A77-54	+	+	−	−			
A77-150	+	ND	−	ND			
78-470-70	+	−	−	−			

[a] Tissue specimens are SIDS cases from Table 1.

[b] Sera 11, 29, and 30 are from mothers of SIDS victims; 2 is a postmortem serum from an SIDS victim; 21 and 20 are from a mother and her apneic child, respectively; 13 is cord serum from an apparently normal newborn. ND, Not determined.

Certain SIDS lungs reacted with all three sera, while others reacted specifically with only one serum (Table 4). At least in these cases selectivity does not result from differences in the sec-

tions cut from the same paraffin block. Single sections were reacted with sera sequentially, initially using those that failed to stain. For example, a single section that failed to stain with sera 11 and 30 did react with serum 2.

Each serum, including that of the victim (if maternal antibody is present), likely contains multiple antibodies, yet these complex sera are each distinctive in reaction to particular specimens. Likewise, there is a diversity in the reactive foreign antigen displayed in various SIDS lungs because some react differently with a single serum (Table 4).

The character of the staining with labeled human sera is similar to that obtained with the anti-human immunoglobulins. Fluorescence was in the basement membrane of the bronchi, bronchioles, desquamated bronchial epithelium, and alveolar septa. This is illustrated in Fig. 3. The lung sections are from a white male victim, age

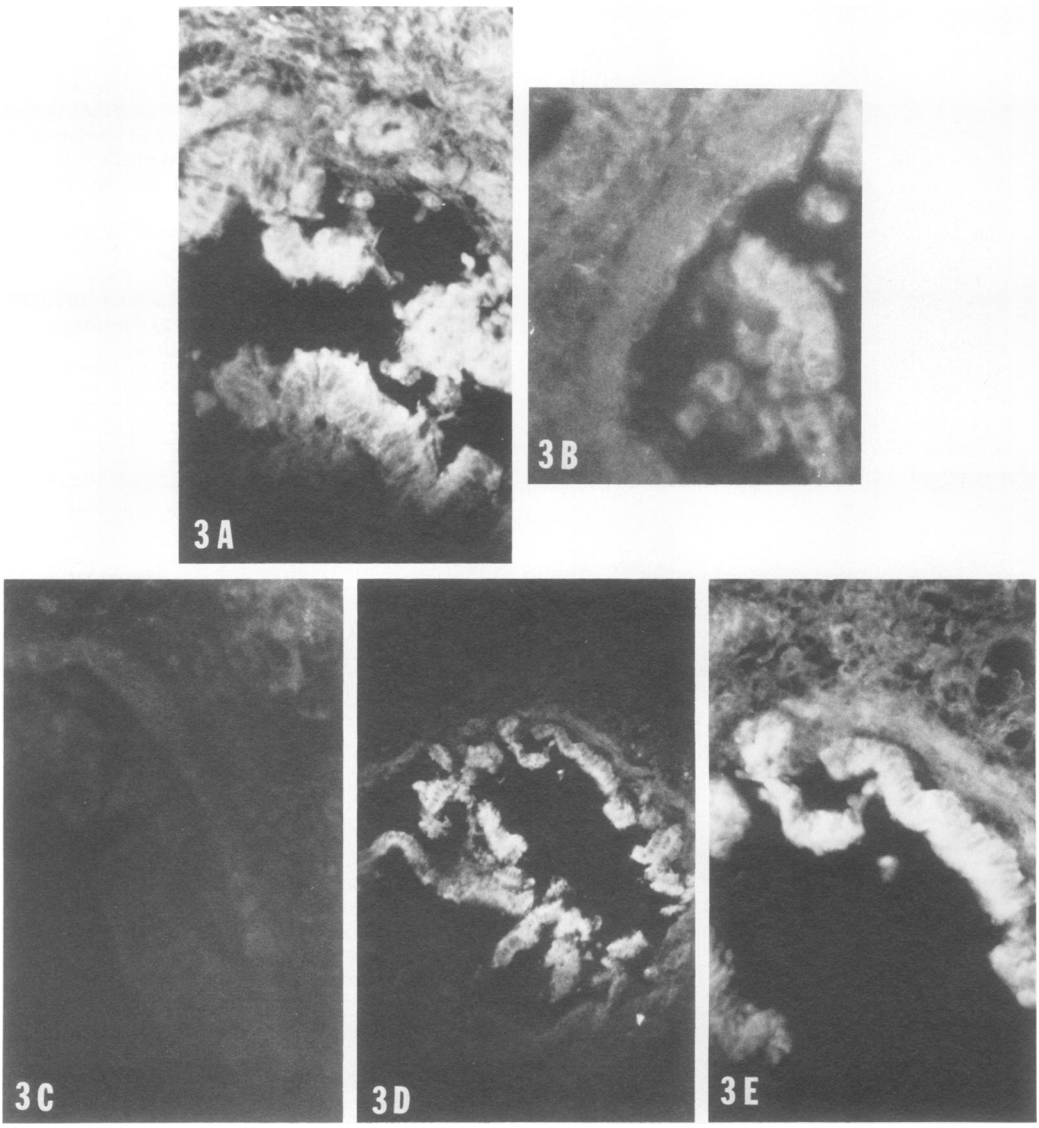


FIG. 3. *Direct immunofluorescent staining of specimen 78-4718: (A) a desquamated bronchiolar epithelium stained with labeled serum from SIDS mother 11; (B) basement membrane and desquamated bronchiolar epithelium stained with serum 10 from mother of case 78-4718; (C) no staining of lung with labeled serum of SIDS mother 29; (D) staining of desquamated bronchiolar epithelium and basement membrane with FITC-labeled goat-specific anti-human IgM. ×100. (E) Same as (D) but at ×250.*

51 days, birth weight, 4 lbs 2 oz (ca. 1.87 kg), who had previously been on a monitor for 2 weeks, case 78-4718. Sections were stained by the direct fluorescent antibody method by using serum 10 (his mother) and serum 11 (another SIDS mother), but not serum 29 (also a SIDS mother). This specimen was one of three cases that contained IgM as well as IgG. It was positive for both K and λ light-chain antigens.

Blocking of staining by labeling human serum using unlabeled serum. Because all of the SIDS lung sections contain bound IgG, the use of the indirect method was not feasible to study their reactivity with human sera. However, the specificity of the direct method is indicated first by the fact that the immunoglobulin from many human sera when conjugated with FITC (to the same dye content) failed to react. Further positive staining could be blocked by combining labeled serum with an aliquot of the same unlabeled serum (one-step blocking method). This is illustrated in Table 5 with two mothers' sera. Staining with serum 30 is completely blocked by unlabeled serum 30. Conjugated serum 11 is partially blocked by unlabeled serum 11, completely blocked by serum 30, but not blocked by an unlabeled cord serum, 113B20.

The in vitro reactions of human sera with SIDS lungs indicate the presence of a reactive antigen, as implied by the in situ bound IgG. The lung antigen(s) might be either endogenous (auto-antigen) or exogenous, presented pre- or postnatally. In very young SIDS victims, antibody is likely maternal suggesting prenatal experience of mothers with SIDS lung antigen. That this is an HLA antigen must be considered, preferably by using serum of a mother as well as cord and postmortem sera of her particular infant. In absence of a perspective study, the present findings do not support this possibility. Sera from three mothers with different specificities for SIDS lungs each failed to react with any of the 10 control lungs. Further, the histological pattern of distribution of fluorescent sites is not supportive.

If the bound IgG is indeed specific antibody, it is interesting to recall that specific humoral antibody, acquired by inactive vaccine (4) or active infection (1), can alter and massively exacerbate the pathological response occurring upon certain subsequent natural viral infections, particularly in infants. There is no evidence here that the antigen is one virus; however, such paradoxical effects of neutralizing antibody upon measles (1) and respiratory syncytial (4) and dengue viruses (2) create doubt as to whether in SIDS, maternally acquired antibody has sensitized the infant for a disastrous encounter with antigen shortly after birth.

TABLE 5. *Blocking of direct fluorescent staining of an SIDS lung (AA-1-77) with labeled human immunoglobulin using unlabeled human sera*

Dilution of sera[a]		Fluorescent reaction
Labeled	Unlabeled[b]	
30 (1:4)	Saline	+
30 (1:4)	30 (1:2)	−
11 (1:32)	Saline	+
11 (1:32)	113B20 (1:2)	+
11 (1:32)	11 (1:2)	Partial
11 (1:32)	30 (1:2)	−

[a] Sera 30 and 11 were from mothers of SIDS victims; 113B20 was a cord serum from an apparently normal newborn.

[b] Blocking was by the one-step method, labeled and unlabeled sera were combined before treatment of the lung section.

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