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New Method To Assess Dilution of Secretions for Immunological and Microbiological Assays

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Accurate quantitation of pathogens and antibody concentrations in secretions has been difficult because of unpredictable dilution of secretion with the diluent at the time of sample collection. We added an inert substance, lithium chloride (LiCl), to the sample diluent and measured its concentration with an atomic absorption spectrometer before and after the specimen was added. LiCl, at a concentration of 2 mmol of Li per liter, has no negative effect on the survival of common respiratory pathogens or on the results of immunoassays. The method is applicable to any sample collecting in which dilution of the specimen is necessary.

Accurate quantitation of antibodies in secretions is a problem when studying mucosal immunity. Absolute results have been obtained with rather cumbersome methods, e.g., aspirating nasopharyngeal secretions under general anesthesia during otolaryngological operations followed by freezing, weighing, and rinsing out the suction device with a known amount of diluent (14). The secretion can be absorbed onto filter paper (8, 10) or dextran (12), with the increase in weight representing the amount of secretion obtained. However, collection of secretion is time-consuming and difficult to carry out with young children, because the filter paper or dextran has to be held in the nostril for 10 s (repeated a few times or held for 5 to 30 minutes at a time), and the weighing needs to be done immediately after collection. Nasal washing is an easier method for collecting nasopharyngeal secretions (5, 11), but the dilution of the secretion is unknown, and only relative amounts of specific antibody or other protein can be measured compared with total immunoglobulin or, e.g., albumin.

In order to be able to study local immunity during respiratory infections of children, we have added an inert substance, lithium chloride (LiCl), to the sample diluent and measured its dilution in the final diluted sample. From this, we can calculate the dilution of the secretion and correct the results of immunoassays performed on the diluted sample. In this paper, we describe the method and our tests of its validity and suitability for bacteriological, virological, and immunological use.

Collection, storage, and treatment of secretions. Middle ear effusion and nasopharyngeal secretion were obtained with a suction device from children (3 months to 6 years of age) with acute otitis media. The diagnostic criteria and clinical details will be published later. The secretion was then rinsed into a tube containing 0.5 to 1.0 ml of LiCl–phosphate-buffered saline (PBS) (2 mmol of Li per liter of LiCl [Merck, Darmstadt, Germany] in PBS, pH 7.4). The sample (diluted secretion) was homogenized by mechanical mixing (Vortex-Genie; Scientific Industries, Inc., Bohemia, N.Y.) for 3 min. Part of the sample was frozen and transported in dry ice to the laboratory for storage at −70°C until use. Before testing, the sample was thawed and mixed again for 3 min and then centrifuged at 12,000 × *g* for 15 min. A total of 30 μl of the supernatant was diluted (1:30) in 870 μl of H_2O to determine the Li concentration, and the rest of the supernatant was used for antibody assays (to be reported elsewhere).

Percentage of the specimen in the sample. The Li concentration was assayed by a flame atomic absorption spectrometer (model 1100 B) equipped with an autosampler (AS-51; both from Perkin-Elmer, Überlingen, Germany), which is available in the clinical chemistry laboratories of most hospitals. The assay rate per sample was 6 s. The percentage of secretion in the sample was then defined as the correction coefficient (CC) and calculated from the formula CC = 1 − (Li in sample/Li in LiCl-PBS).

Validity of the concentration calculation. To test the validity of the calculation of dilution of the specimen, we diluted 10 human serum and 2 human milk specimens in known proportions in LiCl-PBS so that 35 to 100% of the sample consisted of serum or milk and the remainder consisted of LiCl-PBS. The samples were analyzed for Li concentration, and the percentage of serum or milk in the samples was calculated on this basis. This observed percentage was then compared with the expected percentage based on the known dilution. The observed percentage corresponded excellently to the expected percentage: 97 versus 100%, 64 versus 65%, and 37 versus 35% in the serum samples and 97 versus 100%, 80 versus 80%, and 40% versus 40% in the milk samples, respectively. Both the intraassay (standard deviation) and the interassay (coefficient of variation) variation were very satisfactory: the standard deviations are small, both as such and compared with the means (coefficient of variation, 1 to 5%).

LiCl and EIA. To test whether LiCl in the sample would affect the enzyme immunoassay (EIA), we made parallel 65 and 35% dilutions of one human serum specimen in PBS and LiCl-PBS and used these as samples in the EIA for measuring the amounts of anti-pneumolysin antibodies (6). The presence of LiCl did not affect the EIA results; the differences between the PBS and LiCl-PBS groups were not significant ($P > 0.9$).

As further validation of the Li-based calculation method, we used the 65 and 35% dilutions of 10 human serum specimens in LiCl-PBS as samples in the anti-pneumolysin EIA (6). The results of the EIA are presented as EIA units, calculated as follows: the titer of the sample, expressed as

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TABLE 1. LiCl and survival of bacteria[a]

Bacterium	Amt of LiCl-PBS (mmol/liter)	% of live bacteria after time in LiCl-PBS of[b]:			
		1–1.5 h		Overnight	
		Expt 1	Expt 2	Expt 1	Expt 2
S. pneumoniae	0	82	100	18	9
	2	100	100	33	65
	8		133		69
	20	100		45	
M. catarrhalis	0	80		4	
	2	78	63	3	1
	8		83		<0.5
	20	80		7	
H. influenzae	0	83	67	<0.5	<0.5
	2	33	100	<0.5	<0.5
	8	50	150	<0.5	<0.5
S. epidermidis	0	100		100	
	2	75		94	
	20	100		80	
S. aureus	0	100		80	
	2	100		167	
	20	100		133	

[a] Bacteria (~10^4/ml) were kept in LiCl-PBS (0 to 20 mmol/liter) at room temperature for 1 h to overnight before cultivation.
[b] The results are expressed as the percentage of live bacteria from the number of immediately cultivated bacteria.

the reciprocal of sample dilution that gave an optical density value of 0.15, was divided by the titer of the positive control and multiplied by 100. To correct for the dilution of the specimen, the value obtained was divided by the percentage of specimen in the sample (CC). The EIA units corrected by the Li-based (observed) CC were compared with the units corrected by the known (expected) dilution factor. The correlation of the observed and expected values was excellent ($r = 0.995$).

LiCl and survival of bacteria and viruses. Because bacterial and viral isolation should often be carried out from the same secretion samples obtained for antibody determination, we tested the effect of LiCl on bacterial and virus survival. *Streptococcus pneumoniae*, unencapsulated *Haemophilus influenzae*, *Moraxella* ("*Branhamella*") *catarrhalis*, all isolated from nasopharynx of healthy children, and *Staphylococcus aureus* (ATCC 25923) or *Staphylococcus epidermidis* (ATCC 12228), stored in skim milk (20% milk powder in H_2O; Difco 0032-01-1) at −70°C, were grown overnight on blood or chocolate agar plates (2) and suspended in 0.9% NaCl at 10^8 bacteria per ml (density [McFarland] of 1). The bacterial suspension was diluted 1:100 first in 0.9% NaCl and then in PBS containing different concentrations of LiCl (0 to 20 mmol of Li per liter). The suspensions were kept at room temperature for up to 24 h before cultivation onto blood or chocolate agar plates with a 10-μl loop to determine the survival of bacteria in a LiCl diluent over time. All of the bacteria survived well for 1 to 1.5 h (Table 1). After overnight incubation, only staphylococci survived to 100%, whereas the numbers of *S. pneumoniae* decreased to 9 to 69%, those of *M. catarrhalis* decreased to <0.5 to 7%, and those of *H. influenzae* decreased to <0.5%. No differences were found relative to the presence of Li, except with *S. pneumoniae*, for which the survival was somewhat better in LiCl-PBS than in PBS alone.

TABLE 2. LiCl and survival of adenovirus and RSV[a]

Virus	Amt of LiCl-PBS (mmol/liter)	Mean ± SD[b] plaques/well after incubation of:	
		2 h	24 h
Adenovirus	0	258 ± 29	117 ± 31
	2	290 ± 21	144 ± 33
	10	248 ± 33	109 ± 27
	20	237 ± 23	83 ± 30
	40	101 ± 11	18 ± 5
RSV	0	68 ± 6	21 ± 5
	2	80 ± 11	29 ± 3
	10	83 ± 11	39 ± 7
	20	68 ± 10	31 ± 5
	40	48 ± 5	16 ± 5

[a] A total of 100 PFU of adenovirus or RSV was kept in standard virus isolation medium supplemented with LiCl (0 to 40 mmol per liter) at room temperature for 2 or 24 h before inoculation onto cell cultures.
[b] Mean ± standard deviation of quadruplicate wells.

A total of 100 PFU of reference strains of type 2 adenovirus and respiratory syncytial virus (RSV) (15) were diluted into viral isolation medium (0.5% bovine serum albumin and antibiotics in tryptose phosphate broth), which contained different concentrations of LiCl (0 to 40 mmol of Li per liter), and kept at room temperature for 2 or 24 h before inoculation onto A549 (for adenovirus) and human diploid fibroblast (for RSV) cell cultures as quadruplicates in 24-well tissue culture clusters (Costar, Cambridge, Mass.). After 2 days, the cell cultures were fixed and stained with either adenovirus- or RSV-specific monoclonal antibodies (15). The infected cells were visualized as colored plaques of single cells or several cells. The plaques were counted, and the results are expressed as the total number of plaques per well (Table 2). LiCl in concentrations up to 20 mmol of Li per liter did not have a negative effect on survival of either one of the viruses.

LiCl was chosen for the dilution solution for several reasons. First, Li does not exist in human serum or secretions unless used for treatment of psychiatric disorders, and the drug is not used with children under 12 years of age. Second, unlike radioactive or carcinogenic reagents, LiCl is safe for laboratory personnel. Third, measuring Li concentration by an atomic absorption spectrometer is accurate, sensitive, quick, and inexpensive. Fourth, the monovalent Li^+ cation is an inert substance; studies with different laboratory animals in vitro (13) have indicated that Li is not capable of effective chemical binding with plasma proteins. Contrary to the bivalent ions Ca^{2+} and Mg^{2+} and some staining reagents, Li^+ is not known to interact with immunoglobulins or immune complexes.

Li also fulfilled several other requirements for such use: in the concentration used, it did not interfere with the EIA (Table 2) or the survival of bacteria or viruses. In bacteriology, LiCl has been used in high concentrations of 0.2 to 8 mol of Li per liter in preparing cell extracts of meningococci (3) and pneumococci (4) and in concentrations of 0.5 to 15 g of Li per liter in selective media for enrichment of *S. aureus* or *Listeria monocytogenes* (2, 7, 9). However, in the present study, LiCl in concentrations of 2 to 20 mmol of Li per liter, equivalent to 0.08 to 0.8 g of Li per liter, had no deleterious effects on the survival of common respiratory pathogens.

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