Changes in serum pneumoproteins caused by short-term exposures to nitrogen trichloride in indoor chlorinated swimming pools

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Nitrogen trichloride (NCl_3) is an irritant gas released in the air of indoor pools sanitized with chlorine-based disinfectants. In the present study we investigated the effects of NCl_3 on the pulmonary epithelium of pool attendees by measuring the leakage into serum of three lung-specific proteins (pneumoproteins): the alveolar surfactant-associated proteins A and B (SP-A and SP-B) and the bronchiolar 16 kDa Clara cell protein (CC16). These pneumoproteins were measured in the serum of 29 recreational swimmers (16 children and 13 adults) before and after attending a chlorinated pool with a mean NCl_3 concentration of 490 µg m^{-3}. Pneumoprotein changes in serum were also studied in 14 trained swimmers performing an intensive 45 min standardized swimming session in a chlorinated pool (mean NCl_3 concentration of 355 µg m^{-3}) and for the purposes of comparison in a non-chlorinated pool sanitized by the copper/silver method. Serum CC16 was not increased in recreational swimmers, but in trained swimmers serum levels of this protein peaked immediately after strenuous exercise, both in the copper/silver pool and in the chlorinated pool. This acute increase in airway permeability is probably the consequence of the mechanical stress on the epithelial barrier caused by overinflation and/or hyperventilation during intense exercise. Serum levels of SP-A and SP-B were unaffected by strenuous exercise in the copper/silver pool. The two proteins were, however, significantly increased in a time-dependent manner in recreational and trained swimmers attending the chlorinated pool. The intravascular leakage of SP-A and SP-B was already statistically significant after only 1 h of exposure to pool air without exercising and remained elevated for 12 h after. These changes were not associated with decrements in lung function. The ability of NCl_3 to acutely disrupt the lung epithelium barrier was confirmed in mice using serum CC16 and plasma proteins in bronchoalveolar lavage fluid as permeability markers. The significance of these permeability changes induced by NCl_3 in the deep lung is presently unknown. In view of the increasing and widespread human exposure to this gas not only in indoor pools but also in a variety of other situations, these findings warrant further study.

Keywords: nitrogen trichloride, exercise, pneumoproteins, swimming, lung epithelium, Clara cell protein, surfactant-associated proteins, chlorine-based disinfectants, chlorine

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Introduction

Disinfection of water in swimming pools is largely based on the use of chlorine-containing agents, among which the most widely used are sodium or calcium hypochlorite, chlorine gas and dichloroisocyanurates. In pool water, all these compounds release hypochlorous acid, a powerful oxidant that acts as an active biocide. When reacting with organic matter brought by swimmers (e.g. urea, sweat), hypochlorous acid generates a cocktail of potentially harmful disinfection byproducts (DBPs), including trihalomethanes, trihaloacetic acids and chloramines (WHO 2000). Levels of these DBPs in water and air fluctuate greatly with chlorine dose, pool occupancy, bathers' hygiene, and water and air renewal. In the air, the most concentrated DBP is nitrogen trichloride (NCl_3), the final byproduct of the chlorination of nitrogen-containing substances (Jensen 1988). However, in contrast to mono- and dichloroamines, which are soluble in water, NCl_3 is a volatile gas that is almost insoluble in water and is consequently immediately released into the pool air upon its formation. NCl_3 levels in public pools are highly variable and typically fluctuate in the range of 0.1 to 1.5 mg m^{-3} in air sampled 1.5 m above the water's surface (Hery *et al*. 1995), although higher concentrations can be found in recreational and poorly ventilated pools, particularly at times of maximal occupancy.

NCl_3 is a strong irritant and is probably responsible for the eye and upper respiratory tract irritation felt by lifeguards and other pool attendees (Massin *et al*. 1998). In rodents, this gas has the same irritating potency as chlorine or formaldehyde (Gagnaire *et al*. 1994) and causes fatal lung oedema at high doses (Barbee *et al*. 1983). NCl_3 is also responsible for cases of acute lung injury following accidental exposures to chlorine-based disinfectants and cleaning agents (Karnak *et al*. 1996, Tanen *et al*. 1999, Agabiti *et al*. 2001). Although NCl_3 has not specifically been studied, this gas most likely shares the same toxic properties as other chloramines and in particular those of the mono- and dichloroamines. As shown by *in vitro* studies (Tatsumi and Fliss 1994, Nakamura *et al*. 1995, Musch *et al*. 1999), chloramines are membrane-penetrating oxidants that react very rapidly with sulphydryl groups of proteins in the cytoskeleton and extracellular matrix to cause cell retraction, disruption of tight junctions and an almost immediate increase in endothelial and epithelial permeability. Such changes occur *in vivo* during infection and inflammation when inflammatory cells with a myeloperoxidase activity release hypochlorous acid and chloramines as part of their bactericidal function or to facilitate cell recruitment.

New non-invasive tests have recently become available for the detection of epithelium damage caused by air pollutants and other lung toxicants. These tests are based on the determination in serum of lung-specific proteins (pneumoproteins) that reflect the permeability or cellular integrity of the lung epithelial barrier (Hermans and Bernard, 1996, 1998, 1999). So far, three proteins have been validated as non-invasive tests of lung epithelial damage: the anti-oxidant 16 kDa bronchiolar Clara cell protein (CC16) and the alveolar surfactant-associated proteins A and B (SP-A and SP-B). These proteins can be used to detect lung hyperpermeability in a variety of clinical and experimental studies as well as in subjects exposed to air pollutants (Doyle *et al*. 1997, Hermans and Bernard 1999, Broeckaert *et al*. 2000). Serum CC16 can also serve as a marker of Clara cell dysfunction caused by tobacco smoke and other lung irritants (Bernard *et al*. 1994a, Shijubo *et al*. 1997). Because of their sensitivity, serum pneumoproteins

can detect acute or chronic effects on the lung epithelium at a subclinical stage before the onset of respiratory symptoms or lung function impairment (Bernard *et al.* 1994b, Bernard and Van Houte 1996, Broeckaert *et al.* 2000).

In the present study, these new non-invasive biomarkers were used to assess the effects of NCl_3 on the lung epithelium of subjects attending indoor chlorinated pools. The results, confirmed in mice, show for the first time that inhalation of NCl_3 in pool air can produce acute permeability changes in the deep lung parenchyma resulting in intravascular leakage of lung-specific proteins.

Materials and methods

Studies on swimmers

The protocols of the human studies were approved by the Ethical Committee of the Faculty of Medicine, Catholic University of Louvain, Belgium. All subjects were recruited from among the University of Louvain students and staff or their relatives. Subjects participated to the study after having given written informed consent. For children, written approval was obtained from their parents, most of whom also volunteered for the study.

The first study was conducted on recreational swimmers attending an indoor chlorinated pool (using sodium hypochlorite). A group of 16 children aged 5–14 years (10 girls and six boys; mean age 9.6 years) and a group of 13 adults aged 26–49 years (seven women and six men; mean age 36.9 years) participated in the study. All the subjects were healthy and in particular none had a diagnosis or symptoms of asthma. With the exception of one woman, they were all non-smokers. This first study was designed to be representative of usual recreational pool attendance with no specific requirements of exercise. Hence, children were allowed to undertake free activities, playing for the youngest and swimming for the oldest. They performed lung function tests and provided a blood sample just before entering the pool and after staying an average of 2 h in the pool. Adults were asked to stay on the poolside for 1 h without swimming, then to swim freely during the second hour (swimming time varied from 15–45 min depending on the subject). They performed lung function tests before entering the pool and just before leaving the pool. A blood sample was taken before entering the pool, after staying on the poolside for 1 h, and 1 h later after having swum freely for 15–45 min.

The second study was carried out to compare changes in serum pneumoproteins when swimming in a chlorinated indoor pool (the same as above) or in a non-chlorinated indoor pool sanitized by the copper/silver method. Eight male and six female University physical education students, aged 18–23 years (mean age 22.5 years) participated. All the subjects were in good health and were well-trained swimmers, but were not high-level competitors. None was asthmatic or a smoker. Information on their previous exposure to chlorination products in pools during or outside their scholarship was obtained by interview. Subjects were asked to refrain from intense exercise, including swimming, for at least 48 h prior to the experiment. Each subject was asked to perform the same standardized 45 min swimming session in the two pools consisting of 11 exercise bouts over a distance of 1500 m. To assess the exercise intensity, the heart rate was continuously monitored by means of a Polar^{TM} recorder, and oxygen uptake and pulmonary ventilation were measured in six swimmers by means of a portable breath-by-breath analysing system (Cosmed^{TM}). The mean heart beat recorded at the end of the exercise bouts was 170 ± 12 beats min^{-1}, while oxygen uptake and pulmonary ventilation reached 47 ± 7 ml min^{-1} kg^{-1} and 71 ± 13.1 l min^{-1}, respectively. Subjects performed lung function tests and provided a blood sample before entering the pool, immediately after the swimming session and then 11 h later. These two experiments were performed at a 1 week interval.

In the third study, we investigated the kinetics of pneumoprotein changes in the serum in three students (two young men and one young woman) attending an indoor pool disinfected with chlorine gas. The students stayed on the poolside for 1 h without exercising, then performed the same standardized swimming session as above before leaving the pool. They provided a blood sample before entering the pool, after staying 1 h on the poolside, immediately after the swimming exercise, and 2, 4, 6 and 20 h after they left the pool.

Blood samples (7 ml) were obtained by venipuncture and collected in dry tubes. Each sample was allowed to clot for a minimum of 12 h at $4°C$. Samples were then centrifuged at 2000 *g* for 10 min and the serum decanted and stored at $-18°C$ until protein analysis was performed. CC16 was determined by latex immunoassay using rabbit anti-Clara cell protein antibody (Dakopatts, Glostrup, Denmark) and CC16 purified in our laboratory as the standard (Bernard *et al.* 1992). All samples were run in duplicate at two different dilutions. This assay has been validated by comparison with a monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) (Hermans *et al.* 1998). The between- and within-run coefficients of variation ranged from 5–10%. The concentrations of SP-A and SP-B were determined with ELISA inhibition assays, as described in detail previously (Doyle *et al.* 1995) using

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polyclonal antibodies raised against alveolar proteinosis-derived SP-A and mature SP-B, respectively. These antibodies do not react with other plasma or alveolar epithelial lining fluid antigens, and the assays have between- and within-run coefficients of variation lower than 10%. Cystatin C, a small-size protein, was determined in serum by latex immunoassay with the aim of detecting possible variations in glomerular filtration, a potential confounder of serum concentrations of small-size pneumoproteins such as CC16 and SP-B (Newman *et al*. 1994). To increase the accuracy of our measurements, all samples from the same subjects were analysed in the same analytical run and were blinded with respect to the exposure conditions.

A Vitalograph spirometer was used to assess the vital capacity (VC), the forced expiratory volume over 1 s (FEV1) and the peak expiratory flow (PEF). The VC and FEV1 values were the means of the two best acceptable values of lung function, in accordance with the guidelines of the American Thoracic Society (1995).

The mean concentrations of NCl_3 in the air during the experiments in the chlorinated and non-chlorinated pools were measured on the poolside using the method described by Hery *et al*. (1995). The only difference was that air was sampled at a height of 20 cm above the pool's water surface instead of 1.5 m in order to better assess exposure during swimming. The time of sampling was adjusted to that of the experiments and varied between 45 and 120 min.

Studies on mice

Two-month-old female C57 Bl/6 mice (Iffa Credo, l'Asbrele, France) were exposed to NCl_3 in 200 l stainless steel inhalation chambers with an adjustable air flow ($6–16 \ m^3 \ h^{-1}$) and maintained at negative pressure ($2–3 \ mm \ H_2O$) to prevent gas leakage (Gagnaire *et al*. 1994). In the first experiment, groups of nine mice were exposed either to filtered air or to $11.9 \ mg \ m^{-3}$ NCl_3 for 1, 2, 4 or 8 h. The group of mice exposed for 8 h was re-examined 16 h later to evaluate recovery. The targeted NCl_3 concentration in the chamber was $12.2 \ mg \ m^{-3}$, corresponding to the concentration producing a 50% decrease in breathing rate in male OF1 mice (Gagnaire *et al*. 1994).

In a second experiment, groups of 10 mice were exposed for 4 h either to filtered air or to 0.53, 0.8, 3.45 or $13.1 \ mg \ m^{-3}$ NCl_3. NCl_3 was produced in a reactor bubble chamber by continuously mixing two solutions of sodium hypochloride ($0.5 \ mol \ l^{-1}$) and ammonium sulphate ($0.005 \ mol \ l^{-1}$) in phosphoric acid $0.1 \ mol \ l^{-1}$ (pH 2) using peristaltic and high pressure liquid chromatography (HPLC) pumps, respectively. The instantaneously generated NCl_3 was carried by the air flow through a Teflon filter to eliminate possible aerosols and then through a solution of sulphamic acid ($2 \ g \ l^{-1}$) to remove any chlorine that may be present. NCl_3 was then diluted with air to give the required concentration before entry into the exposure chamber. The concentration of NCl_3 in the chamber was determined using the method of Hery *et al*. (1995). An air sample from the inhalation chambers was taken through a vial containing a solution of sodium bicarbonate ($8 \ g \ l^{-1}$) and arsenic trioxide ($0.8 \ g \ l^{-1}$). NCl_3 was transformed into chlorides, which were measured by ionic chromatography and conductimetric detection. Immediately after exposure, the animals were sacrificed with sodium pentobarbitone ($100 \ mg \ kg^{-1}$ intraperitoneally). Blood was collected by cardiac puncture in dry tubes and bronchoalveolar lavage was performed with 2 ml of saline as previously described (Halatek *et al*. 1998). Blood samples were treated as described above for the human samples. Latex immunoassay techniques were used to determine albumin levels in the bronchoalveolar lavage fluid (BALF) and CC16 levels in both serum and BALF (Halatek *et al*. 1998). Total protein and LDH levels in BALF were determined by spectrophotometric methods as described by Arsalane *et al*. 1999.

Statistics

The statistical package SAS/STAT, Version 6, Fourth Edition (SAS Institute Inc., Cary, North Carolina, USA) was used for all analyses. Results were expressed as the mean $\pm$ SE. All statistical tests were performed on log-transformed data. Acute effects of chlorination products on lung function parameters and on pneumoprotein and cystatin C concentrations in serum were assessed using the paired Student's *t*-test. Associations between serum levels of pneumoproteins and cumulated attendance of chlorinated pools were assessed by calculating Pearson's correlation coefficients. *p* values are indicated when lower than 0.1.

Results

Recreational swimmers

Serum pneumoproteins were studied in 16 children and 13 adults before and after attending an indoor chlorinated pool (figure 1 and table 1). The mean concentration of NCl_3 in the pool air during the experiment (120 min) was
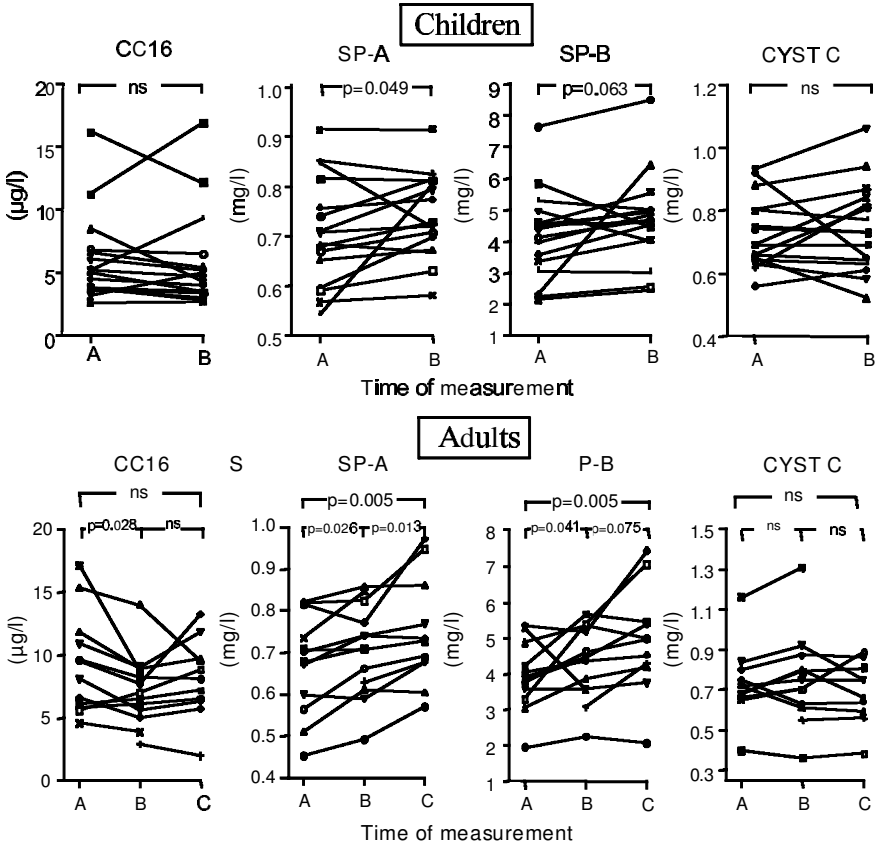
Figure 1.   Concentrations of CC16, SP-A, SP-B and cystatin C (CYST C) in the serum of 16 children (upper panel) and 13 adults (lower panel) attending an indoor chlorinated pool. Values obtained for children: A, before entering the pool; B, 2 h after playing/swimming in the pool. Values observed in adults: A, before entering the pool; B, after a 1 h stay on the pool side without swimming; C, 1 h later after a free 15–45 min swimming session. The mean NCl_3 concentration during the experiment, which lasted 2 h, was 490 $\mu g\ m^{-3}$. The *p* values refer to comparison with pre-exposure levels using the paired Student's *t*-test.

490 $\mu g\ m^{-3}$. In children, no significant change was found between pre- and post-exposure levels of serum CC16. Serum SP-A and SP-B levels, by contrast, increased significantly after attendance at the pool. In adults, the serum CC16 showed a biphasic response, with a significant decrease after 1 h of exposure without swimming and a reversal towards normal 1 h after the swimming session. As in children, serum SP-A and SP-B increased in most of the adult subjects. The increase was already statistically significant after 1 h of exposure without exercising. A further increase was found after the swimming session, resulting in highly significant differences in comparison with pre-exposure levels. Pre-and post-exposure concentrations of serum cystatin C were on average similar in both children and adults, meaning that renal function was not altered. The comparison of lung function tests before and after pool attendance did not reveal any statistically significant differences (table 1). In children, the FEV1, PEF and

Table 1. Serum pneumoproteins, serum cystatin C and lung function parameters (mean ± SD) in children and adults before and after attending an indoor chlorinated pool for 2–3 h with a mean NCl_3 concentration of 490 µg m^{-3}.

| | Children (n = 14) | | | Adults (n = 13) | | | | |
| | | | | | Post-exposure | | | |
	Pre-exposure	Post-exposure (2 h)	p	Pre-exposure	1 h	p	2 h	p
Serum CC16 (µg l^{-1})	6.0 ± 3.5	5.7 ± 3.9	NS	9.3 ± 4.0	7.2 ± 2.8	0.028	8.1 ± 3.1	NS
Serum SP-A (mg l^{-1})	0.71 ± 0.11	0.74 ± 0.08	0.049	0.67 ± 0.12	0.71 ± 0.11	0.026	0.75 ± 0.13	0.005
Serum SP-B (mg l^{-1})	4.2 ± 1.4	4.7 ± 1.5	0.063	3.9 ± 0.96	4.3 ± 1.0	0.041	4.9 ± 1.5	0.005
Serum cystatin C (mg l^{-1})	0.73 ± 0.11	0.74 ± 0.14	NS	0.70 ± 0.2	0.72 ± 0.24	NS	0.69 ± 0.16	NS
VC (% pred.)	93.0 ± 16.3	91.1 ± 17.4	NS	100.4 ± 11.2	—		102.0 ± 16.6	NS
FEV1 (% pred.)	81.7 ± 14.2	92.5 ± 29.1	0.057	91.1 ± 16.1	—		95.9 ± 18.4	NS
FEV1/VC (Tiffeneau)	0.81 ± 0.10	0.93 ± 0.14	0.017	0.80 ± 0.09	—		0.78 ± 0.09	0.097
PEF (% pred.)	70.5 ± 16.3	80.4 ± 13.7	0.057	91.8 ± 28.1	—		95.0 ± 27.8	NS

% pred., % of predicted values. We discarded the results of two young children who did not perform the spirometric tests adequately. The p values refer to comparison with pre-exposure levels using the paired Student's t-test.

Tiffeneau index (FEV1/VC) were, if anything, slightly increased after pool attendance. In adults, also, no significant decrement in lung function was observed.

Trained swimmers

Changes in serum pneumoproteins were studied in trained swimmers performing a standardized swimming session both in an indoor chlorinated pool and in an indoor pool disinfected by the copper/silver method. The mean NCl_3 concentration during the experiments in the chlorinated pool was 355 µg m^{-3} (over 45 min) and was thus lower than in the preceding experiment. The air of the copper/silver pool did not contain detectable amounts of NCl_3. As shown in figure 2 and table 2, the serum CC16 showed an almost immediate increase after the swimming session in both pools. However, this increase in serum CC16 observed in most subjects was transient, since 11 h later all values had completely returned to pre-exposure
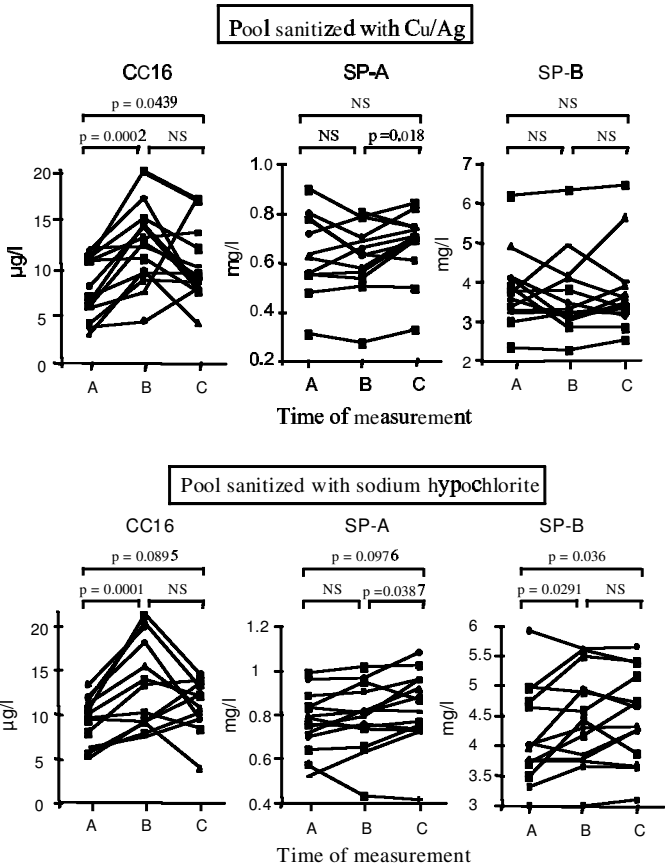

Figure 2.   Concentrations of CC16, SP-A and SP-B in the serum of 14 trained swimmers attending an indoor copper/silver pool (upper panel) and an indoor chlorinated pool (lower panel). A, results observed before entering the pool; B, results obtained after a 45 min standardized swimming session; C, results obtained 11 h after having left the pool. The mean NCl_3 concentration during the whole duration of the experiment, i.e. 45 min, was 355 µg m^{-3}. The *p* values refer to comparison with pre-exposure levels using the paired Student's *t*-test.

Table 2. Serum pneumoproteins and lung function parameters (mean $\pm$ SD) in trained swimmers ($n = 14$) before and after a 45 min swimming session in a copper/silver pool and an indoor chlorinated pool (mean atmospheric NCl$_3$ concentration of 355 µg m^{-3}).

| | Copper/silver pool | | | | | Chlorinated pool | | | | |
| | | Post-exposure | | | | | Post-exposure | | | |
	Pre-exposure	10–20 min	p	11 h	p	Pre-exposure	10–20 min	p	11 h	p
Serum CC16 (µg l^{-1})	7.9 ± 3.2	12.0 ± 4.0	0.0002	9.9 ± 3.7	0.044	9.0 ± 2.8	13.2 ± 5.1	0.0001	10.7 ± 3.0	0.089
Serum SP-A (mg l^{-1})	0.65 ± 0.16	0.63 ± 0.15	NS	0.69 ± 0.14	NS	0.77 ± 0.13	0.79 ± 0.15	NS	0.83 ± 0.17	0.098
Serum SP-B (mg l^{-1})	3.8 ± 0.9	3.6 ± 1.0	NS	3.7 ± 1.1	NS	4.2 ± 0.8	4.4 ± 0.8	0.029	4.5 ± 0.76	0.036
VC (% pred.)	104.8 ± 10.3	106.5 ± 11.1	NS	105.3 ± 8.2	NS	111.0 ± 10.2	108.6 ± 8.8	NS	106.7 ± 11.3	NS
FEV1 (% pred.)	99.0 ± 15.0	105.0 ± 18.4	0.07	99.3 ± 13.8	NS	104.5 ± 12.4	105.0 ± 13.1	NS	101.6 ± 17.1	NS
FEV1/VC (Tiffeneau)	0.78 ± 0.08	0.82 ± 0.88	NS	0.78 ± 0.09	NS	0.78 ± 0.09	0.80 ± 0.08	NS	0.80 ± 0.16	NS
PEF (% pred.)	101.4 ± 16.9	106.3 ± 22.2	NS	98.8 ± 16.3	NS	108.1 ± 18.8	107.4 ± 17.6	NS	99.6 ± 18.1	0.01

0.01 % pred., % of predicted values. The p values refer to comparison with pre-exposure levels using the paired Student's t-test.

levels. No difference was found between the serum levels of CC16 reached after the swimming sessions in the two pools. The patterns of changes in serum SP-A and SP-B in the two pools were clearly different from those of serum CC16. Whilst strenuous swimming in the copper/silver pool did not affect the serum concentrations of SP-A and SP-B, the two proteins were significantly increased after swimming in the chlorinated pool. The increase was also more sustained, since 11 h later the protein levels had not yet completely subsided to pre-exposure values. Despite the intensity of the exercise, pre-and post-swimming concentrations of serum cystatin C were similar in both the copper/silver (0.79 ± 0.034 versus 0.79 ± 0.031 mg l^{-1}) and the chlorinated pool (0.82 ± 0.067 versus 0.79 ± 0.084 mg l^{-1}), indicating again no significant change in the glomerular filtration rate. In the copper/silver pool, swimming exercise did not cause any significant difference in lung function tests, with the exception perhaps of a slight transient increase in FEV1 immediately after the swimming session ($p = 0.07$, table 2). In the chlorinated pool, lung function performances before and immediately after the swimming session were not significantly different. A significant decrease in PEF was observed 11 h later, but other lung function parameters were unchanged.

Kinetics of serum pneumoprotein changes in a chlorinated pool

The time course of serum concentrations of CC16, SP-A and SP-B in a chlorinated pool was studied in three volunteers who were asked to stay for 1 h on the poolside without exercising and then to perform the same standardized 45 min swimming session as in the preceding experiment. The mean NCl$_3$ concentration recorded during this experiment (105 min) was 440 µg m^{-3}. After the session, the subjects left the pool and their pneumoprotein serum levels were followed up for 20 h. As shown in figure 3, the response of the serum CC16 was qualitatively very similar in all three subjects, the protein peaking in the serum immediately after the exercise with a quick return to pre-swimming levels after the subject had left the pool. By contrast, the kinetics of the changes in SP-A and SP-B in the serum were more variable between subjects. With the exception of SP-A in subject 2, the two surfactant-associated proteins increased after the swimming session, either immediately (SP-B in subject 2) or with a delay of about 2–3 h (SP-A and SP-B in subject 1). As in the preceding experiment in the chlorinated pool, the increases in surfactant-associated proteins were more sustained, persisting up to 20 h post-exposure. SP-A and SP-B thus show a slightly delayed and more sustained elevation in serum after swimming in a chlorinated pool than CC16.

Relationships between baseline levels of serum pneumoproteins and cumulated pool attendance

In the kinetic study, our attention was drawn by subject 3 who, albeit a non-smoker, had a very high value of serum SP-B. This value, the highest found in this study, was similar to that found in heavy smokers (Robin *et al*. 2002). Since this subject also had the highest cumulated chlorinated pool attendance of the study (2700 h), this led us to examine the possible relationships between baseline serum levels of pneumoproteins and cumulated chlorinated pool attendance (log–log correlation) in trained swimmers. The cumulated pool attendance ranged from 111
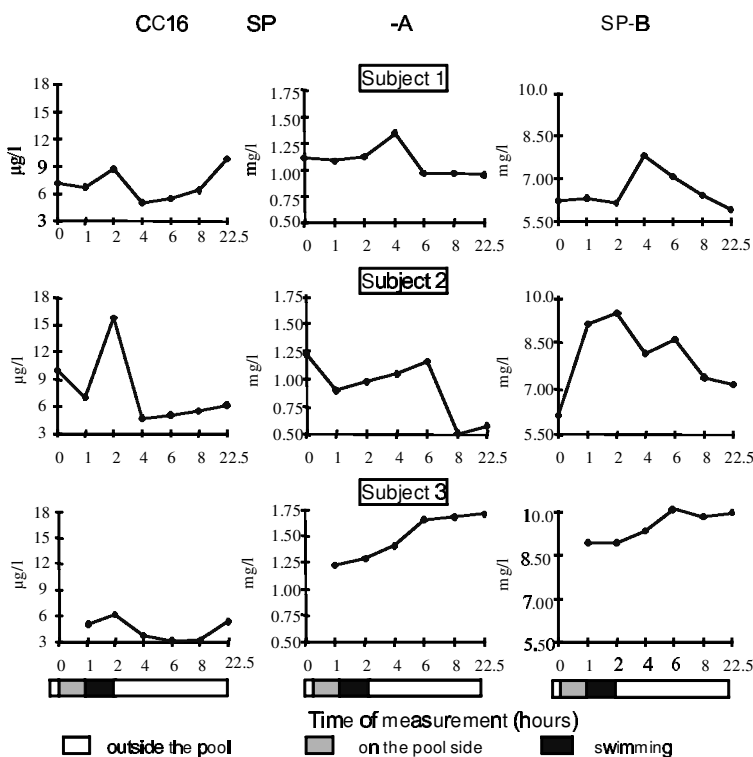
Figure 3. Time course of serum CC16, SP-A and SP-B in three students attending an indoor chlorinated pool. Blood was taken before entering the pool, after a 1 h stay on the poolside without swimming, 1 h later after a 45 min standardized swimming session, and then at regular time intervals after having left the pool. The mean NCl_3 concentration during the pool attendance (105 min) was 440 $\mu g\ m^{-3}$. The first blood sample of subject 3 (before entering the pool) was accidentally lost. The concentrations of CC16, SP-A and SP-B in a blood sample taken 2 weeks later at the same time of the day were 5 $\mu g\ l^{-1}$, 1.12 mg l^{-1} and 9.87 mg l^{-1}, respectively.

to 2740 h ($n = 16$, geometric mean 395 h, median 315 h). The concentration of serum SP-B was weakly positively correlated with pool attendance ($r = 0.47$, $p = 0.068$), whereas serum CC16 showed a weak negative correlation ($r = -0.45$, $p = 0.08$). However, the serum CC16/SP-B ratio, calculated to integrate these two opposite trends, was negatively correlated with chlorinated pool attendance ($r = -0.57$, $p = 0.02$). No association was found between serum SP-A and cumulated pool attendance ($r = 0.27$, $p = 0.31$). These associations were not confounded by age or sex, which did not emerge as determinants.

Mice exposed to NCl_3

The ability of NCl_3 to alter the lung epithelium barrier was confirmed in mice. Female C57 Bl/6 mice were exposed to 11.9 mg m^{-3} NCl_3 for up to 8 h and examined regularly during exposure and then 16 h post-exposure. We determined CC16 in serum and BALF, whilst albumin, total protein and lactate dehydrogenase (LDH) levels were measured in BALF only. As shown in figure 4, serum CC16 increased significantly during exposure, peaking after 4 h at values on average 2.5
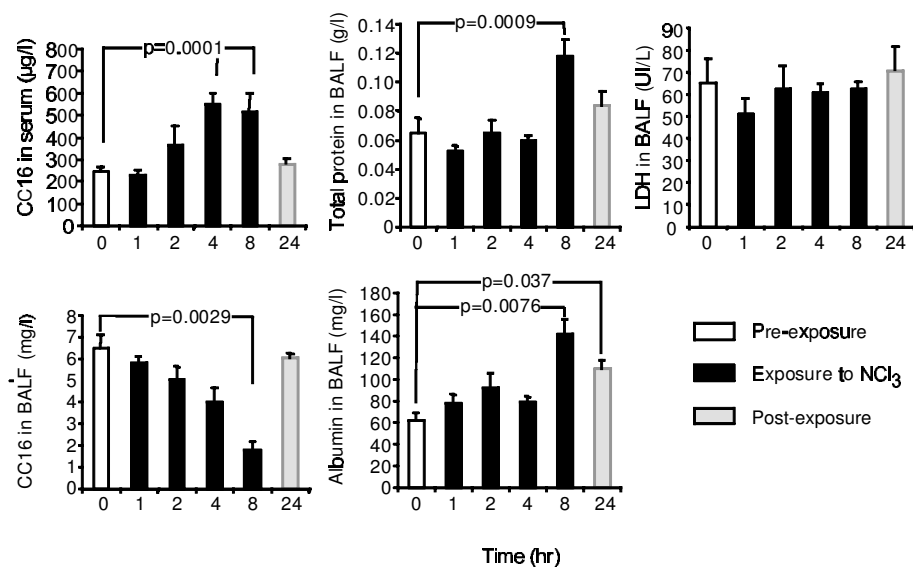
Figure 4. Concentrations of CC16 in serum and BALF, and levels of albumin, total protein and LDH in BALF of mice exposed to a mean NCl_3 concentration of 11.9 mg m^{-3} for 1, 2 or 8 h. Mice exposed for 8 h were also examined 16 h post-exposure. The values given are the mean $\pm$ SE of nine animals in each group. The p values refer to comparison with the pre-exposure group using the Student's t-test

times above pre-exposure levels. In BALF, CC16 showed a completely opposite pattern to that in serum, decreasing in a time-dependent manner with a maximal reduction of about 80% after 8 h of exposure. The increase in serum CC16 and the parallel decrease in CC16 in BALF observed 8 h post-exposure were also accompanied by a statistically significant elevation in both albumin and total protein levels in BALF. At this stage, mice exposed to NCl_3 thus presented an increased bidirectional leakage of proteins across the pulmonary epithelial epithelium. This lung epithelium hyperpermeability caused by NCl_3 was apparently not associated with an overt cell cytotoxicity, since the LDH levels in BALF remained normal throughout the experiment. No direct evidence of lung toxicity was seen on light microscopy (results not shown). These changes were reproduced in the second experiment in mice exposed to 13.1 mg m^{-3} NCl_3, but were not found when animals were exposed to lower concentrations of NCl_3 (3.45, 0.8 and 0.53 mg m^{-3} for 4 h) (results not shown).

Discussion

Our study shows that NCl_3 contaminating the atmosphere of indoor chlorinated pools can produce short-term effects on the lung epithelium of recreational or trained swimmers, resulting in increased intravascular leakage of lung-specific proteins. These permeability changes were already detected after a 1 h exposure to pool air without swimming, excluding confounding by exercise. They were not accompanied by decrements in lung function and no symptoms of respiratory tract irritation were reported by the subjects. The increased epithelial permeability induced by NCl_3 was evidenced by the use of two surfactant-associated proteins

SP-A and SP-B, as markers. Since these proteins are mainly produced by the alveolar epithelium, in contrast to CC16 that is secreted by Clara cells along the tracheobronchial tree, this suggests that, at the exposure levels found in indoor pools, NCl_3 causes permeability changes preferentially affecting the deep lung, largely sparing the airways. These results are fully consistent with the physical properties of NCl_3. Because of its very low solubility in water, NCl_3 does not easily penetrate the ciliated surface and epithelial lining fluid of airways and therefore exerts its action predominantly in the deep lung where cells are non-ciliated and their intercellular junctions much more accessible. Conceivably, the airway epithelium would only be affected after a higher and longer exposure to NCl_3, allowing sufficient amounts of this gas to reach the airway intercellular junctions where pathways for the transepithelial leakage of proteins are located. However, since levels of CC16 in serum may have a dual meaning, reflecting both the epithelial permeability and the integrity of Clara cells, we cannot exclude the possibility that at relatively low exposure levels the increased intravascular leakage of CC16 could be masked by a concomitant decrease in CC16 secretion due to Clara cell dysfunction or damage, as occurs for instance in smokers. The biphasic response of serum CC16 in adults tends to support this interpretation, which of course needs to be substantiated by immunohistochemical or electron microscopy studies.

During strenuous swimming, serum levels of pneumoproteins may be increased either as a result of increased permeability of the lung epithelium or, for small-size proteins such as CC16 or SP-B, as a result of a reduced renal clearance. In the case of surfactant-associated proteins, confounding by exercise was definitively ruled out in our study by the results obtained in the non-chlorinated pool disinfected by the copper/silver method. Despite very strenuous exercise, serum levels of SP-A and SP-B were unchanged during swimming in this pool. These results are in agreement with those of Nanson *et al.* (2001), who found no change in serum SP-A in subjects performing a strenuous treadmill exercise or heavy tasks simulating firefighting tasks. The transport of proteins across the alveolar epithelium thus seems to be unaffected by exercise. By contrast, strenuous exercise, whether in a chlorinated or a non-chlorinated pool, caused an immediate increase in serum CC16. A similar peak in serum CC16 was also reported by Nanson *et al.* (2001) after intense treadmill or firefighting tasks. However, the elevation in serum CC16 induced by swimming was of very short duration, since 2 h after the end of the exercise the serum CC16 had already returned to pre-exercise levels. Since serum cystatin C, a sensitive marker of the glomerular filtration rate, was not increased by the exercise, we can exclude an elevation of serum CC16 due to a decreased renal elimination of this small-size protein. As shown by previous studies in animals and humans, intense exercise can be associated with a disruption of the epithelium and endothelial air/blood barriers, resulting in an increased influx of plasma protein and red cells in BALF (West *et al.* 1991, Hopkins *et al.* 1997). These changes are attributed to mechanical stress on the air/blood barrier due to lung overinflation or hyperventilation. This mechanism is also most likely to be responsible for the peak in serum CC16 observed after intense swimming in both the copper/silver and the chlorinated pool. These stress-induced alterations in airways barriers might present a link with the relatively high incidence of airways diseases found in elite athletes (Weiler *et al.* 1998). The assay of serum CC16 could be a simple non-invasive test to identify those training conditions that most affect the air/blood barrier integrity.

Our studies in mice were limited by the lack of immunoassays specific to surfactant-associated proteins in this species. The only pneumoprotein we can presently measure in mouse is CC16, which in humans does not appear to be the most sensitive marker of the epithelial changes caused by NCl_3. In agreement with the observations on pool attendees, we did not find any increase in serum CC16 in mice exposed to NCl_3 levels similar or close to those found in indoor pools. However, when the animals were exposed to 11.9 mg m^{-3}, a value that is 10–20 times higher than in indoor pools, the serum CC16 was clearly increased after 4 and 8 h of exposure. This increase was associated with an influx of albumin and total protein in BALF, confirming the ability of NCl_3 to disrupt the lung epithelial barrier. The patterns of changes observed for CC16 in both serum and BALF and for albumin and total protein in BALF when mice were exposed to NCl_3 were remarkably similar to those induced in rats or mice by short-term exposures to ozone. As for ozone, the increase in serum CC16 induced by NCl_3 is very transient and is paralleled by a marked decrease in this protein in BALF due to its intravascular leakage (Arsalane *et al.* 1999, Broeckaert *et al.* 2000).

Since very few epidemiological or controlled studies have addressed the possible chronic effects of chlorination products, including NCl_3, in swimmers there are few data to which we can refer to assess the clinical significance of our observations. Although the number of subjects was limited, the concentration of CC16 in the serum of trained swimmers correlated negatively with their cumulated pool attendance, whereas their serum levels of SP-B tended to increase with pool attendance. These associations, which are reminiscent of those found in smokers (Robin *et al.* 2002), suggest that repeated exposure to NCl_3 might cause permanent changes to the lung epithelium. Signs of bronchial hyperresponsiveness and airways inflammation have recently been evidenced in elite swimmers, which also suggests that chlorination products might chronically irritate the airways epithelium (Helenius *et al.* 1998a, b). The long-term consequences of these effects are unknown, but theoretical arguments indicate that repeated or chronic disruption of the lung epithelial barrier could be detrimental to the lung. This might lead to a loss of epithelial secretory proteins, including proteins that are vital for preserving the functional or structural integrity of the lung, and, conversely, a more permeable epithelium might facilitate the exudation of plasma proteins as well as the penetration into the lung interstitium of toxic inhaled agents such as fine particles or allergens (Hermans and Bernard, 1999).

Uncertainties mainly related to exposure measurement preclude any derivation of a safe air level of NCl_3 from the results obtained on pool attendees. The only method currently validated for monitoring NCl_3 in pools is based on air sampling on the poolside to avoid contamination of the filter by chlorinated water. Although in our experiments air samples were taken at a height of 20 cm above the water's surface, we cannot guarantee that NCl_3 concentrations observed at this level accurately reflect the dose of NCl_3 effectively inhaled by the swimmers. Depending on the type of swimming and on the respiration mode, a very active swimmer can also inhale aerosols or droplets containing soluble chlorination products that, even if they are not carried deeply into the respiratory tract, may lead to *in situ* formation of NCl_3 by reacting with the upper airways. It is also conceivable that ingestion of water during swimming may result in the formation of NCl_3 in the oral cavity. In addition, with a highly volatile and reactive gas such as NCl_3, it cannot be excluded that the epithelial effects observed in our study are caused by

short-lived peaks of exposure to NCl_3 undetectable by the current methodology. Another source of uncertainty stems from the fact that most of the volunteers recruited in our study were regular recreational or trained swimmers. Depending on their previous exposure, it cannot be excluded that some of them have developed a form of resistance to chlorination products or already have a certain degree of lung hyperpermeability, making them less sensitive to the acute effects of NCl_3. This view is supported by the baseline levels of pneumoproteins that in trained swimmers but also in recreational swimmers are quite variable and tend to correlate with previous attendance at chlorinated pools.

In conclusion, this study reports for the first time that short-term exposure to NCl_3 in the air of indoor chlorinated pools can produce permeability changes of the deep lung that are undetected by classical lung function tests. In view of the increasing and widespread attendance of chlorinated pools by the population, further research is needed to assess the significance of these effects and to establish safe exposure levels to NCl_3 in indoor pools for the different categories of pool attendees.

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