Serum Clara Cell Protein: A Sensitive Biomarker of Increased Lung Epithelium Permeability Caused by Ambient Ozone

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Ozone in ambient air may cause various effects on human health, including decreased lung function, asthma exacerbation, and even premature mortality. These effects have been evidenced using various clinical indicators that, although sensitive, do not specifically evaluate the O_3-increased lung epithelium permeability. In the present study, we assessed the acute effects of ambient O_3 on the pulmonary epithelium by a new approach relying on the assay in serum of the lung-specific Clara cell protein (CC16 or CC10). We applied this test to cyclists who exercised for 2 hr during episodes of photochemical smog and found that O_3 induces an early leakage of lung Clara cell protein. The protein levels increased significantly into the serum from exposure levels as low as 0.060–0.084 ppm. Our findings, confirmed in mice exposed to the current U.S. National Ambient Air Quality Standards for O_3 (0.08 ppm for 8 hr) indicate that above the present natural background levels, there is almost no safety margin for the effects of ambient O_3 on airway permeability. The assay of CC16 in the serum represents a new sensitive noninvasive test allowing the detection of early effects of ambient O_3 on the lung epithelial barrier. *Key words:* biomarker, CC10, CC16, Clara cell protein, epithelium, lung permeability, ozone. *Environ Health Perspect* 108:533–537 (2000). [Online 26 April 2000]
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Ozone, the main oxidant species of photochemical smog, can produce a variety of acute pulmonary effects, including decrement of lung function, inflammatory reaction, increase of epithelial permeability, and airway resistance. There is also some epidemiologic evidence that current ambient exposures to O_3 are associated with reduced baseline lung function, exacerbation of asthma, and premature mortality (*1,2*). In 1997, the U.S. Environmental Protection Agency revised the National Ambient Air Quality Standards (NAAQS) for O_3 by replacing the 1-hr health-based standard of 0.12 ppm with an 8-hr standard of 0.08 ppm (*3*). Whether this new guideline based on average daily exposure sufficiently protects public health from both chronic and acute effects of O_3 has been debated (*4*).

The assessment of health effects of ambient O_3 has mainly relied on such end points as lung function impairment or respiratory symptoms that, although sensitive, do not permit the evaluation of the extent of oxidative damage caused by O_3 to the pulmonary epithelium. Recently, a new approach for assessing early effects of pollutants on the respiratory tract was developed, based on the assay in serum of lung-derived proteins. One of these proteins is the 15.8-kDa Clara cell protein (CC16 or CC10), which is secreted in large amounts at the surface of airways from where it leaks into the serum, most likely by passive diffusion (*5–8*). The serum concentration of CC16 is a new sensitive marker to detect an increased permeability of the epithelial barrier, which is one of the earliest signs of lung injury induced by air pollutants, including O_3 (*2,9*).

Materials and Methods

Study on cyclists. The study was conducted in Parma, Italy, between 18 June and 31 July 1998 under varying conditions that included episodes of photochemical smog. After providing their informed consent, 24 nonsmoking cyclists (15 women and 9 men) 28.5 (SD, 3.4) years of age participated in the study. Each volunteer performed two 2-hr rides between 0200 and 0400 hr on roads and dates characterized by different levels of air pollution. The rides covered a distance between 30 and 40 km (18 and 25 miles). The speed was moderate (9–13 miles/hr) and the heart rate was relatively stable (122.3 ± 11.8 beats/min). Ozone concentration was monitored every 10 min by six stations of the local monitoring network. Mean O_3 concentrations during the rides measured by these stations varied between 0.033 and 0.103 ppm (mean 0.076 ppm).

Immediately before and after each ride, the subjects provided a blood sample for the assay of serum CC16 and performed respiratory function tests [forced vital capacity (FVC), forced expiratory volume in 1 sec, (FEV_1), peak expiratory flow (PEF), and maximum expiratory flows at 25, 50, and 75% of the vital capacity ($MEF_{25/50/75\%}$)]. We assessed subjects' lung function using a spirometer equipped with a Fleish pneumotachometer (Fukudasangyo Europe, Bologna, Italy). Subjects were tested within 30 min before and after the ride, while wearing nose clips. Mean FEV_1 and FVC values were the means of the two or three best acceptable values tests of lung function, according to the American Thoracic Society (*10*). The concentration of CC16 in serum was determined by an automated immunoassay relying on the agglutination of latex particles (*11,12*). The accuracy of this immunoassay was recently confirmed by comparison with a monoclonal antibody-based ELISA (*13*). Cystatin C, a small-size protein like CC16, was determined in serum to detect possible variations in the glomerular filtration rate (*14*), a potential confounder for CC16 concentrations (*7*).

Study on mice. Two-month-old female C57Bl/6 mice (Iffa Credo, l'Asbrele, France) were exposed to 0.08 ppm O_3 or to filtered air for 4 or 8 hr in inhalation chambers (Sheet Metal Products, Dust Control Systems; Young & Bertke Co., Cincinnati, OH). O_3 was produced from dried and filtered air by a high-voltage generator (Anseros Ozomat; Anseros Klaus Nonnenmacher GmBH, Tübingen, Germany) and continually monitored by an ultraviolet photometric analyzer (Signal Instrument Company, Farington-Oxon, UK). Immediately after exposure, the animals were sacrificed with sodium pentobarbital (100 mg/kg, ip) to collect serum and bronchoalveolar lavage fluids (BALFs). Bronchoalveolar lavage and cell counts were conducted according to the technique described previously (*15*). Briefly, the lung was washed 3 times with a 2-mL volume of saline, then BALFs were centrifuged for 10 min (1,000*g* at 4°C). We used the cell-free supernatant of the first lavage fraction for biochemical measurements, whereas we used the cell pellets of BALFs for total and differential counts. The

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BALF pellets were resuspended in 1 ml NaCl containing 0.1% bovine serum albumin, and we determined the total number of live cells by the trypan blue exclusion method. We determined the cell differential counts of macrophages and polymorphonuclear neutrophils (PMNs) by characterizing 200–250 cells/animal on cytocentrifuge preparations fixed in methanol and stained with Diff Quick (Dade Behring AG, Düdingen, Switzerland). We measured the concentrations of CC16 in BALF and serum by an automated latex immunoassay recently developed for rodent CC16 (*16*). A similar immunoassay was also used for the determination of albumin in BALF (*16*).

Statistics. The results were expressed as mean ± SE. All statistical tests were applied on log-transformed data. We assessed the differences between pre- and postride values using the paired Student's *t*-test. Factors significantly influencing serum concentrations of CC16 or changes in lung function tests were identified by stepwise multiple regression analysis. We assessed the differences between values in quartiles of O_3 concentrations by one-way analysis of variance followed by the Dunnett's multiple comparison test. In the animal study, we compared mean values of control and O_3-exposed groups by the Student's *t*-test. The level of significance was assigned at $p < 0.05$.

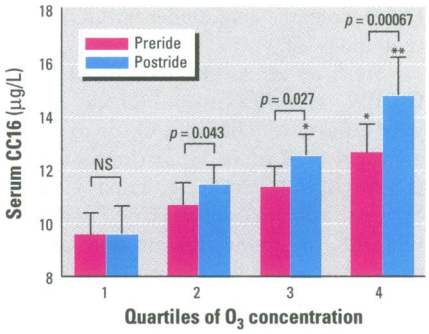

Figure 1. Serum Clara cell protein in cyclists before (preride) and after (postride) a 2-hr ride in Parma (Italy). NS, not significant. The preride serum concentrations of CC16 have been adjusted for a value of 1 mg/L cystatin C on the basis of the slope derived from the multiple regression analysis. Values of CC16 are given as mean ± SE. Quartiles correspond to the following ranges of O_3 concentrations: quartile 1, 0.0325–0.0595; quartile 2, 0.0605–0.084; quartile 3, 0.084–0.0925; and quartile 4, 0.0925–0.103 ppm. Mean O_3 concentrations of the quartiles were 0.048, 0.072, 0.089, and 0.096 ppm, respectively. $n = 12$ riders for each quartile. The *p*-values refer to the comparison of pre- and postride concentrations by the paired samples Student's *t*-test.
Asterisks indicate means that are significantly different from the first quartile. *$p < 0.05$. **$p < 0.01$.

Results

After the ride, the mean ± SE serum concentration of CC16 was significantly increased in both men (12.3 ± 0.9 vs. 11.2 ± 0.8 µg/L, $n = 18$, $p = 0.011$) and women (11.9 ± 1.3 vs. 11.1 ± 0.6 µg/L, $n = 30$, $p = 0.012$). In contrast, pre- and postride concentrations of serum cystatin C were similar (1.22 ± 0.22 vs. 1.20 ± 0.21 mg/L, $n = 48$, $p = 0.162$). Stepwise regression analysis of all data shows that the increase in serum CC16 during the ride (i.e., the difference between post- and preride concentrations) was independent of sex and of cystatin C variations in serum ($r^2 = 0.01$, $p = 0.41$), but correlated with the O_3 concentrations ($r^2 = 0.18$, $p = 0.0024$). We found an even more significant correlation between O_3 levels and the postride concentrations of CC16 in serum ($r^2 = 0.29$, $p < 0.0001$). Interestingly, O_3 levels were also correlated with preride serum CC16 concentrations ($r^2 = 0.13$, $p = 0.011$), most probably because the first blood sample was collected when subjects had been exposed to O_3 before the exercise. These associations were not confounded by the renal function because serum cystatin C emerged as a significant determinant only for the preride serum CC16 levels ($r^2 = 0.1$, $p = 0.02$).

To examine dose–effect relationships, we divided the subjects into quartiles of increasing O_3 levels. Both pre- and postride concentrations showed an exposure-related trend; the rise over the first quartile is significant from the fourth and third quartile onward, for the pre- and postride, respectively (Figure 1). Post-ride CC16 in the fourth quartile was increased by 53% on average as compared to the first quartile. When we compared pre- and postvalues of serum CC16 within each quartile, the postride elevation of serum CC16 was statistically significant from the second quartile, corresponding to O_3 levels between 0.060 and 0.084 ppm.

The comparison of lung function performances before and after the ride on the whole population did not reveal any significant decrement in the FEV_1 and FVC, which are the parameters classically impaired by O_3. However, the $MEF_{75\%}$ ($p = 0.021$) and PEF ($p = 0.006$) were slightly decreased. We also found significant correlations between O_3 concentrations and decreases in FEV_1 ($r^2 = 0.144$, $p = 0.008$), FVC ($r^2 = 0.162$, $p = 0.005$), $MEF_{50\%}$ ($r^2 = 0.142$, $p = 0.032$), and $MEF_{75\%}$ ($r^2 = 0.09$, $p = 0.037$). When the subjects were classified in quartiles of O_3 levels, we found significant decreases in several lung function parameters in quartile 3 (FVC, FEV_1, $MEF_{50\%}$, $MEF_{75\%}$, and PEF) and/or quartile 4 (FVC, $MEF_{75\%}$, and PEF) (Table 1). However, changes in lung function parameters were not correlated with those in serum CC16 levels ($r^2 = 0.05$, $p > 0.15$).

We confirmed the ability of O_3 to alter the lung epithelial barrier in animals at ambient air levels. Female C57Bl/6 mice were exposed to 0.08 ppm O_3 for 4 and 8 hr. We determined CC16 in serum and bronchoalveolar lavage together with classical indicators of lung injury. As shown in Figure 2, O_3 produced an increase in serum CC16 that was already statistically significant after 4 hr of exposure. After 8 hr, this increase was more pronounced and was accompanied by an influx of albumin and of PMNs in BALF. At this stage, the inflammatory response was associated with an enhanced bidirectional leakage of proteins across the pulmonary epithelial barrier, which, by light microscopy, appears morphologically intact (results not shown). The level of CC16 in BALF was unchanged.

Discussion

Our study shows that in both humans and mice, short-term exposures to ambient levels of O_3 induce an early increase of serum CC16 occurring before most other manifestations of lung toxicity. Because CC16 is synthesized and secreted almost exclusively by the lung Clara cells, its elevation in serum

Table 1. Lung function parameters in cyclists before (preride) and after (postride) a 2-hr ride in Parma (Italy) according to quartiles[a] of O_3 concentrations.

Lung function parameter	Q1 ($n = 12$)		Q2 ($n = 12$)		Q3 ($n = 12$)		Q4 ($n = 12$)	
	Preride Mean ± SE	Postride Mean ± SE	Preride Mean ± SE	Postride Mean ± SE	Preride Mean ± SE	Postride Mean ± SE	Preride Mean ± SE	Postride Mean ± SE
FEV_1	3.82 ± 0.22	3.85 ± 0.23	3.81 ± 0.21	3.84 ± 0.58	4.07 ± 0.23	3.99 ± 0.23*	3.70 ± 0.17	3.64 ± 0.19
FVC	4.55 ± 0.27	4.60 ± 0.28	4.58 ± 0.33	4.59 ± 0.31	4.89 ± 0.30	4.84 ± 0.30*	4.53 ± 0.24	4.41 ± 0.26*
PEF	9.54 ± 0.80	9.58 ± 0.75	8.26 ± 0.47	8.12 ± 0.52	10.2 ± 0.65	9.34 ± 0.62*	8.10 ± 0.61	7.83 ± 0.65*
$MEF_{25\%}$	1.86 ± 0.15	1.85 ± 0.17	2.02 ± 0.11	2.02 ± 0.11	1.89 ± 0.14	1.89 ± 0.13	1.86 ± 0.12	1.85 ± 0.13
$MEF_{50\%}$	4.72 ± 0.35	4.85 ± 0.32	4.60 ± 0.21	4.65 ± 0.21	5.26 ± 0.29	5.02 ± 0.30**	4.42 ± 0.30	4.32 ± 0.23
$MEF_{75\%}$	8.69 ± 0.69	8.71 ± 0.75	7.21 ± 0.33	7.24 ± 0.35	9.00 ± 0.56	8.68 ± 0.48*	7.18 ± 0.51	6.81 ± 0.48*

[a]Quartiles correspond to the following ranges of O_3 concentrations: Q1, 0.0325–0.0595 ppm; Q2, 0.0605–0.084 ppm; Q3, 0.084–0.0925 ppm; and Q4, 0.0925–0.103 ppm. Pre- and postride values are compared by the paired Student's *t*-test. *$p < 0.05$. **$p < 0.01$.

can be explained only by assuming an increased intravascular leakage of the protein across the lung epithelial barrier (*5–7,16*). Another possible explanation is a reduced renal clearance, but such a mechanism can be formally ruled out because in our study the renal function was not impaired by the exercise or by O_3. The possibility of an increased synthesis of CC16 causing the elevation of CC16 in serum can also be excluded because the levels of CC16 in BALF in mice were unaffected by O_3 exposure.

As reviewed recently by Hermans and Bernard (*7*), there is now ample evidence that protein transfer across the lung epithelial barrier occurs mainly by passive diffusion through water-filled porous channels in the tight junctions. The increased permeation of proteins across the air–blood barrier observed in lung injury most likely results from a loss of the size selectivity of the epithelial barrier due to an enlargement of paracellular pores or to the appearance of nonrestrictive transepithelial leaks. Alternate mechanisms such as basolateral secretion or transcellular passage, which have been invoked to account for the increased transepithelial flux of proteins, have not received experimental support. The intravascular leakage mechanism for the elevation of serum CC16 is fully consistent with the current understanding of the epithelial toxicity of O_3, considering that the primary effect of O_3 on the lung epithelium is an increased permeability due to the enlargement or the formation of intercellular channels (*9,17,18*). In mice exposed to 0.08 ppm O_3, loss of the size selectivity of the lung epithelial barrier was confirmed by an elevation of albumin in BALF, which is classically interpreted as an evidence of increased permeability to proteins.

In cyclists, both the pre- and postride elevations of serum CC16 showed very significant correlations with the O_3 concentrations in air. These correlations were much higher than those emerging between function deficits and O_3. The association with O_3 was particularly remarkable with the postride values of serum CC16, when the delivered dose of O_3 was increased by both the exercise and the higher ambient O_3 levels in the afternoon. By contrast, no association was found between the increase of serum CC16 and lung function deficits. This is not surprising because at moderate O_3 levels, lung function decrements do not correlate with inflammatory changes in BALF, which usually occur earlier (*19–22*). This earlier increase of epithelial barrier permeability also clearly emerges from our study because the rise of serum CC16 was statistically significant in quartile 2 (0.0605–0.084 ppm), in contrast to lung function changes, which were significantly altered only from quartile 3 (0.084–0.0925 ppm) or 4 (0.0925–0.103 ppm) (Figure 1). However, the most remarkable finding was that serum CC16 in mice shows a sensitivity to O_3 almost identical to that observed in humans. In both species, the protein rose in serum after only a few hours of exposure to average O_3 levels around 0.08 ppm (Figure 2). This indicates that the human lung responds to O_3 with nearly the same sensitivity as the lung of the C57Bl/6 mouse, a mouse strain among the most sensitive to this air pollutant (*23*).

Although the exact mechanisms governing the transepithelial passage of CC16 are still poorly understood, we think that the higher sensitivity of serum CC16 to a disruption of the lung epithelial barrier in comparison with BALF albumin mainly stems from the differences in the concentration gradients that drive the diffusion of these proteins across the bronchoalveolar–blood barrier. Figure 3 illustrates schematically the transepithelial leakage of CC16 and albumin across a terminal bronchiole, the principal site of CC16 secretion (*7*) and also of injury by O_3 (*9,24*). In normal lung (Figure 3A), the epithelium is the main barrier hindering the bidirectional air–blood exchange of proteins; the endothelium offers some resistance only to the passage of proteins the size of albumin or larger (interstitial fluid/plasma albumin ratio estimated at 0.5–0.6) (*25,26*). Proteins entering the lung interstitium are constantly removed by lymphatic drainage. After acute exposure to O_3 (Figure 3B), only the epithelium is damaged; the endothelium remains intact up to levels of 0.7 ppm O_3 (*27*). Under these circumstances, we believe that the leakage of serum CC16 across the epithelial barrier occurs first because it is greatly facilitated by the huge transepithelial concentration gradient of CC16 (around 5,000) as compared to that of albumin (around 2). These gradients are presumably related to the different sizes of the compartments in which leaking proteins are diluted (epithelial lining fluid, 20 mL; CC16 distribution space, 40 L). The small size of CC16 might conceivably make this protein more sensitive than albumin to a slight enlargement of the transepithelial protein pathways induced by O_3.

Our study is the first to demonstrate an increased epithelial barrier permeability in humans exposed to ambient O_3, by applying a new noninvasive test in a field study. So far, such an effect has been reported only in animals and humans after controlled exposures to O_3, in terms of elevation of albumin or total protein concentrations in BALF. In humans, the lowest exposure level of O_3 at which this alteration of the epithelial permeability has been observed is 0.1 ppm for 6.6 hr under conditions of moderate exercise (*28*). In animals, increased permeability occurs from threshold concentrations between 0.1 and 0.2 ppm O_3 (*24*). We found an increased airway permeability in moderately

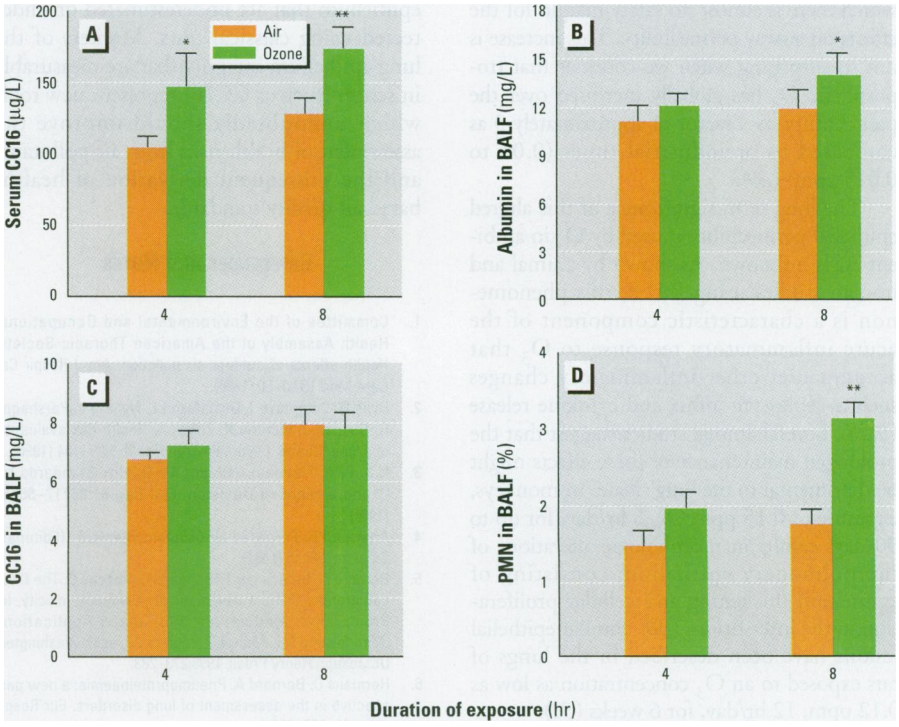

Figure 2. Concentration of Clara cell protein in serum and levels of CC16, albumin, and PMNs in BALF. Bars represent mean ± SE of 6–7 animals.
*$p < 0.05$. **$p < 0.01$.

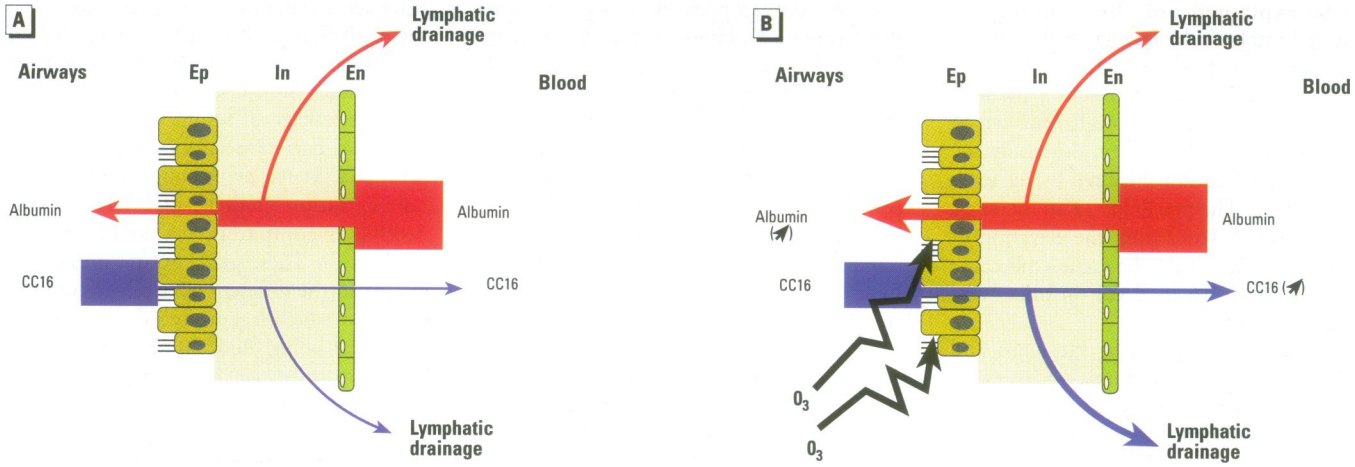

Figure 3. Schematic representation of the passage of albumin and CC16 across the different barriers separating the airways from the blood at the level of a terminal bronchiole (*A*) under normal conditions or (*B*) after acute exposure to O_3. Abbreviations: En, endothelium; Ep, epithelium; In, interstitium. The thickness of the arrows is not proportional to the concentration of the proteins but is used to illustrate the relative permeabilities of the different barriers and the increased fluxes caused by O_3 exposure. The concentrations of albumin in the epithelial lining fluid and blood estimated in normal subjects are approximately 3.5 and 20 g/L, respectively (*24*). The corresponding values for CC16 are approximately 100 mg/L and 15 µg/L, assuming a free exchange of the protein across the endothelium (*7*). For CC16, acute exposure to O_3 (*B*) causes an increased permeability of the epithelial barrier to proteins (*9,17*), resulting in an increased leakage of plasma albumin in the airways and, in the opposite direction, of CC16 into the interstitium from which it is cleared by lymphatic drainage or directly in the blood across the endothelium.

exercising subjects exposed on average to 0.07 ppm O_3 during 2 hr. This level, which is below the new 8-hr standard for O_3 in the United States (*3*), is in the range of the maximum natural levels of ambient O_3 that can be encountered in clean nonurban areas of the United States during the summer season (*29*). These findings are disturbing because they suggest that natural background concentrations of O_3 have now reached levels above which there is almost no safety margin for the effects on airway permeability. This increase is not so surprising when we consider that tropospheric O_3 has globally increased over the past century by a factor of approximately 3 as compared to preindustrial times (0.01 to 0.015 ppm) (*29*).

The long-term significance of this altered epithelial permeability caused by O_3 in ambient air is unknown. As shown by animal and human studies using BALF, this phenomenon is a characteristic component of the acute inflammatory response to O_3 that accompanies other inflammatory changes such as leukocyte influx and cytokine release (*9,28*). Several animal studies suggest that the prolonged maintenance of these effects might be detrimental to the lung tissue. In monkeys, exposure to 0.15 ppm O_3, 8 hr/day, for up to 90 days results in morphologic alterations of the pulmonary epithelium consisting of epithelium thickening and cellular proliferation in the interstitium (*30*). Similar epithelial lesions have been described in the lungs of rats exposed to an O_3 concentration as low as 0.12 ppm, 12 hr/day, for 6 weeks (*31*).

In summary, the application of a new noninvasive test to evaluate the permeability of the lung epithelial barrier shows that the pulmonary epithelium is much more sensitive to O_3 oxidative stress than suggested by previous studies. Alterations of the airway epithelium resulting in an increased leakage of lung proteins into the bloodstream were observed after only 2 hr in moderately exercising subjects exposed to ambient O_3 levels below the new NAAQS standard for O_3 (*3*). These observations indicate that air pollutants can produce effects on the pulmonary epithelium that are underestimated or undetected using classical tests. Markers of the lung epithelium integrity that are measurable in serum (such as CC16) represent new tools which undoubtedly should improve the assessment of health risks from air pollutants and the subsequent derivation of health-based air quality standards.

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