

Cytogenetic damage in buccal epithelia and peripheral lymphocytes of young healthy individuals exposed to ozone

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Ozone (O₃) is an important component of air pollution and a potent oxidant of biomolecules. To address the hypothesis that elevated ambient O₃ can induce cytogenetic damage in healthy people, we collected buccal cells from two groups of students (N = 126) from University of California, Berkeley, in the spring and again in the fall. One group spent their summer in the Los Angeles (LA) area where summer O₃ concentrations are significantly higher than in the San Francisco Bay (SF) area, and another remained in SF. During the school year, all students were exposed to low O₃ levels in SF. The micronucleus assay in a total of 611 000 buccal cells demonstrated that, in the fall, micronuclei (MN) in normal cells for the LA group had increased 39% relative to levels in the spring (1.52 and 0.87 MN/1000 cells, respectively, P = 0.001). Students who spent the summer in SF had a 12.7% increase (P = 0.48). A similar effect of season was seen in degenerated buccal cells for the LA group (3.23 versus 1.88 MN/1000 cells, P = 0.003). LA but not SF subjects also had more degenerated cells in the fall sample (P = 0.003). These findings were paralleled by an increase in MN and nucleoplasmic bridges in lymphocytes and MN in buccal cells in a sub-group of 15 students who underwent a 4-h controlled exposure to 200 p.p.b. O₃. This cytogenetic evidence, along with recent studies linking O₃ exposure to elevated lung cancer risk and mortality, suggest potential public health implications from exposures to high oxidant environments.

Introduction

Ozone (O₃), a major constituent of ambient air pollution, is formed when air pollutants (hydrocarbons and nitrogen oxides) react with oxygen in the presence of sunlight. In the USA, O₃ levels typically rise during the period from May through September, when higher temperatures and sunlight favour O₃ production (1). With the enactment of clean air legislation over the past 20 years, overall ambient O₃ levels have declined, although ~100 million Americans currently live in areas that exceed the air-quality standard for O₃ (85 p.p.b. averaged over 8 h), with the highest levels in the USA reported from California and along the East Coast (<http://www.epa.gov/air/data/index.html>) (2). Over the past two decades, the California South Coast Air Basin averaged 50 days above the state 1-h limit of 90 p.p.b. (3).

O₃ is a potent oxidant of biomolecules. It is extremely reactive and when inhaled is consumed almost entirely in the respiratory tract lining fluid (RTLF) (4). O₃ is degraded quickly into molecular oxygen and oxygen free radicals, which, in turn, combine with water to form highly oxidative hydroxyl radicals that react with nucleic acids, lipids and proteins (5). The peroxidation of lipids and proteins in the RTLF leads to an ongoing chain reaction that generates other reactive oxygen species (6). In addition, the inflammatory response of neutrophils and macrophages to oxidative injury leads to further production of reactive radical species (7). The principal sites of O₃-induced injury are in the proximal conducting airways and the distal small airways of the lungs (8).

The contribution of air pollutant exposure to airway epithelial injury is well documented (9). Increased levels of inflammatory and metaplastic changes were found in the nasal epithelia of children living in Mexico City, which has an atmosphere with a complex mix of air pollutants that includes high concentrations of O₃ (10). An age-related increase in DNA damage has been demonstrated by comet assay (11). DNA strand breaks in the nasal epithelia of newly relocated individuals to Mexico City increased within two weeks of arrival to the city (12). Histopathological changes also were found in individuals after their relocation to Mexico City from low pollution areas. Short-term exposure (<30 days) was associated with loss of normal epithelia, basal cell hyperplasia and mild dysplasia. Individuals exposed for >60 days showed severe loss of normal respiratory epithelia and squamous metaplasia (13). These results suggest that DNA damage in the nasal passage was initiated shortly after arrival to a highly polluted area and remained for the duration of an individual's stay in that environment. An increased level of chromosome aberrations in human lymphocytes has been demonstrated in response to O₃ exposure in controlled experiments and natural settings (14). Enhanced DNA fragmentation was observed by comet assay in the nasal mucosa of individuals who lived in a highly polluted area of Italy compared with those who lived in a low pollution area (15). However, Valverde *et al.* (16) reported no significant difference in DNA damage in buccal cells between exposed and non-exposed subjects using the comet assay.

These studies provide convincing, but not unequivocal, evidence that air pollution, specifically high ambient O₃, can cause DNA fragmentation and histological changes in children and adults exposed to air pollution. However, there is insufficient data as to whether cytogenetic damage is associated with relatively common seasonal changes in ozone levels in chronically exposed young adults.

We chose the end-points of the micronuclei (MN) assay as a primary measure of cytogenetic damage, because this method has proven informative for monitoring genetic damage in blood and exfoliated buccal cells in humans (17). Additionally, MN

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can be analysed in non-dividing cells, which include oral epithelia exposed directly to ozone. Exfoliated buccal cells can be collected non-invasively, which presents a significant advantage for population studies. Association of high MN frequency with elevated risk of cancer has been recently confirmed in a large prospective study (S. Bonassi, personal communication). MN are formed by the condensation of acentric chromosomal fragments or as a result of whole chromosomes lagging behind during cell division (18). Increased MN frequencies have been found in bladder, buccal, nasal, sputum and cervical epithelial cells in adults exposed to tobacco, arsenic, chromium, formaldehyde, paints, air pollution and other environmental factors (19,20). Most studies of children exposed to genotoxic agents observed elevated levels of MN (21). Mexico City residents exposed to high levels of ambient air pollution had a 30% higher MN frequency in nasal epithelia than controls (22). Recently, we reported that regional O₃ levels, highly correlated with season, were strongly associated with MN frequencies in buccal cells and lymphocytes of African-American children and their mothers from Oakland, CA (23). Additionally, MN frequencies were modestly associated with individual measures of traffic pollution in children of this cohort.

Here, we use a longitudinal study approach to explore the role of O₃ exposure in induction of cytogenetic damage in young, healthy students from the University of California, Berkeley (UCB), chronically exposed to different seasonal and geographic levels of O₃ before and after summer vacation in either the LA or the SF area, or acutely exposed to O₃ in a controlled environment. This is part of a study examining the effect of long-term O₃ exposure on lung function in adolescents (24).

Materials and methods

Longitudinal cohort study

The overall design of the study has been presented in detail by Tager *et al.* (24). Briefly, participants were first-year undergraduates at UCB who were recruited in three waves of enrollment between February and the first week of June for each of the years 2000–2002 (total $N = 249$). Eligibility was based on the following: (i) lifelong residence in either the LA or the SF area, (ii) lifetime never-smoking status, (iii) no history of chronic respiratory disease and (iv) no physical impairment that would hinder performance of spirometry. All subjects were screened to confirm that they did not have dental or other X-ray exposures in the three weeks before two buccal cell collections. The first took place during spring enrollment, a period of low O₃ exposure, when all students spent most of their time in the vicinity of UCB. During this visit, complete residential histories were obtained along with general respiratory illness, environmental exposure and dietary histories. Additionally, skin prick tests, anthropometry assessments and spirometry were performed. A second visit occurred in late August, 'fall', in the first 8–12 days of each subject's return from summer-time residences in LA and SF to UCB. During this visit, a complete history of summer-time residences was obtained. Out of the 249 enrolled students, 126 subjects provided buccal cell samples for both collection periods, since the decision to collect the spring sample was made after ~50% of the target cohort was recruited.

Written informed consent was obtained from all subjects. The Committee of the Protection of Human Subjects, UCB, and the Committee on Human Research, University of California, San Francisco, approved all protocols for this study.

Acute, controlled ozone exposure sub-study

Sixty-nine subjects from the larger group were eligible to participate in an acute, controlled chamber exposure to O₃ based on the forced expiratory flow (FEF) between the 25th and 75th percentage volume points of the time–volume curve (FEF_{25–75}) whose values fell in the upper or lower 10th percentile of the distribution of all subject values. Fifteen subjects agreed to participate and provided peripheral blood before exposure and 18 h after exposure. Buccal cells were collected before and 9–10 days after exposure following previously described procedures (25).

Exposure assessment

The details for the assessment of lifetime exposure to O₃ have been described in detail previously (26). A brief description of the method is provided in the online supplement. Estimates of chronic exposure to O₃ prior to each buccal cell collection were calculated on the basis of the 8-h maximum O₃ concentrations 7–14 and 30 days before the date of each buccal collection and moving averages of length 2–7 days, 7–14 days before buccal collection. Exfoliated buccal cells have an ~7–14 day cycle from basal layer to exfoliation (27,28). Thus, a window of 7–14 days was chosen to represent the relevant period of O₃-related oxidant stress to which these cells have been exposed in the basal layer of the oral epithelia.

The protocol for acute exposure has been described in detail elsewhere (29). Briefly, subjects were exposed to 200 p.p.b. O₃ in a chamber (Model W00327-3R, Nor-Lake, Inc., Hudson, WI) filled with filtered air at 20°C and 50% relative humidity. The O₃ was generated from a corona discharge O₃ generator (Model T 408, Polymetrics, Inc., San Jose, CA) and was analysed every 30 s with an ultraviolet light photometer (Model 1008 PC, Dasibi, Glendale, CA). Subjects exercised for 30 min of each hour on a treadmill (Model M9.1, PrecorCo-, Bothell, WA) or a cycle ergometer (Model 9081Bd, Monark, Varberg, Sweden), which were adjusted to produce a targeted ventilatory rate (V_E) of 25 l/min/m² body surface area.

Cytogenetic analysis

Methods of cell collection, processing and scoring criteria for exfoliated cells and peripheral lymphocytes have been described previously (30). Four slides of buccal cells were prepared per subject and stained by May-Grunwald-Giesma. For the longitudinal cohort, 1000 normal cells were scored for each of two slides. Thus, each subject had at least 2000 normal plus all degenerated cells that were found in the same scoring area (~500 degenerated cells or 15–20% of the total cells scored for spring or fall). MN analysis was done separately in normal and degenerated cells to compare respective levels of cytogenetic damage. All subjects in the acute exposure sub-study had ~3000 normal cells and 500 degenerated cells scored for each time point. Additionally, on the basis of criteria suggested by Tolbert *et al.* (31), degenerated cells in the acute sub-group were differentiated into karyolysis, karyorrhexis, pycnotic cells and 'broken eggs' and uneven division as described in ref. (25).

One-thousand binucleated (BN) cells were scored from slides prepared from isolated lymphocyte cultures established from peripheral blood collected before and 18 h after controlled O₃ exposure. Frequencies of MN and nucleoplasmic bridges (NB) were used to assess cytogenetic damage, and replicative index (RI) was calculated to measure cell proliferation in lymphocyte cultures. Before scoring, slides were mixed and coded so that the time and location of collection were randomized. All questionable MN and cell types were double-checked by two of the authors (N.H. and C.C.). Approximately 10% of the slides were re-scored to assure reproducibility. Variability of results did not exceed 25%, which is consistent with criteria of the International Micronucleus Project (32).

Statistical analysis

Comparison of cytogenetic end-points (MN/total cells, MN/1000 normal cells, MN/1000 degenerated cells, percent degenerated cells) for each subject between fall and spring were compared with Student's paired, two-sample *t*-test or the Wilcoxon's signed rank test for paired data. Univariate summaries were prepared for MN/total cells, MN/1000 normal cells, MN/1000 degenerated cells and percent of degenerated cells for all collection times. Spearman rank correlations were used to evaluate the relation between cytogenetic end-points with O₃ levels seven days before collection and estimates of lifetime exposure for each individual generated as previously described by Kunzli *et al.* (33).

For the acute exposure sub-study, the Student's paired two-sample *t*-test or the Wilcoxon's signed rank test was used to evaluate the same four MN end-points in exfoliated buccal cells as noted above for the chronic cohort as well as for several end-points for the MN assay in lymphocytes (MN frequencies per thousand mono-, bi-, tri-, tetranucleated cells; frequency of NB, and RI). Pearson correlation coefficients (*r*) were used to examine the relation between cytogenetic damage in the two cell types, lymphocytes and exfoliated buccal cells.

Lymphocyte MN and NB data were analysed further with adjusted associations based on a Proc Logistic of SAS (v8.2) with inclusion of subject (to account for replicates at each time point) and an interaction with pre- and post-controlled exposure status. For the RI in lymphocytes and the percentage of degenerated buccal cells, Proc GLM of SAS with repeated measures was employed. GEE with Poisson link was used for all count outcomes. Robust standard errors were used for statistical inferences.

Table I. MN assay results for exfoliated buccal cells

	Spring ^a		Fall ^a	
	SF Mean (SD)	LA Mean (SD)	SF Mean (SD)	LA Mean (SD)
(a) Chronic exposure cohort ^a				
Total cells scored	143 012	160 742	146 854	160 350
Average cells/subject				
Normal cells	1998.5 (144.6)	2055.9 (217.1)	2185.9 (173.7)	2038.3 (237.0)
Degenerated cells	425.5 (92.5)	417.0 (80.8)	490.0 (94.3)	486.9 (87.7)
Degenerated (%)	17.2 (4.12) ^b	16.90 (3.45) ^c	19.4 (5.63)	19.3 (5.12)
MN/1000 normal cells	0.76 (0.69)	0.70 (0.53) ^c	0.87 (0.91) ^d	1.17 (1.06)
MN/1000 degenerated cells	2.16 (3.22)	1.88 (2.50) ^c	2.46 (2.78)	3.63 (3.88)
	Pre-exposure mean (SD)		Post-exposure mean (SD)	
(b) Sub-Cohort: controlled acute exposure to 200 p.p.b. O ₃ for 15 subjects				
Total cells scored	55 171		41 607	
Average cells/subject				
Normal	3005 (11.1)		3000.4 (15.1)	
Degenerated	627.4 (212.3)		777.9 (166.9)	
Degenerated (%)	18.3 (7.31)		20.6 (3.59)	
MN/1000 normal cells	1.25 (1.60)		0.82 (0.92)	
MN/1000 degenerated cells	2.38 (4.04) ^e		4.21 (2.13)	

^aSeasonal changes in cytogenetic data for two locations in CA with different oxidant environments for 126 subjects. See methods for description of collection periods.

^bDifference between fall and spring within same geographical group ($P = 0.06$).

^cSignificant difference between fall and spring within same geographical group ($P < 0.05$).

^dSignificant difference between SF/LA within the same season ($P = 0.001$).

^eSignificant difference between pre- and post-exposure, paired two-sample t -test ($P < 0.001$).

Results

Longitudinal study of MN in exfoliated buccal cells

Elevated summer-time O₃ exposure, reflected by the fall collection, resulted in the increased frequency of MN cells in oral epithelia compared with baseline spring levels (Table I). Subjects who spent their summer in LA showed a 39% increase in MN in normal cells (spring: 0.7 cells with MN/1000 cells; 95% CI: 0.53–0.88; fall: 1.17 cells with MN/1000 cells, 95% CI: 0.91–1.44; $P = 0.001$, Figure 1A) while those who spent their summer mainly in SF showed only a 12.7% increase (spring: 0.76 cells with MN/1000 cells, 95% CI: 0.57–0.94; fall: 0.87 cells with MN/1000 cells, 95% CI: 0.63–1.11; P -value = 0.48). A strong effect of season was also observed in MN in degenerated cells, which averaged a 2.8-fold higher prevalence of cells with MN than normal epithelial cells, $P < 0.01$ (Table I). MN in degenerated cells increased ~2-fold in the LA group in the fall in comparison with the spring baseline (spring: 1.88 cells with MN/1000 cells, 95% CI: 1.22–2.46; fall: 3.63 cells with MN/1000 cells, 95% CI: 2.66–4.60), while the SF group showed a modest increase (spring: 2.16 cells with MN/1000 cells, 95% CI: 1.33–2.99; fall: 2.46 cells with MN/1000 cells, 95% CI: 1.34–2.79). This change cannot be explained completely by the variation in the prevalence of degenerated cells, which did not differ between SF and LA groups either in the spring (17.2 versus 16.9%, respectively) or in the fall (19.4 versus 19.3%). The overall percentage of degenerated cells in the fall, however, was slightly (12%) higher than that in the spring collection ($P = 0.13$, paired t -test). Finally, the prevalence of total micronucleated cells (normal + degenerated) cells increased significantly following high O₃ summer exposure for the LA group from 0.89 cells with MN/1000 cells (95% CI: 0.68–1.10) in the spring to 1.63 cells with MN/1000 cells

(95% CI: 1.32–1.94) in the fall ($P < 0.001$, t -test). Subjects who spent their summer in SF also showed a modest 17% increase in the frequency of total micronucleated cells from 1.09 cells with MN/1000 cells (95% CI: 0.76–1.17) in the spring to 1.17 cells with MN/1000 cells (95% CI: 0.84–1.34) in the fall. Thus, the level of cytogenetic response was more pronounced in LA subjects compared with SF subjects (Figure 1A). In addition, LA subjects also showed a 25% higher mean MN in degenerated cells in the fall compared with the SF group (2.46 versus 3.63 MN/1000 cells, SF and LA, respectively) ($P = 0.07$).

Formation of multiple MN in normal and degenerated cells can be a separate indicator of induced cytogenetic damage. Between 80 and 94% of all micronucleated buccal cells contained only one micronucleus with no significant variation by season or location. However, the percentage of degenerated cells with multiple MN more than doubled in the LA group after summer exposure to air pollution, from 6.2 (1.6–13.4) in the spring to 13.6% (6.9–19.8) in the fall ($P = 0.007$, two-sample t -test) and was ~2-fold higher than the fall percentage in the SF group [7.7% (1.6–12.2); $P = 0.07$, two-sample t -test].

To further explore links between O₃ exposure and changes in MN frequency, data from air-quality monitoring stations, based on the residence of the subject, were employed. Frequencies of MN in normal or degenerated cells were not correlated with O₃ averaged over number of days (2, 7, 10, 14 and 30) before sample collection nor lifetime O₃ exposure.

Effect of 4-h controlled exposure to 200 p.p.b. O₃

MN frequency in degenerated cells increased 76.9% following exposure to O₃ ($P = 0.04$, paired t -test) (Table I (b), Figure 1A); there was no significant change in normal buccal cells

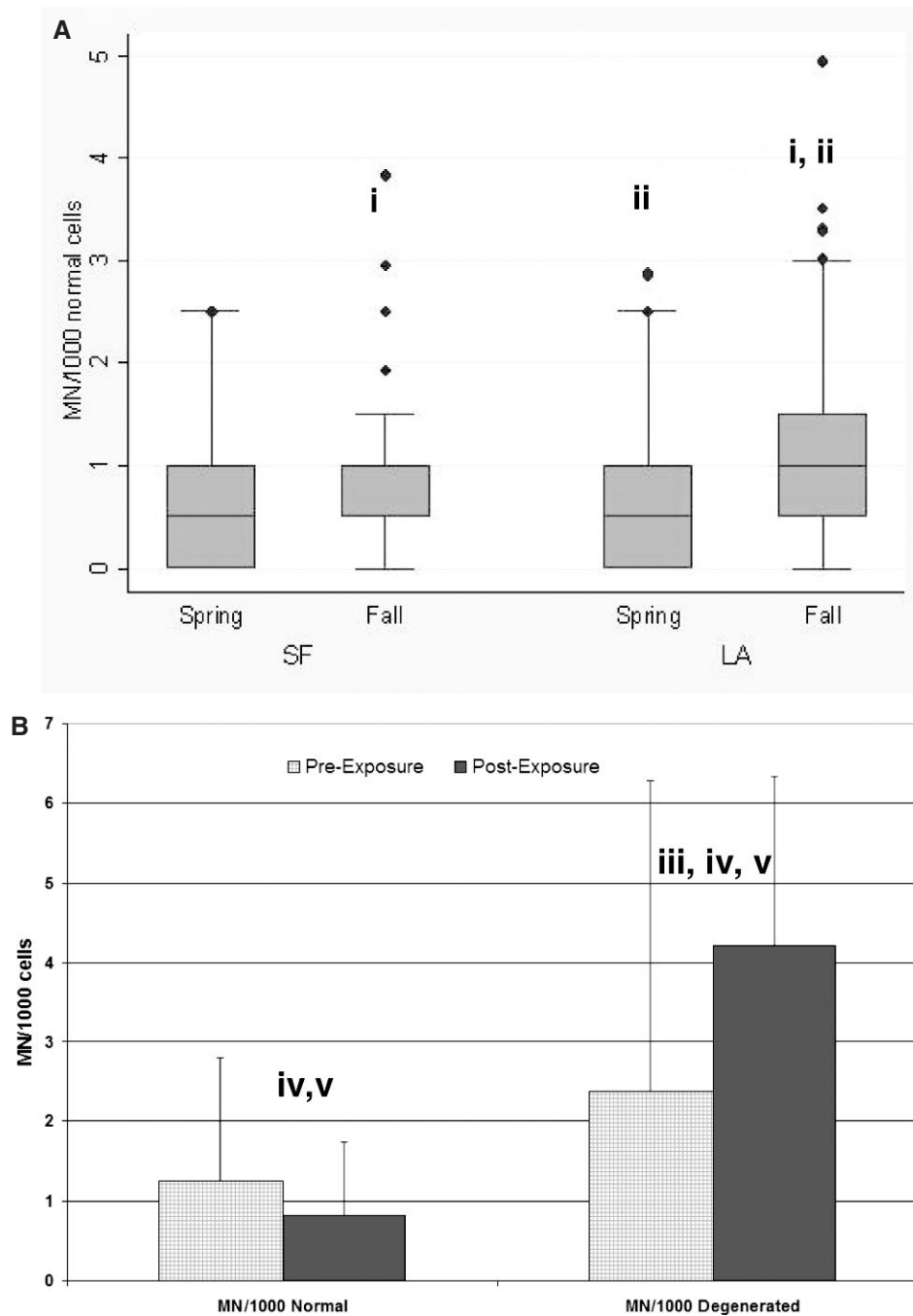


Fig. 1. MN assay in exfoliated buccal cells. (A) Results of normal buccal cells by permanent residence location and season of specimen collection. (B) Results for 15 subjects with controlled, 4-h exposure to 200 p.p.b. O₃. (i) Significant difference comparing MN frequency by season within the same geographical group (spring versus fall), $P < 0.05$ (paired two-sample t -test); (ii) significant difference comparing geographical location within the same season (LA versus SF), $P = 0.04$ (paired two-sample t -test); (iii) significant difference comparing pre- versus post-exposure levels, $P = 0.04$ (paired two-sample t -test); (iv) significant difference comparing normal versus degenerated cells pre-exposure ($P = 0.004$, paired two sample t -test); and (v) significant difference comparing MN frequency in normal versus degenerated cells post-exposure ($P = 0.04$, paired two sample t -test).

(Figure 1B). Similar to the results found in the LA subjects in the chronic exposure cohort after summer exposure to air pollution, the 15 subjects who underwent controlled exposure to O₃ also had a higher MN frequency in degenerated cells (2.4 cells with MN/1000 cells before and 4.2 MN cells/1000 cells after) than in normal cells (1.2 versus 0.8 MN cells/1000 cells, respectively).

The percent of degenerated cells increased slightly after exposure to O₃ from 18.3 (95% CI: 14.9–20.5) before to

20.6% (95% CI: 17.5–23.5) post-exposure ($P = 0.27$, paired t -test). Further analysis revealed that cells with karyorrhexis [58.4 (95% CI: 49.5–68.9) versus 60.4% (95% CI: 50.0–68.2) pre- and post-exposure, respectively] and karyolysis [41.0 (95% CI: 30.5–49.9) versus 37.9% (95% CI: 29.8–48.3) pre- and post-exposure, respectively] were the most common types of degenerated cells, although O₃ exposure did not affect their frequencies. In contrast, pycnotic cells, which reflect cell necrosis, did show a significant increase [0.3 (95%

Table II. Summary of the MN assay in lymphocytes in controlled ozone exposure sub-group

	Pre-exposure		Post-exposure	
	Measure	SD	Measure	SD
Total cells scored	23 297		19 015	
Average BN cells scored/subject	995.23	50.75	936.73	154.07
Measures of cytogenetic damage				
MN/mononucleated cells	2.34	1.78	5.00	6.79
MN/BN cells	5.88	3.75	10.97 ^a	7.95
NB/1000 BN cells	10.44	6.36	18.06 ^b	11.68
Cell proliferation ^c				
Mononucleated cells (%)	41.08	9.15	40.76	16.62
Replicative Index, RI	1.34	0.24	1.49	0.37
Binucleated cells (%)	55.36	6.68	54.19	12.08
Trinucleated cells (%)	1.54	1.43	1.92	1.83
Tetranucleated cells (%)	2.02	3.15	3.12	4.93

^a $P = 0.08$, paired t -test for a difference in MN levels before and after exposure.

^b $P = 0.02$, paired t -test for a difference in NB frequency before and after exposure.

^cPercent cells with different number of nuclei calculated for 500 cells.

CI: -0.1 – 0.5) versus 1.6% (95% CI: 0.9 – 2.7); $P = 0.005$, paired t -test]. Two other types of degenerated cells, ‘broken eggs’ and uneven nuclear division, were rare (0 – 0.15%) and were not affected by controlled O_3 exposure.

Increased cytogenetic damage associated with acute exposure was also observed in cultured peripheral blood lymphocytes of the controlled O_3 -exposed subjects and included an ~ 2 -fold increase in MN in BN lymphocytes, from 5.9 to 11.0 cells with MN/1000 BN cells, $P = 0.08$ (Table II). A small proportion (3%) of BN cells had multiple MN, although there was no change in the frequency of multiple MN before and after O_3 exposure. Mononucleated lymphocytes showed the same pattern of increase in MN following O_3 exposure as BN cells (Table II). However, owing to significant inter-individual variability in MN frequency, this change was not statistically significant ($P = 0.13$). NB primarily reflect chromosomal breakage (18). On average, the NB frequency after O_3 exposure increased 73.0% , from 10.4 (95% CI: 5.9 – 15.3) before to 18.7 NB/1000 BN cells (95% CI: 10.7 – 24.2) after exposure, ($P = 0.02$).

In BN lymphocytes, frequencies of NB and MN were moderately correlated ($r = 0.38$; $P = 0.08$). NB frequency in BN lymphocytes was correlated more strongly with the frequency of MN in buccal cells from the same subjects, both before and after O_3 exposure ($r = 0.74$, $P = 0.02$; $r = 0.62$, $P = 0.05$, respectively). The correlation between MN in buccal cells and MN in BN lymphocytes also was significant post-exposure ($r = 0.62$, $P = 0.05$) but not at the baseline, which suggests that O_3 may have a similar cytogenetic effect in blood lymphocytes and oral epithelial cells.

There was no change in the average proliferation of lymphocytes before or after O_3 exposure as measured by the RI (1.44 versus 1.49 , respectively) (Table II). However, a significant interaction was demonstrated between RI and inter-individual variability, which was largely explained by elevated rate of proliferation in three individuals ($P < 0.05$).

Discussion

The results of this longitudinal study provide suggestive evidence of an association between ambient O_3 and cytogenetic

damage in oral epithelia. Responses seen in the controlled acute exposure sub-cohort further substantiate the hypothesis that O_3 can induce cytogenetic damage in healthy adults, as a similar response was seen in two cell types, blood lymphocytes and buccal cells. Season and residence in a geographic location characterized by elevated exposure to ambient O_3 were identified as important factors in changes in MN frequency. This indicates that, at least for the oral epithelia, effects of a chronic seasonal exposure rather than either a short-term (days) or lifelong O_3 exposure may be more informative for assessment of cytogenetic damage. To our knowledge, this ‘cumulative’ effect of seasonal O_3 exposure has not been shown previously in other longitudinal cytogenetic studies.

Buccal cells are in constant contact with the environment, which suggests that the oral epithelium is an important target site for inhaled toxicants; therefore, it is reasonable to expect that they would exhibit evidence of genotoxicity. Subjects exposed to summer-time O_3 levels in the LA Basin had 39% higher MN levels in normal buccal cells in the fall than in the spring compared with a less significant 13% increase in students exposed to the lower summer-time O_3 concentrations in the Bay area.

Our findings corroborate results of the cross-sectional study in Mexico City, where residents exposed to high levels of air pollution, primarily O_3 , had a 30% higher MN frequency in nasal epithelia compared with controls (22). In another cross-sectional study conducted in Oakland, CA, a significant association between MN frequencies in buccal cells and O_3 levels were observed for mothers and children (23). Thus, MN studies of the effects of ambient pollution, which includes O_3 , complement studies that reported MN induction in traffic policemen and gas-station attendants (19,20). Since O_3 is an outdoor pollutant, the weak correlations we observed between MN levels and ambient O_3 concentrations up to 30 days before the buccal cell collection may be due to home ventilation, time spent outdoors and variation in other components of air pollutants such as NO_x , SO_x and particulates.

Baseline MN frequencies in the chronic exposure cohort (spring) and controlled exposure sub-group (pre-exposure) were similar and ranged from 0.5 to 0.8 MN/1000 normal buccal cells. This is in agreement with the majority of data on the baseline MN frequency in exfoliated epithelial cells in healthy people (34–36).

To the best of our knowledge, no studies of acute O_3 exposure in humans have utilized the MN assay, although a few studies have shown genotoxic effects of hydrogen peroxide (H_2O_2) on human lymphocytes *in vitro* (37–39). The primary toxic cytogenetic effect of O_3 is probably the chromosomal breakage leading to the formation of MN in both buccal cells and lymphocytes and NB in lymphocytes. The fact that NB and MN were highly correlated in our acute O_3 exposure study further supports the hypothesis that NB are the result of dicentric chromosomes being pulled to opposite poles of the cell since ROS-induced DNA breaks form acentric fragments and dicentric chromosomes simultaneously (39).

The use of degenerated cells as one of end-points in the MN assay in exfoliated cells was initially proposed by Tolbert *et al.* (31), and employed in many other studies. It is becoming common to report a prevalence of degenerated cells and also to identify MN frequency. In this context close attention needs to be paid to differentiate MN in degenerated cells from bacteria, fragments of the dyes and fragmentation of the nucleus, which is common for apoptotic cells (19,25,40). Results

presented here show a similar relation between MN and high O₃ levels in normal and degenerated buccal cells. However, MN frequencies were 2–4-fold higher in degenerated cells. Despite the fact that peroxidases present in saliva may inactivate or block the induction of oxidative stress and DNA damage (41), we saw significantly higher frequencies of MN in degenerated cells collected in the fall in both LA and SF groups compared with MN frequencies in the spring. Interestingly, the MN levels in the degenerated cells in the fall in the LA group were similar to those observed in the subset of subjects who underwent acute exposure to O₃ (4.2 versus 3.6 MN/1000 cells, respectively). This is in contrast to our previous report about similarity in the MN levels in normal and degenerated cells (34). This difference can be explained possibly by the complexity of O₃ effects, leading to the formation of MN, which in turn may initiate cell death, or effects of other components of air pollution. Additionally, cell degeneration can occur through other mechanisms, which include apoptosis and necrosis. For example, in our subjects exposed to 200 p.p.b. O₃, there was a change in the proportion of one particular type of degenerated cell, pycnotic cells. Although only 9% of the degenerated cells were pycnotic, the observed 6-fold increase from 0.23 to 1.4 MN/1000 pycnotic cells was statistically significant. Although this suggests that O₃ exposure may initiate cell death in human buccal cells, most probably caused by necrosis, the exact mechanism of pycnosis is still unclear (31).

In addition, we observed a relatively small but statistically significant increase in the presence of degenerated cells in the fall for both SF and LA groups, and a similar increase after acute exposure. However, the latter was not significant because of a smaller number of subjects in the acute sub-group. This observation corroborates data from the residents of Mexico City who also had a significantly higher percentage of degenerated cells than controls (22).

It is noteworthy that a cytogenetic response to inhaled O₃ was observed not only in buccal cells but also in lymphocytes of exposed volunteers, a finding that is probably due to systemic oxidative stress. These results suggest that oxidative stress may play a role in the association of short-term increases in ambient pollutants (42–44) and increases in cardiopulmonary morbidity and mortality (45,46). In particular, a recent study has documented an increased daily mortality associated with summer-time O₃ levels (47).

An increased level of MN may also be a precursor to carcinogenesis (S. Bonassi, personal communication). Casartelli *et al.* (48) have demonstrated that the MN frequency increased from normal mucosa to pre-neoplastic lesions to carcinomas. In addition, 90% of all human cancers arise from epithelial tissues (49). These data provide suggestive evidence to corroborate recent reports of an increased risk of lung cancer in lifetime residents of California, which has relatively high O₃ levels compared with the rest of the country (50,51).

In conclusion, this study confirms that an assessment of cytogenetic damage in buccal epithelia by MN assay is a sensitive approach to monitor the toxic effects of oxidant pollutants in humans. The suggestive evidence of MN induction by summer-time air pollution, characterized by elevated ambient O₃, was observed in a longitudinal study of healthy young adults and was further supported by results from the controlled O₃ chamber study. In addition to published data showing effects of ambient O₃ exposure on DNA damage, common diseases and mortality in humans, cytogenetic data by the

MN assay in human lymphocytes and exfoliated cells also indicate a possibility that high oxidant environments may pose a greater threat to public health than previously thought. Thus, more research into the specific role of O₃ and other components of air pollution in cytogenetic damage in healthy adults and especially in susceptible sub-populations such as children, elderly and asthmatics is warranted.

Supplementary material

Supplementary material is available at *Mutagenesis* online.

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