Ozone-induced Inflammation Is Attenuated with Multiday Exposure

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It is well known that ozone (O_3) causes acute lung inflammation. What is not known is whether there is progression of the inflammatory response in humans with repeated short-term exposures. Our study was designed to test the hypothesis that repeated exposures to a high-ambient concentration of O_3 (0.2 ppm) over several days would cause more inflammation than a single exposure. Fifteen healthy volunteers were exposed in random fashion to 0.2 ppm ozone for 4 h on a single day and to 0.2 ppm O_3 for 4 h on 4 consecutive days while exercising moderately for 30 min of each hour. Pulmonary function tests were obtained immediately before and after each 4-h exposure. Bronchoscopy was performed 20 h after the completion of each exposure arm to obtain bronchoalveolar lavage (BAL) for measurement of markers of inflammation. Our results show initial progression followed by attenuation of the acute physiologic response to O_3 with repeated daily exposures. We found a significant difference in percent change in FEV_1, FVC, and specific airway resistance (SRaw) across the single-day exposure when compared with the change across Day 4 of the 4-d exposure. Bronchial fraction (the first 15 ml of BAL return) and BAL were analyzed for the following end points: total and differential cell counts, total protein, lactate dehydrogenase (LDH), fibronectin, interleukin-6 (IL-6), interleukin-8 (IL-8), and granulocyte-macrophage colony-stimulating factor (GM-CSF). In the bronchial fraction the number of polymorphonuclear cells (PMN)s and fibronectin concentration were significantly decreased after 4-d exposure compared with single-day exposure. In BAL, significant decreases in the number of PMNs, fibronectin, and IL-6 were found after 4-d exposure versus single-day exposure. These results suggest that there is attenuation of the O_3-induced inflammatory response in both proximal airways and distal lung with repeated daily exposures. Christian DL, Chen LL, Scannell CH, Ferrando RE, Welch BS, Balmes JR. Ozone-induced inflammation is attenuated with multiday exposure. AM J RESPIR CRIT CARE MED 1998;158:532–537.

Ozone (O_3) is a major component of urban air pollution which millions of people are periodically exposed to in North America. It is well documented through extensive controlled human exposures that short-term inhalation of O_3 causes dose-dependent decrements in spirometric parameters of lung function, including FEV_1 and FVC, and relatively smaller increases in specific airway resistance (SRaw) (1–4). Several human studies have demonstrated that short-term O_3 exposures capable of causing acute changes in lung function can also cause cellular and biochemical evidence of injury and inflammation in both proximal airways and in the distal lung (5–8). Markers of injury/inflammation that are typically elevated after O_3 exposure are neutrophils, total protein, fibronectin, and lactate dehydrogenase (LDH). The results of studies that have examined the relationship of O_3-induced acute changes in FEV_1 and FVC to subsequent airway inflammation (as much as 18 h after exposure) have shown either no correlation or a negative one (7, 8), although we showed in one study that acute increases in SRaw did correlate with subsequent airway inflammation (9) and Weinmann and coworkers (10) showed a correlation between isovolumetrically calculated $FEF_{25-75\%}$ and BAL fibrinogen concentration. With repeated short-term exposures to O_3, animal studies have consistently shown evidence of attenuation of pulmonary function responses but somewhat conflicting findings regarding airway inflammation and injury (11, 12). Repeated daily exposures to O_3 in humans have caused decrements in lung function on the first and second days of exposure, but with succeeding daily exposure, this physiologic response is attenuated (13, 14). What has not been adequately characterized in humans is the nature of the inflammatory response to O_3 with repeated short-term exposures. Our study was designed to test the hypothesis that repeated daily exposures in humans causes a progression of injury and/or inflammation. We compared the effects of a single 4-h exposure to 0.2 ppm O_3, a high ambient level, with those of repeated daily 4-h exposures to this concentration.

(*Received in original form September 8, 1997 and in revised form January 14, 1998*)

Supported by Contract No. 93-303 from the California Air Resources Board and by the National Center for Research Resources RR00083-35 from the General Clinical Research Center at San Francisco General Hospital.

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Am J Respir Crit Care Med Vol 158. pp 532–537, 1998
Internet address: www.atsjournals.org

TABLE 1

SUBJECT CHARACTERISTICS

Subject No.	Sex	Age (*yr*)	Ht (*cm*)	FEV_1* (*L*)	FEV_1 (*%*)	FVC* (*L*)	FVC (*%*)	SRaw†	PC_{20}‡ (*mg/ml*)
02	M	31	178	5.29	126	6.92	133	5.1	6.1
03	F	34	161	2.89	103	4.21	123	6.1	320
04	F	34	159	2.56	93	3.23	97	2.9	52.8
05	M	31	175	4.69	116	5.40	108	2.9	320
06	F	29	165	3.59	115	4.46	118	4.5	1.6
07	M	29	175	5.25	128	6.34	125	3.5	162.1
09	M	32	171	4.21	109	4.81	101	1.4	320
12	F	37	163	3.80	135	4.34	127	2.1	160
13	M	25	175	4.92	116	6.11	117	3.0	320
15	F	28	171	3.13	97	3.72	94	2.4	101.2
17	M	30	178	4.04	96	4.98	95	2.1	10.6
19	M	25	177	4.58	107	5.75	109	2.4	320
20	M	25	180	4.67	102	5.48	102	1.8	320
23	F	23	166	3.22	101	4.31	111	3.6	20.0
27	M	24	180	5.14	113	5.91	110	2.0	> 25
Mean		29.1	171.6	4.13	110.5	5.06	111.3	3.1	173.9§
SD		4.2	7.1	0.9	12.4	1.04	12.3	1.3	140.5

* Mean of best three of six baseline values on exposure days.
† Mean of five baseline values on exposure days.
‡ Concentration of methacholine required to produce a 20% decrease in FEV_1 from baseline calculated by log linear interpolation.
§ n = 14.

Because large populations reside in areas with high levels of O_3 for extended periods of time, it is important to determine whether progression of O_3-induced airway inflammation occurs with repeated exposures.

METHODS

Subjects

Although 27 subjects were initially enrolled in this study protocol, only 15 completed both single-day and 4-d exposures to O_3. Of the original enrollees, one dropped out because of inability to complete the exercise, two were excused after violating the protocol, four dropped out after the first exposure/bronchoscopy, and five dropped out because of illness and/or scheduling difficulties. This protocol required a substantial time commitment as well as the ability to tolerate repeated periods of exercise. All results are based on the 15 subjects who completed both exposure arms. These healthy, nonsmoking volunteers were informed of the risks of the experimental protocol, and they signed consent forms approved by the Committee on Human Research of the University of California, San Francisco. The subjects, six female and nine male, ranged from 23 to 37 yr of age and specifically denied a history of pulmonary or cardiac disease or respiratory infection within 6 wk of the onset of each exposure sequence. No subject used supplemental vitamins C or E during the study. All of the subjects received financial compensation for their participation. Characteristics of individual study participants are listed in Table 1.

Pulmonary Function Measurements

SRaw was determined as the product of airway resistance and thoracic gas volume, both having been measured in a constant-volume body plethysmograph (Warren E. Collins, Braintree, MA). SRaw was calculated as the average of five measurements taken 30 s apart. Spirometry was performed on a dry rolling-seal spirometer (S400; Spirotech Division, Anderson Instruments, Atlanta, GA). Mean values for FVC and FEV_1 were calculated from three acceptable FVC maneuvers (15) obtained approximately 30 s apart.

Experimental Protocol

After a telephone interview, subjects were scheduled for an initial visit to the laboratory where consent was obtained and a medical history questionnaire was completed. Baseline pulmonary function tests, a methacholine challenge test, and a 15-min exercise test designed to determine a work load that generated the target ventilatory rate of 25 L/min/m^2 body surface area were also completed on the initial visit. The subjects were randomly exposed to 0.2 ppm O_3 for 1 d or for 4 d, with a minimum break of 4 wk between exposure protocols. Each subject had his or her baseline SRaw, FVC, FEV_1, and peak flow measured 5 to 10 min before undergoing each daily exposure. Exposures were for 4 h on each study day, with subjects exercising for the first 30 min and then resting for the following 30 min of each hour. Each subject was given the option to exercise on a treadmill (Model M9.1; Precor Co., Bothell, WA) and/or a cycle ergometer (Model 818E; Monark, Varberg, Sweden). Tidal volume, respiratory rate, and ventilatory rate were measured 10 and 20 min into each 30-min exercise period and the work load was adjusted as needed to maintain the target ventilatory rate. Ventilation was not measured during rest periods; however, peak flow was measured 10 min into each 30-min rest period to monitor the pulmonary status of subjects during the 4-h exposure period. Subjects remained inside the chamber for the entire 4-h exposure period. Symptom questionnaires consisting of a 5-point rating scale (0 = not noticeable to 4 = severe) were administered immediately before and immediately after each exposure period. Subjects rated the following 11 symptoms: anxiety, chest discomfort or chest tightness, chest pain on deep inspiration, cough, eye irritation, headache, nasal irritation, nausea, phlegm or sputum production, shortness of breath, throat irritation, and wheezing.

Exposure Chamber and Atmospheric Monitoring

All exposures took place in a chamber ventilated with filtered air at 20° C and 50% relative humidity to which O_3 was added. The stainless steel and glass chamber, $2.5 \times 2.5 \times 2.4$ m (Model W00327-3R; Nor-Lake, Hudson, WI), was custom-built and designed to maintain chamber temperature and relative humidity within 1.0° C and 2%, respectively, of the set points (DSC 8500; Johnson Controls, Poteau, OK)

TABLE 2

EXPOSURE CHARACTERISTICS*

	1-day Ozone	4-day Ozone
O_3, ppm	0.196 ± 0.003	0.195 ± 0.004
Temperature, °C	20.5 ± 1.7	20.8 ± 1.3
Relative humidity, %	49.9 ± 2.9	50.7 ± 2.1

* Values are mean ± SD.

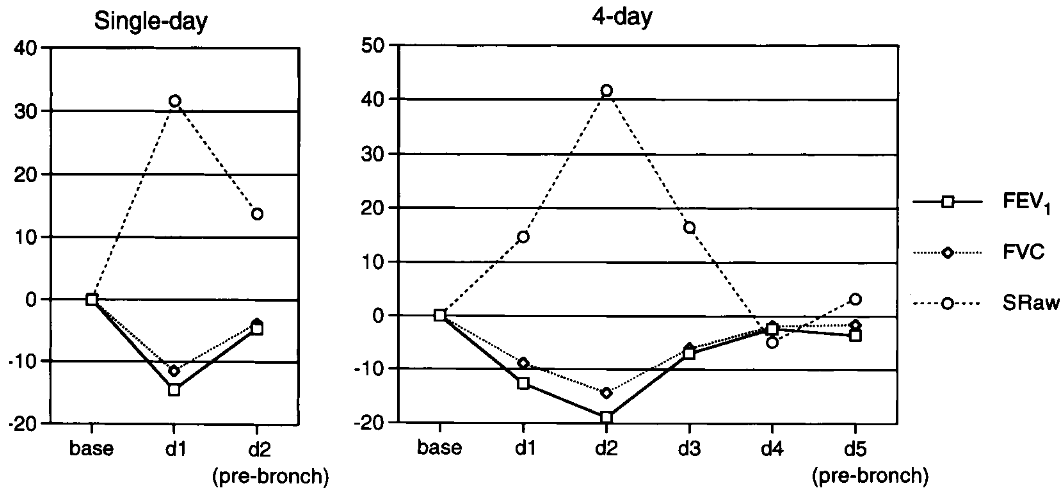

Figure 1. Mean percent changes in pulmonary function values from baseline of Day 1 (zero values) and percent change across each day (preexposure to postexposure) of single-day and 4-d exposure to O_3. Prebronchoscopy data points = percent change calculated from baseline of day prior to bronchoscopy; n = 15.

(16). Relative humidity and temperature were recorded every 30 s and averaged over each exposure.

O_3 was produced with a corona-discharge O_3 generator (Model T 408; Polymetrics, Inc., San Jose, CA) and analyzed with an ultraviolet light photometer (Model 1004AH; Dasibi, Glendale, CA). O_3 concentration was measured every 30 s, displayed in real-time (Labview 2; National Instruments, Austin, TX), and stored by a microcomputer (Model IIsi; Apple Computer Inc., Cupertino, CA). The O_3 analyzer was calibrated biannually with an O_3 transfer standard (Model 1003PC; Dasibi) by the California Air Resources Board and precision-checked on a monthly basis.

Mean ± SD for O_3 concentration, temperature, and percent relative humidity for the two exposure arms are listed in Table 2. Mean exposure conditions were not significantly different on any day, and there were no differences in mean temperature, relative humidity, or O_3 between the single-day exposure and the 4-d exposures.

Bronchoscopic and Lavage Procedures

Bronchoscopies were performed 19.7 ± 0.8 h (SD) after single-day O_3 exposure and 19.9 ± 0.8 h (SD) after 4-d O_3 exposure, in a dedicated room at San Francisco General Hospital. The procedures of bronchoscopy and BAL have been discussed previously in detail (8). Briefly, intravenous access was established, supplemental O_2 was delivered, and the upper airways were anesthetized with topical lidocaine. The bronchoscope (FB 18x; Pentax Precision Instruments Corp., Orangeburg, NY) was introduced through the mouth and into the airways. The bronchoscope was then directed into the right middle lobe where BAL was performed with three 50-ml aliquots of 0.9% saline warmed to 37° C. The first 15 ml of fluid returned from the first 50-ml aliquot was separated and labeled bronchial fraction, whereas the remaining fluid returned was labeled BAL. All lavage samples were immediately put on ice and taken to the laboratory for processing. After bronchoscopy, each subject was transported to the General Clinical Research Center (GCRC) at San Francisco General Hospital for a two-hour or more recovery period.

Total cells were counted on uncentrifuged aliquots of bronchial fraction and BAL using a hemacytometer. Differential cell counts were obtained from slides prepared using a cytocentrifuge (Cytospin 2; Shandon Southern Products, Ltd, Astmoor, UK), 25 *g* × 5 min, and stained with Diff-Quik (American Scientific Products, McGaw Park, IL) as previously described (16). Bronchial fraction and BAL fluids were then centrifuged at 180 *g* for 15 min, and the supernatant was separated and recentrifuged at 1,200 *g* for 15 min to remove any cellular debris prior to freezing at −70° C.

Measurement of Biochemical Constituents of Lavage Fluid

Lavage fluid and biochemical constituents were measured in bronchial fraction and BAL. Lactate dehydrogenase (LDH) was measured within 30 min of lavage time using a commercially available reagent (No. 228-20; Sigma Chemical Co., St. Louis, MO) and a spectrophotometer (DU 65; Beckman Instruments Inc., Fullerton, CA). The other biochemical assays were performed on lavage supernatants that had been frozen at −70° C. Total protein was assayed by a modification of the Lowry procedure (17). Lavage concentrations of fibronectin were determined with an antibody-capture immunoassay, as described by Miles and Hales (18), with minor modifications (16). Interleukin-6

TABLE 3

LAVAGE END POINTS

	Normal Values*	Single-day O_3†	4-day O_3†
Bronchial fraction			
Total leukocytes, $\times 10^4$/ml	12–17	28.9 ± 4.0	32.2 ± 4.4
Macrophages, % leukocytes	90	55.0 ± 3.8	66.9 ± 4.2‡
Neutrophils, % leukocytes	3.5–7.0	35.7 ± 4.3	25.3 ± 4.4‡
Total protein, mg/ml	0.11–0.13	0.224 ± .014	0.226 ± 0.021
LDH, U/L†	12.0–15.0	18.13 ± 1.86	23.65 ± 4.35
Fibronectin, ng/ml	N/A	351.1 ± 82.7	204.8 ± 55.7†
IL-6, pg/ml	1.8	5.9 ± 0.7	5.2 ± 0.7
IL-8, pg/ml	90	202.6 ± 46.1	214.6 ± 41.4
GM-CSF, pg/ml	N/A	2.11 ± 0.31	1.49 ± 0.25
BAL			
Total leukocytes, $\times 10^4$/ml	14–18	23.6 ± 2.6	19.6 ± 1.9‡
Macrophages, % leukocytes	90	76.1 ± 3.0	85.9 ± 2.2‡
Neutrophils, % leukocytes	2.5–4.5	16.4 ± 3.0	7.6 ± 1.3‡
Total protein, mg/ml	0.07–0.11	0.161 ± .012	0.147 ± 0.013
LDH, U/L§	7.0–10.0	11.49 ± 1.37	11.10 ± 1.52
Fibronectin, ng/ml	120	364.3 ± 65.1	205.7 ± 51.1‡
IL-6, pg/ml	2.3	3.6 ± 0.6	2.26 ± 0.3‡
IL-8, pg/ml	36	76.3 ± 25.9	43.4 ± 9.5
GM-CSF, pg/ml	N/A	4.21 ± 0.55	3.43 ± 0.72

Definition of abbreviations: LDH = lactate dehydrogenase; IL-6 and IL-8 = interleukin-6 and -8; GM-CSF = granulocyte-macrophage-colony-stimulating factor.

* Values obtained from previous studies of unexposed, healthy, nonsmoking volunteers in our laboratory.

† Values are means ± SD.

‡ $p < 0.05$.

§ n = 13.

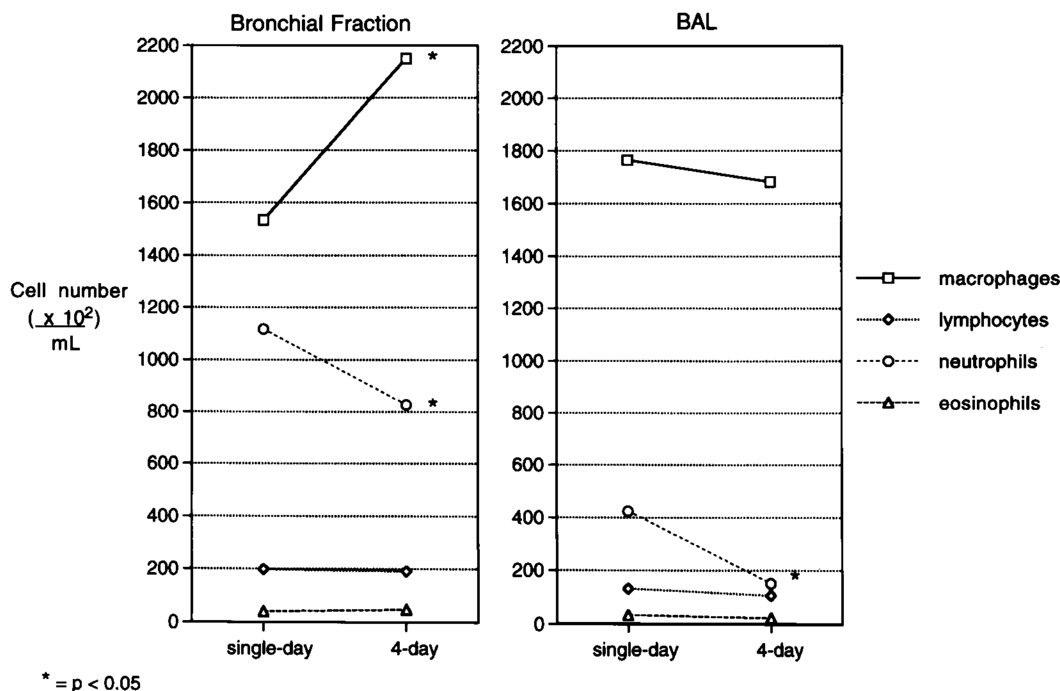

Figure 2. Mean leukocyte counts for bronchial fraction and BAL; n = 15, *asterisks* indicate p < 0.05.

(IL-6), interleukin-8 (IL-8), and GM-CSF were measured with commercially available immunoassays (R&D Systems, Minneapolis, MN).

Statistical Analysis

All data analyses were performed using Statview 4.5 software (Abacus Concepts, Inc., Berkeley, CA) and data were summarized as means ± SD or SE. If the measured variables had a normal distribution, the Student's paired *t* test was used to compare paired data within the 4-d exposures and between the exposure arms. If the variable did not have a normal distribution, Wilcoxon's signed rank test was used for comparisons.

The lower respiratory symptom scores that we analyzed included chest discomfort or chest tightness, chest pain on deep inspiration, cough, phlegm or sputum production, shortness of breath, and wheezing. Symptom score differences were determined by subtracting the preexposure symptom score from the postexposure symptom score. The sum of symptom-score differences across the single-day O_3 exposure versus the sum across Day 4 of the 4-d exposure were compared using

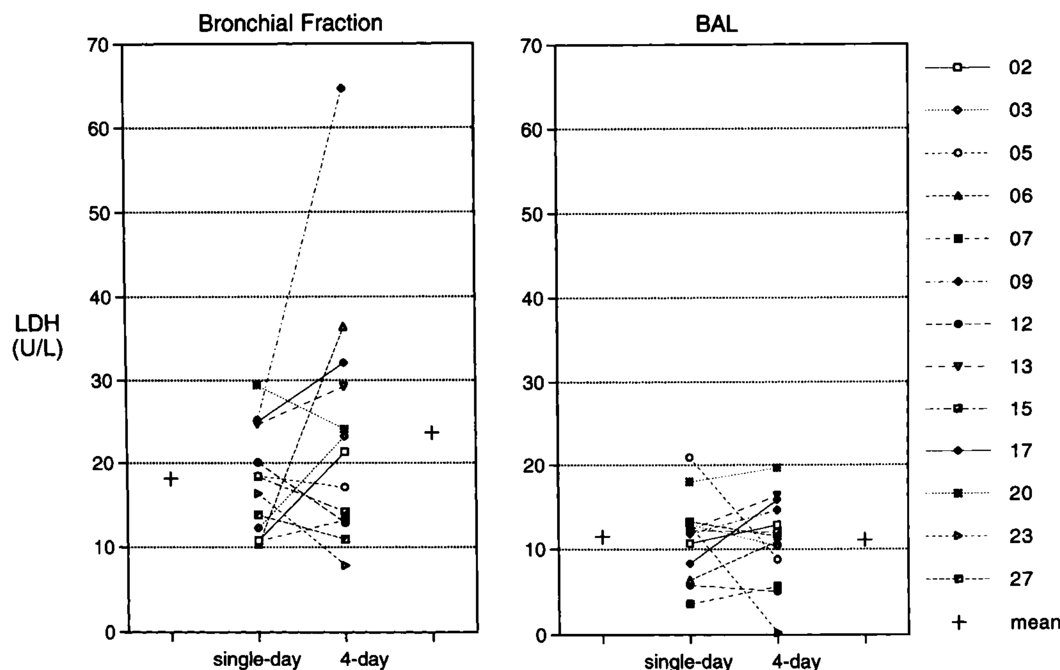

Figure 3. Individual LDH values for subjects from bronchial fraction and BAL after single-day versus 4-d O_3 exposure. Group means are indicated by *plus signs* next to each column of data points; n = 13.

Wilcoxon's signed rank test. A p value of 0.05 was considered statistically significant in all data analyses.

RESULTS

Pulmonary Function Results

The mean percent change across each study day for FEV_1, FVC, and SRaw are shown in Figure 1.

No differences were found in preexposure baseline measurements for each day of the single-day and 4-d arms of the study protocol. As expected, significant decreases ($p < 0.001$) were found in the preexposure to postexposure FEV_1 and FVC across the single-day exposure and Day 1 of the 4-d exposure. Significant preexposure to postexposure decreases in FEV_1 and FVC were also found across Days 2 ($p < 0.0001$) and 3 ($p \leqslant 0.024$), but not across Day 4 ($p \geqslant 0.10$) of the multiday protocol. Although SRaw increased across exposures on Days 1 to 3, none of these increases achieved statistical significance. Significant differences consistent with attenuation were noted when we compared mean percent change in FEV_1 ($p < 0.0001$), FVC ($p < 0.0001$), and SRaw ($p < 0.04$) across Day 1 of the single-day exposure with the mean percent change across Day 4 of the 4-d exposure.

There were no differences in minute ventilation data when comparing means from all exposure days. During the course of each daily 4-h exposure, ventilation patterns changed in response to O_3, with increased respiratory rate and decreased tidal volume, but the subjects still achieved target ventilatory rates.

Symptom Results

The mean change $\pm$ SD in lower respiratory symptom scores across the single-day exposure was 4.7 ± 4.2. In the 4-d exposure arm, the mean changes were 4.3 ± 2.8, 4.7 ± 3.8, 1.9 ± 2.7, and 0.6 ± 1.1, across Days 1 to 4, respectively. The mean lower respiratory score changes on Days 1 and 2 were significantly higher than on Days 3 and 4.

Lavage Fluid Results

The mean bronchial fraction and BAL data $\pm$ SE after single-day and 4-d exposures are shown in Table 3. Leukocyte cell counts for bronchial fraction and BAL are shown in Figure 2.

In the bronchial fraction we found the following significant differences when comparing the results after 4-d exposure to those after single-day exposure: lower number of neutrophils, higher number of macrophages, and a lower fibronectin concentration ($p = 0.03$, 0.03, and 0.002, respectively). In BAL, significantly lower values after 4-d exposure compared with after single-day exposure were found in total cell count, neutrophil count, fibronectin, and IL-6 concentrations ($p = 0.02$, 0.006, 0.01, 0.005, respectively). In addition, a higher percentage of macrophages was found after 4-d exposure ($p = 0.011$). Only one lavage fluid end point showed an increase after 4-d exposure consistent with progressive injury, mean LDH concentration in the bronchial fraction ($p = 0.18$). We graphed individual LDH data to determine if a subset of subjects was responsible for this mean increase in the bronchial fraction (Figure 3). Of the 13 subjects for whom bronchial fraction LDH data were available, seven showed increases (four with $\geqslant$ 2-fold increase).

DISCUSSION

Contrary to our hypothesis, the results of this study did not show progression of the acute inflammatory response to O_3 with repeated short-term exposures. The BAL end points analyzed in our study tended to follow the pattern of attenuation observed previously in the pulmonary function and symptom responses to repeated exposures to O_3. Neutrophils (both in absolute number and as a percentage of total cells), fibronectin, and IL-6 were significantly decreased in BAL after the 4-d exposure. There was lack of attenuation, but no evidence of progressive increases in BAL total protein and LDH. Although there was attenuation of some bronchial fraction end points after the 4-d O_3 exposure (e.g., neutrophils and fibronectin), there was no attenuation of IL-6, IL-8, total protein, and LDH.

When we designed this study, there were no published data available regarding BAL findings in human subjects after multiday O_3 exposure. Recently, however, Devlin and colleagues (19) reported the results of a study comparing lavage end points in 16 subjects after exposure to 0.4 ppm O_3 for 2 h on five consecutive days to those obtained after exposures to filtered air for five consecutive days. The results from this study were compared with the results of previous studies of single-day O_3 exposure by this group. Although the investigators found attenuation of a number of inflammatory end points in BAL, including percentage of neutrophils and levels of IL-6 and fibronectin, there was no attenuation of LDH, IL-8, and total protein responses. Although the different design of the study of Devlin and colleagues precludes direct comparison with ours, the results of the two studies are consistent in showing dampening of O_3-induced neutrophil recruitment to the airways with repeated short-term exposures. The results of the study of Devlin and colleagues also support our finding of persistent O_3-induced airway epithelial injury with multiday exposure.

Attenuation of O_3-induced pulmonary function and BAL neutrophil responses with repeated exposures has been previously reported in animal studies (11, 20). Increased macrophages, which we observed in bronchial fraction, have been demonstrated in rats exposed to O_3 for 7 d (21). Animal data also support our finding of the lack of attenuation of O_3-induced release of LDH with repeated exposures (20). At least two animal studies have shown histologic evidence of progressive tissue injury at the level of the terminal bronchioles after 5 to 7-d of exposure (11, 22).

The major limitation of our study was the relatively small sample size. This is a generic problem in controlled human exposure studies involving bronchoscopy. Despite the small sample size, however, we still had adequate power to detect significant differences in several lavage end points between the single-day and 4-d O_3 exposures. If we had studied more subjects, the trends toward decreases in IL-8 and GM-CSF that were observed in BAL after 4-d exposure as compared with single-day exposure may have reached statistical significance.

In conclusion, this study indicates that although O_3-induced neutrophil recruitment to the respiratory tract is attenuated with repeated short-term exposures, airway epithelial injury may continue to occur. Persistence of such injury may lead to airway remodeling, which has been observed in several animal studies. Our results suggest the need for further investigation of the potential for recurrent or chronic exposures to O_3 to cause structural damage to the respiratory tract in humans.

Acknowledgment: The writers thank Robert Montanti for his assistance with bronchoscopy and Shankar Prasad, M.D., of the South Coast Air Quality Management District, for his ongoing advice and support.

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