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IMPACT OF COEXPOSURE TO OZONE ON THE CARCINOGENIC POTENTIAL OF INHALED CHROMIUM. I. EFFECTS ON RETENTION AND ON EXTRA- AND INTRACELLULAR DISTRIBUTION

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A health hazard to welders is development of lung cancer. It is believed that this is likely due, in part, to the presence in welding fumes of several hexavalent chromium (Cr[VI]) species, whose solubility depends primarily on which process (i.e., manual metal arc verus metal–inert gas) is used. However, inhalation of Cr alone is uncommon in this setting. Thus, an examination of potential contributions from other coinhalants in creating or enhancing conditions whereby inhaled fume-associated Cr (primarily the insoluble forms) may initiate cancer is critical to increasing our understanding and preventing this particular occupational disease. One major chemical species formed and released during welding is ozone (O_3). Though implications of adverse pulmonary effects from individual exposure to Cr or O_3 have been investigated, those from simultaneous exposure are unclear. To begin to address whether the carcinogenic potential of insoluble Cr[VI] agents might be enhanced in hosts inhaling mixtures of Cr and O_3 versus Cr alone, analyses of total lung Cr burden, Cr retention in lung epithelium and interstitium, and potential shifts in lung cell distribution of Cr from the cytoplasm to nuclei were undertaken in F-344 rats exposed nose-only (5 h/d, 5 d/wk for up to 48 wk) to an extrapolated occupationally relevant level of Cr (360 μg Cr/m^3 as calcium chromate) alone and in combination with 0.3 ppm O_3. Overall, there was only a nominal effect from O_3 on Cr retention or on distribution of Cr particles among extracellular sites and within lung cells. However, there were O_3-related effects upon mechanisms for clearing the Cr from the deep lung, specifically at the levels of particle uptake and postphagocytic/endocytic processing by macrophages. This O_3 exposure-related shift in normal pulmonary clearance might potentially increase the health risk in workers exposed to other insoluble or poorly soluble carcinogenic Cr compounds.

Electric arc welding, a commonly used procedure in several major industrial and construction processes, involves high-temperature fusion of work pieces. The technique results in production of a plume containing metal fumes, the nature of which depends upon the specific welding process, along with several

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gases, including ozone (O_3) (Stern et al., 1986; Pedersen et al., 1987; Van der Wal, 1990). The generation of localized, high levels of several biologically active substances presents a potential source of risk to workers. Significant respiratory health problems have been associated with exposure to welding fumes, including pneumoconiosis, chronic bronchitis, metal fume fever, and, of greatest significance, lung cancer (Fleschig, 1988; Sferlazza & Beckett, 1991; Sinomato et al., 1991; Hansen et al., 1996). The increased relative risk of lung cancer among welders is 30–50% compared to the general population or to nonwelding workers in the same industry (IARC, 1990); there is also suggestive evidence that welding involving specific metals increases cancer risk over that observed in the total welding population.

Welding processes involving stainless and high-alloy steels are widely used. One component of fumes derived from these processes that has been implicated in lung cancer is chromium (Cr). There is a correlation between increased cancer risk in welders and increasing length of time since first exposure to such Cr-containing fumes (Becker et al., 1985, 1991; Simonato et al., 1991). Commonly, the Cr present is in its strongly oxidative hexavalent state, Cr(VI) (Pasanen et al., 1986; Stern, 1982; Stern et al., 1986; Gray, 1987), with insoluble forms being more likely to give rise to cancer formation (IARC, 1990; Shi et al., 1999). Overall, the pulmonary toxicity and carcinogenicity of Cr(VI) compounds has been documented in experimentally exposed animals and epidemiological studies of occupationally exposed humans (Cohen et al., 1993; Cohen & Costa, 1998, 1999).

The toxicologic effects in general, and the carcinogenic effect in particular, of Cr might be modulated by the presence of other toxic materials, and clearly one component of welding effluent that may act in this regard is O_3. Exposure to O_3 alone has been shown to result in altered pulmonary morphology, physiology, and biochemistry, including effects on pulmonary macrophages and related particle clearance processes (Bhalla, 1999; Bhalla & Gupta, 2000; Bassett et al., 2000).

Although the pulmonary toxicity from exposure to either Cr or O_3 individually has been investigated, a determination of the effects from simultaneous exposure, as would occur under actual working conditions, is lacking. By altering normal mechanisms by which inhaled and deposited Cr(VI) is handled by the lungs, coinhalation of O_3 may create conditions that could potentially modulate the carcinogenic potential of co-present insoluble Cr-containing agents. These could include alterations in Cr particle retention and/or clearance or in the persistence of the Cr(VI) ion in the hexavalent state. The goal of this study was to begin to examine whether and how O_3 might impact upon the course of Cr carcinogenicity in the lungs by examining the first of these two phenomena—that is, to determine whether exposure to O_3 results in alterations in retention and/or bioavailability of Cr(VI). To this end, an assessment of Cr(VI) particle burden within the lungs, the distribution of inhaled Cr within specific pulmonary compartments, and the relative distribution of Cr between nuclear and cytoplasmic regions of cells of the pulmonary epithelium and interstitium was performed, using rats repeatedly

exposed to atmospheres containing insoluble Cr particles in the presence or absence of O_3.

MATERIALS AND METHODS

Chemicals

Calcium chromate ($CaCrO_4$, 99% pure) was obtained from Aldrich (Milwaukee, WI), RPMI 1640. Hanks balanced salt solution (HBSS) and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY). Reagents described for use in sample preparation and their subsequent analyses by graphite atomic absorption were obtained from Fisher (Springfield, NJ).

Experimental Animals

Male F-344 rats (pathogen free; 200–250 g, Charles River, Raleigh, NC) were used. Rats were quarantined for 2 wk prior to exposure, housed individually in Cr-free cages in temperature (20 °C)/humidity (50% relative humidity; RH)-controlled rooms, and provided Purina rodent chow and water ad libitum.

Experimental Design

For each exposure protocol, cohorts of rats were exposed nose-only, 5 h/d, 5 d/wk for 4, 8, 12, 24, or 48 wk to: filtered air, O_3 only, $CaCrO_4$ only, or $CaCrO_4 + O_3$. Each cohort consisted of 30 rats/exposure group/duration. Within each given exposure duration, all four groups were exposed simultaneously; however, initiation of the exposures were staggered for logistical purposes. To avoid assessing acute effects of the final exposure, subsets of rats were euthanized by injection of sodium pentobarbital (120 mg/kg) 3 d after their respective final exposures. The lungs were processed using protocols previously employed in this laboratory (Miller et al., 1991; Cohen et al., 1997) for analysis of either total Cr burden, Cr levels within three designated pulmonary compartments [i.e., lavageable cells, acellular lavage fluid, and postlavage lung tissue (epithelium and interstitium)], or intracellular distribution of Cr between the cytoplasmic and nuclear compartments of cells within the epithelium and interstitium.

Generation and Characterization of Exposure Atmospheres

Aerosols were generated by nebulizing aqueous (pH 7) $CaCrO_4$ suspensions, as previously described (Cohen et al., 1997, 1998). The nebulizer was connected in series with a nose-only, flow-through-design exposure system capable of housing up to 49 rats simultaneously. Hourly sequential atmosphere samples were collected on filters using dedicated ports during each exposure to determine Cr levels. Particle size was assessed using a Mercer cascade impactor. Ozone was generated by an ultraviolet (UV) O_3 generator and levels during exposure were continuously monitored using a UV photometer (Dasibi model 1008-PC). The target O_3 concentration was 0.3 ppm, a level regularly

encountered during welding (Van der Wal, 1990). The target concentration of Cr was 360 μg Cr/m^3, and the actual generated particle size (mass median aerodynamic diameter, MMAD) of the $CaCrO_4$ was 0.6 μm (σ_g=1.7), similar to that in relevant welding fumes. The Cr level was calculated such that daily Cr deposition (with respect to total lung surface area) in the rat would be ~10-fold higher than that in workers exposed during an 8-h shift to, at a minimum, the threshold level value (TLV) (i.e., 50 μg Cr/m^3), and assuming minute ventilations of 0.2 and 7.5 L/min for rats and humans, respectively, and equivalent deep lung deposition efficiencies (i.e., ~20%) for particles of appropriate diameter (Schlesinger, 1995).

Preparation of Biological Samples for Measurement of Cr Burden

To avoid potential contributions from any Cr within red blood cells trapped in the tissues at the time of sacrifice, lungs were removed en bloc using Cr-free surgical tools and then perfused. In this procedure, 50 ml phosphate-buffered saline (PBS) was introduced via the pulmonary artery and displaced blood exited via an opening in the left ventricle.

To assess whole-lung Cr burdens in one subset of exposed rats, lungs were trimmed of extraneous tissue, blot-dried, weighed, and frozen at −70 °C for later atomic absorption spectroscopy (AAS) analysis. To determine Cr distribution among the three pulmonary compartments in each rat from a parallel subset, the trachea was exposed and a cannula inserted and ligated. Eight milliliters warm (37 °C) Ca^{2+}, Mg^{2+}-free HBSS/0.6 m*M* ethylenediamine tetraacetic acid (EDTA) was then instilled and the solution drawn and reinjected 8 times. Lavages with warm HBSS (without EDTA) were then performed 12 additional times. The aliquots were pooled and contents pelleted by centrifugation at 1000×g for 15 min at 4 °C; the initial 8-ml lavage was similarly processed. The pellets from the two protocols were combined, washed twice with cold RPMI, and resuspended in RPMI/10% FBS. Total cell numbers and viabilities were assessed by hemacytometer counting and trypan blue exclusion, respectively. To determine lavaged cell profiles, 2.5×10^5 cells were removed and differentials performed on Diff Quik-stained cytocentrifuge preparations. Remaining cells were repelleted and the sample containing both viable and dead cells was stored at −70 °C for later AAS analysis. After their volumes were recorded, acellular lavages from each rat were frozen at −70 °C for later analyses. All solutions used for lavage procedures were also analyzed by AAS to determine the extent, if any, of Cr that might have potentially been introduced into lavage samples; any such contributions were then subtracted from each result prior to data analyses.

Analysis of Intracellular Cr Distribution Analyses

Cells in the epithelium and interstitium were isolated using a modification of a method by Cohen et al. (2002). Portions (0.5–1 g) of lavaged lung were placed in a dish containing 10 ml digest solution (1 mg Type I collagenase/ml

in HBSS:0.1 *M* HEPES, pH 7.6) and finely minced with Cr-free scissors. The suspension (along with 10 ml digest solution used to rinse processing dish) was placed in a 37 °C waterbath for 1 h, then in a 37 °C air bath (120 oscillations/min) for 30 min, and finally passed through a 100-μm nylon mesh. Released cells were centrifuged at 1000 × g for 15 min at 4 °C; pelleted cells were resuspended in 2 ml 0.83% ammonium chloride solution (2 min, 25 °C) to lyse any erythrocytes not originally displaced by perfusion. This suspension was layered over 12 ml ice-cold FBS, centrifuged (500 × g, 10 min, 4 °C), and the pellet was suspended in 2.5 ml RPMI/5% FBS. These cells were counted on a hemacytometer and equal aliquots were distributed to designated tubes for determination of total Cr present in all (i.e., "live + dead") cells, or for determination of Cr distribution between the cytoplasm and nucleus of the isolated cells.

To determine intracellular Cr distribution, cells were centrifuged (1000 × g, 10 min, 4 °C), resuspended in 20 ml ice-cold pH 7.5 lyse buffer (10 m*M* Tris, 10 m*M* NaCl, 5 m*M* $MgCl_2$, 1 m*M* phenylmethanesulfonyl fluoride [PMSF]), and placed on ice for 15 min. Cells were then pelleted at 500 × g for 5 min at 4 °C; the supernatant generated was transferred to a tube designated for cytoplasmic material. The cells were resuspended in 7.5 ml lysis buffer supplemented with 0.5% Nonidet P-40, and homogenized in a Dounce tube with 15 strokes of a loose-fitting glass pestle. Nuclei in the homogenate were pelleted by centrifugation at 700 × g for 5 min at 4 °C; the supernatant was transferred to the cytoplasmic materials tube. Isolated nuclei were resuspended in 6 ml pH 7.5 buffer (10 m*M* Tris, 5 m*M* $MgCl_2$, 1 m*M* PMSF, 0.25 *M* sucrose), layered atop 5 ml of this buffer containing 0.88 *M* sucrose, and centrifuged (1000 × g, 10 min, 4 °C) to remove cytoplasmic contaminants. Supernatant generated was transferred to the cytoplasmic material tube and the final total volume was recorded. Both this solution and the nuclei were stored at −70 °C for later AAS analysis. To determine the extent, if any, of Cr that might be introduced by the various buffers used, cell-free samples were processed in parallel.

Chromium Analysis

Tissue, cell, and fluid Cr levels were determined by graphite AAS. Samples were heated first in concentrated nitric acid (HNO_3) and then in acid containing 50% (v/v) hydrogen peroxide to aid matrix breakup. At near dryness, samples were removed from the heat and brought to room temperature; HNO_3 was then added and samples reheated to near dryness. This process was repeated twice to ensure breakdown of any lipid droplets. The final sample was then reconstituted in ultrapure (18 MΩ double distilled) water for processing on a Solar model M6 atomic absorption spectrophotometer (with D_2O/Zeeman background correction) equipped with a GF95 graphite furnace and autosampler. In each analysis, calibration curves were generated with certified NIST-traceable Cr standards. The minimal detectable concentration of Cr using this system was 10 ppb or 0.01 μg Cr/ml sample fluid.

Data Analysis

Data were analyzed to determine if any differences between exposure groups in the measured endpoints were a function of exposure duration or (with respect to effects of Cr) the presence of O_3. This was done using two-way analysis of variance (ANOVA, with duration and exposure atmosphere as variables), followed, when appropriate, by a least-square means test to determine differences between specific groups. Results were considered significant at $p < .05$.

RESULTS

Average concentrations of Cr in each exposure regimen are shown in Table 1. There were no differences related to duration of exposure in Cr levels generated among groups of rats exposed to the $Cr+O_3$ mixtures, with values ranging from ~340 to 357 μg Cr/m³. Values ranged from 315 to 363 μg Cr/m³ among rats in the Cr-only groups, with the lower levels occurring primarily during the shorter exposure (i.e., 4- and 8-wk) regimens. Although there are statistically significant differences among the levels of Cr generated in the Cr-only rats, the actual differences between absolute levels (excluding that of the 4-wk regimen) were ≤7%. Ozone levels in the O_3-only and in the $Cr+O_3$ mixtures ranged consistently from 0.305 to 0.310 ppm (±0.002–0.004 SD) over the course of all exposures.

Table 2 shows the total cell recoveries from the lavages performed after each exposure period; there were no exposure-related differences in cell viability, with viabilities of recovered cells for all exposure groups averaging between 80 and 90%. Among all Cr-exposed rats, cell recoveries significantly increased as the cumulative exposure increased beyond 12 wk; no such effect was evident among rats in either the air or O_3-only controls. Figure 1, A and B, presents the relative percentages of two cell types present in BAL recovered following

TABLE 1. Atmospheric Cr Concentrations (μg Cr/m³)

Exposure regimen (wk)	Cr only	O_3 + Cr
4	315.14 ± 7.77	341.11 ± 16.86
8	336.85 ± 10.83	340.04 ± 8.58
12	362.66 ± 5.63[a]	339.71 ± 5.61[c]
24	360.71 ± 3.99[a]	343.74 ± 4.77
48	340.98 ± 4.21[b]	356.84 ± 5.78[c]

Note. Values are mean (±SE) concentration value.

[a]Within a given exposure group, value is significantly ($p < .05$) different from that of its 4- and 8-wk counterparts.

[b]Within a given exposure group, value is significantly ($p < .05$) different from that of its 4-, 12-, and 24-wk counterparts.

[c]Value is significantly ($p < .05$) different from its time-matched Cr-only counterpart.

TABLE 2. Profile of Pulmonary Immune Cells in Lavage

Exposure regimen (wk)	Total cells	Macrophages	Neutrophils	Total cells	Macrophages	Neutrophils
		Cr only			O_3 + Cr	
4	2.90 ± 0.47	2.47 ± 0.45	0.26 ± 0.06[b,d]	3.27 ± 0.43	2.88 ± 0.39[b]	0.22 ± 0.07
8	2.83 ± 0.26[d,e]	1.94 ± 0.19[b,d,e]	0.81 ± 0.12[b,d,e]	3.00 ± 0.34[e]	2.33 ± 0.25[e]	0.61 ± 0.10[d,e]
12	3.94 ± 0.33	2.86 ± 0.34[e]	0.78 ± 0.11[b,d,e]	3.08 ± 0.22[e]	2.22 ± 0.30[d,e]	0.61 ± 0.09[d,e]
24	5.79 ± 0.49[c,e]	1.88 ± 0.24[b,d,e]	3.14 ± 0.36[c,d,e,f]	5.22 ± 0.36[c,e]	2.20 ± 0.16[d,e]	2.36 ± 0.30[c,d,e]
48	7.58 ± 0.49[c,d,e]	2.93 ± 0.33[d,f]	3.78 ± 0.25[d,e]	6.98 ± 0.65[c,e,f]	2.01 ± 0.20[d,e]	4.29 ± 0.53[c,d,e]
		Air only			O_3 only	
4	3.56 ± 0.19[c,b]	3.00 ± 0.28[c]	0.03 ± 0.01	3.16 ± 0.35[b]	2.66 ± 0.32	0.10 ± 0.08
8	4.40 ± 0.42	3.57 ± 0.47	0.01 ± 0.01[b]	5.22 ± 0.82[a,c]	4.26 ± 0.76[c]	0.04 ± 0.02
12	3.86 ± 0.31	3.31 ± 0.29	0.02 ± 0.01	4.31 ± 0.37	3.95 ± 0.35	0.02 ± 0.01
24	4.92 ± 0.42	3.86 ± 0.24	0.17 ± 0.05[b,c]	3.67 ± 0.20[d]	3.05 ± 0.19[d]	0.02 ± 0.01
48	4.88 ± 0.41	4.24 ± 0.37	0.09 ± 0.02	4.85 ± 0.35	3.78 ± 0.31	0.14 ± 0.05

Note. Values are mean (±SE) number ($\times 10^6$) of cells recovered in lavages from 5 rats per cohort.
[a] Within a given exposure group, value is significantly ($p < .05$) different from that of its 24-wk counterpart.
[b] Significantly different from 48-wk counterpart.
[c] Significantly different from counterparts in all shorter regimens.
[d] Within a given exposure length, value is significantly ($p < .05$) different from that of air-exposed rats.
[e] Significantly different from O_3-only-exposed rats.
[f] Significantly different from O_3 + Cr-exposed rats.

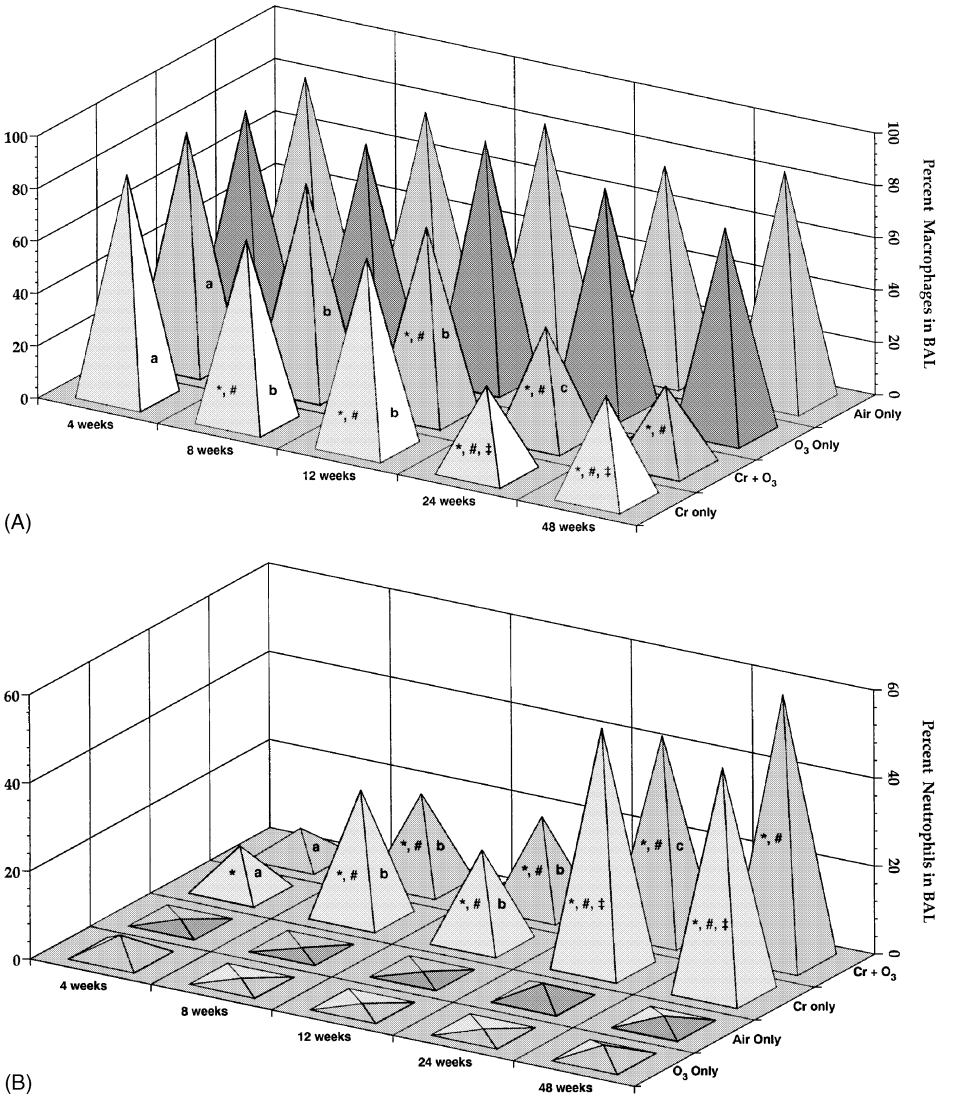

FIGURE 1. Relative percentages of (A) PAM and (B) PMN as a function of exposure duration as well as exposure atmosphere. Each spike value represents the mean (±SE) percentage of the cell type in the lavages of five rats per exposure group. Within each exposure group, values significantly ($p < .05$) different from counterparts at (a) all later time points, (b) 24 and 48 wk or (c) 48 wk are indicated. In a given exposure duration, asterisk indicates values among all Cr-exposed rats significantly different from counterparts in air exposed groups and in O_3-only-exposed groups; values that significantly differ between Cr-only-exposed rats and $CaCrO_4 + 0.3$ ppm O_3 counterparts are denoted by double dagger.

exposures: pulmonary alveolar macrophages (PAM) and neutrophils (PMN). The data suggests that as soon as the end of 8 wk of exposures (and consistently thereafter), the percentages of PAM in the lungs of rats exposed to Cr only were significantly reduced compared to those in air controls, reaching a

minimum by wk 24 of exposure. Among rats exposed to the $Cr+O_3$ mixture, this decrease relative to controls also became evident, but required an added period (4 wk; i.e., until wk 12) of exposure. Additional time (undefined, but <24 wk total) was also necessary for the values in these rats to reach the minima attained by their Cr-only counterparts at wk 24. With PMN, all Cr-only rats had significantly elevated percentages of cells compared to air controls; these values progressively increased in tandem with cumulative exposure and again appeared to plateau after wk 24 of exposure. Rats exposed to $Cr+O_3$ mixtures displayed these significant elevations only after 8 wk of exposures and again did not achieve levels of their counterparts exposed to Cr alone until somewhere between wk 24 and 48 of exposure. As a result of these significant changes in the percentages of these two cell types, even at time points where total cell recoveries appeared unaffected by exposure to Cr alone or with O_3, absolute numbers of PAM and PMN were still significantly altered (Table 2). Exposure to O_3 alone for any of the experimental periods did not result in any change in percentages or absolute numbers of either cell type as compared to that in air controls.

Figure 2 shows the total Cr burdens (as μg Cr/g lung weight) in the lungs following each exposure series. In all cases, lung total wet weight increased as the rats aged; however, for any given exposure duration, there were no significant differences in lung weights between rats exposed to Cr alone and those exposed to the $Cr+O_3$ mixture. It can be seen that, irrespective of exposure regimen, amounts of Cr retained in the lungs progressively and significantly increased in tandem with exposure duration.

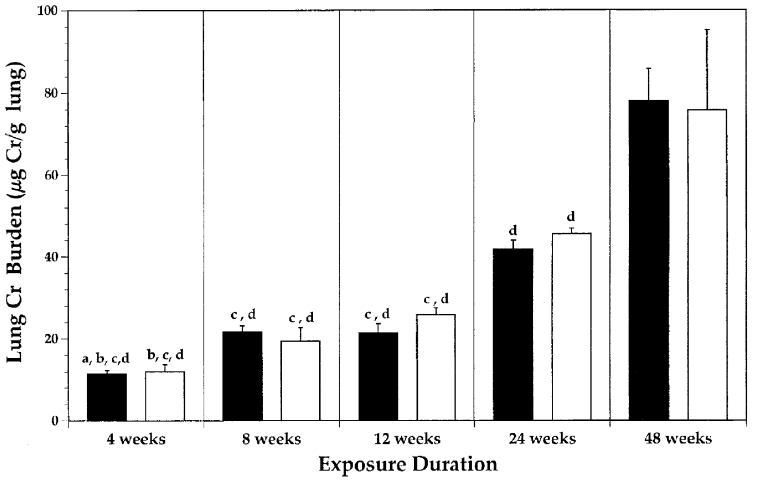

FIGURE 2. Total burdens of Cr in the lungs as a function of exposure duration. Each bar value represents the mean (±SE) of a total of five rats per exposure group. Bar fills: black = rats exposed to $CaCrO_4 + 0.3$ ppm O_3, white = rats exposed to $CaCrO_4$ only. Within each exposure group, values significantly ($p < .05$) different from their counterparts at 8, 12, 24, or 48 wk are indicated by use of superscript a, b, c, or d, respectively.

When lungs were analyzed for relative distribution of Cr among three compartments—lavageable cells, BAL fluid, and nonlavageable sites corresponding to the epithelium and interstitium—it was noted that the majority (>94%) of all Cr present was localized in this last compartment (Table 3) as early as after 4 wk of exposure. Only within the two shortest periods of exposure (i.e., 4 and 8 wk) did there seem to be any difference in distribution patterns of Cr as a result of the copresence of O_3. Specifically, coinhalation of O_3 seemed to initially cause an increase in percentages of the Cr present to be localized in those cells recoverable by lavage. Consequently, the presence of O_3 also initially led to a decrease in percentages of total lung Cr in acellular BAL, suggesting that less Cr was localized in lung extracellular spaces. If, however, the data are analyzed in the context of the mass of Cr recovered in each compartment (Table 4), it seems that, in fact, the absolute amounts of Cr found in all lavaged cells and recovered fluids did not differ as a result of copresence of O_3. In all studies, levels of Cr detected in cells lavaged from control rats never exceeded

TABLE 3. Relative Distribution of Cr (Percent of Total Lung Burden) in Designated Compartments

Exposure regimen (wk)	Cr only	O_3 + Cr
Lavaged cells		
4	5.57 ± 0.49^{c}	$7.38 \pm 0.54^{a,e}$
8	5.20 ± 0.70^{c}	5.26 ± 0.50^{d}
12	3.46 ± 0.43^{d}	4.16 ± 0.40
24	1.76 ± 0.25^{d}	2.57 ± 0.25^{d}
48	1.86 ± 0.20	1.09 ± 0.04^{d}
Lavage fluid (BAL)		
4	$0.20 \pm 0.04^{a,b,c}$	$0.16 \pm 0.04^{b,c}$
8	0.27 ± 0.05	$0.14 \pm 0.02^{b,c,e}$
12	0.43 ± 0.05	0.41 ± 0.02^{e}
24	0.37 ± 0.03	0.40 ± 0.04
48	0.34 ± 0.05	0.37 ± 0.04
Non lavageable sites (interstitium and epithelium)		
4	94.22 ± 0.49^{c}	$92.45 \pm 0.56^{a,e}$
8	94.53 ± 0.73^{c}	94.59 ± 0.51^{d}
12	$96.10 \pm 0.43^{c,d}$	95.43 ± 0.40
24	97.86 ± 0.28^{d}	97.03 ± 0.26^{d}
48	97.80 ± 0.19	98.53 ± 0.07^{d}

Note. Values are mean (±SE) percentage values as determined from samples recovered from each of five rats per cohort.

[a] Within a given exposure group, value is significantly ($p < .05$) different from that of its 12-wk counterpart.

[b] Significantly different from 24-wk counterpart.

[c] Significantly different from 48-wk counterpart.

[d] Significantly different from counterparts in all shorter regimens.

[e] Value is significantly ($p < .05$) different from that of its time-matched Cr-only counterpart.

TABLE 4. Total Amounts (μg) of Cr in Designated Compartments

Exposure regimen (wk)	Cr only	O_3 + Cr
Lavaged cells		
4	0.578 ± 0.051[a,b]	0.798 ± 0.059[b]
8	0.997 ± 0.077[d]	1.067 ± 0.102[c]
12	0.863 ± 0.107	0.863 ± 0.083[b]
24	0.911 ± 0.131	1.186 ± 0.115[c,e]
48	1.276 ± 0.136[d]	0.742 ± 0.030[e]
Lavage fluid (BAL)		
4	0.021 ± 0.004[c]	0.018 ± 0.004
8	0.047 ± 0.008[c]	0.029 ± 0.004
12	0.108 ± 0.012[c,d]	0.086 ± 0.004[d]
24	0.192 ± 0.017[d]	0.184 ± 0.018[d]
48	0.234 ± 0.034	0.251 ± 0.028[d]
Non lavageable sites (interstitium and epithelium)		
4	9.78 ± 0.05	9.99 ± 0.06[e]
8	16.32 ± 0.13[d]	19.17 ± 0.10[d,e]
12	23.95 ± 0.11[d]	19.79 ± 0.08[d,e]
24	50.58 ± 0.14[d]	44.75 ± 0.12[d,e]
48	67.05 ± 0.13[d]	66.69 ± 0.05[d,e]

Note. Values are mean (±SE) levels of Cr in samples recovered from each of five rats per cohort.

[a] Within a given exposure group, value is significantly ($p < .05$) different from that of its 12-wk counterpart.

[b] Significantly different from 24-wk counterpart.

[c] Significantly different from 48-wk counterpart.

[d] Significantly different from counterparts in all shorter regimens.

[e] Value is significantly ($p < .05$) different from that of its time-matched Cr-only counterpart.

1 ng total Cr; levels in BAL fluids were either undetectable or <5–10 ng total Cr in one "worst" case. Lastly, background levels of Cr in any solution used for the lavage protocols were never more than 1 ng Cr/ml.

Since cancers induced in the lungs as a result of exposure to Cr compounds are most likely to evolve in the cells of the epithelium or interstitium, as opposed to cells that are lavageable or normally exit during mucociliary clearance, the relative burdens of Cr in these cells were assessed to determine if copresence of O_3 might affect this endpoint. As can be seen in Table 5, burdens were relatively constant in the cells from rats in both exposure regimens over the first 12 wk of exposure, ranging from 0.05 to 0.08 pg Cr/cell; only results from cells obtained from rats exposed to Cr alone for 4 wk appeared aberrant. As the period of exposure increased further, cellular burdens were significantly increased in rats from both regimens. In no case did coexposure to O_3 affect the numbers of cells recoverable from the lavaged lung tissues or their relative cellular Cr burdens (data not shown).

TABLE 5. Burden of Cr (pg Cr/Cell) in Rat Lung Epithelium and Interstitium

Exposure regimen (wk)	Cr only	O_3 + Cr
4	0.119 ± 0.011[a]	0.054 ± 0.004[c]
8	0.079 ± 0.005	0.080 ± 0.008
12	0.058 ± 0.010	0.084 ± 0.013
24	0.105 ± 0.016[a]	0.139 ± 0.024[b]
48	0.286 ± 0.146[b]	0.243 ± 0.012[b]

Note. Values are mean (± SE) burdens from samples isolated from lungs of each of five rats per cohort.

[a] Within a given exposure group, value is significantly ($p < .05$) different from that of its 12-wk counterpart.

[b] Significantly different from counterparts in all shorter regimens.

[c] Within a given exposure length, value is significantly ($p < .05$) different from that of its matched Cr-only counterpart.

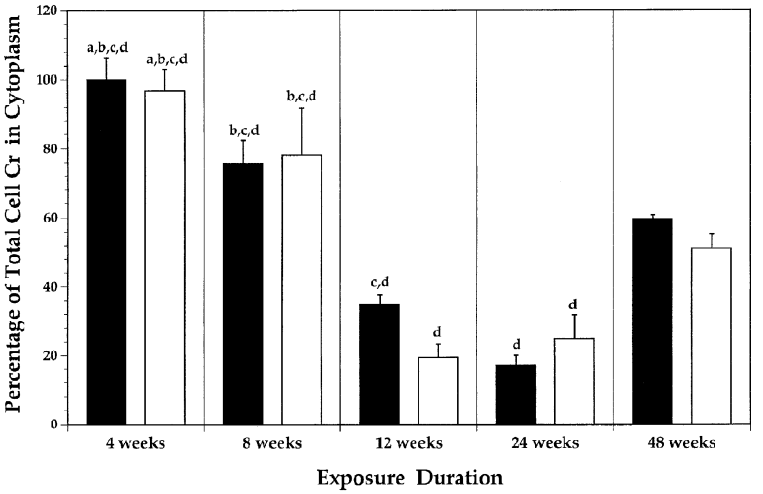

FIGURE 3. Relative percentage of interstitial/epithelial cell Cr burdens remaining in the cytoplasm over the course of the cumulative exposures. Each bar value represent the mean (± SE) of individual samples from a total of five rats per exposure group. Bar fills: black = rats exposed to $CaCrO_4$ + 0.3 ppm O_3, white = rats exposed to $CaCrO_4$ only. Within each exposure group, values significantly ($p < .05$) different from their counterparts at 8, 12, 24, or 48 wk are indicated by use of superscript a, b, c, or d, respectively.

To ascertain whether there were effects from O_3 coexposure upon the intracellular localization of Cr, these cells from the epithelium or interstitium were also analyzed for relative Cr content within their cytoplasm and nuclei. As seen in Figure 3, there was a continuous shift of the relative burden of cellular Cr from the cytoplasm to the nucleus as the period of exposure progressed to 12 wk. After this, the percentage of Cr found associated with the cytoplasm plateaued and then began to rise in cells recovered from rats exposed to Cr alone. Among rats exposed to the Cr + O_3 mixture, the shift in

percentage Cr toward the nucleus continued over the period from 12 to 24 wk of exposure, and only thereafter started to shift back. Apart from these differences in temporal patterns of intracellular distribution of Cr, there were no exposure regimen-related differences in these values at any of the time points examined.

DISCUSSION

The goal of this study was to examine whether the retention and distribution of Cr within the lungs were affected by the copresence of O_3. This evaluation represented the first step in a study to ascertain whether one aspect of the pulmonary toxicity of insoluble Cr agents, their carcinogenic potential, could be affected by the copresence of this oxidant gas as commonly occurs in welding fumes. The results showed that while there is only a nominal effect from coexposure to 0.3 ppm O_3 on overall retention or on distribution of Cr particles among extracellular sites and within cells of the lungs, there seem to be effects from coexposure on mechanisms normally involved in clearing insoluble or poorly soluble Cr from the deep lung.

Unlike soluble Cr, which can traverse the epithelium and enter the blood or lymph (unless the ions are bound by epithelial biomolecules), clearance of insoluble Cr relies primarily on PAM phagocytosis and mucociliary transport. Thus, it would be reasonable to expect that the extent to which coinhaled O_3 affects retention of inhaled Cr of a given solubility would then be dependent on the severity of the impact of the O_3 on lung epithelium integrity or PAM number/functionality. Because of the known toxicity of O_3 toward PAM (Bhalla, 1999; Balla & Gupta, 2000; Bassett et al., 2000), it was unexpected that $CaCrO_4$ + 0.3 ppm O_3-exposed rats had lung Cr burdens similar to that in rats exposed to $CaCrO_4$ only. Our earlier studies using insoluble $BaCrO_4$ (barium chromate) showed that copresence of 0.3 ppm O_3 during repeated exposures for up to 4 wk resulted in significant increases in relative burdens (Cohen et al., 1997). Conversely, when soluble K_2CrO_4 (potassium chromate) was used, a copresence of O_3 caused decreased Cr retention relative to that in rats breathing K_2CrO_4 alone. Solubility of nonhydrated $CaCrO_4$ is 0.71 mmol/kg H_2O (110.8 mg/L, at 25 °C; Katz & Salem, 1993); that of $BaCrO_4$ and K_2CrO_4 is 0.017 mmol/kg (4.4 mg/L) and 3.24 mol/kg (629 g/L), respectively. Thus, the results of the present study suggest that the potential for O_3 to affect Cr retention is apparently more closely related to the solubility of Cr agents than had been previously surmised. That $CaCrO_4$ is more soluble (~40 times) than $BaCrO_4$ yet far less so than K_2CrO_4 suggests that at a coexposure level of 0.3 ppm O_3, a solubility of ~100 mg/L may represent a threshold level for an O_3 effect on Cr retention; that is, at solubilities increasingly greater than 100 mg/L, Cr agents are not as well retained during coinhalation of 0.3 ppm O_3, while retention is increasingly enhanced as values fall further below the value. If so, this would potentially have a significant impact on retention of other poorly soluble carcinogenic Cr(VI) agents like lead, nickel, and zinc chromates. Ongoing studies in

our laboratory are examining whether this effect on Cr retention is consistent over a wider range of O_3 levels (i.e., 0.1 and 0.6 ppm); future studies using Cr or other metals agents with a broader solubility range, alone and in combination with varied levels of O_3, will help more precisely define this relationship.

It is of note that lavage profiles indicated that although all Cr-exposed rats had significant decreases (relative to air or O_3-only rats) in percentages of PAM as the cumulative exposure duration increased, rats breathing $Cr+O_3$ required longer durations to both first express this effect and then to reach a nadir. It is reasonable to assume that as more PAM should then be present (and thus able to partake in uptake/clearance) as a result of these lags, total Cr burden values should then be lower in mixture-exposed rats. That values were still equivalent to those in rats inhaling Cr alone suggests that the toxicity of O_3 to PAM might impact on the role of these cells in clearance, so as to permit Cr retention to be greater than expected for any given number of PAM in situ. Exposure to O_3 has been shown to induce alterations in PAM phagocytic activity (Driscoll et al., 1986; Dormans et al., 1996), surface receptors (Prasad et al., 1988; Dormans et al., 1990; Cohen et al., 2002), lysosome and phagosome structure/activity (Hurst et al., 1970; Lum et al., 1983), and cytoplasmic pH regulation (Chen et al., 1993).

In the current study, had O_3 acted to induce depressed particle uptake, there should be increased retention of Cr in the interstitium, increased amounts of Cr in lavage fluid, and decreased PAM Cr burdens relative to those in rats inhaling $CaCrO_4$ alone. While lavage levels of Cr appeared unaffected by coinhaled O_3, nonlavageable site Cr levels were greater in $Cr+O_3$-exposed rats through the first 8 wk of exposure but then fell below those of Cr-only rats by wk 12 and remained so thereafter. Lavaged cell burdens did not seem to demonstrate any consistent effect from the O_3 exposure. If, however, cell burden data were analyzed in the context of relative immune cell populations, it became evident that total levels of Cr associated with PAM (calculated from product of pg Cr/lavaged cell × total number PAM in lavage) at each time point (except 48 wk) were greater in rats breathing the mixture (data not shown). This would suggest that, even with any O_3-induced decrease in phagocytic/endocytic activity, there were likely also effects on intraphagosomal processing of whatever $CaCrO_4$ was internalized. These effects on phagosomes would then allow PAM to become (over)laden with unprocessed $CaCrO_4$ as the exposures continued, thus rendering the cells less capable of ingesting subsequent particles deposited in the lungs and forcing more of the material into the interstitium and the pulmonary extracellular spaces.

Because these studies were part of a project to ascertain whether coinhalation of O_3 might enhance the carcinogenic potential of insoluble/poorly soluble Cr agents also present in welding fumes, the impact of O_3 on localization of Cr to the nuclei of cells in the interstitium/epithelium was also analyzed. As a consequence of responses to induced oxidative/peroxidative damage to the cells (Hueter & Fritzhand, 1977), O_3 exposure can affect the intracellular levels of several low-molecular-weight compounds [e.g., cysteine, methionine, glutathione, NAD(P)H, ascorbate] that could be used in the detoxification of

Cr(VI) ions liberated by intraphagosomal processing. While a lowering of cellular reducing equivalent levels should be conducive to an enhanced longevity of Cr(VI) in lung cells, it could be concluded that this decrease in reductant availability should then present a means for decreasing the carcinogenic potential of the Cr; that is, there would be less cytoplasmic generation of highly reactive penta- [Cr(V)] and tetravalent [Cr(IV)] intermediates. However, reduction of Cr(VI) may be seen either as a toxification or a detoxification process with respect to mechanisms of Cr carcinogenesis (Shi et al., 1999). Ultimately, Cr toxification or detoxification depends on the site in the cytoplasm where reduction occurs; activation is more likely if reduction occurs near the nucleus, and detoxification if reduction occurs in cytoplasmic organelles or sites distal to the nuclear envelope. Under conditions wherein O_3 is present, there would then be an increased opportunity for the Cr(VI) liberated from phagosomes to overwhelm the reducing capacity of the cytoplasm and so elude reduction/detoxification (DeFlora & Wetterhahn, 1989). Thereafter, the increased amounts of Cr(VI) ions in the cytoplasm could then translocate to the nucleus, where (perhaps following reduction near/within the nucleus) they interact directly with DNA to induce damage or to oxidize/complex with select amino acids in the nucleus to give rise to increased formation of DNA–protein cross-links (Zhitkovich et al., 1996).

In the current study, the copresence of O_3 did not seem to cause any acceleration in the translocation of Cr to the nuclei of cells of the interstitium and epithelium. The only effect that was notable was that, similar to other endpoints previously discussed, there appeared to be a lag: that is, higher nuclear Cr burdens were achieved in the rats exposed to Cr alone at a time point well before that of the rats that breathed the $Cr+O_3$ mixtures. Similarly, a rebounding in the percentage of Cr found in the cytoplasm also occurred earlier in cells from rats exposed to Cr alone. The precise mechanism underlying this shift back toward the cytoplasm is not clear; it is possible that the observed increasing cell burdens of Cr eventually overwhelm intracellular processing mechanisms such that liberation of Cr(VI) ions is retarded, thereby decreasing the opportunity overall for Cr to cross the nuclear envelope. With respect to the apparent delay effect induced by coinhalation of O_3, as indicated in the analyses of PAM Cr burden studies, it is likely that intracellular processing by some cells of the interstitium was also inhibited by the presence of O_3 during exposures. As a result, in a manner similar to the already postulated effect from Cr itself, the rate of liberation of Cr(VI) ions might be retarded further (compared to that in cells from rats exposed to Cr alone), thus lessening more so the opportunity for the cellular Cr to reach the nucleus.

In summary, the results of this study indicate that, in part through effects on the mechanisms critical to efficient removal of insoluble/poorly soluble Cr particles from the lungs, coinhalation of 0.3 ppm O_3 (as might occur during welding fume exposure) may ultimately have the ability to impact upon the carcinogenic potential of these Cr agents. As in our earlier studies, it was found that when O_3 is inhaled concurrently with Cr, it is the compound solubility

that substantially influences overall lung Cr retention. Furthermore, this study showed that coinhalation of O_3 appears to affect clearance of Cr particles, with effects seeming to occur at the levels of particle uptake and of postphagocytic/endocytic processing by PAM. While this O_3-dependent shift in normal pulmonary clearance could portend potential problems for those exposed to insoluble or poorly soluble Cr(VI) carcinogens, this study failed to demonstrate that expected effects of O_3 on cellular reducing equivalents might cause increased delivery of Cr ions to the nuclei of lung cells. It is possible that 0.3 ppm is not an adequately high level of O_3 to cause this latter effect; it is equally plausible that lower levels of O_3 may be more effective (i.e., while levels of reducing equivalents could be lessened, intraphagosomal processing might proceed more normally). Because localized concentrations of O_3 in welding fumes can often be below or well over 0.3 ppm (Stern et al., 1986; Howden, 1988; van der Wal, 1990), ongoing exposure studies are examining other levels (i.e., 0.1 and 0.6 ppm) of O_3 for their effects on Cr translocation.

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