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Metal Oxide Nanoparticles Induce Unique Inflammatory Footprints in the Lung: Important Implications for Nanoparticle Testing

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BACKGROUND: Metal oxide nanoparticles (NPs) have been widely used in industry, cosmetics, and biomedicine.

OBJECTIVES: We examined hazards of several well-characterized high production volume NPs because of increasing concern about occupational exposure via inhalation.

METHODS: A panel of well-characterized NPs [cerium oxide (CeO2NP), titanium dioxide (TiO2NP), carbon black (CBNP), silicon dioxide (SiO2NP), nickel oxide (NiONP), zinc oxide (ZnONP), copper oxide (CuONP), and amine-modified polystyrene beads] was instilled into lungs of rats. We evaluated the inflammation potencies of these NPs 24 h and 4 weeks postinstillation. For NPs that caused significant inflammation at 24 h, we then investigated the characteristics of the inflammation. All exposures were carried out at equal-surface-area doses.

RESULTS: Only CeO2NP, NiONP, ZnONP, and CuONP were inflammogenic to the lungs of rats at the high doses used. Strikingly, each of these induced a unique inflammatory footpath both acutely (24 h) and chronically (4 weeks). Acutely, patterns of neutrophil and eosinophil infiltrates differed after CeO2NP, NiONP, ZnONP, and CuONP treatment. Chronic inflammatory responses also differed after 4 weeks, with neutrophilic, neutrophilic/lymphocytic, eosinophilic/fibrotic/granulomatous, and fibrotic/granulomatous inflammation being caused respectively by CeO2NP, NiONP, ZnONP, and CuONP.

CONCLUSION: Different types of inflammation imply different hazards in terms of pathology, risks, and risk severity. In vitro testing could not have differentiated these complex hazard outcomes, and this has important implications for the global strategy for NP hazard assessment. Our results demonstrate that NPs cannot be viewed as a single hazard entity and that risk assessment should be performed separately and with caution for different NPs.

KEY WORDS: eosinophilic inflammation, intratracheal instillation, in vitro assay, lymphocytic inflammation, metal oxide nanoparticles, neutrophilic inflammation, risk assessment, surface area dose, Wistar rat. Environ Health Perspect 118:1699–1706 (2010). doi:10.1289/ehp.1002201 [Online 20 August 2010]

Compared with bulk materials, nanoparticles (NPs) have unique and novel properties and thus offer great opportunities for development of new industrial applications (Borm et al. 2006). Many NPs are already in use or have potential to be widely used in a range of applications (Nohynek et al. 2007). Maynard et al. (2006) and Nel et al. (2006) have called for risk assessment for the environment and humans before widespread industrial application of NPs. Such risk assessment requires hazard identification and dose–response data.

In vitro assays have limitations and have not necessarily been validated for NPs (Kroll et al. 2009). In addition, the traditional mass dose does not well reflect the biologically effective dose for NPs, and surface area combined with surface reactivity is more likely a better descriptor of potential to cause inflammation at the site of particle deposition (Donaldson et al. 2008; Duffin et al. 2007; Tran et al. 2000).

The commonly proposed pathogenic mechanisms initiated by NPs are dominated by inflammation-driven effects, including fibrosis, oxidative stress, and DNA damage, making inflammation a target for toxicological testing (Borm et al. 2006; Lu et al. 2009; Nel et al. 2006, 2009). Inflammation is a complex, concerted group of responses that, although defensive against infection, is harmful when induced chronically by environmental stimuli such as inhaled particles (Donaldson et al. 2006; Mroz et al. 2008; Oberdorster et al. 2005). The type, harmfulness, and outcome of inflammation vary depending on the nature of the stimulus initiating the inflammation; the affected tissue; the nature of the cellular exudates; its chronicity, severity, and potential to resolve; and the genetic susceptibility of the individual.

Inflammation cannot be replicated by in vitro models because it depends on an intact vascular system and a huge assortment of cellular and humoral interactions. Although in vitro studies with NPs can claim to demonstrate “proinflammatory effects,” such studies cannot demonstrate anything more than a general indication that such articles are likely to elicit “some sort” of inflammation; they cannot predict the form of inflammation or its tempo, persistence, or tendency to resolve.

We studied the acute pulmonary toxicity of a large panel of NPs (cerium oxide (CeO2NP), titanium dioxide (TiO2NP), carbon black (CBNP), silicon dioxide (SiO2NP), nickel oxide (NiONP), zinc oxide (ZnONP), copper oxide (CuONP), and amine-modified polystyrene beads) and found that four of them possessed significant acute inflammation: CeO2NP, NiONP, ZnONP, and CuONP. We went on to study these four NPs further and found very different patterns of inflammation (in terms of time), resolution, and cellular exudates with each of the four different particle types.

Materials and Methods

Characterization of NPs. We used a panel of eight NPs (CeO2NP, TiO2NP, CBNP, SiO2NP, NiONP, ZnONP, CuONP, and Beads) purchased from commercial sources, as noted in Table 1. These NPs are typical of those most commonly used in industry. The surface area of NPs [Brunauer-Emmett-Teller (BET) method] was determined by ParticlesCIC Ltd. (Leeds, UK) using a Micromeritics TriStar 3000 analyzer (Micromeritics Ltd., Dunstable, Bedfordshire, UK). All exposures took place using NPs dispersed in rat serum. NP stock solution was prepared at 6,000 cm²/mL in distilled water and sonicated with a probe sonicator (Philip Harris Scientific, Lichfield, UK) to break up aggregates. Rat serum (final concentration, 5%) was added for dispersion. The stock solution of NPs was then diluted with phosphate-buffered saline (PBS) to the final concentration. The hydrodynamic size and zeta potential of the NPs were analyzed in PBS containing 5% rat serum with a Brookhaven 90Plus particle size analyzer.
Female Wistar rats (200–250 g) obtained from Harlan Laboratories (Hillcrest, UK) were maintained and handled under a specific license granted by the UK Home Office to one of the authors (K.D.) that ensures humane treatment and alleviation of suffering in all animal experiments.

Intratracheal instillation of NPs and bronchoalveolar lavage. Female Wistar rats (200–250 g) obtained from Harlan Laboratories (Hillcrest, UK) were maintained and handled under a specific license granted by the UK Home Office to one of the authors (K.D.) that ensures humane treatment and alleviation of suffering in all animal experiments. NPs dispersed in saline containing 5% rat serum were prepared at surface area doses of 100 and 300 cm²/rat for 24 h and handled under a specific license granted by the UK Home Office to one of the authors (K.D.) that ensures humane treatment and alleviation of suffering in all animal experiments.

Intratracheal instillation of NPs, and lavages were performed using methods previously described by Lu et al. (2009). Briefly, 10,000 cells were centrifuged at 200 g for 10 min and then dehydrated and mounted. Immunohistochemical staining for CD3 as a T-cell marker and CD45RA as a B-cell marker was performed in the lungs of NIONP on 4 weeks postinstillation on parallel sections. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide at room temperature for 15 min. For antigen retrieval, Borg Decloaker (Biocare Medical, Holtsville, NY) was applied for 2 min. Slides were then blocked with normal goat serum, and anti-rat CD3 antibody or anti-mouse IgG from the Envision kit (Dako, Cambridgeshire, UK). After slides were washed three times, diaminobenzidine substrate (Vector Laboratories, Peterborough, UK) was applied.

**Table 1.** Characterization of the eight NPs.

| Nominal chemistry | CeO₂/NP | TiO₂/NP | CBP⁺/NP | SiO₂/NP | ZnONP⁺/NP | CuONP⁺/NP | NIONP⁺/NP | Beads⁺/NP |
|-------------------|---------|---------|---------|---------|-----------|-----------|-----------|-----------|
| Diameter (nm)     | 20–30   | 30–40   | 14      | < 10    | < 50      | 10–20     | 36        |
| Surface area (μm²/μg)² | 24.1    | 27.5    | 254     | 523     | 48.2      | 27.3      | 172       | 54.5      |
| Mass (μg) per 150 cm² | 625     | 545     | 59      | 25      | 310       | 515       | 163.5     | 94        |
| Mass (μg) per 50 cm² | 208     | 182     | 19.7    | 8.3     | 103       | 172       | 172       | 54.5      |
| Hydrodynamic size (nm) | 88.1 ± 29.3 | 119.1 ± 39.6 | 78.0 ± 41.8 | 377.5 ± 170.2 | 306.3 ± 102.7 | 112.1 ± 17.3 | 92.5 ± 4.1 | 96.1 ± 11.8 |
| Polydispersity (%) | 0.148 ± 0.04 | 0.21 ± 0.07 | 0.18 ± 0.01 | 0.27 ± 0.05 | 0.27 ± 0.07 | 0.21 ± 0.03 | 0.21 ± 0.08 | 0.13 ± 0.02 |
| Zeta potential (mV) | −32.4 ± 5.6 | −28.5 ± 5.2 | −25.0 ± 5.0 | −12.9 ± 3.7 | −27.1 ± 1.4 | −26.7 ± 1.25 | −26.0 ± 5.0 | 40.3 ± 8.5 |
| Endotoxin (pg/mL) | ND      | ND      | ND      | ND      | ND        | ND        | ND        | ND        |

ND, not detectable. Values are mean ± SD from four independent experiments. All chemicals were provided by Nanostructural and Amorphous Materials Inc. (Houston, TX, USA) except where noted.

**Statistical analysis.** Data are expressed as mean ± SD (n = 4) and were analyzed with GraphPad InStat software (version 3; GraphPad Software, Inc., La Jolla, CA, USA). We used one-way analysis of variance with post hoc Tukey’s pairwise comparisons to compare each treatment group. The value of p < 0.05 was taken to be statistically significant.

**Results.**

**Characterization of NPs.** The physicochemical characteristics of the panel of eight NPs are summarized in Table 1. Hydrodynamic size showed that all NPs had small aggregates, but the hydrodynamic size of SiO₂NP and ZnONP (~300 nm) was larger than that of the other NPs (~100 nm). All NPs except Beads were negatively charged.

**Cytological analysis of the BALF. Twenty-four hours.** Total cells in BALF of rats exposed to CeO₂NP, NiONP, or CuONP were significantly increased (Figure 1) compared with vehicle controls. Total cells were also significantly higher in rats treated with 150-cm² versus 50-cm² NiONP. The numbers of PMN in BALF were significantly higher than those in vehicle controls after exposure to CeO₂NP, NiONP, ZnONP, or CuONP, but we saw a significant positive dose response only with NiONP. Eosinophils were significantly increased in BALF of rats treated with ZnONP or CuONP. Other NPs did not increase the number of eosinophils in BALF.

**Four weeks.** Total cell numbers were significantly higher after NiONP or ZnONP treatment compared with vehicle controls (Figure 2). PMN were significantly increased with CeO₂NP or NiONP treatment, and lymphocytes were significantly increased in the
NiONP treatment groups. In addition, eosinophils were still significantly present in BALF at 4 weeks in the high-dose ZnONP group but not in the low-dose group. We observed giant cells, a marker for chronic granulomatous inflammation, in lungs after treatment with ZnONP or CuONP. Interestingly, the neutrophilic inflammation caused by NiONP was much more severe at 4 weeks than at 24 hr. Low-dose NiONP recruited the same types of inflammatory cells in the BALF at 4 weeks after instillation as the high dose. We observed no significant increases in inflammatory cells 4 weeks after treatment with CuONP.

**LDH and total protein concentration in BALF.** Twenty-four hours. LDH, a marker of cell death, and total protein, a marker of cell permeability, were significantly increased in BALF relative to vehicle controls 24 hr after treatment with CeO$_2$NP, NiONP, ZnONP, or CuONP [see Supplemental Material, Figure 1 (doi:10.1289/ehp.1002201)]. These findings were paradoxical for ZnONP given that treatment was not associated with a very substantial leukocyte influx. We observed significant positive dose responses for LDH and total protein levels after NiONP and ZnONP treatment. Protein concentration was significantly higher and LDH concentration was significantly higher over controls 4 weeks after treatment with CeO$_2$NP, NiONP, ZnONP, or CuONP [see Supplemental Material, Figure 3 (doi:10.1289/ehp.1002201)]. Interestingly, we did not observe giant cells and fibrosis—which are commonly associated with chronic inflammation—after NiONP treatment. CeO$_2$NP induced minimal to mild neutrophilic inflammation and rare granulomas in alveoli and interstitium (Figure 4; see also Supplemental Material, Figure 4). In contrast, ZnONP induced alveolar and interstitial inflammatory cell infiltration, including eosinophils, macrophages, and giant cells, with severe fibrotic lesions that were predominantly in collapsed and contracted alveoli. CuONP induced granulomatous inflammation in alveoli and also induced interstitial fibrosis that was less severe than that associated with ZnONP.

**Four weeks.** Histological analysis of lung sections obtained 4 weeks after NiONP installation showed lipoproteinaceous material in the alveolar spaces, foamy macrophages (presumed to contain lipoprotein) in the alveolar spaces (Figure 4), and many organized lymphocyte aggregates in the perivascular and peribronchial interstitium that consisted of B cells and T cells [see Supplemental Material, Figure 3 (doi:10.1289/ehp.1002201)]. Interestingly, we did not observe giant cells and fibrosis—which are commonly associated with chronic inflammation—after NiONP treatment. CeO$_2$NP induced minimal to mild neutrophilic inflammation and rare granulomas in alveoli and interstitium (Figure 4; see also Supplemental Material, Figure 4). In contrast, ZnONP induced alveolar and interstitial inflammatory cell infiltration, including eosinophils, macrophages, and giant cells, with severe fibrotic lesions that were predominantly in collapsed and contracted alveoli. CuONP induced granulomatous inflammation in alveoli and also induced interstitial fibrosis that was less severe than that associated with ZnONP.
Measurement of proinflammatory mediators. To evaluate the cytokine profile underlying inflammation, we selected and measured representative inflammatory mediators for neutrophilic (TNF-α, IL-1β, and MIP-2), eosinophilic (eotaxin and IL-13), and lymphocytic (IFN-γ) inflammation in BALF.

Twenty-four hours. We observed no significant difference in TNF-α or IFN-γ in any treatment group compared with the vehicle control (data not shown). IL-1β was significantly increased in the high-dose CeO₂NP, NiONP, and ZnONP and high- and low-dose CuONP treatment groups (Table 2; see also Supplemental Material, Figure 5 [doi:10.1289/ehp.1002201]). MIP-2 levels in BALF were significantly increased in the low- and high-dose CeO₂NP, NiONP, and CuONP groups, but MIP-2 was not increased with either dose of ZnONP. ZnONP treatment was associated with significant increases in eotaxin and IL-13, consistent with the severe acute eosinophilia observed after ZnONP treatment. CuONP treatment, which resulted in modest eosinophilia, was associated with significantly increased eotaxin only.

Four weeks. At 4 weeks after instillation, levels of MIP-2 were significantly increased in the high-dose CeO₂NP group and both NiONP dose groups (see Supplemental Material, Figure 6 [doi:10.1289/ehp.1002201]). Levels of IFN-γ were significantly increased after treatment with low- and high-dose NiONP and high-dose ZnONP. In contrast to the increases in IL-1β and MIP-2 observed after 24-hr treatment with CuONP, the levels of these cytokines 4 weeks after installation were not significantly different from vehicle controls.

Effects of dispersion media on eosinophil recruitment by ZnONP. To evaluate the effects of rat serum on the recruitment of eosinophils, we instilled ZnONP with several dispersants. After 24 hr, ZnONP treatment was associated with comparable numbers of eosinophils in BALF when dispersed using rat serum (5.95 ± 2.74, × 10⁵), human serum (5.52 ± 1.45, × 10⁵), and rat BALF (5.40 ± 1.57, × 10⁵).

Discussion
We dispersed NPs in 5% rat serum using an equal-surface-area dose as the exposure metric and instilled mass doses ranging from 50 to 1,250 μg, which are roughly comparable with the predicted alveolar retained mass of 50 μg that would result from 1 month of exposure to a 250-μg/m³ cloud of NPs with aerodynamic diameter of 0.25 μm (consistent with typical NPs included in our study) according to the multiple-path particle dosimetry deposition model (Cassée et al. 2002). No single characteristic, including zeta potential, clearly predicted the inflammogenic potency of the eight NPs.

Using the rat model, we found that four metal oxide NPs each induced a different type of inflammation characterized by different types of infiltrating cells, inflammatory mediators, time course, and cytotoxicity. The vector diagrams in Figure 5 show the “inflammatory footprints” of each NP 24 hr and 4 weeks after instillation. After 24 hr of exposure, CeO₂NP and NiONP were associated with a...
neutrophilic/mild cytotoxic pattern; ZnONP was associated with an eosinophilic/severe cytotoxic pattern; and CuONP was associated with a neutrophilic eosinophilic/severe cytotoxic pattern. Four weeks after instillation, differences among NPs and from the 24-hr patterns were evident from the chronic inflammation patterns we observed, including a modest residual neutrophilic/mild cytotoxic inflammation with CeO₂NP; a greatly amplified immunological/severely cytotoxic inflammation with NiONP; and the almost total resolution of acute inflammation with CuONP. We also observed a modest residual eosinophilic/ immunological signal in BALF from ZnONP-exposed rats after 4 weeks.

It is important to note the contrast between the BALF results and the histology of the lung sections. Although the BALF inflammatory profile had waned dramatically by 4 weeks in both ZnONP- and CuONP-exposed rats, the histological assessments showed fibrosis in both cases, although the specific histological pattern differed between the ZnONP and CuONP groups. In contrast, the inflammation in the NiONP group, which had amplified greatly between 24 hr and 4 weeks, was not associated with fibrosis, although alveolar lipoproteinosis was present indicating severe, ongoing epithelial injury. It is possible, even likely, that fibrosis could develop in the longer term in NiONP-exposed lungs, as documented in previous studies with NiO particles and NPs showing late-developing pulmonary fibrosis (Ogami et al. 2009; Ozaki et al. 2002). The histological lesions 24 hr and 4 weeks after CeO₂NP instillation were consistent with BALF analysis (neutrophilic and mild cytotoxic inflammation) except for the rare granulomas present at 4 weeks, which seem to be associated with areas of high particle deposition. We used instillation in this study.
study as a substitute for inhalation; although instillation is known to produce a localized high dose and a high dose rate, it is a useful method for comparing responses to different particle types (Dricoll et al. 2000).

CeO$_2$NP- and NiONP-induced neutrophilic/mildly cytotoxic inflammation at 24 hr was associated with elevated IL-1$\beta$, MIP-2, and LDH in BALF, and both IL-1$\beta$ and MIP-2 are mediators of acute neutrophilic inflammation (Ulich et al. 1991; Wolpe et al. 1989). Four weeks after CeO$_2$NP instillation, a mild neutrophilic/cytotoxic inflammation associated with modestly increased MIP-2 expression was still evident. CeO$_2$NP has been used as a fuel additive for diesel engines (B. Park et al. 2008), and in vitro studies have shown that CeO$_2$NP increased production of reactive oxygen species in A549 cells (Lin et al. 2006) and BEAS-2B cells (E.J. Park et al. 2008). In contrast, CeO$_2$NP has been reported to be non-inflammatory (Lu et al. 2009) after pulmonary instillation, or cytoprotective in vivo by means of scavenging free radicals or reactive oxygen species in vivo.

Table 2. Summary of inflammatory mediators, LDH, and total protein in BALF 24 hr (acute) and 4 weeks (chronic) after instillation of high-dose (150 cm$^2$/rat) metal oxide NPs into rat lung.

| BALF measure          | VC       | CeO$_2$NP | NiONP   | ZnONP   | CuONP   |
|-----------------------|----------|-----------|---------|---------|---------|
| **Acute**             |          |           |         |         |         |
| IL-1$\beta$ (pg/mL)   | 2.8 ± 1.4| 8.4 ± 6.6 | 2.0 ± 2.3| 0 ± 0   | 0 ± 0   |
| MIP-2 (pg/mL)         | 82 ± 1.3 | 31.9 ± 10.2* | 39.5 ± 9.0* | 3.7 ± 3.3 | 7.3 ± 4.5 |
| Eotaxin (pg/mL)       | 0 ± 0    | 0 ± 0     | 1.9 ± 1.4| 0.2 ± 0.4| 0 ± 0   |
| IFN-\(\gamma\) (pg/mL)| 1.5 ± 1.2| 1.2 ± 0.8 | 0.8 ± 0.9| 1.8 ± 1.1| 1.6 ± 0.4|
| LDH$^a$               | 1.0 ± 0.01 | 3.3 ± 0.1$^a$ | 3.5 ± 0.2$^a$ | 12.3 ± 3.0$^a$ | 6.4 ± 0.9$^a$ |
| Total protein (mg/mL) | 0.19 ± 0.03 | 0.40 ± 0.13$^{**}$ | 0.42 ± 0.08$^{**}$ | 2.62 ± 0.63$^a$ | 2.81 ± 1.14$^a$ |
| Inflammation (BALF and histology) | None | Neutrophilic/cytotoxic | Neutrophilic/cytotoxic | Eosinophilic/cytotoxic | Neutrophilic/eosinophilic/cytotoxic |
| **Chronic**            |          |           |         |         |         |
| IL-1$\beta$ (pg/mL)   | 2.0 ± 0.01 | 3.3 ± 0.1# | 3.5 ± 0.2# | 12.3 ± 3.0# | 6.4 ± 0.9# |
| MIP-2 (pg/mL)         | 2.0 ± 0.01 | 3.3 ± 0.1# | 3.5 ± 0.2# | 12.3 ± 3.0# | 6.4 ± 0.9# |
| Eotaxin (pg/mL)       | 0 ± 0     | 0 ± 0     | 1.9 ± 1.4| 0.2 ± 0.4| 0 ± 0   |
| IL-13 (pg/mL)         | 0.17 ± 0.07 | 0.42 ± 0.34 | 3.24 ± 0.39$^f$ | 0.34 ± 0.01 | 0.37 ± 0.04 |
| Total protein (mg/mL) | 0.17 ± 0.07 | 0.42 ± 0.34 | 3.24 ± 0.39$^f$ | 0.34 ± 0.01 | 0.37 ± 0.04 |
| Inflammation (BALF and histology) | None | Neutrophilic/cytotoxic | Neutrophilic/cytotoxic | Eosinophilic/granulomatous/cytotoxic fibrosis | Granulomatous/fibrosis |

VC, vehicle control. Data are mean ± SD (n = 4 per group). For full LDH and total protein levels, see Supplemental Material, Figures 1 and 2 (doi:10.1289/ehp.1002201); for the full cytokine profile, see Supplemental Material, Figures 5 and 6.

$^a$Levels of LDH were expressed as fold changes compared with vehicle control. *p < 0.05, **p < 0.01, and $^f$p < 0.001, compared with VC.

Figure 5. Vector diagrams showing trends and types of acute (A) and chronic (B) inflammation after instillation of NPs (150 cm$^2$/rat). The various inflammatory parameters are shown as vectors, where radial axes depict the relative magnitude of any vector quantity. By joining the vector quantities, a unique pattern is produced for any treatment. The left upper quadrant shows lymphocytic parameters (lymphocytes and IFN-\(\gamma\)); right upper quadrant, neutrophilic parameters (PMN, MIP-2, and IL-1$\beta$); lower right quadrant, cytotoxic parameters (LDH and total protein); and lower left quadrant, eosinophilic parameters (eosinophils, eotaxin, and IL-13). VC, vehicle control. In A (24 hr after instillation), CeO$_2$NP and NiONP showed neutrophilic inflammation with mild cytotoxicity. ZnONP and CuONP, respectively, showed eosinophilic inflammation with severe cytotoxicity and neutrophilic/eosinophilic inflammation with severe cytotoxicity to the lungs of rats. In B (4 weeks after instillation), CeO$_2$NP still showed neutrophilic/mild cytotoxic inflammation. In contrast, NiONP showed neutrophilic/lungfibrotic inflammation with severe cytotoxicity, and ZnONP and CuONP showed eosinophilic inflammation without cytotoxicity and no inflammatory responses, respectively.
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