Role of Residual Additives in the Cytotoxicity and Cytokine Release Caused by Polyvinyl Chloride Particles in Pulmonary Cell Cultures

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Occupational exposure to polyvinyl chloride (PVC) dust has been linked to pulmonary disease. The aim of the present study was to investigate, in vitro, the role of additives in the cytotoxicity and the release of inflammatory mediators caused by PVC particles in different cells. We compared two types of emulsion PVC particles (E3 and E8) with their washed (hence, “additive-free”) counterparts (W3 and W8). A positive control (crystalline SiO2, Min-U-Sil) and the pure additives, sodium lauryl sulfate (A3) and sodium alkybenzenesulfonate (A8), were tested concurrently. Cytotoxicity (MTT assay) was assessed in primary cultures of rat alveolar macrophages, rat type II pneumocytes, and human alveolar macrophages, rat type II pneumocytes, and human alveolar macrophages (h-AM), was measured by ELISA after 4, 16, 24 and/or 48 h of exposure. Cytotoxicity and hemolytic activity of the washed particles were abolished or markedly decreased compared with their nonwashed forms. In A549 cells, E3 and E8 (2.5 mg/ml) caused a 3-fold increase in IL-8 release and a more than 10-fold increase in IL-6 release, whereas W3 and W8 did not elicit any significant response at similar concentrations. Compared with Min-U-Sil (0.1, 0.5, and 2.5 mg/ml), the response to E3 and E8 occurred later and was slightly lower (IL-8) or much more pronounced (IL-6). A3 and A8 exhibited similar responses to E3 and E8, at concentrations corresponding to those present in the particles. In conclusion, the in vitro cytotoxicity and inflammatory potential of some PVC particles appear to be mostly due to their residual additives.

Key Words: polyvinyl chloride; PVC; additive; silica; particle; cytotoxicity; IL-8; IL-6; TNF-α; A549; THP-1.

Polyvinyl chloride (PVC) is among the most widely used plastic materials. The physical properties of PVC can be readily modified through the introduction of additives and plasticizers, leading to diverse applications in construction, tubing, coating, and packaging (Lewis, 1999). Occupational exposure to PVC dust has been associated with lung disorders in sporadic case reports of “PVC pneumoconiosis” (Antti et al., 1986; Arnaud et al., 1978; Studnicka et al., 1995; Szende et al., 1970) and in epidemiological studies, indicating that occupational PVC dust exposure might affect pulmonary function and lead to a higher prevalence of small opacities on chest X-ray (Lee et al., 1991; Lloyd et al., 1984; Ng et al., 1991; Niels et al., 1989; Soutar and Gauld, 1983).

Dust exposure is often associated with airway inflammation. In chronic exposure the inflammation is generally recognized as having adverse effects and contributing fibrogenic mediators to lung fibrosis (Bassett and Bhalla, 2000). Both alveolar macrophages and epithelial cells act as a first-line defense against inhaled agents; they can recruit inflammatory cells to the airways via the release of chemo-attractants or cytokines. Interleukin-8 (IL-8), Interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) mediate a variety of inflammatory effects in pulmonary inflammation. IL-8 and IL-6 are produced and released from both epithelial cells and alveolar macrophages, while the major source of TNF-α in the lung is alveolar macrophages (Lohmann-Mathes et al., 1994; Martin et al., 1997).

In a previous study, we tested 14 types of PVC particles and 16 relevant additives sampled from normal industrial processes, and found that some PVC particles produced by the emulsion process showed moderate in vitro cytotoxicity. The most toxic particles contained the most toxic additives (Xu et al., 2002). To elucidate the role of residual additives, two of these emulsion PVC particles were chosen for the present study, and they were compared with their “additive-free” counterparts and with the pure additives sodium lauryl sulfate and sodium alkybenzenesulfonate. We investigated the cytotoxicity and the release of cytokines caused by PVC particles with or without additives in different relevant cells. Cytotoxicity was assessed in primary rat alveolar macrophages and type II pneumocytes and primary human alveolar macrophages (h-AM), as well as in cells from the human-derived A549 and THP-1 cell lines. Hemolytic potential was assessed in human erythrocytes. The effect on cytokine production was assessed in A549 cells, THP-1 cells, and h-AM.

We found that unwashed PVC particles exhibited the potential to damage pulmonary cells and to induce the release of inflammatory mediators in vitro. These particles might also...
have hazardous potential to the lung under excessive occupational exposure. The present study proposes that the hazardous potential of these PVC particles is most probably related to their residual additives.

**MATERIALS AND METHODS**

**Particles and additives.** PVC particles (E3, E8), their “additive-free” counterparts (W3, W8), additive A3 (sodium lauryl sulfate, 1% in E3), and additive A8 (sodium alkylbenzenesulfonate, 0.85% in E8) were provided by the Association of Plastics Manufacturers Europe (APME). The preparation of W3 and W8 was done by an industrial laboratory of a member company of APME, as follows: E3 and E8 were washed four successive times in 8 h with methanol boiled under reflux (fresh methanol being used for each stage), then dried by filtration after each stage. First drying was at room temperature and final drying in an oven under vacuum at 40°C until constant weight. More than 92% of the additives of E3 and E8 were washed off by this procedure, as checked by titration of anionic groups with hyamine on samples before and after washing.

Min-U-Sil (crystalline SiO2), kindly provided by Prof. B. Fubini (Facoltà di Farmacia, Università di Torino, Italy), was used as a positive particle control. The median size of particles was around 2 μm, as measured by means of a Coulter LS particle size analyzer at Vlaamse Instelling voor Technologisch Onderzoek (VITO), Belgium.

Samples for electron microscopy were dispersed in ultrafiltered ethanol, using a vortex mixer. Droplets of the resulting suspension were applied to aluminum specimen holders, dried-down, sputter-coated with gold, and examined in an ISI DS130 scanning electron microscope. The remaining suspension was mixed (1 + 1) with ultrafiltered, ultrapure water and mixed again. Droplets of this suspension were applied to nickel grids, precoted with a film of formvar/carbon, and examined in a Zeiss 902A transmission electron microscope.

**Reagents and solutions.** Waymouth’s 752/1 medium, RPMI 1640 medium, penicillin-streptomycin solution (10,000 units, 10,000 μg/ml), fungizone (ampicillin 250 μg/ml), L-glutamine (200 mM), HEPES, and fetal bovine serum (FBS) were purchased from GibcoBRL company (N.V. Life Technologies, Merelbeke, Belgium). DMSO (dimethyl sulfoxide), MTI (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), Percoll, lipopolysaccharide (LPS), polymyxin B sulfate, polyoxyethylenesorbitan mono- laurate (Tween 20), trypsin type I (EC3.4.214. cat. No. T-8003), and phorbol myristate acetate (PMA) were purchased from Sigma Belgium (Bornem, Belgium). Deoxyribonuclease (DNAse) I was purchased from Boehringer (Belgium). Deoxyribonuclease (DNAse) I was purchased from Boehringer (Belgium). Deoxyribonuclease (DNAse) I was purchased from Boehringer (Belgium). Deoxyribonuclease (DNAse) I was purchased from Boehringer (Belgium). Deoxyribonuclease (DNAse) I was purchased from Boehringer (Belgium). Deoxyribonuclease (DNAse) I was purchased from Boehringer (Belgium). Deoxyribonuclease (DNAse) I was purchased from Boehringer (Belgium).

**Primary human alveolar macrophages (h-AM).** Human surgical specimens were obtained from the nontumoral part of the lung of three patients undergoing lobectomy for lung cancer. The ages of the patients were 59, 55, and 71 years and the Ethical Committee of the Faculty of Medicine approved the use of such tissue. Alveolar macrophages were isolated based on the methods described previously (Hoet et al., 1999; Xu et al., 2002). The tissue was first sliced and then washed with NaCl (0.9%). The washing fluid contained mainly macrophages and erythrocytes. In order to eliminate the erythrocytes, the washing fluid was suspended in an isotonic shock solution containing 155 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA, pH 7.4 (Roos and Loos, 1970), for 10 min at 4°C. The remaining cells were washed three times in PBS. This yielded 5–10 x 10^5 alveolar macrophages (more than 92% pure) per gram of tissue with a viability of more than 95%. Cells were transferred to 96-well plates (100,000 cells/200 μl/well) and incubated in the same medium as for A549 cell cultures. Four h after plating, test samples were added when the cell culture reached confluence.

**Human red blood cells (h-RBC).** The cells were obtained, as in our previous study (Xu et al., 2002), from volunteers by venipuncture, using Vacutainers® (Becton Dickinson Co.) containing heparin. The serum and white cells were removed and the erythrocytes were washed three times with veronal-buffered saline (VBS). H-RBC were suspended at approximately a 2% concentration in VBS. In order to avoid donor differences, the initial h-RBC suspensions were adjusted to equal hemoglobin concentrations (Nolan et al., 1981).

**Exposure of adherent cells (A549, r-AM, r-TII, h-AM, THP-1) to particles and additives.** The particles/additives were freshly (i.e., 1–2 h before addition to the cells) suspended/dissolved in vehicle (Waymouth medium or RPMI 1640 medium + 5% ethanol), then vortexed for 1 min and placed in an ultrasonication bath (Branson 1200, Branson Ultrasonics Corp., working frequency: 47 kHz, HF-output power: 30 W, 15 min). The suspensions/solutions were pipetted into the cell culture (20 μl of sample + 180 μl culture medium per well); the wells containing control cells received 20 μl of vehicle and 180 μl culture medium. Final working concentrations were 2.5–0.004 mg/ml (1 mg/ml = 0.2 mg/10^5 cells = 6 μg/mm²) for particles and 0.04–0.0025 mg/ml for additives, with 4- to 5-fold dilution steps. During exposure of the cells to test compounds, the culture medium was serum-free (Waymouth’s medium or RPMI 1640 medium + 1% glutamine + 1% penicillin-streptomycin + 0.5% fungizone). Cytotoxicity was determined after 20–24 h or 48 h of incubation. Aliquots of A549 cell culture supernatant (after 4-, 16-, 24-, and 48-h exposure), THP-1 cell culture supernatant (after 48-h exposure), and h-AM cell culture supernatant (after 24-h exposure) were frozen at −80°C for subsequent determination of IL-8, IL-6, or TNF-α concentrations.

**Exposure of h-RBC to particles and measurement of hemolysis.** The particles were suspended in EV (VBS containing 10% ethanol) on the day of the experiment. A volume of 1 ml h-RBC suspension was added to the glass tubes containing 3 ml sample suspension. The mixtures were placed in a water bath (37°C) for a period of 2 h. The tubes were inverted every 30 min by hand to resuspend the settled cells and particles. At the end of 2 h incubation, all tubes were centrifuged (1600 g) for 20 min and the absorbance of the supernatant was determined.
Background controls (1 ml h-RBCS + 3 ml EV = 0% lysis) and total lysis controls (1 ml h-RBCS + 3 ml distilled water = 100% lysis) were treated identically in each experiment. Working concentrations were 0.25, 0.5, 1, 2, and 4 mg/ml.

The absorbance of liberated hemoglobin, or hemolysis, was recorded at 540 nm, using a Beckman Model DU-65 spectrophotometer. Viability was expressed as 100% minus the percentage hemolysis versus vehicle control.

**Measurement of cellular toxicity in adherent cells.** As an index of cell toxicity, the decline in MTT reduction was measured (Mosmann, 1983). MTT solution (0.5 mg/ml) was added to each culture well (200 μl/well) and incubated for 1 h (37°C, 5% CO2). Then the formation of the purple formazan was assessed spectrophotometrically (Benchmark Microplate Reader, Bio-Rad) at 550 nm, with reference wavelength 650 nm. Viability of cells was expressed as a percentage versus vehicle control.

**Measurement of cytokine (IL-8, IL-6, and TNF-α) production.** Cytokine proteins in the cell culture supernatants released from treated A549 cells, THP-1 cells, or h-AM were measured by ELISA using kits from BIOSOURCE (HU IL-8 cytose, HU IL-6 cytose, and HU TNF-α cytose). Each assay was performed according to manufacturer’s directions and read on a Bio-Rad microplate reader at 450 nm (with reference filter at 655 nm). Standards and samples were pipetted into 96-well microplates (NUNC™, Nunc-Immuno™, Module, MaxiSorp™ surface) precoated with coating antibody, followed immediately by addition of biotinylated antibody. After a room temperature incubation step, unbound antigen was washed off and streptavidin-horseradish peroxidase conjugate was added. Following another incubation and wash step, substrate was added and color development was in proportion to the amount of cytokine added to the wells. The lower limits of detection for IL-8, IL-6, and TNF-α were 8.2, 10.2, and 10.2 pg/ml, respectively.

**Endotoxin measurements.** Endotoxin measurements of the tested particles were performed using the Limulus Amebocyte Lysate (LAL) assay (E-TOXATE Kit, Sigma).

**Data presentation and analysis.** At least 3 separate experiments were performed with each substance, concentration, and cell system. The results were analyzed with Prism 3.0 Graphpad software (San Diego, CA). Statistical differences were assessed by two-way analysis of variance (ANOVA). Two-tailed tests of significance were employed and significance was assumed at p < 0.05.

**RESULTS**

**Particle morphology.** The samples of Min-U-Sil contained fragments typical of ground silica, mostly ranging from 0.5–3.0 μm in the largest dimension (Fig. 1A). The majority of E3 particles were spheroidal, and ranged from 0.2–2.0 μm in the largest diameter (Fig. 1B). Some of these particles formed compact spheroidal aggregates of up to 50 μm in the largest diameter, which could not be dispersed by prolonged (15-min) agitation with a vortex mixer or with an ultrasonic bath (Fig. 1C). Smaller, irregular clumps were also present in these samples, but they were less common after prolonged agitation. A similar range of particles was observed in washed (W3) samples but the irregular clumps of particles were more common, even after prolonged agitation (Fig. 1D). Samples of E8 particles were similar to those in E3, but there was a marked preponderance of spherical particles both 1.0 μm and 260 nm in diameter. This was particularly evident when grids were examined by transmission electron microscopy, using an energy filter to highlight only those regions with a thickness of approximately 100–750 nm (Fig. 1E). The same technique confirmed the presence of irregular clumps of particles in the samples of W8, even after prolonged agitation. These were smaller than those in W3 but still largely devoid of regions thinner than 750 nm (Fig. 1F).

Thus, washing did not alter the preponderance of individual particles or groups of particles less than 2.0 μm in the longest dimension, but it did result in some of the irregular clumps of particles being more resistant to dispersion.

**Reduced cytotoxicity and hemolytic activity with additive-free particles.** Confirming our previous findings (Xu et al., 2002), significant cytotoxicity and hemolysis were observed for PVC particles E3 and E8, and their relevant additives (A3 and A8) in the different cells used. As shown in Figure 2, the cytotoxicity and hemolytic activity of E3 and E8 particles were abolished or markedly decreased after removal of their additives.

**Cytokine release from A549 cells.** The effects of the particles E3 and W3, and additive A3 on cytokine release from A549 cells were similar to the effects of E8, W8, and A8, respectively. No detectable TNF-α was measured from A549 cell culture supernatant after exposure to either Min-U-Sil, PVC particles, or additives. However, an increased release of IL-8 and IL-6 was found after exposure to E3, E8, A3, and A8 (Fig. 3). Because of the high interexperimental variation of IL-8 release from untreated A549 cells (values between 50–200 pg/ml at 4 h, 150–500 pg/ml at 16 h, 200–800 pg/ml at 24 h, and 400–1200 pg/ml at 48 h), although the intra-experimental levels were quite stable (variation within 10% among six control wells, Table 1), we took mean control levels as a baseline (100%) for each separate experiment in the case of IL-8. For IL-6, the release from control cells was just around or lower than the detection limit. Thus, we present actual measured concentrations (pg/ml) in the case of IL-6. IL-8 and IL-6 release were induced by exposure to E3 and E8 at 2.5 mg/ml, whereas W3 and W8 did not elicit any significant response at similar concentrations. A3 and A8 exhibited similar responses as E3 and E8 but at approximately one hundred-fold lower concentrations (Fig. 3). This corresponds to the concentrations of A3 present in E3 (1%) and of A8 in E8 (0.85%).

We found different dose and time courses of cytokine release from A549 cells after exposure to PVC particles and Min-U-Sil. Min-U-Sil induced an earlier IL-8 and IL-6 release compared with PVC particles (Fig. 4; as shown in Fig. 3, the effects of E3 and E8 on cytokine release were similar, therefore, only results of E3 are shown in Fig. 4). The induction was already significant at 4 h with Min-U-Sil, but not with PVC particles. For Min-U-Sil, the highest induction was reached at a concentration of 0.156 mg/ml rather than the two higher concentrations.

Quantitatively, the response to PVC particles was slightly lower for IL-8 but much more pronounced for IL-6, compared to the response with Min-U-Sil (Fig. 4).
were consistent across the three batches of macrophages (Fig. 5). Within patients, the variability in cytokine release was higher and led to SD of up to 45% in IL-8 release in control wells \((n = 8)\). Macrophages from patient 1 had no IL-8 response to Min-U-Sil and PVC particles, cells from patient 2 appeared to “overreact” to all concentrations of Min-U-Sil and PVC particles, while only the cells from patient 3 showed a “decent” dose-response reaction to Min-U-Sil. The variability in response to the particles precluded any meaningful statistical analysis and interpretation, and this is why we chose to work with a macrophage cell line.

**Cytokine release from THP-1 cells.** No detectable IL-6 release was measured in THP-1 cell culture supernatants after a 48-h exposure to either particles or additives. TNF-\(\alpha\) and IL-8 were measurable (mean level: 374.2 pg/ml and 19.5 ng/ml in the control wells, respectively), but no significant increase was found after a 48-h exposure to PVC particles (Fig. 6). However, there was an increased release from THP-1 cells exposed to Min-U-Sil at a concentration of 0.1 mg/ml. At higher concentrations, there was decreased release of TNF-\(\alpha\) and IL-8, but at these concentrations viability also had become lower than 25% of control.
FIG. 2. Cell viability, as assessed by MTT reduction after 20–24 h (r-AM, r-TII, h-AM, A549) or 48 h (THP-1) incubation or by hemolysis test (viability being expressed as 100% minus the percentage of hemolysis versus vehicle control) after 2-h incubation (h-RBC) with emulsion PVC particles (E3 and E8), their additive-free counterparts (W3 and W8), or pure additives (A3 and A8). Mean ± SD, n = 3–4 (absence of bars for SD indicates SD is smaller than symbol). r-AM, rat alveolar macrophages; r-TII, rat type II pneumocytes; h-AM, human alveolar macrophages; h-RBC, human red blood cells; THP-1, differentiated THP-1 cells; A549, A549 cell line. Concentration: 1 mg/ml = 0.2 mg/10^7 cells = 6 μg/mm^2. Results for A3 and A8 in r-AM, r-TII, h-AM, and h-RBC have been presented previously (Xu et al., 2002, Fig. 2).
Exclusion of LPS as a cause of the effects. Possible endotoxin contamination of the particles was checked by the LAL assay. The sensitivity of this semiquantitative test is 0.06 EU/ml (i.e., endotoxin standard is positive at 0.06 EU/ml and negative at 0.03 EU/ml). We assessed the endotoxin levels in our particles (at 2.5 mg/ml, the highest concentration used) and found that only E3 and Min-U-Sil had positive results with endotoxin levels between 0.06 and 0.125 EU/ml.

Two concentrations of LPS (0.25 and 5 EU/ml) were applied in the experiments to examine the effect of LPS on cytokine release from the cells. At these concentrations, LPS did not induce cytotoxicity in A549 or THP-1 cells. No significant increases of IL-6 or TNF-α release from A549 cells were found after 4 – 48 h of exposure to LPS. However, the release of IL-8 was significantly increased after 24 or 48 h of exposure to LPS at 5 EU/ml, but not at 0.25 EU/ml, which is higher than the highest endotoxin level found in our particle suspensions (Table 1). We also tested the effect of suspending the particles with polymyxin B (10 μg/ml) to neutralize the effect of LPS (Dong et al., 1996; Morrison and Jacobs, 1976) before addition to the cell cultures, but the cytokine release remained similar (data not shown). Neither measurable IL-6 release nor significant decrease of cell viability were found in THP-1 cells after a 48-h exposure to LPS. However, the release of TNF-α and IL-8 were significantly increased after 48 h of exposure to LPS at 5 EU/ml, but again not at 0.25 EU/ml (Table 1). Thus, we concluded that any increased cytokine release after exposure to EU/ml (i.e., endotoxin standard is positive at 0.06 EU/ml and negative at 0.03 EU/ml.). We assessed the endotoxin levels in our particles (at 2.5 mg/ml, the highest concentration used) and found that only E3 and Min-U-Sil had positive results with endotoxin levels between 0.06 and 0.125 EU/ml.

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### TABLE 1
Cytokine Release from Cells in Response to LPS

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Duration</th>
<th>Control</th>
<th>0.25 EU/ml</th>
<th>5 EU/ml</th>
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<tbody>
<tr>
<td>A549</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>IL-8</td>
<td>4 h</td>
<td>100 ± 5</td>
<td>98 ± 9</td>
<td>103 ± 16</td>
</tr>
<tr>
<td></td>
<td>16 h</td>
<td>100 ± 4</td>
<td>108 ± 9</td>
<td>171 ± 57</td>
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<td></td>
<td>24 h</td>
<td>100 ± 8</td>
<td>113 ± 13</td>
<td>220 ± 86*</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>100 ± 7</td>
<td>116 ± 19</td>
<td>277 ± 107**</td>
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<tr>
<td>THP-1</td>
<td></td>
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<tr>
<td>TNF-α</td>
<td>48 h</td>
<td>100 ± 9</td>
<td>146 ± 29</td>
<td>373 ± 77**</td>
</tr>
<tr>
<td>IL-8</td>
<td>48 h</td>
<td>100 ± 5</td>
<td>129 ± 68</td>
<td>313 ± 158*</td>
</tr>
</tbody>
</table>

Note. LPS, lipopolysaccharide. Cells were exposed to LPS for 4 – 48 h. IL-6 and TNF-α were nondetectable in A549 cell culture supernatant from control wells or cells treated with LPS; IL-6 was nondetectable in THP-1 cell culture supernatant from control wells or cells treated with LPS, n = 3–5, values are % (± SD) compared to the mean of 6–8 control wells in each experiment. *p < 0.05, **p < 0.01, compared to control.
particles was not due to their possible endotoxin contamination.

**DISCUSSION**

The present study confirmed that some emulsion PVC particles (e.g., E3, E8) exhibited cytotoxicity (Xu et al., 2002) and showed, additionally, that these particles induced the release of inflammatory mediators in pulmonary cells in vitro. Removing their residual additive considerably decreased their cytotoxic effect and ability to induce the release of inflammatory mediators. The pure additives exhibited similar responses in cytotoxicity and cytokine release at concentrations corresponding to those present in the particles. Earlier experimental studies by Richards and his coworkers also found that the hemolytic activity of some PVC particles was reduced after washing with saline but some retained similar hemolytic activity after washing (Richards et al., 1975, 1976). In another study, the lethality towards peritoneal macrophages was reduced after alcohol washing, but not after saline washing (Pigott, 1976). However, in the study, the authors doubted whether the washing procedure they used by centrifugation would result in the loss of some very fine PVC particles (Richards et al., 1976). It should

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**FIG. 4.** Release of IL-8 and IL-6 from A549 cells incubated with emulsion PVC particle E3 or Min-U-Sil at different concentrations. Cells were exposed to these particles for 4, 16, 24, or 48 h, then the supernatants were collected and IL-8 and IL-6 protein levels were measured by ELISA. Mean ± SD, n = 3.
be noted that the washing procedure used here was different and much more drastic. Nevertheless, the distribution of the particles did not substantially change after the washing procedure, as observed by electron microscopy. Figure 1 shows that the PVC samples were mainly composed of respirable particles, as well as larger clumps. Therefore, we suggest that the hazardous potential of these PVC particles is most probably associated with their residual additives. Our findings support the concept that chemical surface characteristics are an important determinant of the biological effects of particles (Kuschner et al., 1997; Nemmar et al., 2002).

Cytokine measurement and cell cultures. IL-8 is an essential factor for neutrophil infiltration in most inflammatory reactions (Sekido et al., 1993). However, according to a recent review “no clear-cut IL-8 homolog in rat or mouse has been identified so far, although macrophage inflammatory protein-2 (MIP-2) and KC may be functional homologs of IL-8 in mice” (Iizasa and Matsushima, 2000, 1065). In the rat there are at least three members of the CXC chemokine family that are potent chemotactic factors for neutrophils: cytokine-induced neutrophil chemoattractant-1 (CINC-1), CINC-2 and CINC-3/MIP-2 (Shibata et al., 2000). Not all above-mentioned cytokine assays are available for rat. Therefore, we preferred to assess the release of cytokines from human cells in the present study. We have attempted to assess this in primary human alveolar macrophages obtained from surgical specimens, as in our previous study (Xu et al., 2002). Figure 5 shows that although the cell viability experiments were consistent across the three batches of macrophages that we had isolated from three patients, there were large variations in cytokine release within and between individual subjects. Such variability is not unusual when working with patient-derived tissues. However, because this variability complicated the interpretation of our results, we chose to use more stable cell lines in the present study.

Endotoxin level and cytokine release. Endotoxin is known to have an effect on induction of cytokine production (Thorne et al., 1999), but it was not responsible for the induction of the cytokine release by cells exposed to PVC particles or silica in our study, since we demonstrated that LPS at 0.25 EU/ml, which is higher than the endotoxin level found in our particle suspensions, could not induce cytokine release from A549 cells or THP-1 cells. Nevertheless, the elevated release of cytokines by both types of cells after exposure to a higher LPS concentration (5 EU/ml) verified that LPS could effect an inflammatory response.

Differential cytokine release in response to particles and additives: Comparison of A549 cells and THP-1 cells. Cytokine release, upon exposure to particles, exhibited a nonlinear dose-response relationship. At a concentration of 0.156 mg/ml, Min-U-Sil led to the highest enhancement of cytokine release in both A549 cells and THP-1 cells (Figs. 4 and 6). However, less than half of the cells survived at higher concentrations, as assessed by the MTT assay. Consequently the apparent decrease of cytokine production at higher concentrations is most...
likely an effect of fewer cells available to produce it. This may also explain the decreased cytokine level in THP-1 cell cultures after exposure to cytotoxic concentrations of particles and additives (Fig. 6).

The additives are highly toxic, but the original PVC particles only contain less than 1% of these additives. The responses of cells after treatment with PVC particles or the additives alone were qualitatively similar, but the magnitude of difference between effective concentrations of PVC particles and their additives was approximately 100-fold lower. Limited experiments (data not shown) were performed using the supernatant obtained after centrifugation of suspended PVC particles. Supernatant alone induced slight toxicity but the major toxic effect remained with resuspended particles, indicating that the additives were not readily released into the medium. In fact, even after four successive methanol washings, 5–8% of the additives still remained associated with the particles.

The cytokine release from A549 cells was induced by PVC particles and Min-U-Sil, but only Min-U-Sil induced cytokine release from THP-1 cells (Figs. 4 and 6). The magnitude of the increases in IL-6 and IL-8 release after particle exposure in A549 cells is significantly greater. Also after exposure to highly toxic Min-U-Sil, A549 cells still produced more cytokines, but THP-1 cells did not. We speculate that A549 cells have a stronger ability to produce cytokine in response to particles than THP-1 cells, and alveolar epithelial cells respond in a cell-specific way to this type of trigger. It is possible that the potential of THP-1 cells to produce additional cytokines was exhausted by their prior incubation with phorbol esters, thus resulting in a less marked response to the subsequent particle exposure. The IL-8 and TNF-α levels in control THP-1 cell supernatants were more than 20 times higher than those of control A549 cells.

Comparison of PVC particles with Min-U-Sil. The induction of cytokine release was rapid by Min-U-Sil, with cytokine protein elevated 4 h after exposure, and it was also more potent, except for IL-6 (Figs. 4 and 6). This is compatible with the notion that crystalline silica is one of the most fibrogenic dusts (Fubini, 1997). However, we were surprised to find that the magnitude of the increase in IL-6 release by A549 cells exposed to PVC E3 or E8 particles was significantly greater than after exposure to Min-U-Sil. IL-6 acts on proliferating B cells to promote differentiation into antibody forming cells (plasma cells) and it stimulates antibody secretion. It has been described as a “hepatocyte stimulating factor” and strongly stimulates hepatocytes to make acute-phase proteins in response to inflammation (Zhang et al., 1995). It is also an
effective inducer of tissue factor upregulation and may trigger endothelial cells to change their antithrombotic properties into a procoagulant, clot-promoting state (Grignani and Maiolo, 2000). IL-6 stimulates fibrinogen expression both in vivo and in vitro (Castell et al., 1990; Rokita et al., 1993). The apparently specific effect of PVC particles on IL-6 production and its relevance for pulmonary disease remain to be explored, particularly with regard to granulomatous lung disease.

Relevance of the study. In humans, PVC pneumonoisosis has some features of granulomatous lung disease (Antti-Poika et al., 1986; Arnaud et al., 1978; Studnicka et al., 1995; Szende et al., 1970). Rats developed focal pulmonary fibrosis after a 15-week inhalation of PVC dust at a “nuisance” level, i.e., 10 mg/m³ (Richards et al., 1981). Inflammation is likely to be part of the mechanisms, although this was not investigated in the study. The initial steps of the airway inflammation include stimulation of resident cells, release of chemotactic agents, and recruitment of inflammatory cells, which ultimately results in the development of chronic lung disease (Bassett and Bhalla, 2000). In the present study, unwashed PVC particles (E3 and E8) exhibited the potential to damage pulmonary cells and to induce the release of inflammatory mediators, such as IL-8 and IL-6. IL-8 enhances inflammation, by enabling immune cells to migrate into tissue (Huber et al., 1991). IL-6 plays a critical role in the progression of lung inflammation/injury (Yu et al., 2002). Therefore, excessive occupational exposure to these PVC particles might cause cellular damage and chronic inflammation. According to our in vitro study, reducing the amount of residual toxic additive might prevent these processes. Additional studies in vivo will follow to investigate whether these PVC particles can induce lung inflammation and whether the in vivo response is correlated with the in vitro results.

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