

# The Capacity of Particles to Increase Allergic Sensitization Is Predicted by Particle Number and Surface Area, Not by Particle Mass

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Particle exposure has traditionally been monitored as mass concentration of PM<sub>10</sub> (particles with an aerodynamic diameter less than 10  $\mu\text{m}$ ), more recently also as PM<sub>2.5</sub>. The mass concentration is strongly influenced by the large particles. Therefore, particle mass is a poor measure for characterizing the amount of the small, possibly more biologically potent particles. We used polystyrene particles (PSP) ranging in diameter from 0.0588 to 11.14  $\mu\text{m}$ , carbon black (CB), and diesel exhaust particles (DEP), to study the adjuvant effect of particles on the immune response to the allergen ovalbumin (OVA) after sc injection into the footpad of BALB/cA mice. At a given mass dose, the small particles (0.0588 and 0.202  $\mu\text{m}$  PSP, CB, and DEP) increased the allergen-specific IgE serum levels to a substantially higher degree than the larger particles (1.053, 4.64, and 11.14  $\mu\text{m}$  PSP). Further, in the draining lymph node during the primary response, the fine particles (0.202  $\mu\text{m}$ ) with OVA increased cell numbers, expression of surface markers (CD19, MHC class II, CD86, and CD23) and *ex vivo* production of IL-4 and IL-10, whereas the largest (11.14  $\mu\text{m}$ ) particles did not. Linear regression analyses indicated that the IgE response was not predicted by particle mass ( $R^2 = 0.06$ ), but was predicted by the total particle surface area ( $R^2 = 0.64$ ), number of particles ( $R^2 = 0.62$ ), and particle diameter ( $R^2 = 0.58$ ). In conclusion, we found that fine particles exerted stronger adjuvant effects on allergic responses than larger particles at equal mass doses. Consequently, the dose described as total particle surface area or particle number predicts the adjuvant effect of particles better than the currently used particle mass.

**Key Words:** PM; particle size; adjuvant effect; allergy; mice; IgE.

Ambient air particles have recently received much interest because of increasing epidemiological and experimental evidence for their effects on pulmonary and cardiovascular health (Zanobetti *et al.*, 2003). Airborne particles are a complex mixture of particles differing by size, chemical composition, and structure. However, which characteristics of particulate pollution are responsible for the health effects are not clear. Several epidemiological studies have indicated that the fine and ultrafine particles are more responsible for the respiratory

effects than the coarse particles (Penttinen *et al.*, 2001a; Peters *et al.*, 1997; Schwartz and Neas, 2000). Traditionally, particle exposure has been monitored as mass concentration of PM<sub>10</sub> (particulate matter with an aerodynamic diameter less than 10  $\mu\text{m}$ ), more recently also as PM<sub>2.5</sub>. These particle mass measurements are strongly influenced by the large particles in the respective fractions. Therefore, the particle mass concentration is a poor measure for characterizing the amount of the fine, possibly more biologically potent particles. Accordingly, an increasing number of investigators therefore challenge the traditional sampling of mass PM<sub>10</sub> or PM<sub>2.5</sub>, and suggest measuring particle numbers (Penttinen *et al.*, 2001a,b; Peters *et al.*, 1997) or particle surface area (Lison *et al.*, 1997; Maynard and Maynard, 2002; Moshhammer and Neuberger, 2003; Tran *et al.*, 2000) as more relevant parameters in further investigations.

Numerous studies indicate that several types of particles increase the allergic response, both in mice and humans (reviewed in Granum and Lovik, 2002). Both the particle core and the organic and inorganic substances on the particles have been shown to increase allergic sensitization (Granum *et al.*, 2001; Heo *et al.*, 2001; Lovik *et al.*, 1997; Siegel *et al.*, 2004; van Zijverden *et al.*, 2000), and particle composition and biosolubility have been shown to play a role in allergic sensitization (Lambert *et al.*, 2000). However, few investigators have assessed the role of the different physical characteristics of particles in relation to allergic responses. Granum *et al.* (2000) studied the adjuvant effect of polystyrene particles (PSP, a surrogate for the insoluble particle core) with diameters between 0.1 and 0.99  $\mu\text{m}$  on the production of IgE, which is a hallmark of allergic disease. After ip injection of allergen and constant mass dose PSP in BALB/cA mice, the adjuvant effect on allergen-specific IgE levels in serum increased with decreasing particle size. Within the limited size range used, the enhanced IgE antibody levels showed covariation with the total particle number and the particle surface area but not with particle mass. We have recently shown (Nygaard *et al.*, in press) that PSP with a diameter of 0.1  $\mu\text{m}$  exerted an adjuvant effect on allergen-specific IgE antibody responses in mice after sc injection into the footpad. Moreover, the IgE adjuvant potency of ambient air particles from four European cities have been

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screened in this model, and the fine fraction (0.1–2.5  $\mu\text{m}$ ) generally was more potent than the coarse fraction (2.5–10  $\mu\text{m}$ ) (Lovdal *et al.*, 2003). The footpad drains exclusively to the popliteal lymph node (PLN; Tilney, 1971), and the PLN cells therefore is particularly well suited for studies of the lymph node immune regulation, which is important also after particle exposure in the lung (Toews, 1997). Moreover, the retained dose varies less after sc injection than after exposure in the airways, giving less experimental variation.

In the present study we used the mouse footpad model to test the hypotheses that (1) fine particles exert a stronger adjuvant effect on the allergic response than coarse particles and (2) if so, particle surface area or particle numbers predict the increased IgE response better than particle mass. We studied allergen-specific antibody responses after injection of a broad size range of PSP (diameters 0.0588 to 11.14  $\mu\text{m}$ ) together with the allergen ovalbumin (OVA). Carbon black particles (CB) and diesel exhaust particles (DEP) were included as reference particles. To investigate if the fine and coarse particles influenced the primary cellular responses in a qualitative different way (i.e., by different mechanisms), we studied the lymphocytes from the draining lymph node five days after injection of OVA with PSP with diameters of 0.202, 1.053, and 11.14  $\mu\text{m}$ . In order to decide which particle characteristics could best predict the responses, we performed linear regression analyses for all measured parameters versus the particle characteristics mass, size, number or surface area. Our results support the notion that total particle surface area and particle numbers, better than the currently used mass concentration of PM10 or PM2.5, predict the biological effects of particulate matter.

## MATERIALS AND METHODS

**Animals.** Female inbred BALB/cA mice (Taconic M&B A/S, Ry, Denmark) were housed, 5–8 animals per cage, on BeeKay bedding (B&K Universal AS, Nittedal, Norway) in type III macrolon cages in filter cabinets (Scantainers). The animals were allowed to rest for at least one week before entering the experiments at 6–7 weeks old. The mice were exposed to a 12-h/12-h light/dark cycle (30–60 lux in cages), regulated room temperature ( $20 \pm 2^\circ\text{C}$ ), and 40–60% relative humidity. The animals were given pelleted food (RM1, SDS, Essex, U.K.) and tap water *ad libitum*. The experiments were performed in conformity with the laws and regulations for experiments with live animals in Norway, and they were approved by the Experimental Animal Board under the Ministry of Agriculture in Norway.

**Particles and allergen.** Diesel Exhaust Particles (DEP, Standard Reference Material 2975) were obtained from the National Institute of Standards & Technology (Gaithersburg, MD). As models for the insoluble particle core, Carbon Black particles (CB, Regal250, Cabot Corporation, Leiden, The Netherlands) were used, as well as polystyrene particles (PSP) of different sizes (diameters of 0.0588, 0.202, 1.053, 4.64, and 11.14  $\mu\text{m}$ ) (Polybead Polystyrene Microspheres; Polysciences Europe GmbH, Eppelheim, Germany). The characteristics of the polystyrene particles used are given in Table 1. The mean particle diameters  $\pm$  standard deviations are given as specified by the manufacturer. The total number and total surface area for the administered mass dose were calculated assuming spherical particle shape and a smooth surface (confirmed by transmission electron microscopy, data not shown). All PSP suspensions were

**TABLE 1**  
**Particle Mean Diameter ( $\pm$  SD), Total Particle Mass, Total Particle Surface Area, and Particle Number for the Polystyrene Particles Used in the Experiments**

| Diameter $\pm$ SD<br>( $\mu\text{m}$ ) | Low dose (40 $\mu\text{g}$ PSP)      |                       | High dose (200 $\mu\text{g}$ PSP)    |                       |
|----------------------------------------|--------------------------------------|-----------------------|--------------------------------------|-----------------------|
|                                        | Total surface area ( $\text{cm}^2$ ) | Number                | Total surface area ( $\text{cm}^2$ ) | Number                |
| 0.0588 $\pm$ 0.0023                    | 38.9                                 | $3.58 \times 10^{11}$ | 194                                  | $1.79 \times 10^{12}$ |
| 0.202 $\pm$ 0.01                       | 11.3                                 | $8.83 \times 10^9$    | 56.5                                 | $4.41 \times 10^{10}$ |
| 1.053 $\pm$ 0.01                       | 2.17                                 | $6.23 \times 10^7$    | 10.8                                 | $3.12 \times 10^8$    |
| 4.64 $\pm$ 0.314                       | 0.492                                | $7.28 \times 10^5$    | 2.46                                 | $3.64 \times 10^6$    |
| 11.14 $\pm$ 0.37                       | 0.205                                | $5.26 \times 10^4$    | 1.03                                 | $2.63 \times 10^5$    |

practically free of endotoxin (less than 0.04 ng per injection) as determined by Limulus Amebocyte Lysate Kinetic – QCL Kit (Bio-Whittaker, Walkersville, MD). The spherulite diameter of DEP has been estimated to be 0.030  $\mu\text{m}$  (Lovik *et al.*, 1997), and according to the manufacturer the specific surface area was 91  $\text{m}^2/\text{g}$ , determined by gas adsorption. The spherulite diameter of the CB particles was 0.035  $\mu\text{m}$  and the specific surface area was 60  $\text{m}^2/\text{g}$  (Lovik *et al.*, 1997).

As allergen, ovalbumin (OVA, Gal d2; chicken egg albumin, grade VII, Sigma, St. Louis, MO) was given in a dose of 10  $\mu\text{g}$  per injection. Earlier dose-response-studies with 0.1  $\mu\text{m}$  diameter PSP and OVA showed that the optimal OVA dose was 10  $\mu\text{g}$ . The adjuvant effect of particles was increased by increased mass dose, 40  $\mu\text{g}$  giving a medium but marked effect (unpublished data). In the present experiments, all particles were given as a low (40  $\mu\text{g}$ ) and a high (200  $\mu\text{g}$ ) dose per injection. In the case of injections into both footpads, the total mass dose per animal was 80 and 400  $\mu\text{g}$  PSP, respectively, and 20  $\mu\text{g}$  OVA. All solutions were made in Hank's balanced salt solution (HBSS; PAA Laboratories GmbH, Linz, Austria). Twenty  $\mu\text{l}$  of the different solutions were injected subcutaneously into the hind footpad (heel-toe direction) using a 100  $\mu\text{l}$  Hamilton syringe (Hamilton Bonaduz AG, Switzerland) with a 30 G sterile needle (BD Medical Systems, Ireland).

**Experimental design.** The antibody experiments were performed with groups of eight mice, injected into the right hind footpad with buffer (HBSS), ovalbumin (OVA, 10  $\mu\text{g}$ ), or OVA + particles of low (40  $\mu\text{g}$ ) or high (200  $\mu\text{g}$ ) dose. The particles used were polystyrene particles (PSP) of different sizes (0.0588, 0.202, 1.053, 4.64, and 11.14  $\mu\text{m}$  diameter), carbon black (CB) and diesel exhaust particles (DEP). On day 21, all mice were given OVA alone (10  $\mu\text{g}$ ) in the same footpad. On day 26, the mice were exsanguinated by heart puncture under  $\text{CO}_2$  anesthesia. Serum was stored at  $-20^\circ\text{C}$  until analyzed for OVA-specific IgE and IgG2a, measured by enzyme-linked immunosorbent assay (ELISA).

To study if fine and coarse particles affected the primary cellular response in a qualitatively different way, the response to PSP particles with the diameters 0.202, 1.053, and 11.14  $\mu\text{m}$  was further examined. We have previously found day 5 to be the day of maximum response after injection of OVA and 0.1  $\mu\text{m}$  PSP (Nygaard *et al.*, in press). Therefore, five days after injection of HBSS, OVA, or OVA + particles in high or low doses (as above) into both hind footpads, the animals were killed by cervical dislocation under  $\text{CO}_2$  anesthesia, and both popliteal lymph nodes (PLN) were excised. Cell surface molecules were measured in separate experiments than *ex vivo* cytokines, cell proliferation, and total cell numbers. Practical considerations limited the group sizes to five and nine mice, respectively. To obtain sufficient cell numbers per well for determination of *ex vivo* cell proliferation and cytokine production, PLN cells from three animals were pooled. In all experiments, the values from two independent experiments were combined in the statistical analyses (see data analyses section).

**ELISA for detection of IgE and IgG2a anti-OVA antibodies.** IgE anti-OVA antibodies were detected in a capture ELISA as previously described (Ormstad *et al.*, 1998a). Detection of IgG2a anti-OVA antibodies was performed as follows: polystyrene microtiter plates (Costar EIA/RIA Plate, Corning Inc.,

Corning, NY) were coated with 100  $\mu$ l of OVA (2  $\mu$ g/ml) in 0.05 M carbonate/bicarbonate buffer, pH 9.6. The coated plates were incubated for 1 h at room temperature and thereafter at 4°C overnight. After washing with Tris/Tw (0.1 M Tris/HCL buffer, pH 7.4, containing 0.05% Tween20), the plates were incubated with 100  $\mu$ l of the mouse sera in duplicate, diluted in Tris/Tw containing 1% Bovine Serum Albumine (Biotest, Dreieich, Germany). The plates were incubated for 2 h at room temperature, thereafter at 4°C overnight. After washing with Tris/Tw, the plates were incubated for 2 h at room temperature with biotinylated rat anti-mouse IgG2a antibody (clone R19-15; BD PharMingen, San Diego, CA) diluted 1:500 in Tris/Tw, 100  $\mu$ l per well. Thereafter, the plates were washed with Tris/Tw, and incubated for 1 h at room temperature with pre-formed complexes of streptavidin and biotinylated alkaline phosphatase (Dako StrepABComplex AP, Dako, Glostrup, Denmark), 1:50 dilution in Tris/HCL buffer. Again the plates were washed, and incubated at room temperature with *p*-nitrophenyl phosphate disodiumhexahydrat (Sigma 104 Phosphatase Substrate, Sigma, St. Louis, MO). For both the IgE and IgG2a assays, dilutions of a standard mouse OVA-immune serum pool were included on each plate for standard curve generation. OD was measured at 405 nm on a MRX Microplate Reader (Dynatech Laboratories, Chantilly, VA) connected to a PC using BioLinX software (Dynatech Laboratories), and the antibody concentrations are given in arbitrary units (AU) per ml.

**Lymphocyte preparation.** The popliteal lymph node was excised and kept on ice in HBSS with 2% fetal calf serum (FCS; GibcoBRL, Auckland, New Zealand). Single cell suspensions were prepared from each lymph node, as described in Nygaard *et al.* (in press). After pooling the two PLN cell suspensions from the same animal, the cell concentration was measured with a Coulter Counter Z1 (Beckman Coulter Incorporated, FL), and presented as the total cell number ( $10^6$ ) per PLN.

**Thymidine incorporation.** Cell proliferation was determined by  $^3$ H-labelled thymidine incorporation *ex vivo* without any stimulant added, as previously described (Nygaard *et al.*, in press). The median value from each cell culture triplet was used for data presentation.

**Cell phenotyping.** Lymph node cells were prepared and stained as described elsewhere (Nygaard *et al.*, in press). In short,  $5 \times 10^6$  cells were stained in 100  $\mu$ l staining buffer containing the optimal (pre-diluted) concentration of FITC- or PE-conjugated monoclonal antibodies against CD19 (B lymphocyte marker), CD86, CD23 (BD PharMingen, San Diego, CA), MHC class II (Southern Biotech, Birmingham, AL) or the appropriate isotype controls. A minimum of 10,000 lymphocytes were analyzed on an EPICS XL instrument (Coulter, FL) connected to a PC using EXPO32 Software (Applied Cytometry Systems, Sheffield, U.K.). Expression of surface markers were determined in two ways as previously described (Nygaard *et al.*, in press): (1) the relative number of positive cells and (2) the relative amount of epitopes per cell measured as median fluorescence intensity. Three separate lymph node cell populations were observed, mainly separated by different forward light scatter (FS). The population with low FS were dead cells, as verified by propidium iodide staining, and was excluded from the analyses. The population with medium FS contained about 92% of the live cells from control animals, and analyses of the surface markers were performed by selective gating on this main lymphocyte population. The population with high FS, hereafter called lymphoblasts, was by high baseline surface marker expression identified as strongly activated lymph node cells (data not shown), in agreement with Tuschl *et al.* (2002). The relative numbers of lymphoblasts were calculated by dividing the number of lymphoblasts by the number of live lymphocytes.

**Cytokine quantification.** *Ex vivo* cytokine release by the lymph node cells was determined as previously described (Nygaard *et al.*, in press). In short, the cells from three mice were pooled and cultured with 5  $\mu$ g/ml ConA (Concanavalin A, Sigma, MO) for 24 h. Thereafter, the cell supernatant levels of IL-4, IL-10, IFN- $\gamma$ , and IL-2 were determined by sandwich ELISA (Mouse DuoSets; R&D Systems Inc., MN).

**Data analysis.** All statistical analyses were performed with SigmaStat Statistical Analysis System for Windows Version 2.03 (Jandel Scientific, Erkrath, Germany). The antibody data, the lymph node cell numbers,

proliferation and cytokine levels were log<sub>10</sub>-transformed to obtain normality and equal variance, whereas the levels of cell surface molecules were used untransformed. In order to determine whether the different particles induced significantly different responses, the data from two independent experiments were analyzed in a two-way ANOVA with “day” (experiment 1 and 2) and “treatment” (the different treatments) as the two factors. As the two-way ANOVAs for all parameters showed statistical differences between treatments ( $p \leq 0.001$ ), pairwise comparisons were performed by Tukey’s post hoc test in order to determine which groups were significantly different, averaging over the experiments. Overall, the following comparisons were regarded: All OVA + PSP versus OVA, OVA versus HBSS, and between all OVA + PSP given the same particle mass dose. Experimental groups were considered statistically different if  $p \leq 0.05$ . The response patterns in the two independent experiments were consistent, thus data from one experiment are shown in the figures, whereas the indicated statistical significances are based on data from the two independent experiments (as described above).

Linear regression analyses of the antibody and cellular data versus the particle characteristics were performed using log<sub>10</sub>-transformed data (to achieve normally distributed residuals with stabilized variances) as well as log<sub>10</sub>-transformed particle mass, number, surface area or diameter (to obtain a suitable distribution of the particle number or surface area data points). The linear regressions were performed on the pooled data from the two individual experiments.  $R^2$  was used as a measure of how well the data fit the regression line.

## RESULTS

### *OVA-Specific IgE and IgG2a after Injection of OVA*

#### *Together with CB, DEP, or PSP of a Broad Size Range*

Low and high mass doses of all particles together with OVA, as well as OVA alone, were injected into the footpad on day 0, followed by an OVA injection on day 21. On day 26, OVA with CB, DEP, or PSP of diameters 0.0588 and 0.202  $\mu$ m strongly increased the OVA-specific IgE levels compared to OVA alone ( $p < 0.001$ , Fig. 1A). OVA with the high dose of the 1.053 and 4.64  $\mu$ m PSP marginally increased the IgE levels ( $p = 0.006$ ). Furthermore, 0.0588  $\mu$ m and 0.202  $\mu$ m PSP, CB, and DEP with OVA induced significantly higher IgE levels than the 1.053, 4.64, and 11.14  $\mu$ m PSP with OVA ( $p < 0.001$ ). The response pattern for the Th1-associated IgG2a antibodies differed from the Th2-associated IgE responses. Only OVA together with 0.0588  $\mu$ m PSP or CB particles significantly increased the OVA-specific IgG2a levels compared to OVA alone ( $p < 0.001$ , Fig. 1B).

### *The Primary Cellular Response in the Draining Lymph*

#### *Node after Injection of OVA with PSP of Different Sizes*

To study whether the cellular parameters were more affected by the fine than the coarse particles, we measured selected cell parameters on PLN cells five days after a single injection into both hind footpads of HBSS, OVA, or OVA together with a low or high dose of 0.202, 1.053, or 11.14  $\mu$ m PSP.

The total lymph node cell numbers were significantly higher in all groups given OVA + PSP than in the group given OVA alone, except for the group given the low dose of 11.14  $\mu$ m PSP ( $p < 0.001$ , Fig. 2A). For both doses, the increase in cell

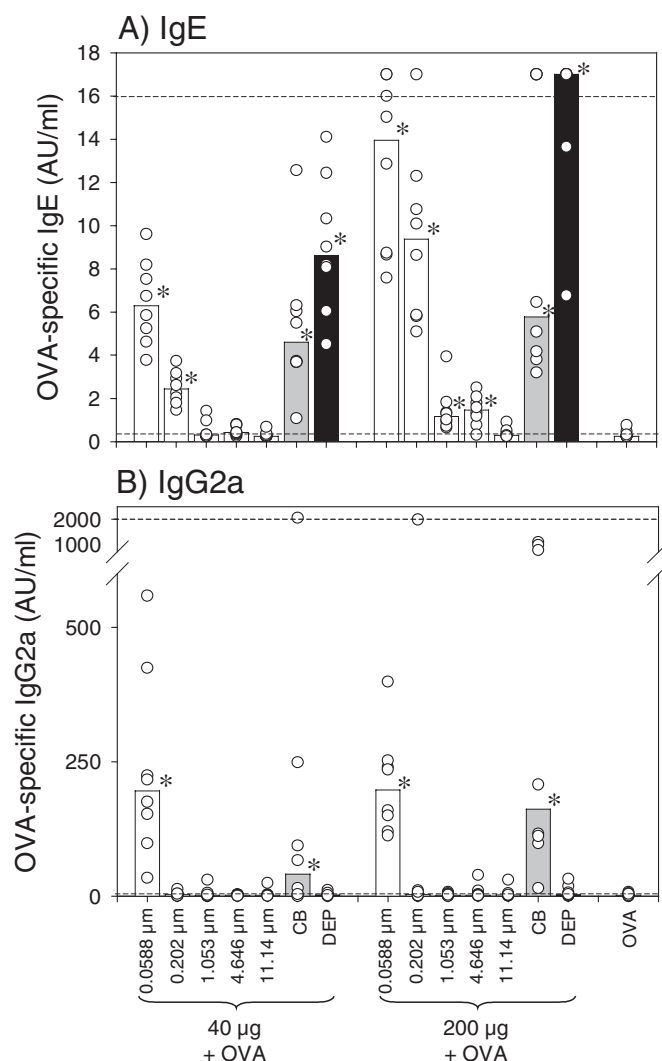


**FIG. 1.** Levels of OVA-specific IgE (A) and IgG2a (B) on day 26 after injection into one hind footpad of 10 µg OVA + 40 or 200 µg particles, or 10 µg OVA alone. Particles used were polystyrene particles (PSP) with diameter 0.0588, 0.202, 1.053, 4.64, and 11.14 µm, carbon black particles (CB, 0.035 µm) and diesel exhaust particles (DEP, 0.03 µm). On day 21 all mice were boosted with 10 µg OVA in the footpad. Values (arbitrary units, AU) for individual mice (circles) and median values (columns) for groups of eight mice are shown. The dotted lines indicate the lower and upper detection limits for the ELISA assays. Data shown are from one single experiment, however the stars denote statistical significance versus OVA alone ( $p < 0.05$ ) based on a two-way ANOVA and Tukey's multiple comparison test performed on data from two independent experiments.

numbers was significantly larger in mice given OVA + 0.202 µm PSP than in mice given OVA + 1.053 µm or OVA + 11.14 µm PSP. Furthermore, the cell numbers were significantly larger in mice given OVA + 1.053 µm PSP than OVA + 11.14 µm PSP. The cell proliferation was significantly increased in the groups given OVA with the low and high dose of 0.202 µm PSP both compared to the groups given OVA alone ( $p < 0.001$ ) and OVA with the two larger particles ( $p < 0.015$ , Fig. 2B). The relative

number of lymphoblasts (determined by flow cytometry, data not shown) increased from about 7% in the control groups to near 12% for the groups given OVA + 0.202 µm PSP (low and high dose). Also, the relative number of lymphoblasts in the groups given OVA + 1.053 µm PSP seemed higher than in the OVA group.

The relative numbers of CD19<sup>+</sup> cells (B lymphocytes) were significantly increased by OVA together with both low and high doses of 0.202 µm PSP and with high dose of 1.053 µm PSP, compared to OVA alone ( $p < 0.001$ , Fig. 2C). Further, the relative numbers of B lymphocytes were significantly higher in the groups given OVA with 0.202 µm PSP than with the two larger particles, and higher in the group given OVA with high dose of 1.053 µm PSP than high dose of 11.14 µm PSP ( $p < 0.001$ ). While this pattern was consistently similar for expression of MHC class II and CD86 on B cells (Figs. 2D and 2E), the expression of CD23 on B cells appeared to be increased only by the fine 0.202 µm PSP (Fig. 2F).

We determined the culture supernatant concentration of IL-4, IL-10, IFN-γ, and IL-2 after *ex vivo* stimulation with ConA. Both the production of IL-4 and IL-10 was significantly higher in the groups given OVA with low and high doses of 0.202 µm PSP than in the groups given OVA alone or together with the two larger particles ( $p < 0.001$ , Figs. 3A and 3B). OVA alone weakly increased the IL-4 (n.s.) and IL-10 ( $p < 0.001$ ) levels compared to the HBSS group. As for IL-4 and IL-10, the IFN-γ production from the PLN cells tended to gradually increase with reduced particle size (Fig. 3C). Even though only the high dose of 0.202 µm PSP with OVA induced significantly higher IFN-γ levels than OVA alone ( $p < 0.001$ ), OVA + 0.202 µm PSP induced significantly higher IFN-γ levels than OVA + 11.14 µm PSP ( $p \leq 0.02$ ). In contrast, the production of IL-2 by the PLN cells was significantly lower in the groups given OVA + 0.202 µm PSP (low and high dose) than in the OVA group ( $p < 0.002$ , Fig. 3D).

With the exception of the total cell number, injection of OVA + 11.14 µm PSP (low or high dose) altered none of the measured cellular parameters compared to the levels in the OVA group.

#### Particle Mass, Size, Number, and Surface Area in Relation to the IgE Adjuvant Effect

As seen from Figure 1A, equal mass of the different PSP gave highly variable increases in the OVA-specific IgE levels. Thus, the particle mass on its own could not explain the differences in IgE response between the different sizes of PSP. Therefore, we analyzed the IgE levels in relation to the corresponding PSP dose in terms of particle mass, total particle surface area, particle number, and particle size (diameter). Linear regressions for the IgE response versus the different particle characteristics were performed on the pooled data points for all PSP sizes from the two experiments (Fig. 4). After log-transformations of both the IgE values and the particle characteristics values,

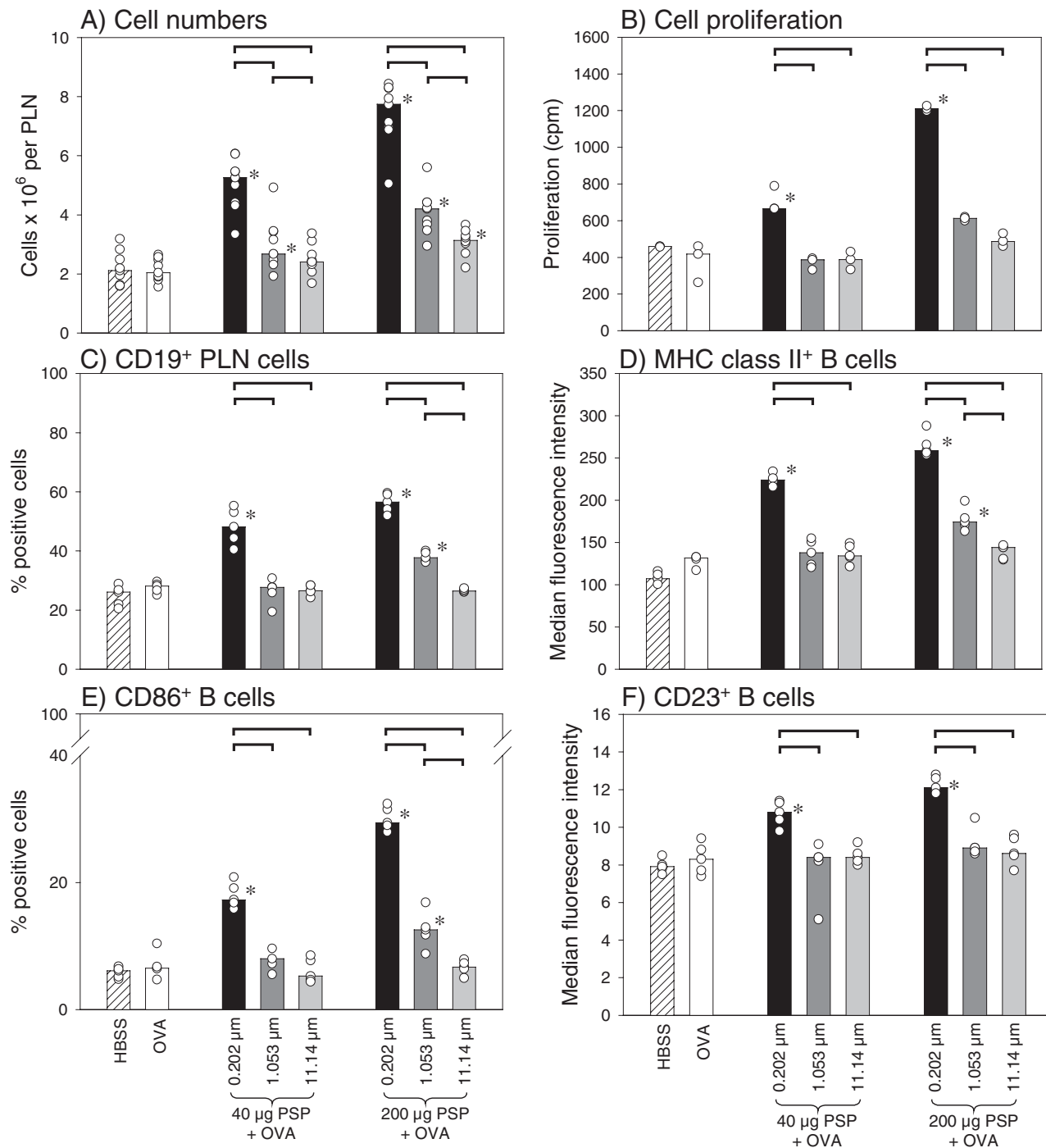


**FIG. 2.** Total cell numbers, *ex vivo* cell proliferation and expression of surface markers on lymph node cells five days after a single injection into both hind footpads of HBSS, 10 µg OVA, or 10 µg OVA + 40 or 200 µg polystyrene particles (PSP) with diameter 0.202, 1.053, or 11.14 µm. Single cell suspensions were prepared, and cell numbers (A) and cell proliferation (B) were determined. The cells were stained with antibodies against CD19 (C), CD19 and MHC class II (D), CD19 and CD86 (E), CD19 and CD23 (F), and the cell surface molecule expression was determined by flow cytometry. Data presentation and statistics as described in Figure 1. Brackets denote statistically significant differences between groups given the same particle mass dose ( $p < 0.05$ ).

the regression analyses indicated that the total particle surface area explained 64% ( $R^2 = 0.64$ ) of the variance in the IgE-levels. Based on particle number, 62% of the variance was explained, whereas particle diameter explained 58% of the variance. In

contrast, particle mass explained only 6% of the variance in the IgE-levels.

After log-transformation of the IgG2a levels, the linear regression line of the total particle surface area, particle number and

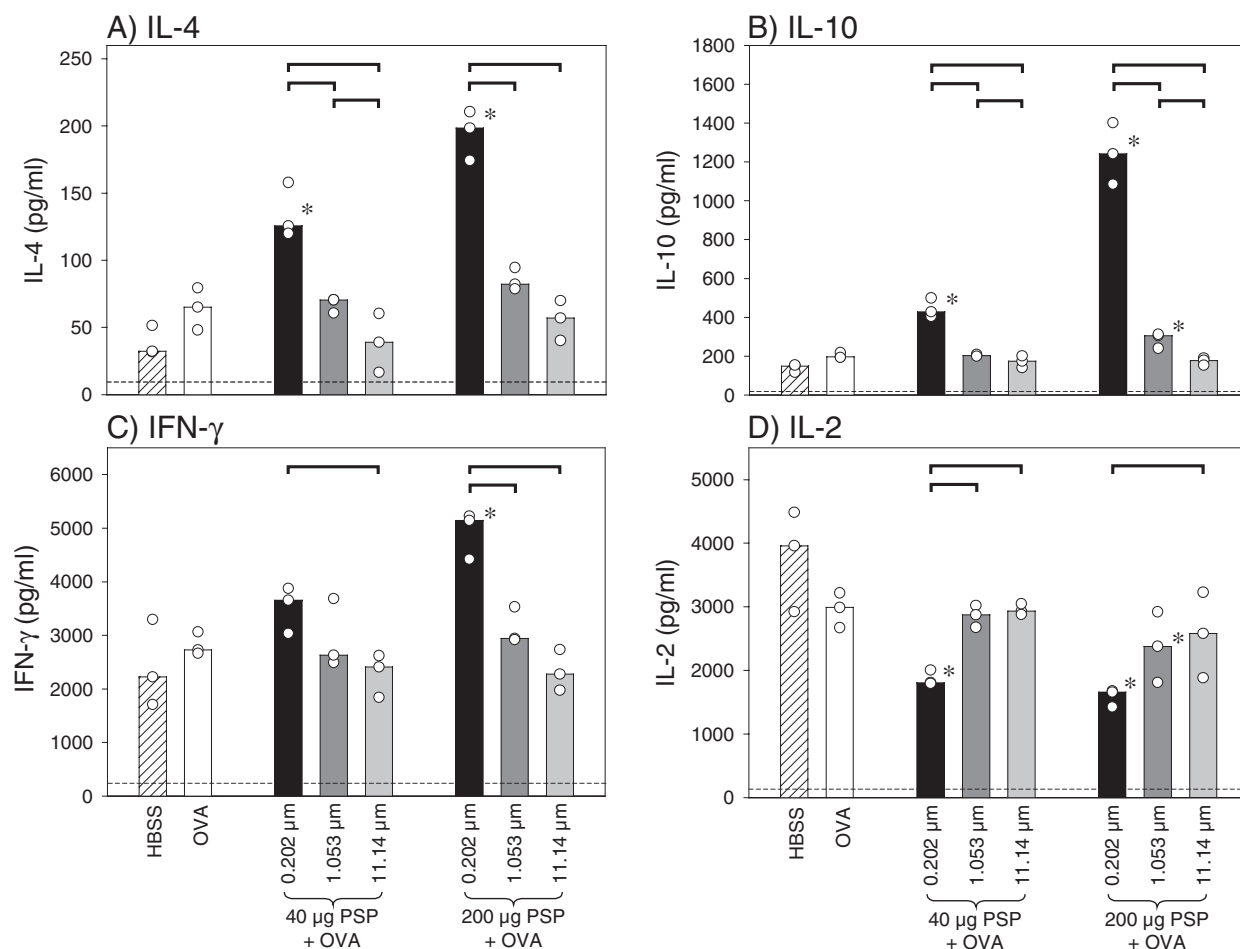


**FIG. 3.** *Ex vivo* cytokine production by popliteal lymph node cells cultured with ConA. Five days after a single injection into both hind footpads of HBSS, 10 μg OVA, or 10 μg OVA + 40 or 200 μg polystyrene particles (PSP) with diameter 0.202, 1.053, or 11.14 μm, the draining lymph nodes were excised and single cell suspensions were prepared. Cells from three animals were pooled and cultured with ConA (5 μg/ml) for 24 h. IL-4 (A), IL-10 (B), IFN-γ (C), and IL-2 (D) in the supernatants were measured by ELISA. Dotted lines indicate the lower detection limits for the ELISA assays. Each point represents a pool of three animals (circles) and the median values (columns) for each group of mice are shown. Data presentation and statistics as described in Figure 1. Brackets denote statistically significant differences between groups given the same particle mass dose.

particle diameter explained only a small part of the variation (36, 38, and 37%, respectively;  $p < 0.001$ , data not shown). Regression analyses based on particle mass did not predict the IgG2a levels with statistical significance. For all the PLN cellular parameters except CD23 expression, the total particle surface area, number and diameter explained 60 to 80% of the variation ( $p < 0.001$ , data not shown). The total particle surface area, number and diameter explained the variation in CD23 expression to similar degrees, however with lower regression coefficients ( $R^2$  about 0.4). Again, particle mass did not predict any of the cellular parameters with statistical significance.

## DISCUSSION

Fine and ultrafine polystyrene particles (PSP) in our experiments had stronger IgE adjuvant effects than coarse particles at

equal mass concentrations. Accordingly, the particle IgE adjuvant effect in this model was best predicted by particle surface area or particle number, and not by particle mass. Ambient air particle distributions differ with respect to mass, number, and surface area concentrations. The particle number concentration mainly reflects the amount of ultrafine particles ( $<0.1$  μm diameter), whereas the particle mass reflects both the accumulation mode (0.1–2 μm) and the coarse mode ( $>2.5$  μm) (Junker *et al.*, 2000; Lighty *et al.*, 2000). The surface area best reflects the particles between 0.1–1 μm (Harrison *et al.*, 2000; Moshhammer and Neuberger, 2003). Consequently, there is a need for defining which physical characteristics of the particles are important in inducing adverse health effects before concluding what methods for particle measurements are optimal.

The underlying causes of the biological effects from ambient air particles are not clear. The chemical composition of particles has been extensively studied, and transition metals,

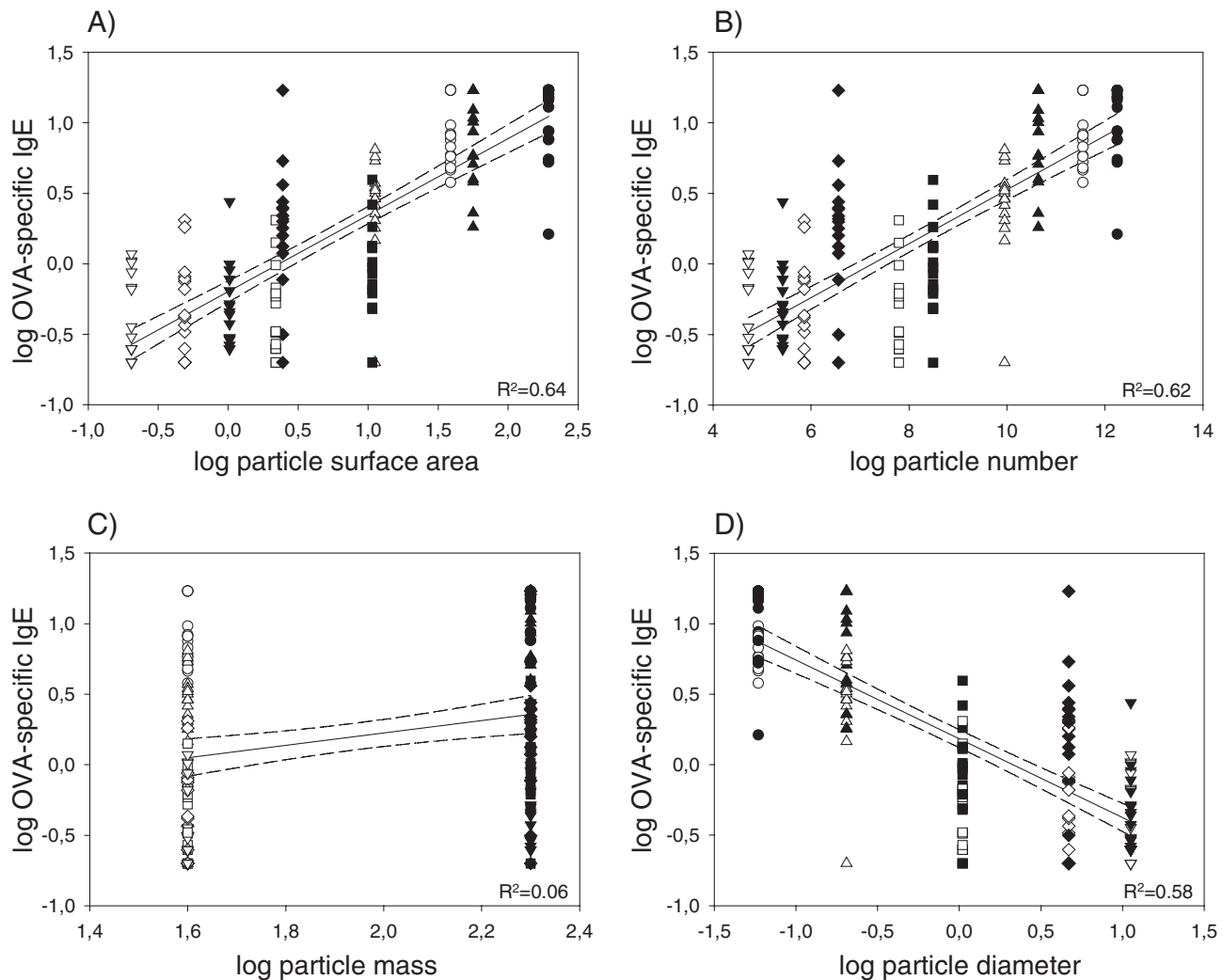


**FIG. 4.** Linear regression plots for the  $\log_{10}$ -transformed IgE values versus the  $\log_{10}$ -transformed particle characteristics: total surface area (A), particle number (B), particle mass (C), and particle diameter (D). The IgE levels on day 26 (Fig. 1) were log-transformed to meet the assumptions for linear regression, and particle characteristics were log-transformed to obtain a suitable distribution of the data points. Thereafter, linear regression analyses were performed, and the regression line (solid) and the 95% confidence interval (dotted lines) are shown.  $R^2$  is shown as a measure of how well the data fit the regression line. The symbols represent the low (open) and high (black) mass doses of particles with diameters of 0.0588  $\mu\text{m}$  ( $\circ$ ), 0.202  $\mu\text{m}$  ( $\triangle$ ), 1.053  $\mu\text{m}$  ( $\square$ ), 4.64  $\mu\text{m}$  ( $\diamond$ ) and 11.14  $\mu\text{m}$  ( $\nabla$ ).

polyaromatic hydrocarbons and ultrafine particle surfaces have been suggested to induce oxidative stress causing inflammation (Donaldson *et al.*, 2003). Another theory is that the physical characteristics (e.g., numbers, size, shape, and surface structure) are important for the biological effects. Several experimenters have reported that ultrafine particles of different, poorly soluble materials induce stronger inflammatory responses in the lung than their larger counterparts (Brown *et al.*, 2001; Donaldson *et al.*, 2000; Gilmour *et al.*, 2004; Oberdorster *et al.*, 1994). In contrast, the coarse fraction of collected ambient air particles has been reported to induce stronger inflammatory responses than the fine fraction both *in vitro* and *in vivo* (Becker *et al.*, 2003; Osornio-Vargas *et al.*, 2003; Schins *et al.*, 2004). The presence of bacterial endotoxin or other bacterial products on ambient air

particles may help explain this discrepancy. Endotoxins have been observed mainly in the coarse fraction (Schins *et al.*, 2004; Soukup and Becker, 2001), and the proinflammatory effects of  $\text{PM}_{10}$  in several studies was shown to act by endotoxin-dependent mechanisms (Becker *et al.*, 2003; Osornio-Vargas *et al.*, 2003). With respect to the stronger IgE adjuvant effect of fine than coarse particles, the present results are in accordance with previous studies from our group using both PSP and urban particulate matter from European cities (Granum *et al.*, 2000; Lovdal *et al.*, 2003), and the numerous studies showing that DEP and CB increase allergic responses (reviewed in Granum and Lovik, 2002; Nel *et al.*, 1998). On a more general level, our results are consistent with a number of studies showing that fine or ultrafine particles are more strongly associated with

respiratory health effects than the coarse particles (Penttinen *et al.*, 2001a; Peters *et al.*, 1997; Schwartz and Neas, 2000). Because we injected the suspensions subcutaneously, we suggest that the stronger adjuvant effect of fine particles demonstrated here, in the lung would add to the higher deposition and interstitial retention of fine and ultrafine particles (Churg and Becker, 1997; Daigle *et al.*, 2003; Oberdorster *et al.*, 1994), further increasing the health effects of fine particles.

At a given mass dose, the adjuvant effect of PSP on the Th2-associated IgE response appeared to increase with decreasing particle diameter, the ultrafine 0.0588  $\mu\text{m}$  PSP having the largest adjuvant capacity. In contrast, only the 0.0588  $\mu\text{m}$  PSP exerted an adjuvant effect on the Th1-associated IgG2a response, explaining the low regression analyses coefficients. Unlike the other PSP, the suspension of OVA + 0.0588  $\mu\text{m}$  PSP consisted of few single particles after dilution, but instead particle aggregates with diameters up to 10  $\mu\text{m}$  (as determined by transmission electron microscopy, data not shown). The total particle surface area, particle number, and particle size will be altered by particle aggregation. Although we do not know the persistence of these presumably loose aggregates after injection *in vivo*, the particle characteristics given in Table 1 might be altered for the 0.0588  $\mu\text{m}$  PSP if the aggregates persist. Therefore, we also performed the regression analyses excluding the 0.0588  $\mu\text{m}$  PSP data, and found that the particle surface area, number, diameter and mass then explained 57, 55, 51, and 6% of the variation in IgE, respectively. Thus, although the IgE variation was explained to a somewhat lesser degree without the 0.0588  $\mu\text{m}$  PSP, the particle surface area, number, and diameter still explained the IgE variation far better than the particle mass. Additionally, these aggregated ultrafine PSP (0.0588  $\mu\text{m}$ ) are a good model for the solid part of combustion particles in ambient air. Solid combustion particles, like DEP, exist as aggregated individual carbonaceous particles, the aggregates varying greatly in size (Lighty *et al.*, 2000; Murphy *et al.*, 1999). The IgE adjuvant capacity of CB particles was on the same level as the 0.0588 and 0.202  $\mu\text{m}$  PSP, whereas DEP appeared even more potent (n.s.), as we have seen previously (Lovik *et al.*, 1997). Our data suggest that the combustion particle core is responsible for a substantial part of the IgE adjuvant effect, in accordance with others (Al Humadi *et al.*, 2002; Granum *et al.*, 2001; Heo *et al.*, 2001; Lovik *et al.*, 1997; van Zijverden *et al.*, 2001). The chemicals on the DEP surface may further increase the response or affect the Th1/Th2 balance (reviewed in Nel *et al.*, 1998).

Toxicological and inflammation studies *in vivo* imply a close association between particle surface area and biological responses (Brown *et al.*, 2001; Lison *et al.*, 1997; Tran *et al.*, 2000). Tran *et al.* (2000) found that the particle surface area explained the inflammatory effects far better than the particle number. In our linear model, the particle surface area, number, and size predict the IgE response to similar degrees, although the surface area appear slightly better than particle number and particle size. For the smooth, spherical PSP used in the present experiments, the particle physical characteristics are highly

correlated. The surface to mass ratio will be much larger for particles with a heterogeneous surface structure. Therefore, the predictive differences between the particle surface area and particle number would probably be larger than observed here if using particles with heterogeneous surface structure and different sizes, as is the case for ambient air particles. Apparently, the relationships are not linear for all parameters, and the ability for the different particle characteristics to predict IgE could also be found different when using more complex models.

The number and size of particles will logically be of importance for effects on the individual cell. However, number measurements do not include the complexity of particle morphologies.

Increased particle surface area is likely to augment particle-cell contact and thus the cellular response. In addition, the amount of irritating or proinflammatory substances (Dreher, 2000), electrical charge (Veronesi *et al.*, 2002), or allergens (Ormstad *et al.*, 1998b) carried on the particle depend on the particle surface area. One important mechanism for the adjuvant activity of particles could be increased allergen exposure to antigen presenting cells (APC). The relatively larger allergen-binding surface of fine and ultrafine particles than of larger particles may bring more allergen into APCs. Regardless of these mechanistic questions, we have demonstrated that functionally the strongest IgE adjuvant effect is with the small particles at a given mass. This is important when selecting methods for particulate air pollution surveillance, and for regulatory measures regarding sources of particle emission.

To investigate if the fine and coarse PSP affected the lymph node cells in a qualitatively different way, we measured several cellular parameters presumed to play a role in the allergic immune response. These parameters have previously been shown to increase after injection of OVA with 0.1  $\mu\text{m}$  PSP (Nygaard *et al.*, in press). Overall, all measured cell parameters tended to be gradually increased/decreased with decreasing particle size, most significant for the smallest particle tested in these experiments, OVA + 0.202  $\mu\text{m}$  PSP. The largest PSP (11.14  $\mu\text{m}$ ) with OVA affected none of the parameters except for the lymph node cell numbers. Equal mass doses with PSP of different sizes appeared not to selectively affect any particular of the chosen cellular parameters, suggesting that the mechanisms for the adjuvant effect of the different sizes of particles were not different. Since the fine particles increased the cellular parameters which tended to be weakly altered by OVA alone, we suggest that fine PSP act by intensifying the inherent immune response towards OVA. The regression analyses of all the cellular parameters (except for CD23) further support the conclusions from the IgE responses, that particle surface area, number and diameter, but not particle mass, to a large degree explain the responses.

In conclusion, we found that ultrafine and fine polystyrene particles had stronger IgE adjuvant effects than coarse particles at equal mass concentrations. The particle mass could not predict the IgE adjuvant effect of the particles of different sizes, in

contrast to the total particle surface area, the particle number and particle size. Ambient air particles usually consist of particles of a wide size range. Whole size distributions are complicated to include in regression analyses, and the commonly used mean or median size would be a poor measure of the complex size distribution. We therefore suggest that future studies of particle effects in relation to allergy should include measurements of particle number and surface area.

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