

Adjuvant Activity of Ambient Particulate Matter in Macrophage Activity-Suppressed, *N*-Acetylcysteine-Treated, iNOS- and IL-4-Deficient Mice

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In previous studies, we have shown strong adjuvant activity for Ottawa dust (EHC-93) after coexposure of the BALB/c mouse to EHC-93 and ovalbumin. Mice were intranasally sensitized at days 0 and 14 with 200 μ g ovalbumin and 150 μ g EHC-93, and challenged with ovalbumin at days 35, 38, and 41 with 200 μ g ovalbumin. Mice were autopsied at day 42. This adjuvant activity was shown for the antibody response to ovalbumin (immunoglobulins E, G₁, and G_{2a}), histopathological lesions in the lung, cytokines, and the numbers of eosinophils in lung lavages. To study the mechanisms of this adjuvant activity, mice (BALB/c C.D2-Vil6) with natural-resistance-associated macrophage protein (Nramp1^s), BALB/c mice pretreated with the antioxidant *N*-acetylcysteine (NAC), mice (B6.129P2-Nos2tmLau) deficient in inducible nitric oxide synthase (iNOS), and mice with interleukin-4 (IL-4) deficiency (BALB/c Il4^{<tm2Nnt}) were coexposed to ovalbumin and EHC-93. Our studies have shown that the adjuvant activity induced after such coexposure does not change if the macrophage activation of the mice is disturbed or if the mice have been pretreated with *N*-acetylcysteine. In addition, the adjuvant activity does not develop through the pathway in which inducible nitric oxide synthase is involved. Because the histopathological lesions are statistically significant less in the IL-4 knockout strain in comparison with the wild type, we conclude that interleukin-4 might play an important role in the adjuvant activity caused by EHC-93.

Epidemiological and experimental studies have shown that ambient particles induce adverse health effects (Sarnat et al., 2001; Dockery et al., 1993). Pulmonary toxicity of particulate matter (PM) has been demonstrated (Cassee et al., 2002; Goldsmith et al., 2002; Kodavanti et al., 2000; Saldiva et al., 2002; Sarnat et al., 2001). Penetration of PM in the circulation and crossing the blood–brain barrier (Calderon-Garciduenas et al., 2003) has been shown. The adjuvant activity of particles in atopic diseases, however, is the most eye-catching adverse

health effect (Calderon-Garciduenas et al., 2003; Fujimaki et al., 1997; Hajat et al., 2001; Heo et al., 2001; Kramer et al., 2000; Rusznak et al., 1997; Wyler et al., 2000). There is still a lack of understanding of the mechanism by which the toxic constituents of these particles act to induce adjuvant activity. In general, we recognize four levels on which the adjuvant activity induced by ambient particles is triggered: macrophage activation, induction of oxidative stress, induction of nitric oxide (NO) synthesis, and interleukin (IL)-4 production.

Genes controlling antibacterial resistance may be important in the hygiene hypothesis, which states that lack of bacterial infection during childhood would favor the development of allergic diseases (Strachan, 2000). As macrophages play an important role in this hypothesis, studies have examined mice susceptibility [in natural-resistance-associated macrophage protein (Nramp1^s) or resistance (in Nramp1^r) to intracellular bacteria with combinations of ovalbumin and mycobacteria (Smit et al., 2003a, 2003b, 2003c). This murine polymorphism of Nramp1 is apparent as either low resistance (designated as “s” for susceptibility) or high resistance (r) of macrophages to the growth of intracellular organisms. Other investigators

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suggest that polymorphisms in the genomic region of the human *Nramp1* gene are associated with risk of atopy in bacille Calmette-Guérin (BCG)-vaccinated children (Alm et al., 2002). As *Nramp1*^S macrophages are less capable of releasing iron than *Nramp1*^R macrophages, high cytoplasmic iron levels in the *Nramp1* macrophages may cause the reduced activation of *Nramp1*^S macrophages (Lafuse et al., 2000; Zwilling et al., 1999; Weiss, 1999). Therefore, we hypothesize that these two genes show a difference in the adjuvant activity induced by Ottawa dust (EHC-93).

Another possible explanation of the EHC-93-induced adjuvant activity is the damage to the bronchial and alveolar epithelial surface caused by the generation of reactive oxygen species (ROS) (Ichinose et al., 1995; Kumagai et al., 1997; Lim et al., 1998; Sagai et al., 1993). These ROS are responsible for protein oxidation, lipid peroxidation, and DNA damage in target cells such as macrophages and epithelial cells (Becker et al., 1996; Martin et al., 1997; Nel et al., 1998). These effects will result in an aspecific inflammatory response and release of cytokines from the bronchial cells, macrophages, inflammatory cells, and nitric oxide (NO). There is evidence that pretreatment of mice with the antioxidant *N*-acetylcysteine (NAC) can prevent the adjuvant activity that usually takes place after coexposure to ovalbumin and diesel exhaust particles (DEP) (Whitekus et al., 2002).

Cytokine-inducible nitric oxide synthase (iNOS) is implicated in a variety of diseases, including allergic asthma. The presence of iNOS in epithelial cells, macrophages, endothelial cells, Kupffer cells, neutrophils, fibroblasts, astrocytes, and vascular smooth muscle cells has been established (Barnes & Liew, 1995; Moncada et al., 1991). Inducible NOS is induced by interferon γ (IFN γ), tumor necrosis factor α (TNF α) (Ding et al., 1988), and IL-1 β (Suschek et al., 1994). Asthma patients exhale increased concentrations of NO (Dillon et al., 1996; Hamid et al., 1993; Kharitonov et al., 1995c; Lundberg et al., 1996), and they have airway infections (de Gouw et al., 1998; Kharitonov et al., 1995b), allergic rhinitis (Henriksen et al., 1999; Lim et al., 1998; Martin et al., 1996; van Amsterdam et al., 2003a, 2003b); and bronchiectasis (Kharitonov et al., 1995a). Epidemiological studies have shown that more NO is exhaled by schoolchildren in urban areas than in suburban areas (Steenenberg et al., 2001). Moreover, studies with DEP (an important fraction of urban air pollution), which induce airway inflammation and hyperreactivity in mice, have shown the involvement of superoxide and NO (Lim et al., 1998). Therefore, we tested iNOS in this study as a possible mediator in the adjuvant activity of EHC-93.

Finally, IL-4 has a central role in the regulation of atopic diseases because it is a key factor in the immunoglobulin (Ig) E-isotype-switching B cells (Coffman et al., 1993; Lebman & Coffman, 1988); it induces and sustains the development of T helper (Th) 2 cells from precursor Th0 lymphocytes (Swain et al., 1990); and it enhances airway inflammation by upregulation of cytokines (Bochner & Schleimer, 1994). In previous studies, we examined the increase of IL-4 protein found by

bronchoalveolar lavage (BAL) after coexposure to ovalbumin, DEP, and airborne particles. Most of our studies show a slight increase in the concentration of IL-4 (Steenenberg et al., 2003b, 2003c). However, this slight increase does not predict whether IL-4 plays a crucial role in the adjuvant effect we observed. After coexposure to PM and allergens, damage leading to allergens leaking through basal membranes and becoming more accessible to the immune system might skew the immune response to Th2 cytokine production (Diaz-Sanchez et al., 1997, 1999; Ohta et al., 1999). It is evident that all the players in this cascade of events will contribute equally to increase the response to this allergen coexposed with PM.

In a previous study (Steenenberg et al., 2004) we have shown that adjuvant activity of EHC-93 (Ottawa dust) and other ambient PM samples could be demonstrated using this ovalbumin mouse model. To understand the mechanism of adjuvant activity after coexposure to allergen and EHC-93, we examined specific key factors that may be responsible for the adjuvant activity of PM in the ovalbumin-mouse model. For this purpose, we used wild-type mice and macrophage-stimulation-deprived mice, iNOS-deficient mice, mice pretreated with NAC, or IL-4 deficient mice.

MATERIALS AND METHODS

Animals

Six- to eight-week-old male BALB/cByJ.ico mice were obtained from Charles River (L'Ardresle, France). Male BALB/*cj*, BALB/*cIl4(tm2Nnt)* (deficient in IL-4), C57Bl/6, and B6.129P2-Nos2tmLau (deficient in iNOS) mice were obtained from Jackson Laboratory, Bar Harbor, ME. BALB/*c*/AnPt and C.D2-Vil6 (reduced macrophage activation) mice were obtained from the Dutch National Institute for Public Health and the Environment, Bilthoven, the Netherlands.

The animals were housed in Macrolon type III cages. Food pellets and tap water were available ad libitum, and all animals were allowed to adapt for at least 1 wk before the experiments started. All experiments were performed according to international and institutional guidelines for animal care.

Allergens Sensitization and Challenge

Ovalbumin. The mice were randomly placed in groups ($n = 8$). The mice were slightly anaesthetized with halothane before intranasal sensitization on days 0 and 14. At the moment of awakening, 0.4 mg/ml ovalbumin (grade V, Sigma Chemical, St. Louis, MO) in a total of 50 μ l saline solution was administered in both nostrils of each mouse. The challenge on days 35, 38, and 41 consisted of intranasally exposing the mice to ovalbumin at the same concentration again. To study the effects of the particles, the suspension of EHC-93 and ovalbumin was mixed to a final concentration of 0.4 mg/ml ovalbumin and 3 mg/ml particles for the sensitization phase. The animals were sacrificed on day 42.

In previous studies, we used the BALB/c mouse as a Th2 responder to show adjuvant activity after coexposure to ovalbumin and PM. Because the iNOS-deficient mice were limited to the C57Bl/6 strain that behaves as a Th1-like responder (Herz et al., 1998), we pretested the optimal dose of ovalbumin for inducing this adjuvant activity on the wild-type C57Bl/6 strain. We found that 2 mg/ml was comparable to 0.4 mg/ml used on the BALB/c mice. Therefore, this concentration was used for comparing results from the wild-type C57Bl/6 mice and the iNOS-deficient mice.

N-Acetylcysteine (NAC). To study the effects of the antioxidant NAC (Sigma Chemical, St. Louis, MO), mice were injected with 320 mg/kg NAC intraperitoneally before intranasal exposure to ovalbumin and/or particles on days 0 and 14.

Particles. The Ottawa dust (EHC-93) was a gift of Dr. R. Vincent (Environmental and Occupational Toxicology Division, Environmental Health Directorate, Health Canada, Ottawa, Ontario). In brief, EHC-93 contains profiles and amounts of polycyclic aromatic hydrocarbons, ions, and metals comparable to those of the standard reference materials 1648 (urban particulate matter) and 1649 (organics) of the National Institute of Standards and Technology (Gaithersburg, MD), and to a range of fine particulate materials recovered by field sampling (Bouthillier et al., 1998).

Autopsy

Before autopsy, the animals were anaesthetized with ketamine/Rompun [0.1 ml/100 g body weight from a mix of 0.85 ml of 100 mg/ml ketamine (Aesculaap, Bostel, the Netherlands) and 0.65 ml of 20 mg/ml Rompun (Bayer, Leverkusen, Germany)], and blood was taken by orbital puncture. A cannula was inserted in the trachea, and lung lavage was performed with 1 ml phosphate-buffered saline (PBS) solution, (pH 7.2, 37°C). The lungs were lavaged three times with the same fluid and identical volumes. Then the lungs were intratracheally fixed with formalin.

Serum Samples

After collection, blood was drawn and kept at room temperature for 2–4 h or overnight at 4°C to allow for clotting. The blood samples were centrifuged for 5 min at 13,000 rpm at room temperature. The serum was removed and stored in small aliquots at –20°C until use.

Bronchoalveolar Lavage Fluid

Fluid collected from the BAL of each animal was centrifuged at 400× g and 4°C for 10 min. Cell-free fluid from the lavage was used for analyses of cytokines, including IL-4, IL-5, IFN γ , and tumor necrosis factor (TNF α). The sediments were used to determine the total number of cells [0.1 ml of the cell suspension with 2.9 ml of the isotone (Beckman Coulter, Mijdrecht, the Netherlands) was mixed]. The sediments were also used to produce cytospin preparations for differential cell counts after

staining with May–Grunwald Giemsa. Four hundred cells per cytospin preparation were counted.

The interleukins TNF α and IL-5 were purchased from BioSource (Woerden, the Netherlands); IL-4 and IFN γ were purchased from R&D, ITK Diagnostics (Uithoorn, the Netherlands), and the assays were performed according to the manufacturers' instructions.

ELISA for Specific Immunoglobulins

Briefly, the enzyme-linked immunosorbent assay (ELISA) was performed with serum in flat-bottom, 96-well, polystyrene microtiter plates with a high binding capacity (MaxiSorp, Number 439454, Nunc, Denmark, or number 655092, Greiner, Germany) (Steerenberg et al., 2003b). The microwells were coated and stored overnight at 4°C with monoclonal rat anti-mouse IgE, IgG1, and IgG2a (Jackson, PA) in Na₂CO₃ buffer solution (0.1 M; pH 9.6). Mouse serum samples (1:4 diluted) were incubated for 1 h at 37°C in PBS solution. We measured the binding of IgE, IgG1, and IgG2a by incubating the ovalbumin-extract-DIG solution (0.01 μ g/ml in blocking reagent) for 1 h at 37°C, and then incubating anti-DIG Fab fragments *poly*-labeled with peroxidase (PO) (number 1633716; Boehringer, Mannheim, Germany; 50 U/ml; 1:625) and an incubation for 3 min at 20°C in the dark with the substrate tetramethyl benzidine (TMB) and dimethyl sulfoxide (DMSO). We stopped the enzyme reaction by adding 50 μ l of 2 M H₂SO₄, and we read the optical density at 450 nm on a multichannel photometer. A pooled positive serum with antibodies to ovalbumin was derived from mice sensitized and challenged in a different way: intraperitoneal sensitization by injection with 20 μ g ovalbumin emulsified in 2.25 mg aluminium hydrochloride in a total volume of 100 μ l on day 0 and challenged on days 35, 38, and 41 by exposure to ovalbumin solution (aerosol: 1% in 7 ml PBS nebulized by an ultrasonic nebulizer for 20 min). Extinction values of samples were expressed as percentage of the standard.

Histopathology

The excised lungs were inflated with 4% phosphate-buffered formaldehyde (pH 7.2) with a syringe inserted into the trachea. After full inflation, the trachea was ligated, and the lungs were immersed in fixative and embedded in paraplast. Sections of 5 μ m were prepared and stained with hematoxylin and eosin (H&E). The histopathological lesions (peribronchiolar infiltrate, perivascular infiltrate, hypertrophy of bronchiolar mucous cells, alveolitis, eosinophils influx) were semiquantitatively and blindly scored as absent (0), minimal (1), slight (2), moderate (3), marked (4) or strong (5). Both the incidence and the severity of the lesions were incorporated into this score. Examples of the scoring values are illustrated in Steerenberg et al. (2003b).

Statistical Analysis

We compared the induced serum titers and the number of BAL cells after sensitization and challenge with ovalbumin,

and after sensitization with combined ovalbumin and EHC-93. We determined the statistical differences between the groups with an analysis of variance (ANOVA). Student's two-sample *t*-test was used to derive the 95% confidence interval of each parameter for each treatment group. The histological parameters between groups within one experiment were statistically tested with the Wilcoxon test. We considered the result significant when the *p* value was less than .05.

RESULTS

Natural-Resistance-Associated Macrophage Protein 1

BALB/*c* wild-type mice and macrophage-stimulation-deficient *Nramp1*^r and *Nramp1*^s mice were coexposed to ovalbumin and EHC-93 to study the effect of impaired antigen-processing capacity (*r* stands for unimpaired oncogenic mice and *s* for impaired antigen-processing capacity). Mice immunized only with ovalbumin evoked antibody (IgE, IgG1, and IgG2a) responses that tended to be less in *Nramp1*^s than in *Nramp1*^r mice (data not shown). The increased production of IgE, IgG1, and IgG2a antibodies to ovalbumin were similar for all the different strains of mice coexposed to EHC-93 and ovalbumin and for the mice exposed only to ovalbumin (Figure 1, Table 1). The increase of histopathological lesions was statistically significant after coexposure of the wild type, while the macrophage-stimulation-deficient types showed only a tendency toward increased response (Table 2). Finally, BAL cell, IL-4, IFN γ , TNF α , and IL-5 concentrations were similar in the wild-type and macrophage-stimulation-deficient strains of mice (not shown).

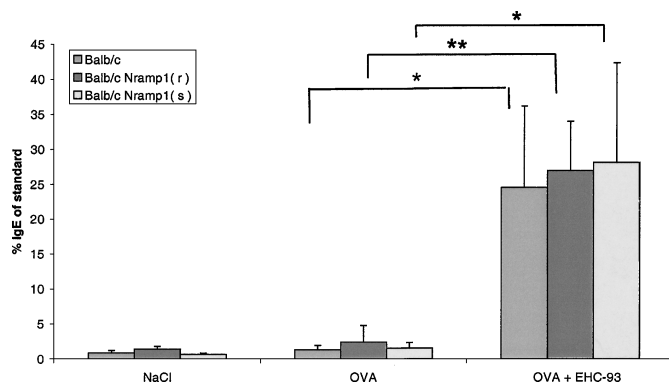


FIG. 1. IgE antibodies to ovalbumin in serum of BALB/*c* wild-type, macrophage-stimulation-deficient *Nramp1*^s and *Nramp1*^r mice that were intranasally exposed to NaCl, ovalbumin (0.4 mg/ml), or both ovalbumin and EHC-93 (3 mg/ml) during the sensitization phase (days 0 and 14) and challenged with ovalbumin only (days 35, 38, and 41). Asterisk indicates significant at *p* < .05; double asterisk, *p* < .01 (significant difference from ovalbumin-exposed group).

TABLE 1

Comparison of wild-type and deficient mice and *N*-acetylcysteine pretreatment on the antibody response after coexposure to ovalbumin and EHC-93

	<i>Nramp1</i>	NAC	iNOS	IL-4
IgE	NS	NS	NS	<i>p</i> > .01 –
IgG1	NS	NS	<i>p</i> > .01 +	<i>p</i> > .05 –
IgG2a	NS	<i>p</i> > .05 +	<i>p</i> > .01 +	NS

Note. –, Statistical decrease, +, statistical increase of parameter in deficient strain vs. wild type; Ig, immunoglobulin; IL, interleukin; iNOS, inducible nitric oxide synthase; NAC, *N*-acetylcysteine; NS, not significant between wild-type and deficient strain; *Nramp*, natural-resistance-associated macrophage protein.

N-Acetylcysteine Pretreatment

BALB/*c* mice coexposed to ovalbumin and EHC-93 showed strong adjuvant activity for IgE, IgG1, and IgG2a (Figure 2 and Table 1). The IgG2a concentration was significantly greater in the NAC-pretreated mice than in the untreated mice (Table 1). Similar results for the two treatment groups were found for the histopathological lesions (Table 2), the cell analyses, and the IL-4 concentrations in BAL (not shown).

Inducible Nitric Oxide Synthase

Wild-type and iNOS-deficient mice were coexposed to ovalbumin and EHC-93 to study the effect of impaired iNOS production. The increased production of IgE antibodies to ovalbumin was similar in both groups of mice (Figure 3). For IgG1 and IgG2a, the antibody response of the iNOS-deficient mice was twice as great as that of the wild-type mice (Table 1). In the wild-type and iNOS-deficient strains, the same increased histological response was found after coexposure to ovalbumin and EHC-93 (Table 2). After coexposure to ovalbumin and EHC-93, no differences in BAL cells were observed between the strains (not shown). Finally, the cytokines examined in the BAL showed no differences between the strains (not shown).

TABLE 2

Comparison of wild-type and deficient mice and *N*-acetylcysteine pretreatment on the histological lesions in the lung after coexposure to ovalbumin and EHC-93

	<i>Nramp1</i>	NAC	iNOS	IL-4
Peribronchiolar infiltrate	NS	NS	NS	<i>p</i> > .05 –
Perivascular infiltrate	NS	NS	NS	<i>p</i> > .05 –
Hypertrophy mucous cells	NS	NS	NS	NS
Alveolitis	NS	NS	NS	NS
Eosinophils influx	NS	NS	NS	<i>p</i> > .01 –

Note. –, Statistical decrease of parameter in deficient strain vs. wild type. IL, interleukin; iNOS, inducible nitric oxide synthase; NAC, *N*-acetylcysteine; NS, not significant between wild-type and deficient strain; *Nramp*, natural-resistance-associated macrophage protein.

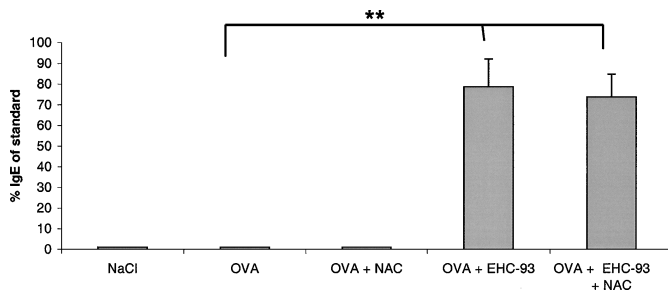


FIG. 2. IgE antibodies to ovalbumin in the serum of the non-pretreated and NAC-pretreated mice. BALB/c mice were injected with 320 mg/kg NAC intraperitoneally before intranasal exposure to ovalbumin (0.4 mg/ml) and/or EHC-93 (3 mg/ml) on days 0 and 14. They were challenged with ovalbumin on days 35, 38, and 41. Double asterisk indicates significant difference from ovalbumin-exposed group, $p < .01$.

Interleukin-4

Wild-type and IL-4-deficient BALB/c mice were coexposed to ovalbumin and EHC-93 to study the effect of impaired IL-4 production. The increased production of IgE antibodies to ovalbumin observed in the wild-type mice was completely absent in the IL-4-deficient mice (Figure 4). The antibody response to ovalbumin for IgG1 was also absent. In contrast, the adjuvant activity for IgG2a was still present in both strains of mice to the same degree (Table 1). Histopathological lesions increased after immunization with ovalbumin in both strains (data not shown). However, coexposure to ovalbumin and EHC-93 in the wild type showed a statistically significantly greater histological response except for alveolitis, while the IL-4-deficient mice had only increased hypertrophy of the bronchiolar mucous cells. (Table 2). Finally, no differences between the two strains were observed in BAL cells, or in the cytokines except for IL-5. Interleukin-5 concentration was slightly (but not sta-

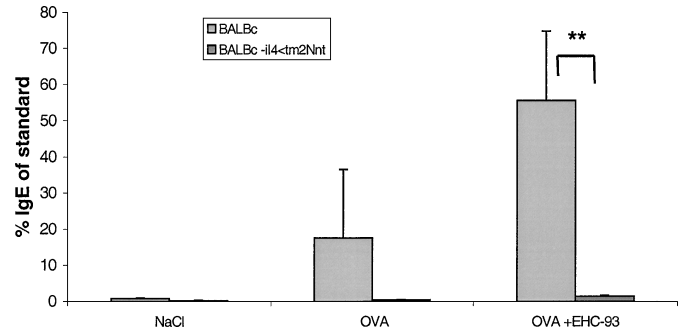


FIG. 4. IgE in the serum of BALB/c wild-type and IL-4-deficient mice. BALB/c and BALB/c-IL-4<tm2Nnt mice were intranasally exposed to NaCl, ovalbumin (0.4 mg/ml), or both ovalbumin and EHC-93 (3 mg/ml) during the sensitization phase (days 0 and 14) and challenged with ovalbumin only (days 35, 38, and 41). Double asterisk indicates significant difference from ovalbumin-exposed group, $p < .01$.

tistically) increased in the wild type, but was significantly low in IL-4-deficient mice (not shown).

DISCUSSION

Our studies show that the adjuvant activity induced after coexposure to ovalbumin and EHC-93 is not affected by disturbance of the macrophage stimulation in the Nrp1 mice or the decreased oxidative stress capacity prevented after pretreatment with NAC. Furthermore, the adjuvant activity induced by EHC-93 does not develop through the pathway in which iNOS is involved. However, IL-4 is an important player in the development of adjuvant activity expressed in antibody response and histological lesions after coexposure to ovalbumin and EHC-93.

In our study, we hypothesized that the adjuvant activity is due to an activation of macrophages. Activated macrophages can process and present antigens better resulting in increased antibody response. Our results show that the Nrp1^f and Nrp1^s of wild-type BALB/c mice elicited similar adjuvant activities of antibodies to ovalbumin in the subclasses of IgE, IgG1, and IgG2a. In contrast, the histopathological lesions of the wild-type group showed a significant increase due to the coexposure to ovalbumin and PM, while the adjuvant activity was not statistically significantly increased in the Nrp1^f and Nrp1^s mice. Similar results were found for the eosinophils in the BAL. The adjuvant activity for antibodies to ovalbumin seems not to be due to the macrophage activating stage, as no differences in adjuvant activity appear in these Nrp1^f and Nrp1^s strains of mice. Our results are comparable with those of others (Smit et al., 2003b), and they show a tendency toward reduced responses in Nrp1^s mice for most of the parameters used. However, this reduced response did not hinder adjuvant activity after coexposure to ovalbumin and EHC-93, which was comparable to the adjuvant activity in Nrp1^f mice.

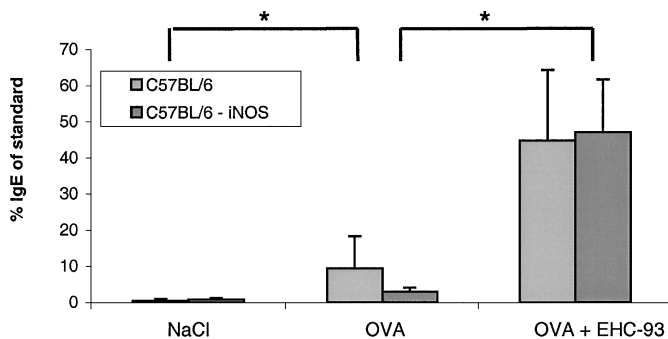


FIG. 3. IgE in the serum of C57BL/6 wild-type and iNOS-deficient mice that were intranasally exposed to NaCl, ovalbumin (2 mg/ml), or both ovalbumin and EHC-93 (3 mg/ml) during the sensitization phase (days 0 and 14) and challenged with ovalbumin only (days 35, 38, and 41). Asterisk indicates significant difference from ovalbumin-exposed group, $p < .05$.

Whitekus et al. (2002) showed that thiol oxidants prevent the adjuvant activity of coexposure to ovalbumin and DEP. Administration of NAC directly suppressed complexes to electrophilic DEP chemicals and exerted additional antioxidant effects at the cellular level (Whitekus et al., 2002; Xiao et al., 2003). This contrasts with our study, in which coexposure of ovalbumin and EHC-93 after pretreatment with NAC showed adjuvant activity in antibody response to ovalbumin in all subclasses. Moreover, the IgG2a response was significantly greater than that of the non-pretreated group. In addition, histopathological lesions and the influx of eosinophils were still statistically significantly greater than those of the non-pretreated group. The discrepancy may be due to the different schedule of the immunization and challenge. In our study, we used a protocol with 42 days, while Whitekus et al. (2002) used 10 days. Furthermore, we injected the mice only just before sensitization when ovalbumin was coexposed with PM (days 0 and 14), while Whitekus et al. (2002) applied NAC during the whole study. Finally, we used EHC-93 that contains only a small portion of DEP. From our results, we conclude that pretreating mice with NAC cannot diminish the adjuvant activity observed after coexposure to ovalbumin and EHC-93. This suggests that the adjuvant activity of ambient particles in this study is not due to antioxidant activity of EHC-93.

Inducible-NOS-deficient mice were used to study the role of NOS in the process of adjuvant activity of coexposure to ovalbumin and PM. Our results show that the adjuvant activity expressed in IgE production was comparable in both strains, and the production of IgG1 and IgG2a antibodies to ovalbumin were even greater in the iNOS-deficient mice. Moreover, the histopathological lesions and the influx of inflammatory cells in the BAL reflected an equal adjuvant activity of coexposure to ovalbumin and EHC-93 in both strains. Our data do not fit the hypothesis that iNOS and inducible NO play a role in the skewing of Th2 response resulting from adjuvant activity. This may contrast with the observations of Feder et al., who were able to reduce the pulmonary eosinophilia by *N*- ω -nitro-L-arginine methyl ester (L-NAME) and aminoguanidine, both selective inhibitors of iNOS (Feder et al., 1997). They proposed that NO had no effect on the recruitment of the bone-marrow cells, which play a role in the extravasation of eosinophils from the circulation into the lung tissue. In our study, we did not see a statistically significant reduction of eosinophils in either the lung tissue or the BAL. The difference in observation might be explained by an L-NAME influence on the eosinophilia cascade at more levels. However, our data agree with those of others (Koarai et al., 2002), who show similar levels of eosinophil infiltration into the airways in wild-type and iNOS-deficient mice subjected to the same ovalbumin sensitization and challenge protocol. In the light of this observation, the Steerenberg et al. (2003a) results, in which a relationship was found between increased numbers of eosinophils and exhaled NO, might be due to independent mechanisms. Thus, we conclude that not only is the induction and excitation phase independent of iNOS, but so

is the adjuvant activity induced after coexposure to ovalbumin and EHC-93.

We used an IL-4-deficient mouse, in which recruitment of airway eosinophils in response to aeroallergens is diminished but not abolished. No antibody response can be elicited after ovalbumin has been inhaled; however, with epicutaneous application, the immunological response remains intact (Herrick et al., 2000). Moreover, the characteristic airway damage and hyperreactivity normally resulting from allergen inhalation are not attenuated (Hogan et al., 1997). Indeed, in our study, no antibodies (IgE and IgG1) to ovalbumin were measured in the IL-4-deficient mice even after coexposure to ovalbumin and EHC-93. Immunoglobulin G2a was produced, but no adjuvant activity was determined in this Th1 compartment of the immune system; these findings (no IgE and IgG1 response, in contrast with IgG2a) agree with those of others (Hogan et al., 1997). Our data show that coexposure to EHC-93 could not circumvent the reduced IgE response as shown for epicutaneous application (Herrick et al., 2000). As IgE in this respiratory model depends on IL-4, we cannot conclude that the adjuvant activity is IL-4 dependent. However, histopathological lesions were observed after respiratory application of ovalbumin to wild-type and IL-4-deficient mice, while no increased lesions (except for hypertrophy of mucous cells) were found after coexposure to ovalbumin and EHC-93 in IL-4-deficient mice. We conclude that IL-4 is a key player in the adjuvant activity based on histopathological lesions, but other processes involved, such as hypertrophy of the mucous cells, do not seem to be under the control of IL-4. Interleukin-5 was still present, but there was less of it than in the wild-type group coexposed to ovalbumin and EHC-93. This reduction of IL-5 may reflect the reduced eosinophilia in the lung, as seen in our study.

Altogether, this survey of models, of which the mechanisms of adjuvant activity after coexposure have been studied, yields evidence that IL-4 is involved in EHC-93-induced stimulation of ovalbumin-specific responses, but Nramp1, NOS, and ROS are not.

REFERENCES

- Alm, J. S., Sanjeevi, C. B., Miller, E. N., Dabadghao, P., Lilja, G., Pershagen, G., Blackwell, J. M., and Scheynius, A. 2002. Atopy in children in relation to BCG vaccination and genetic polymorphisms at SLC11A1 (formerly NRAMP1) and D2S1471. *Genes Immun.* 3:71–77.
- Barnes, P. J., and Liew, F. Y. 1995. Nitric oxide and asthmatic inflammation. *Immunol. Today* 16:128–130.
- Becker, S., Soukup, J. M., Gilmour, M. I., and Devlin, R. B. 1996. Stimulation of human and rat alveolar macrophages by urban air particulates: Effects on oxidant radical generation and cytokine production. *Toxicol. Appl. Pharmacol.* 141:637–648.
- Bochner, B. S., and Schleimer, R. P. 1994. The role of adhesion molecules in human eosinophil and basophil recruitment. *J. Allergy Clin. Immunol.* 94:427–438.
- Bouthillier, L., Vincent, R., Goegan, P., Adamson, I. Y., Bjarnason, S., Stewart, M., Guenette, J., Potvin, M., and Kumarathasan, P. 1998.

- Acute effects of inhaled urban particles and ozone: Lung morphology, macrophage activity, and plasma endothelin-1. *Am. J. Pathol.* 153:1873–1884.
- Calderon-Garciduenas, L., Maronpot, R. R., Torres-Jardon, R., Henriquez-Roldan, C., Schoonhoven, R., Acuna-Ayala, H., Villarreal-Calderon, A., Nakamura, J., Fernando, R., Reed, W., Azzarelli, B., and Swenberg, J. A. 2003. DNA damage in nasal and brain tissues of canines exposed to air pollutants is associated with evidence of chronic brain inflammation and neurodegeneration. *Toxicol. Pathol.* 31:524–538.
- Cassee, F. R., Boere, A. J. F., Bos, J., Fokkens, P. H. B., Dormans, J. A. M. A., and van Loveren, H. 2002. Effects of diesel exhaust enriched concentrated PM_{2.5} in ozone preexposed or monocrotaline-treated rats. *Inhal. Toxicol.* 14:721–743.
- Coffman, R. L., Lebman, D. A., and Rothman, P. 1993. Mechanism and regulation of immunoglobulin isotype switching. *Adv. Immunol.* 54:229–270.
- de Gouw, H. W., Grunberg, K., Schot, R., Kroes, A. C., Dick, E. C., and Sterk, P. J. 1998. Relationship between exhaled nitric oxide and airway hyperresponsiveness following experimental rhinovirus infection in asthmatic subjects. *Eur. Respir. J.* 11:126–132.
- Diaz-Sanchez, D., Tsien, A., Fleming, J., and Saxon, A. 1997. Combined diesel exhaust particulate and ragweed allergen challenge markedly enhances human in vivo nasal ragweed-specific IgE and skews cytokine production to a T helper cell 2-type pattern. *J. Immunol.* 158:2406–2413.
- Diaz-Sanchez, D., Garcia, M. P., Wang, M., Jyrala, M., and Saxon, A. 1999. Nasal challenge with diesel exhaust particles can induce sensitization to a neoallergen in the human mucosa. *J. Allergy Clin. Immunol.* 104:1183–1188.
- Dillon, W. C., Hampl, V., Shultz, P. J., Rubins, J. B., and Archer, S. L. 1996. Origins of breath nitric oxide in humans. *Chest* 110:930–938.
- Ding, A. H., Nathan, C. F., and Stuehr, D. J. 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J. Immunol.* 141:2407–2412.
- Dockery, D. W., Pope, C. A., Xu, X., Spengler, J. D., Ware, J. H., Fay, M. E., Ferris, B.-G. J., and Speizer, F. E. 1993. An association between air pollution and mortality in six U.S. cities. *N. Engl. J. Med.* 329:1753–1759.
- Feder, L. S., Stelts, D., Chapman, R. W., Manfra, D., Crawley, Y., Jones, H., Minnicozzi, M., Fernandez, X., Paster, T., Egan, R. W., Kreutner, W., and Kung, T. T. 1997. Role of nitric oxide on eosinophilic lung inflammation in allergic mice. *Am. J. Respir. Cell Mol. Biol.* 17:436–442.
- Fujimaki, H., Saneyoshi, K., Shiraishi, F., Imai, T., and Endo, T. 1997. Inhalation of diesel exhaust enhances antigen-specific IgE antibody production in mice. *Toxicology* 116:227–233.
- Goldsmith, C. A., Ning, Y., Qin, G., Imrich, A., Lawrence, J., Murthy, G.-G. K., Catalano, P. J., and Kobzik, L. 2002. Combined air pollution particle and ozone exposure increases airway responsiveness in mice. *Inhal. Toxicol.* 14:325–347.
- Hajat, S., Haines, A., Atkinson, R. W., Bremner, S. A., Anderson, H. R., and Emberlin, J. 2001. Association between air pollution and daily consultations with general practitioners for allergic rhinitis in London, United Kingdom. *Am. J. Epidemiol.* 153:704–714.
- Hamid, Q., Springall, D. R., Riveros-Moreno, V., Chanez, P., Howarth, P., Redington, A., Bousquet, J., Godard, P., Holgate, S., and Polak, J. M. 1993. Induction of nitric oxide synthase in asthma. *Lancet* 342:1510–1513.
- Henriksen, A. H., Sue-Chu, M., Lingaas-Holmen, T., Langhammer, A., and Bjerner, L. 1999. Exhaled and nasal NO levels in allergic rhinitis: Relation to sensitization, pollen season and bronchial hyperresponsiveness. *Eur. Respir. J.* 13:301–306.
- Heo, Y., Saxon, A., and Hankinson, O. 2001. Effect of diesel exhaust particles and their components on the allergen-specific IgE and IgG1 response in mice. *Toxicology* 159:143–158.
- Herrick, Ch. A., MacLeod, H., Glusac, E., Tigelaar, R. E., and Bottomly, K. 2000. Th2 responses induced by epicutaneous or inhalational protein exposure are differentially dependent on IL-4. *J. Clin. Invest.* 105:765–775.
- Herz, U., Braun, A., Ruckert, R., and Renz, H. 1998. Various immunological phenotypes are associated with increased airway responsiveness. *Clin. Exp. Allergy* 28:625–634.
- Hogan, S. P., Mould, A., Kikutani, H., Ramsay, A. J., and Foster, P. S. 1997. Aeroallergen-induced eosinophilic inflammation, lung damage, and airways hyperreactivity in mice can occur independently of IL-4 and allergen-specific immunoglobulins. *J. Clin. Invest.* 99:1329–1339.
- Ichinose, T., Furuyama, A., and Sagai, M. 1995. Biological effects of diesel exhaust particles (DEP). II. Acute toxicity of DEP introduced into lung by intratracheal instillation. *Toxicology* 99:153–167.
- Kharitonov, S. A., Wells, A. U., O'Connor, B. J., Cole, P. J., Hansell, D. M., Logan-Sinclair, R. B., and Barnes, P. J. 1995a. Elevated levels of exhaled nitric oxide in bronchiectasis. *Am. J. Respir. Crit. Care Med.* 151:1889–1893.
- Kharitonov, S. A., Yates, D., and Barnes, P. J. 1995b. Increased nitric oxide in exhaled air of normal human subjects with upper respiratory tract infections. *Eur. Respir. J.* 8:295–297.
- Kharitonov, S. A., Yates, D., Springall, D. R., Buttery, L., Polak, J., Robbins, R. A., and Barnes, P. J. 1995c. Exhaled nitric oxide is increased in asthma. *Chest* 107:156S–157S.
- Koarai, A., Ichinose, M., Sugiura, H., Tomaki, M., Watanabe, M., Yamagata, S., Komaki, Y., Shirato, K., and Hattori, T. 2002. iNOS depletion completely diminishes reactive nitrogen-species formation after an allergic response. *Eur. Respir. J.* 20:609–166.
- Kodavanti, U. P., Mebane, R., Ledbetter, A., Krantz, T., McGee, J., Jackson, M. C., Walsh, L., Hilliard, H., Chen, B. Y., Richards, J., and Costa, D. L. 2000. Variable pulmonary responses from exposure to concentrated ambient air particles in a rat model of bronchitis. *Toxicol. Sci.* 54:441–451.
- Kramer, U., Koch, T., Ranft, U., Ring, J., and Behrendt, H. 2000. Traffic-related air pollution is associated with atopy in children living in urban areas. *Epidemiology* 11:64–70.
- Kumagai, Y., Arimoto, T., Shinyashiki, M., Shimojo, N., Nakai, Y., Yoshikawa, T., and Sagai, M. 1997. Generation of reactive oxygen species during interaction of diesel exhaust particle components with NADPH-cytochrome P450 reductase and involvement of the bioactivation in the DNA damage. *Free Radical Biol. Med.* 22:479–487.
- Lafuse, W. P., Alvarez, G. R., and Zwilling, B. S. 2000. Regulation of Nramp1 mRNA stability by oxidants and protein kinase C in RAW264.7 macrophages expressing Nramp1(Gly169). *Biochem. J.* 351 Pt 3:687–696.

- Lebman, D. A., and Coffman, R. L. 1988. Interleukin 4 causes isotype switching to IgE in T cell-stimulated clonal B cell cultures. *J. Exp. Med.* 168:853–862.
- Lim, H. B., Ichinose, T., Miyabara, Y., Takano, H., Kumagai, Y., Shimojyo, N., Devalia, J. L., and Sagai, M. 1998. Involvement of superoxide and nitric oxide on airway inflammation and hyperresponsiveness induced by diesel exhaust particles in mice. *Free Radical Biol. Med.* 25:635–644.
- Lundberg, J. O., Nordvall, S. L., Weitzberg, E., Kollberg, H., and Alving, K. 1996. Exhaled nitric oxide in paediatric asthma and cystic fibrosis. *Arch. Dis. Child.* 75:323–326.
- Martin, L. D., Krunkosky, T. M., Dye, J. A., Fischer, B. M., Jiang, N. F., Rochelle, L. G., Akley, N. J., Dreher, K. L., and Adler, K. B. 1997. The role of reactive oxygen and nitrogen species in the response of airway epithelium to particulates. *Environ. Health Perspect.* 105(suppl. 5):1301–1307.
- Martin, U., Bryden, K., Devoy, M., and Howarth, P. 1996. Increased levels of exhaled nitric oxide during nasal and oral breathing in subjects with seasonal rhinitis. *J. Allergy Clin. Immunol.* 97:768–772.
- Moncada, S., Palmer, R. M., and Higgs, E. A. 1991. Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* 43:109–142.
- Nel, A. E., Diaz-Sanchez, D., Ng, D., Hiura, T., and Saxon, A. 1998. Enhancement of allergic inflammation by the interaction between diesel exhaust particles and the immune system. *J. Allergy Clin. Immunol.* 102:539–554.
- Ohta, K., Yamashita, N., Tajima, M., Miyasaka, T., Nakano, J., Nakajima, M., Ishii, A., Horiuchi, T., Mano, K., and Miyamoto, T. 1999. Diesel exhaust particulate induces airway hyperresponsiveness in a murine model: Essential role of GM-CSF. *J. Allergy Clin. Immunol.* 104:1024–1030.
- Rusznak, C., Bayram, H., Devalia, J. L., and Davies, R. J. 1997. Impact of the environment on allergic lung diseases. *Clin. Exp. Allergy* 27(suppl. 1):26–35.
- Sagai, M., Saito, H., Ichinose, T., Kodama, M., and Mori, Y. 1993. Biological effects of diesel exhaust particles. I. In vitro production of superoxide and in vivo toxicity in mouse. *Free Radical Biol. Med.* 14:37–47.
- Saldiva, P. H. N., Clarke, R. W., Coull, B. A., Stearns, R. C., Lawrence, J., Murthy, G.-G. K., Diaz, E., Koutrakis, P., Suh, H., Tsuda, A., and Godleski, J. J. 2002. Lung inflammation induced by concentrated ambient air particles is related to particle composition. *Am. J. Respir. Crit. Care Med.* 165:1610–1617.
- Sarnat, J. A., Schwartz, J., and Suh, H. H. 2001. Fine particulate air pollution and mortality in 20 U.S. cities. *N. Engl. J. Med.* 344:1253–1254.
- Smit, J. J., van Loveren, H., Hoekstra, M. O., Karimi, K., Folkerts, G., and Nijkamp, F. P. 2003a. The Slc11a1 (Nrp1) gene controls efficacy of mycobacterial treatment of allergic asthma. *J. Immunol.* 171:754–760.
- Smit, J. J., van Loveren, H., Hoekstra, M. O., Nijkamp, F. P., and Bloksma, N. 2003b. Influence of the macrophage bacterial resistance gene, Nrp1 (Slc11a1), on the induction of allergic asthma in the mouse. *FASEB J.* 17:958–960.
- Smit, J. J., van Loveren, H., Hoekstra, M. O., Schijf, M. A., Folkerts, G., and Nijkamp, F. P. 2003c. Mycobacterium vaccae administration during allergen sensitization or challenge suppresses asthmatic features. *Clin. Exp. Allergy* 33:1083–1089.
- Steenenbergh, P. A., Nierkens, S., Fischer, P. H., van Loveren, H., Opperhuizen, A., Vos, J. G., and van Amsterdam, J. G. 2001. Traffic-related air pollution affects peak expiratory flow, exhaled nitric oxide, and inflammatory nasal markers. *Arch. Environ. Health* 56:167–174.
- Steenenbergh, P. A., Janssen, N. A., Meer, G. de, Fischer, P. H., Nierkens, S., van Loveren, H., Opperhuizen, A., Brunekreef, B., and van Amsterdam, J. G. 2003a. Relationship between exhaled NO, respiratory symptoms, lung function, bronchial hyperresponsiveness, and blood eosinophilia in school children. *Thorax* 58:242–245.
- Steenenbergh, P. A., van Dalen, W. J., Withagen, C. E., Dormans, J. A., and van Loveren, H. 2003b. Optimization of route of administration for coexposure to ovalbumin and particle matter to induce adjuvant activity in respiratory allergy in the mouse. *Inhal. Toxicol.* 15:1309–1325.
- Steenenbergh, P. A., Withagen, C. E., Dormans, J. A., van Dalen, W. J., van Loveren, H., and Cassee, F. R. 2003c. Adjuvant activity of various diesel exhaust and ambient particles in two allergic models. *J. Toxicol. Environ. Health A* 66:1421–1439.
- Steenenbergh, P. A., Withagen, C. E., van Dalen, W. J., Dormans, J. A. M. A., Cassee, F. R., Heisterkamp, S. H., and van Loveren, H. 2004. Adjuvant activity of ambient particulate matter of different sites, sizes and seasons in a respiratory allergy mouse model. *Toxicol. Applied Pharmacol.*, in press.
- Strachan, D. P. 2000. Family size, infection and atopy: The first decade of the “hygiene hypothesis.” *Thorax* 55(suppl. 1):S2–S10.
- Suschkew, C., Fehsel, K., Kroncke, K. D., Sommer, A., and Kolb-Bachofen, V. 1994. Primary cultures of rat islet capillary endothelial cells. Constitutive and cytokine-inducible macrophagelike nitric oxide synthases are expressed and activities regulated by glucose concentration. *Am. J. Pathol.* 145:685–695.
- Swain, S. L., Weinberg, A. D., English, M., and Huston, G. 1990. IL-4 directs the development of Th2-like helper effectors. *J. Immunol.* 145:3796–3806.
- van Amsterdam, J. G., Bischoff, E. W., Klerk, A. de, Verlaan, A. P., Jongbloets, L. M., van Loveren, H., Opperhuizen, A., Zomer, G., Hady, M., Spijksma, F. T., Dormans, J. A., and Steenenbergh, P. A. 2003a. Exhaled NO level and number of eosinophils in nasal lavage as markers of pollen-induced upper and lower airway inflammation in children sensitive to grass pollen. *Int. Arch. Occup. Environ. Health* 76:309–312.
- van Amsterdam, J. G., Janssen, N. A., de Meer, G., Fischer, P. H., Nierkens, S., van Loveren, H., Opperhuizen, A., Steenenbergh, P. A., and Brunekreef, B. 2003b. The relationship between exhaled nitric oxide and allergic sensitization in a random sample of school children. *Clin. Exp. Allergy* 33:187–191.
- Weiss, G. 1999. Iron and anemia of chronic disease. *Kidney Int. Suppl.* 69:S12–S17.
- Whitekus, M. J., Li, N., Zhang, M., Wang, M., Horwitz, M. A., Nelson, S. K., Horwitz, L. D., Brechun, N., Diaz-Sanchez, D., and Nel, A. E. 2002. Thiol antioxidants inhibit the adjuvant effects of aerosolized diesel exhaust particles in a murine model for ovalbumin sensitization. *J. Immunol.* 168:2560–2567.
- Wyler, C., Braun-Fahrlander, C., Kunzli, N., Schindler, C., Ackermann-Liebrich, U., Perruchoud, A. P., Leuenberger, P., and

- Wuthrich, B. 2000. Exposure to motor vehicle traffic and allergic sensitization. The Swiss Study on Air Pollution and Lung Diseases in Adults (SAPALDIA) Team. *Epidemiology* 11:450–456.
- Xiao, G. G., Wang, M., Li, N., Loo, J. A., and Nel, A. E. 2003. Use of proteomics to demonstrate a hierarchical oxidative stress response to diesel exhaust particle chemicals in a macrophage cell line. *J. Biol. Chem.* 278:50781–50790.
- Zwilling, B. S., Kuhn, D. E., Wikoff, L., Brown, D., and Lafuse, W. 1999. Role of iron in Nramp1-mediated inhibition of mycobacterial growth. *Infect. Immun.* 67:1386–1392.