JOURNAL OF AEROSOL MEDICINE AND PULMONARY DRUG DELIVERY
Volume 21, Number 2, 2008
© Mary Ann Liebert, Inc.
Pp. 000–000
DOI: 10.1089/jamp.2007.0672

Anti-inflammatory Effect from Indomethacin Nanoparticles Inhaled by Male Mice

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ABSTRACT

The respiratory system provides entry for drug nanoparticles to cure systemic diseases. The modern devices that are available on the market of therapeutic aerosol delivery systems have a number of disadvantages. There remains a need for an alternative means that is low cost, convenient, and capable of producing small-sized particles. On the other hand, one-third of the modern drugs are poorly water soluble. Many currently available injectable formulations of such drugs can cause side effects that originate from detergents and other agents used for their solubilization. The aerosol lung administration may by a good way for delivery of the water-insoluble drugs. We present here a new way for the generation of drug nanoparticles suitable for many water insoluble substances based on the evaporation–condensation route. In this paper the indomethacin nanoaerosol formation was studied and its anti-inflammatory effect to the outbred male mice was examined. The evaporation–condensation aerosol generator consisted of a horizontal cylindrical quartz tube with an outer heater. Argon flow was supplied to the inlet and the aerosol was formed at the outlet. The particle mean diameter and number concentration were varied in the ranges 3 to 200 nm and 10^3 to 10^7 cm^{-3}, respectively. The liquid chromatography and X-ray diffraction methods have shown the nanoparticles consist of the amorphous phase indomethacin. The aerosol lung administration experiments were carried out in the whole-body exposure chamber. Both the lung deposited dose and the particle deposition efficiency were determined as a function of the mean particle diameter for mice being housed into the nose-only exposure chambers. The anti-inflammatory action and side pulmonary effects caused by the inhalation of indomethacin nanoparticles were investigated. It was found that the aerosol administration was much more effective than the peroral treatment. The aerosol route required a therapeutic dose six orders of magnitude less than that for peroral administration.

Key words: indomethacin, nanoparticles, aerosol drug administration, particle lung deposition, mice, anti-inflammatory effect

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INTRODUCTION

THE ADMINISTRATION OF DRUGS directly into the respiratory tract has been used in a number of therapeutic areas. The field for aerosolized drug application includes treatment of lung diseases, like asthma, chronic obstructive pulmonary disease, cystic fibrosis, and lung cancer.[1] The aerosol delivery has expanded also into the field of systemic drug delivery.[2] One of the good examples of the expanding role of aerosol therapy is the development of insulin as an aerosol to treat diabetes.[3,4] The optimal target within the lungs for delivery of drugs to the systemic circulation is the alveolar region. For rapid delivery, the alveolar drug administration has a number of advantages including the large absorptive surface area, easy permeability of the alveolar walls resulting in the fast passage from the alveolar airspace to the pulmonary capillary bed, direct connection between the pulmonary circulation and the systemic circulation. The aerosol delivery for some drugs (like, e.g., zanamivir[1] or amantadine hydrochloride[5]) is much more efficient then the peroral administration because of low oral bioavailability. The modern devices that are available on the market of therapeutic aerosol delivery systems can be subdivided into three groups: nebulizers, dose-metering inhaler systems, and dry powder inhalers.[1,6] All these inhalers have evident shortcomings.[1] One of the main problems for the jet nebulizers is their limited portability due to the need for compressed gas supply. Also, it is important that the nebulizer solutions should not be stored in nebulizer reservoirs due to the possible growth of bacteria. The disadvantages of pressurized dose-metering inhaler systems include high aerosol velocity (which results in substantial oropharyngeal deposition), limited single dose, etc. Different facilities including extension tubes and spacers were applied to overcome some of these problems; however, new problems have appeared like large spacer volume and particle electric charge.[7] One of the problems for dry powder inhalers is that the dry powder formulation should be appropriately prepared. The powder properties are shelf life dependent. The crucial question is hygroscopicity. Thus, the aerosol properties and the lung deposition efficiency can be functions of the powder storage time.

The lung deposition efficiency for the above systems normally does not exceed 10%.[1] All these devices are able to generate the particles as small as a few microns in diameter. However, the alveolar deposition efficiency is a strong function of the particle size. The particles 10 to 20 nm in size deposit to the alveolar region about four times more efficiently than those several microns in diameter.[1,8–10] Therefore, a possible way to achieve higher deposition efficiency (and, probably, to overcome some of the traditional inhaler shortcomings) is to develop a new-type generator for nanometer-size range. The possible alter-

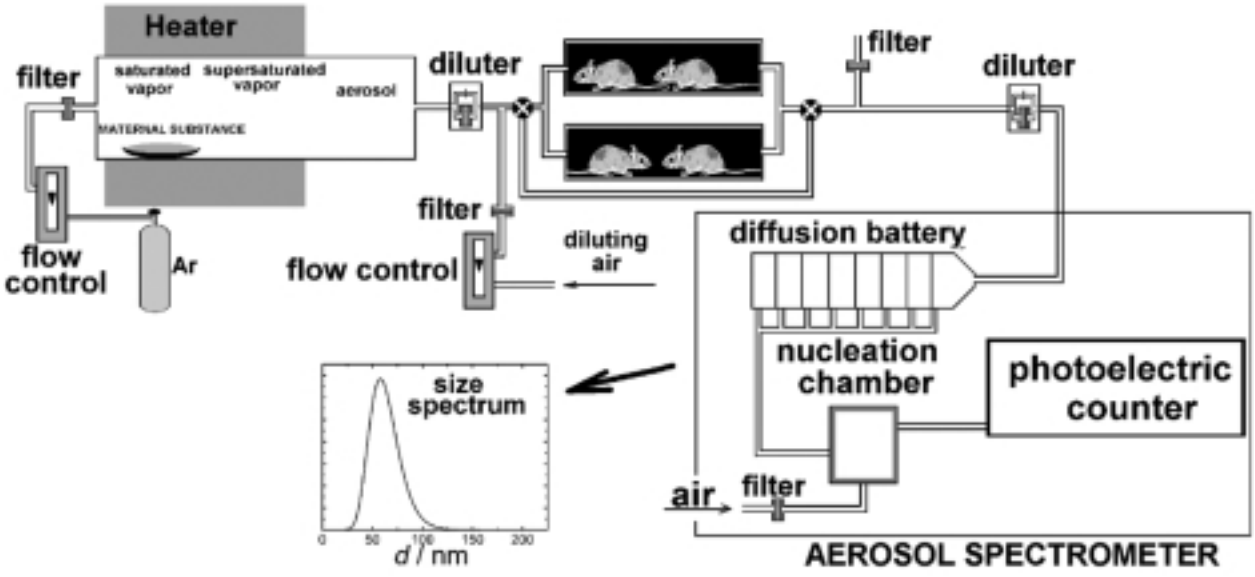

FIG. 1. Scheme of the experimental setup for inhalation experiments.

native to nebulizers, metered-dose, and dry powder inhalers is evaporation–condensation route,[11,12] which combines the possibility to generate a fine aerosol with the particle diameter 1 to 100 nm and the number concentration as high as 10^8 cm^{-3}.[13] The problem is that the evaporating substance must be thermally stable. In addition, different formulations require different heating temperature. Thus, the evaporation–condensation way of aerosol delivering is waiting for the detailed investigation.

Approximately one-third of the modern drugs are water insoluble or poorly water soluble. Many currently available injectable formulations of such drugs can cause side effects that originate from detergents and other agents used for their solubilization. Also, water-solubility problems delay or completely block the development of many new drugs and other biologically useful compounds. Thus, the lung deposition route can be a good alternative for the administration of poorly soluble substances. Indomethacin, which has low water solubility of 25 mg/L, is one of the candidates considered for the lung delivery.[14] Indomethacin is a well-known nonsteroid anti-inflammatory drug for use against a wide range of diseases such as rheumatoid arthritis, spondylosis, chondrosis. However, its side effects can cause serious disorders such as bleeding and perforation of gastrointestinal tract, depression, drowse, mental disorder, increased blood pressure, congestive heart failure, etc. One may hope that the aerosol lung administration of indomethacin may be an alternative route which would diminish side effects and decrease the therapeutic dose. However, new side effects like pulmonary emphysema are possible. Therefore, it is necessary to estimate the therapeutic benefit as well as possible risks from indomethacin aerosol administration.

In this paper we study the evaporation–condensation formation of indomethacin nanoparticles and its anti-inflammatory effect, as well as side effects to the outbred male mice.

MATERIALS AND METHODS

The scheme of inhalation experiment is shown in Figure 1. The evaporation–condensation aerosol

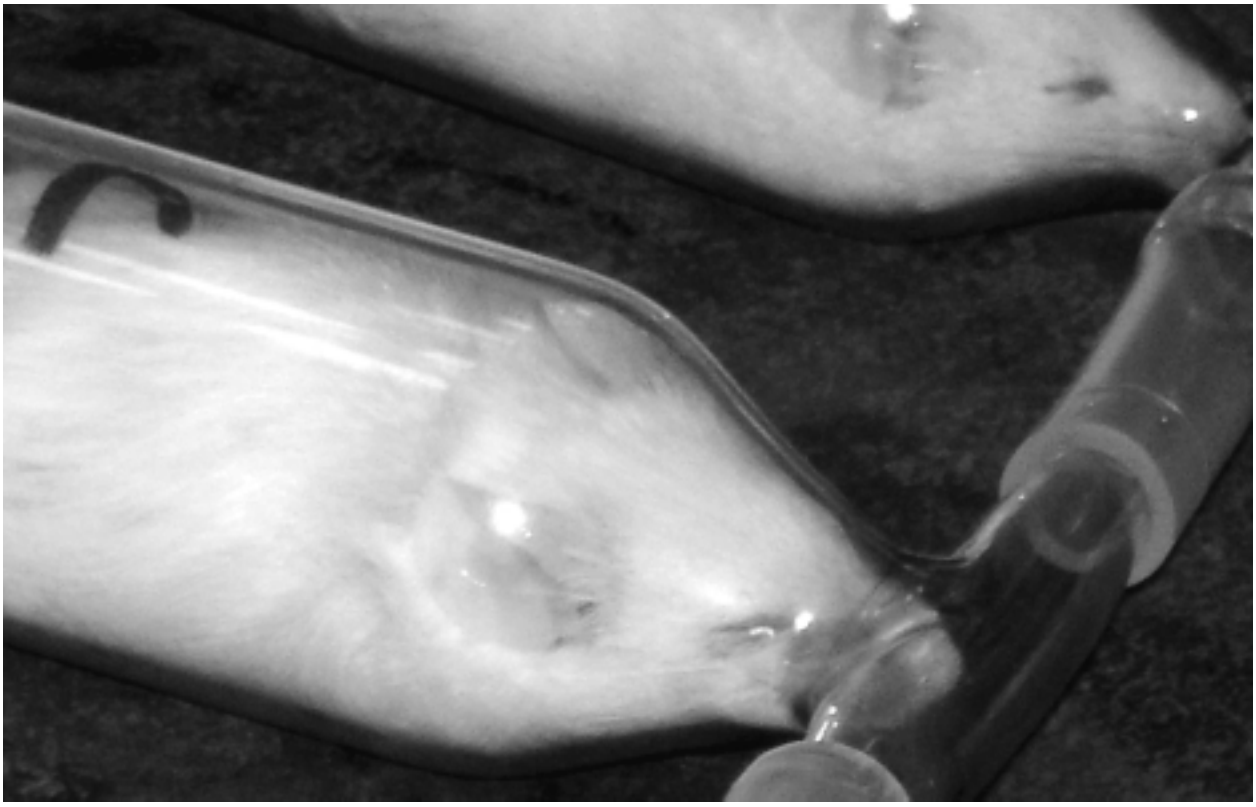

FIG. 2. Nose-only exposure glass chambers.

generator consists of a horizontal cylindrical quartz tube (with the inner diameter of 1.50 cm) with an outer heater. Argon flow is supplied to the inlet of the generator at the rate of 8 cm^3/sec (at standard temperature and pressure). The maternal substance (Indomethacin, ICN-190217) is put to the hot zone inside the tube. The saturated vapor is formed inside the generator. The temperature drops down at the outlet of the tube resulting in vapor supersaturation and, as a consequence, in the homogeneous vapor nucleation. Then the aerosol is diluted up to the necessary concentration using an aerosol diluter (based on flow splitting, filtering one of the subflows and mixing the subflows again). The final aerosol is mixed with the air flow (at the flow rate of 100 cm^3/sec) and admitted into the plastic whole-body (WB) exposure chambers 300 cm^3 volume. Two chambers were used in parallel; thus, the aerosol flow rate was about 50 cm^3/sec in each chamber. Each chamber contained two mice during the experiment. The outbred laboratory male mice of 25 to 30 g body weight were used in the aerosol inhalation experiments. The inhalation time in all the experiments was 20 min. The aerosol concentration and size distribution were measured with the aerosol spectrometer designed and built at the Institute of Chemical Kinetics and Combustion, Novosibirsk, Russia.[15] This aerosol spectrometer consists of an automatic diffusion battery, condensation chamber, and photoelectric counter. The spectrometer measured the aerosol number concentration and particle size distribution at the chamber inlet and outlet during the exposition time. The aerosol depletion in the WB chambers did not exceed a few percent. Therefore, later, when evaluating the lung deposited dose, we will put the aerosol concentration in the WB chambers as equal to the inlet concentration.

To determine the lung deposited dose special experiments were carried out. Nose-only exposure (NOE) glass chambers were used to minimize the skin or fur effect. The laboratory animals were confined so that only nose was exposed to the aerosol (Fig. 2). The aerosol flow was switching between two parallel lines. Each line contained six chambers in tandem (Fig. 3). One line was loaded with the animals; the other one had empty chambers. The aerosol depletion due to the mice breathing was determined by comparing the indomethacine particle concentration at the outlets of loaded and unloaded lines as measured by the aerosol spectrometer.

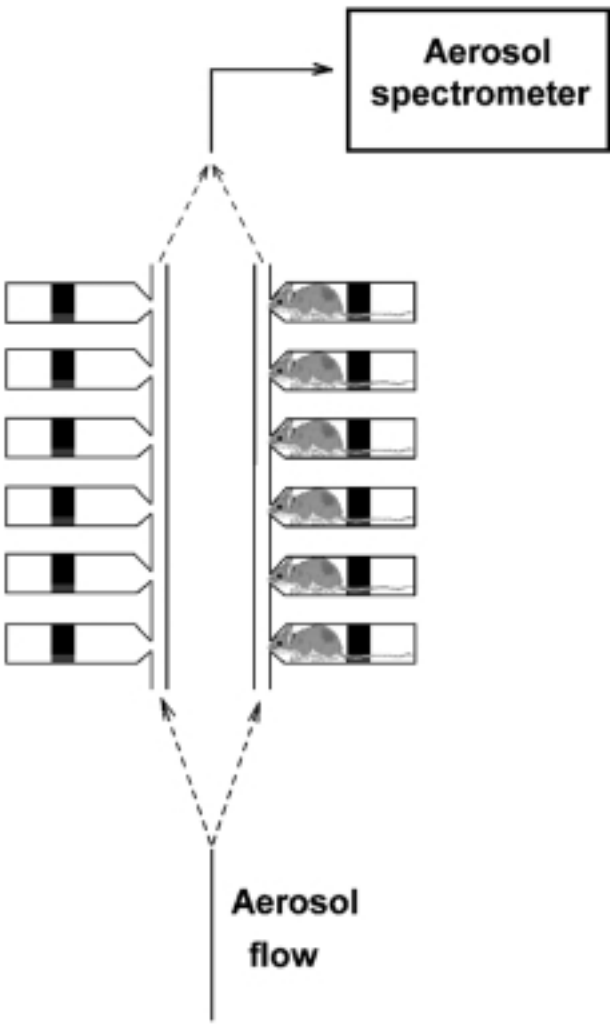

FIG. 3. Scheme of the experimental setup for the lung deposited dose measurements.

The breathing frequency of the mice being loaded to the NOE chambers was measured as shown in Figure 4. The air flux seeded with NaCl tracing particles about 0.3 μm in diameter was supplied through the chamber. The particles were generated by the collison-type nebulizer. Initially, this device generated the liquid NaCl/water solution particles that transform to the solid NaCl particles due to the immediate water evaporation when coming out of the nebulizer. The particle number concentration was as low as about 10^3 cm^{-3} to decrease the mouse airway irritation to a minimum. The flow rate through the chamber was modulated by the mouse breathing. To measure the flow rate, the seeding particles were il-

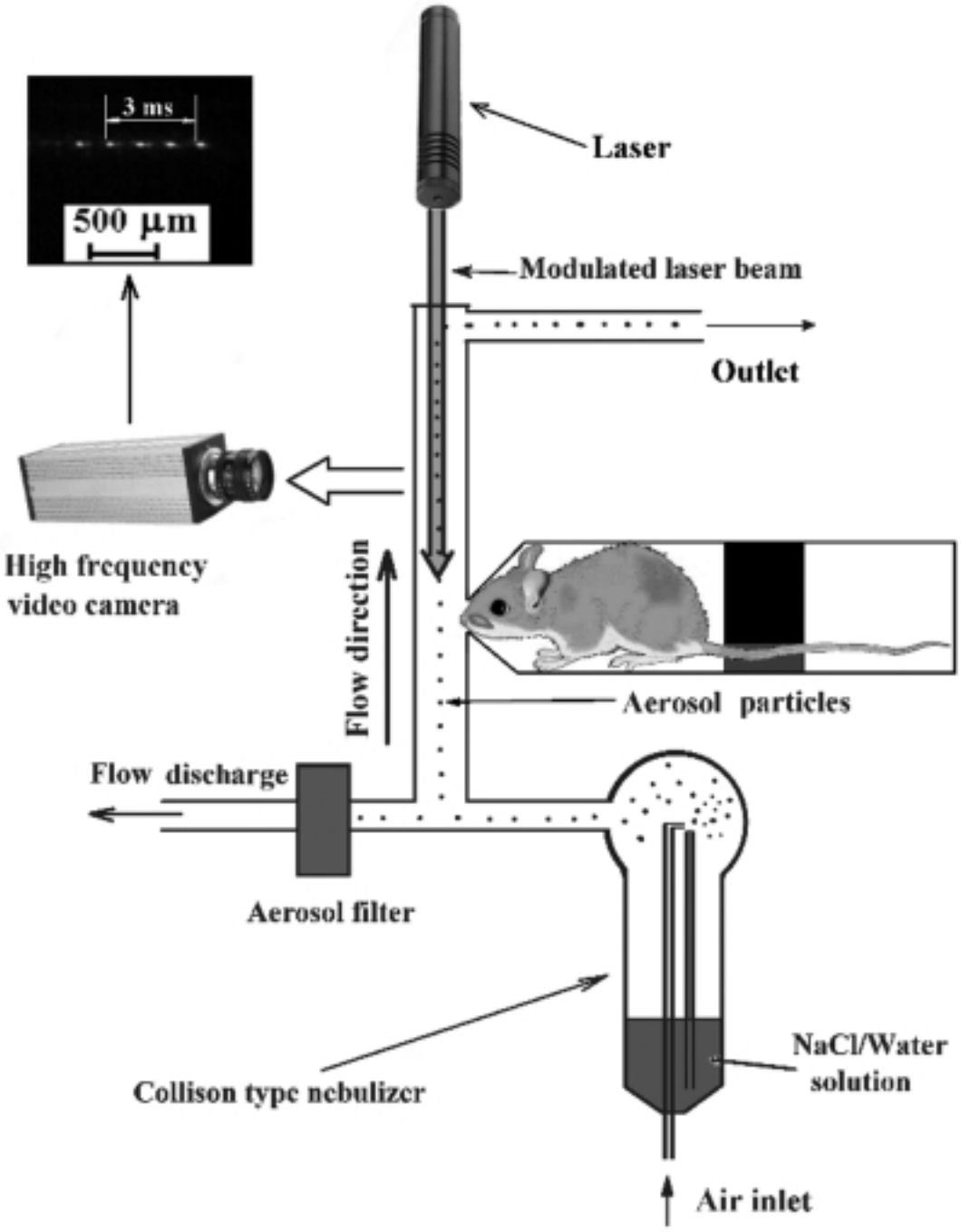

FIG. 4. Scheme of the experimental setup to measure the mice breathing pattern.

luminated by the laser beam modulated with the frequency of 1 kHz. A semiconductor amplitude modulated laser KLM-M650-24 was used. The illuminated particles were observed with a high-frequency video camera "Videoscan" at the angle of 90° (see insert in Fig. 4) to determine the flow velocity. Figure 5 shows a representative breathing pattern.

A histologic analysis was performed to observe the indomethacin aerosol effect on the mice lungs morphology. The mice were killed 6 h after exposure. Lungs were fixed in 4% paraformaldehyde in the phosphate buffer (pH 7.2–7.4). The fixed tissues were treated in a standard way using histological equipment "MICROM" (Carl Zeiss, Thornwood, NY) and then embedded to

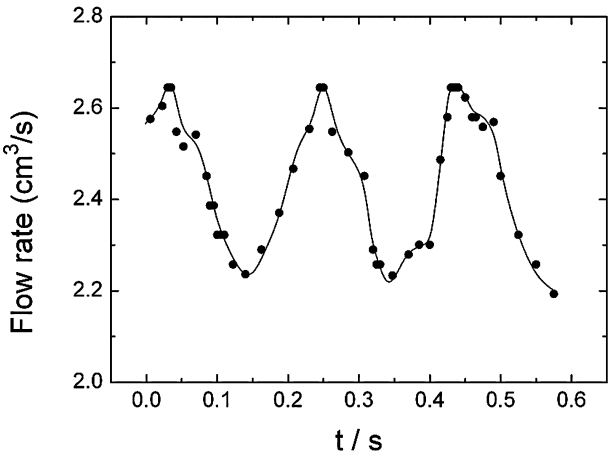

FIG. 5. Typical mouse breathing pattern as obtained from the video observation of the breathing-modulated air flow rate.

paraffin. Sections 3–4 μm thick were stained with hematoxylin and eosin. Slides were examined under the light microscope Axioskop 40 (Carl Zeiss).

RESULTS AND DISCUSSION

Aerosol size, concentration, and composition

The mean particle size and number concentration (as measured at the WB chamber inlet) are shown in Figure 6 as a function of generator temperature. The typical size spectrum is shown in Figure 7. The chromatographic analysis of the aerosol particles was performed to make sure that there was no thermal decomposition of indomethacin during evaporation–condensation. For this purpose, the aerosol particles were sampled by passing the aerosol flux through the Petrianov high-efficiency aerosol filter.[16] Then the deposit was dissolved in acetonitrile. High-performance liquid chromatograph Milikhrom-1 equipped with a UV-spectrophotometric detector was used with a standard column packed with LiChrosorb C18 sorbent. The eluent was acetonitrile–H_2O–$LiClO_4$–H_3PO_4. Elution rate was 50 μL/min. The chromatographic analysis showed that the chromatogram from nanoparticles was identical to that from the original indomethacin sample (Fig. 8). Thus, one may suppose that the nanoparticles are chemically identical to the original substance.

The crystal phase analysis of nanoparticles was carried out using X-ray diffractometer system Bruker-AXS D8 Discover with GADDS area detector. The powder X-ray diffraction patterns of nanoparticles and γ-indomethacin original powder are compared in Figure 9. The XRD pattern of nanoparticulate material contains a broad halo uncorrelated with the crystalline peaks, which is typical for the amorphous indomethacin.[17]

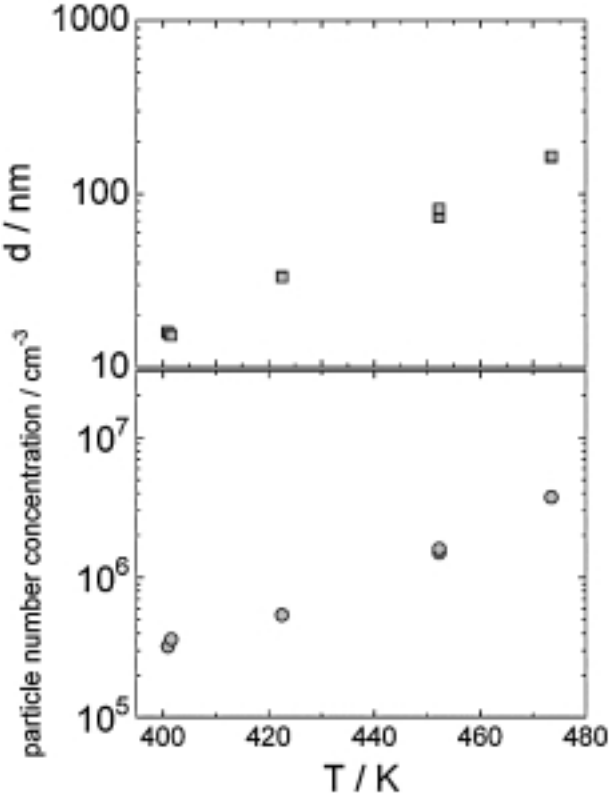

FIG. 6. Mean nanoparticle diameter and number concentration (as measured at the WB chamber inlet) versus the temperature of aerosol generator.

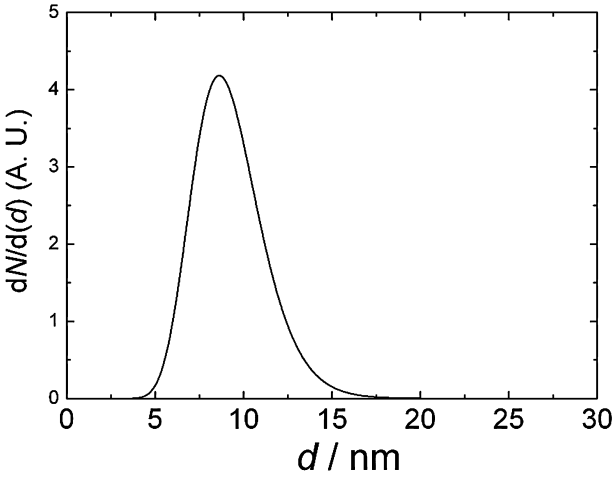

FIG. 7. Typical diameter distribution for indomethacin particles.

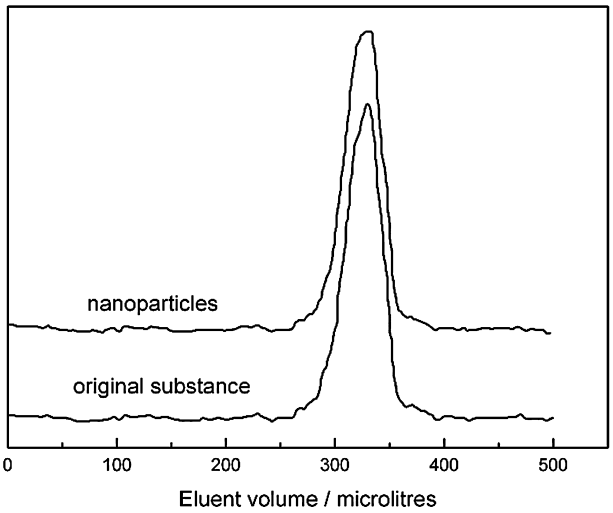

FIG. 8. Comparison between the chromatograms of the original indomethacin sample and nanoparticles formed by evaporation–condensation route.

Thus, the evaporation–condensation route results in the formation of amorphous nanoparticles.

Lung deposited dose

To determine the lung deposited dose we have measured the fraction α of particles that were consumed per chamber due to the mouse breathing:

$$\alpha = 1 - \left(\frac{n}{n_0}\right)^{1/N} \tag{1}$$

where n and n_0 are aerosol number concentrations at the outlets of the loaded and unloaded NOE lines, respectively, N is number of chambers in the tandem (see Fig. 3). The rate D [sec^{-1}] of particle lung deposition per mouse can be written as:

$$D \approx F\alpha n_0, \tag{2}$$

where F is flow rate though the NOE chambers tandem.

One can also use the relative deposition rate

$$D_0 = \frac{D}{n_0}. \tag{3}$$

On the other hand:

$$D_0 \approx fV_T\varepsilon \tag{4}$$

where f, V_T, and ε are average mouse breathing frequency, tidal volume, and particle lung deposition efficiency (the number of exhaled to the number of inhaled particles ratio), respectively. Figure 10 shows the relative deposition rate D_0 as a function of the mean particle diameter. The logistic regression analysis applied to the relative deposition rate data showed that there was a statistically significant correlation between the particle diameter and the relative deposition rate ($R^2 = 0.96$). The fitted curve is shown as solid line.

To determine the particle deposition efficiency one should know both the mouse breathing frequency and the tidal volume. Using the tracing particles technique (Figs. 4 and 5) we determined the mean respiration frequency for the mice used in the inhalation experiments: $f = 5.0 \pm 0.2$ c^{-1}. We used the value of tidal volume $V_T = 0.16$ cm^3

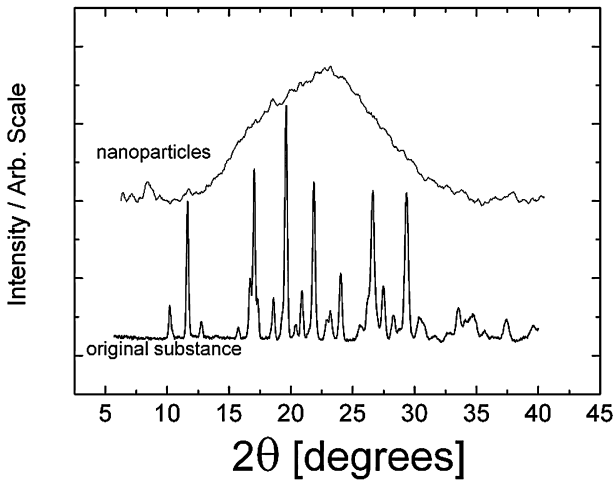

FIG. 9. Comparison between the X-ray diffraction patterns from original γ-indomethacin powder and nanoparticles formed by evaporation–condensation.

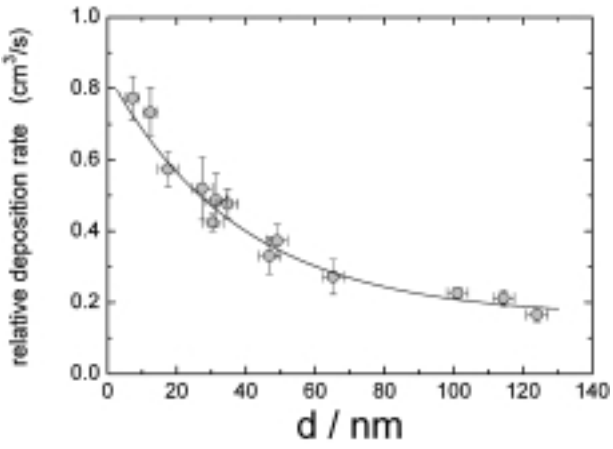

FIG. 10. Relative deposition rate per mouse versus mean particle diameter. Bars indicate standard error. Line is logistic regression analysis result.

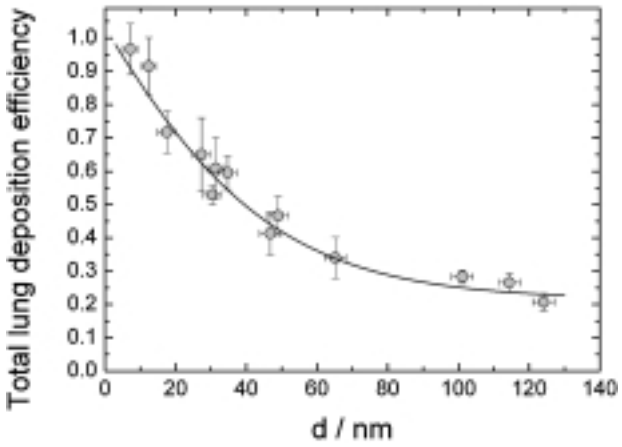

FIG. 11. Mouse respiratory deposition efficiency versus mean particle diameter. Bars indicate standard error. Line is logistic regression analysis result.

as determined in plethysmographic measurements for CD-1 and HA/ICR mice with the body weight of 25–30 g.[18] Then the particle lung deposition efficiency ε was evaluated from Equation (4) using the experimentally measured D_0. The particle deposition efficiency as a function of the mean particle diameter is shown in Figure 11. The logistic regression analysis applied to the total lung deposition efficiency data showed that there was a statistically significant correlation between the particle diameter and ε ($R^2 = 0.96$). The fitted curve is shown as solid line. One can see that ε tends to unity at small diameter values, which is in good agreement with numerical simulations for the particle lung deposition.[9] Thus, the good agreement between the literature simulation results and our evaluated ε testifies that we used the correct value for V_T.

Anti-inflammatory effect of indomethacin nanoparticles

Each animal was used only once in the inhalation procedure and sacrificed at the end of the experiment. The mice were separated into three groups (8–10 animals in each). The animals of the first group (untreated) were not exposed to indomethacin (neither peroral nor by aerosol inhalation); the animals of the second group (peroral) were treated orally with the water–Tween suspension of indomethacin with 15 mg

TABLE 1. EDEMA INDEX FOR TREATED AND UNTREATED ANIMALS

Group number	*Animal number*	*Weight of paw, mg*		*Edema index, %*	*Mean edema index, %*	*Relative edema index*
		Treated	*Untreated*			
1	1	193	160	20.6	20.3 ± 1.9	
(untreated)	2	186	150	24.0		
	3	187	145	29.0		
	4	150	130	15.4		
	5	203	177	14.7		
	6	170	149	14.1		
	7	204	168	21.4		
	8	179	145	23.4		
2	1	187	167	12.0	12.2 ± 1.6	
(peroral)	2	183	173	11.6		
	3	173	160	8.1		
	4	178	156	14.1		
	5	174	162	7.0		
	6	170	154	10.4		
	7	204	176	15.9		
	8	174	163	6.7		
	9	179	144	24.3		
	10	152	170	11.8		
3	1	200	177	13.0	7.3 ± 1.2	
(aerosol	2	171	157	8.9		
group)	3	159	155	2.3		
	4	160	147	8.4		
	5	178	170	4.7		
	6	157	151	4.0		
	7	179	165	8.5		
	8	158	171	8.2		0.36 ± 0.06

Standard errors are shown in the last column.

per kg bodyweight (bw); the animals from the third group (aerosol group) were subjected to the aerosol inhalation. The mice from all the three groups were put to the WB chambers for 20 min. The mice from the first and the second groups were exposed to the pure air; the mice of the third group were exposed to the aerosol (the average lung deposited mass was determined from the NOE chambers experimental data using the amorphous indomethacin density of 1.3 g/cm^3). One hour after the chamber exposition, 0.05 mL of 0.1% histamine solution in water was injected into the subplanar surface of the mouse hind paw. Six hours later the mice were killed by cervical dislocation. Then the mouse's paws were cut off at the ankle joint and weighed. The ratio of the difference in weight between the treated and untreated hind paws to the weight of the untreated hind paw was used as an index of paw edema. Table 1 gives an example of the edema index for the groups 1 to 3. The particle number concentration and mean diameter were 7×10^5 cm^{-3} and 37 nm, respectively, in these experiments, which corresponded to the average lung deposited dose of 1.4×10^{-5} mg per mouse (5.1×10^{-4} mg per kg bw). One can see a considerable anti-inflammatory effect from both aerosol and oral forms of indomethacin—the mean edema index (MEI) for the groups 2 and 3 is considerably less than that for the control group 1. The indomethacin aerosol form is more effective than the indomethacin peroral treatment (MEI = 7.1% and 12.2%, respectively), while the lung deposited dose is a few orders of magnitude less than the peroral dose. It is more convenient to use the rel-

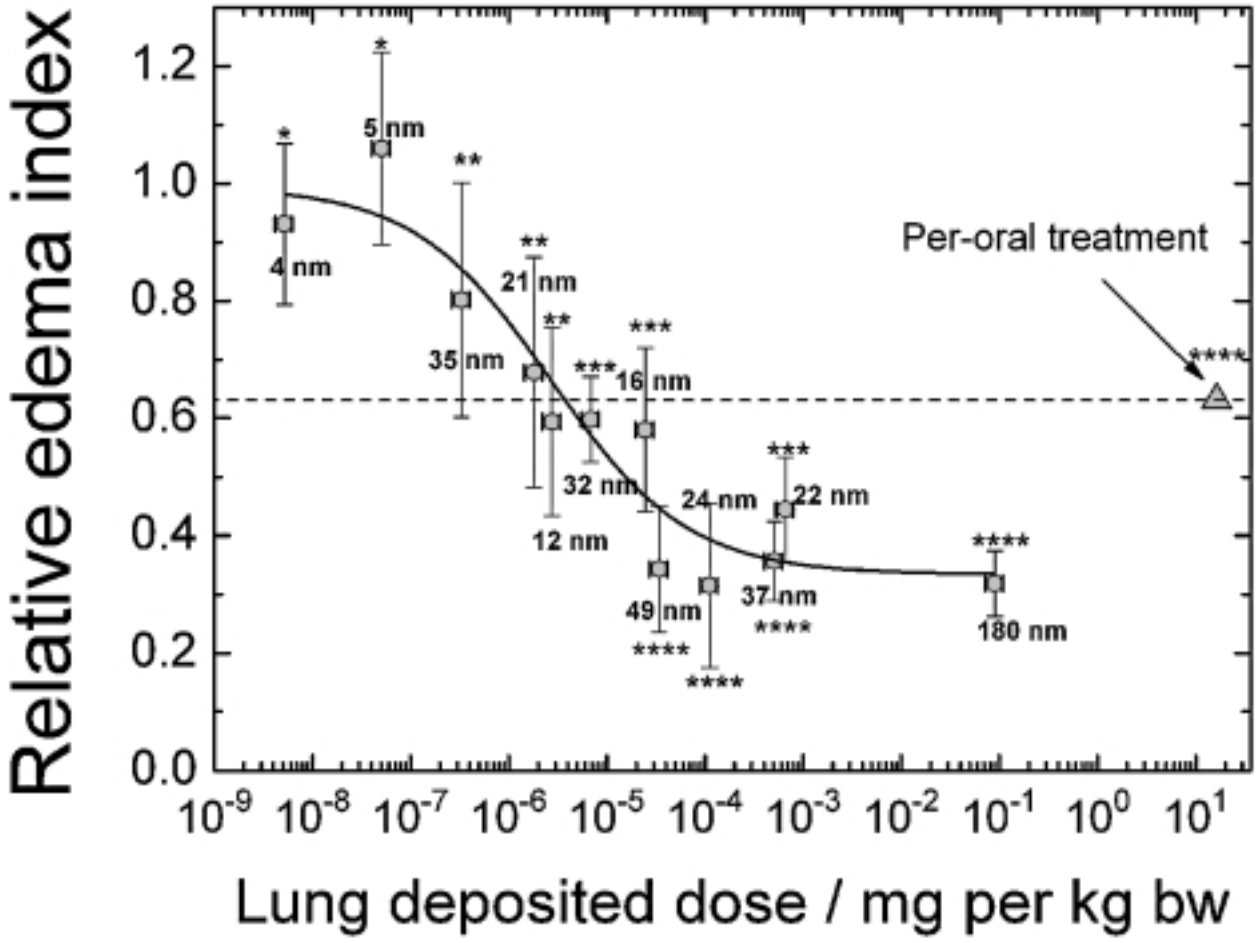

FIG. 12. Mean relative edema index (REI) versus the lung deposited dose (circles). Bars indicate standard error. The particle mean diameter is shown at every point. The levels of statistical significance of differences of the edema indexes between the aerosolized and untreated groups were calculated using Students *t*-test and are indicated as *1 < $p < 0.5$, **0.5 < $p < 0.05$, ***0.05 < $p < 0.001$, ****$p < 0.001$. The fitted dose–response curve is shown as solid line. Triangle is REI for peroral treatment; this point is a mean value throughout six experimental trials (each trial included groups of 8 to 10 animals).

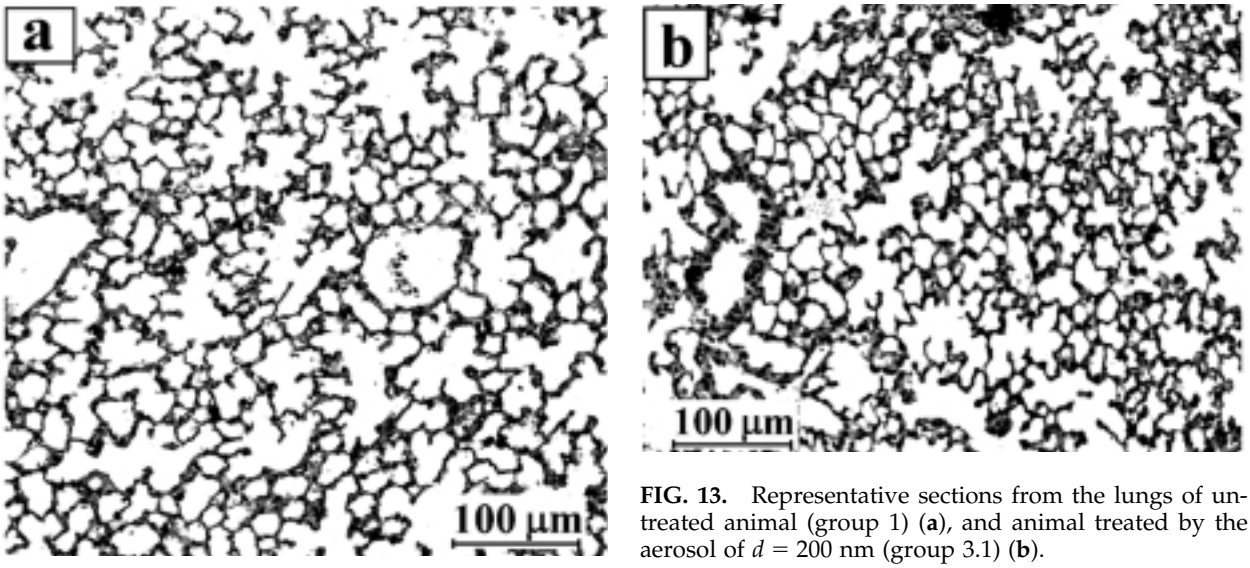

FIG. 13. Representative sections from the lungs of untreated animal (group 1) (**a**), and animal treated by the aerosol of $d = 200$ nm (group 3.1) (**b**).

ative edema index (REI), that is, the ratio between the mean edema indexes for the aerosolized group (or peroral group) and untreated group. Figure 12 shows the mean REI for the aerosolized animals versus the lung deposited dose as well as REI for the orally treated animals. These data were obtained during six experimental trials. Each trial involved one un-

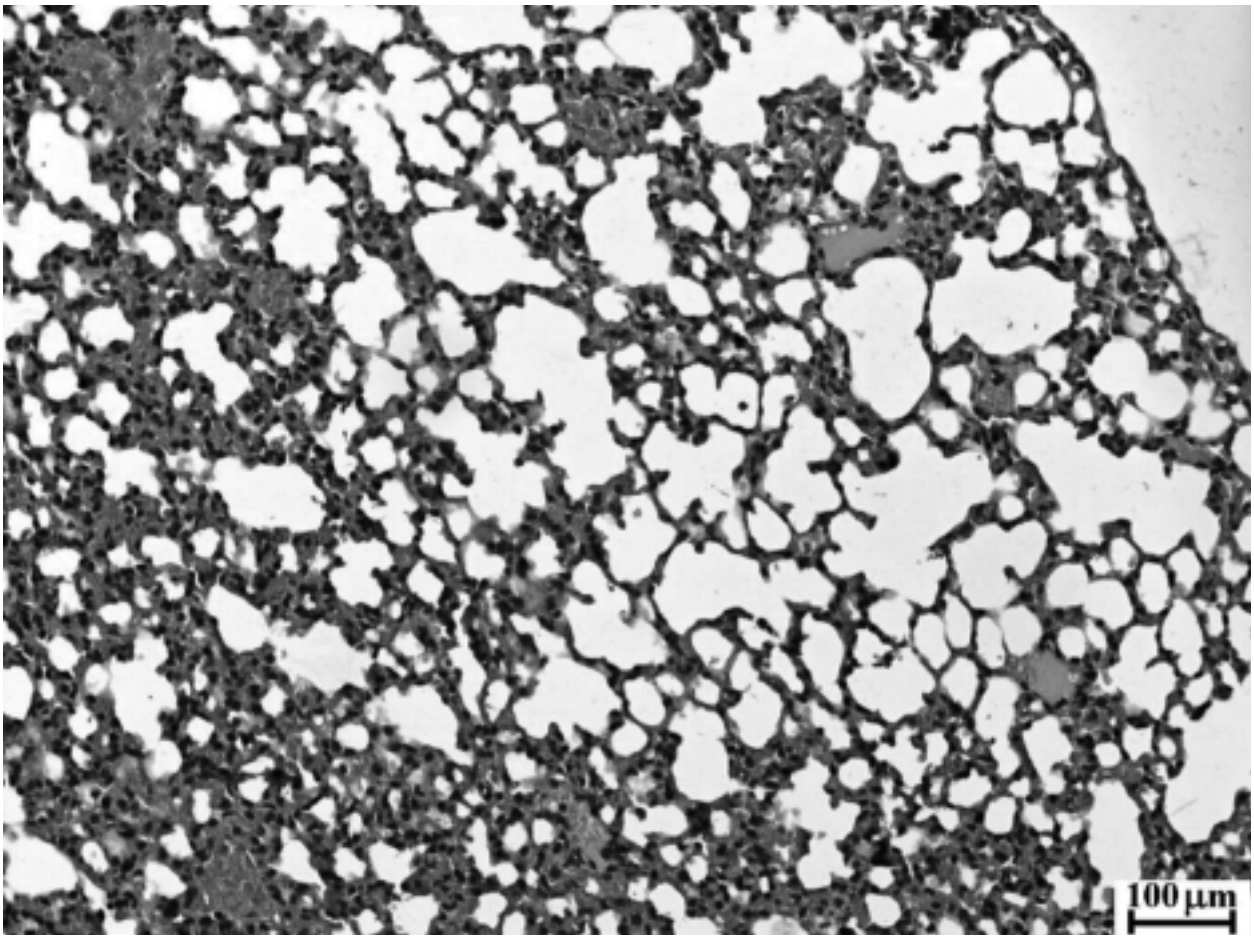

FIG. 14. A cross-section of the lung from a mouse treated with 200-nm nanoparticles showing enlarged airspaces.

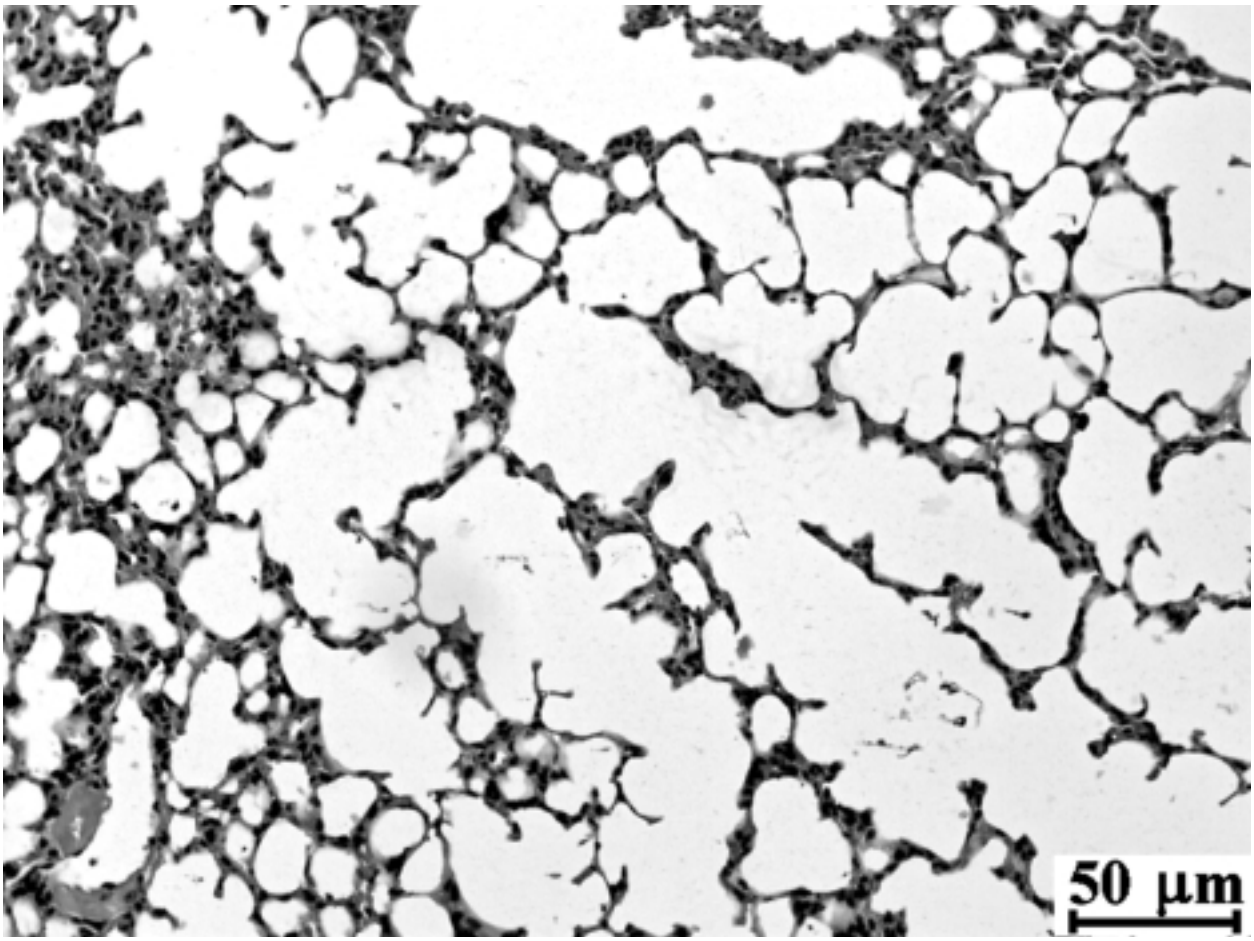

FIG. 15. A cross-section of the lungs of mice treated with 9-nm nanoparticles.

treated group, one oral group, and two aerosolized groups treated with particles of different size. Thus, there are 12 points for the inhalation treatments and one point for the peroral treatment (averaged over six treatments). In total, the data presented in Figure 12 involve about 210 animals. The REI data were analyzed for the dose–response relationship. The fitted curve is shown as the solid line. The regression analysis applied to the REI results showed that there was a statistically significant dose response ($R^2 = 0.91$). The EC50 was 2.7×10^{-6} mg per kg bw. The triangle symbol gives the mean REI for peroral animals for comparison. One can see that the aerosol administration is more effective (gives less REI) than the peroral treatment even at the lung deposited dose six orders of magnitude less than the peroral dose. The mean particle diameter is indicated for each point in the graph. It is hardly possible to find any dependence on the particle size. Thus, the points for the nearby diameters (e.g., 21, 22, 24 nm and 32, 35, 37 nm) seem to be following the basic tendency within the experimental accuracy. On the other hand, the points differing essentially by size (180 and 37 nm; 35 and 4–5 nm) also follow the same tendency.

Histology of the lungs

A histologic analysis was performed to observe possible hemodynamic abnormalities and pulmonary edema after the aerosol treatment. The animals were exposed to two kinds of aerosol of $d = 200$ nm, with the lung deposited dose of 2.3×10^{-5} mg per kg bw (group 3.1) and $d = 9$ nm, with the dose $= 1.2 \times 10^{-5}$ mg per kg bw (group 3.2). The aerosol number concentrations in the WB chambers were 7.1×10^2 and 7.4×10^5 cm^{-3}, respectively. Group 1 again included untreated mice being exposed to the pure air in the chambers. Each group consisted of 8 to 10 animals. The lungs of animals from both group 3.1 and group 3.2 as well as from group

1 have a normal structure without any destructive and hemodynamic pathologic changes (Fig. 13). However, a moderate venous and arterial hyperemia was observed for all the animals in group 3.1. Two animals of this group have revealed the focal emphysematous dilatation of the respiratory bronchioles without any visible vascular bed reduction (Fig. 14). All the animals from group 3.2 have demonstrated the vascular and capillary hyperemia (which was considerably stronger than in the case of group 3.1). An homogeneous venous deposition (presumably fibrin) was observed. Besides, typical emphysematous signs, stronger than in group 3.1, were observed, that is, the dilatation of bronchioli and alveolar channels, alveolar wall thinning, and partial capillary bed reduction (Fig. 15).

CONCLUSIONS

The anti-inflammatory action and side pulmonary effects caused by the inhalation of indomethacin nanoparticles were investigated. To this end, an evaporation–condensation system was developed that was able to generate aerosol nanoparticles within the size range of $3 < d < 200$ nm. The chromatographic analysis showed that the aerosol particles were chemically identical to the maternal substance (i.e., there was no thermal decomposition during the substance evaporation). The X-ray diffraction analysis showed that the indomethacin nanoparticles were amorphous.

The lung deposited dose versus nanoparticle diameter was measured using the nose-only exposure chambers. Using the mean mouse respiration frequency as determined in the breathing modulated flow rate measurements and the literature value for the tidal volume, the total lung deposition efficiency was evaluated as a function of the mean particle diameter.

The inhalation experiments show that the aerosol administration is more effective (results in less edema index) than the peroral treatment even at the lung deposited dose six orders of magnitude less than the peroral dose. However, the lung histology analysis for the mice treated with the small particles ($d = 9$ nm) at dose as small as 10^{-5} mg per kg bw has revealed emphysematous signs like the dilatation of bronchioles and alveolar channels, alveolar wall thinning, and partial capillary bed reduction.

ACKNOWLEDGMENTS

Financial support for this work was provided by the Siberian Branch of Russian Academy of Sciences (Interdisciplinary Integration Project no. 78), the Russian Foundation for Basic Research (RFBR) (project no 07-03-00643a).

AUTHOR DISCLOSURE STATEMENT

All authors declare that no competing financial interests exist. AU1

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Received on October 15, 2007
in final form, February 20, 2008

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