A Critical Appraisal of Carbon Monoxide Uptake Measurements for the Follow-up of Experimental Respiratory Diseases in the Laboratory Mouse

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Summary
Adaptation of double-chamber plethysmography to the laboratory mouse was recently proven to yield stable and reliable pulmonary function values. This approach to investigation of the respiratory function in mice owes its success to its decisive advantages in terms of non-invasiveness, practical implementation and generation of quantitative flow/volume measurements and undisputed airway resistance calculation. When implemented to screen the resistance/susceptibility patterns to pathogens displayed by a panel of mouse inbred strains, the resistance value obtained was indeed able to detect tracheobronchic inflammation and to quantify its severity. However, extension of the pathological process to the most distal parts of the respiratory system did not result in further alteration of resistance, suggesting that its value reflects constraints acting on airflow in the airways rather than pathologic processes located in the more distal parts of the lungs. In this context, we hypothesized that a more exhaustive functional picture could be obtained, still non-invasively, by combining double-chamber plethysmography with carbon monoxide (CO) uptake measurements. The feasibility of CO-uptake measurements in mice was demonstrated and the conditions under which reproducibility can be maximized were defined. Differences linked to strain, somatic growth, and sex were examined and discussed, and reference values in growing male and female conscious and healthy BALB/cBy, SJL/J, C57BL/6, C3H/HeN, DBA/2 and 129/Sv mice were given. Finally, double-chamber plethysmography and CO-uptake values were proven to be exquisitely complementary in assessing and dissecting the functional impact of Sendai virus pneumonia in the laboratory mouse.

Introduction
Mice have become a permanent part of biomedical research laboratories (*Malakoff, 2000*). They owe this success to the low costs incurred, the existence of thousands of inbred strains and the fact that the mouse is the species with the greatest density of known genetic markers which facilitates the tracing of the alleles partly or wholly responsible for complex phenotypes (*Malo & Skamene, 1994*). In this respect, numerous recent examples in both pulmonary toxicology (*Kleeberger et al, 1997*) and physiology (*Tankersley et al, 1998*) or in the field of asthma (*De Sanctis et al, 1999*) and infectious diseases (*Fortier et al, 2005*) clearly demonstrate that in the future there will be a constantly increasing need for practical and fast respiratory function investigative techniques yielding accurate results independent of external factors. In this area, there is the classical physiological approach based on the analysis of transpulmonary pressure and nasal flow. This gold standard provides values relating to ventilatory mechanics (compliance and resistance) that are invaluable in terms of understanding and quantifying the phenomena involved. Unfortunately, the

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use of this approach implies anaesthesia, intubation, pleural catheterisation and artificial ventilation, all of which are procedures that generate significant artefacts (*Chong et al, 1998 ; Glaab et al, 2001*). Moreover, it is clearly not possible to consider screening a large number of animals in this way, and this approach provides single-point measurements which do not allow follow-up studies.

A second option is to use the low-frequency forced oscillation technique (LFOT), which has been recently validated in mice (*Gomes et al, 2000 ; Petak et al, 2001*). It provides direct, separate and high-grade assessment of airway and tissue mechanics but, unfortunately, also implies the use of an anaesthetised, intubated and artificially ventilated mouse preparation.

The third option is to use single-chamber barometric plethysmography, whereby the respiratory function is assessed on the basis of the characteristics of the pressure wave generated by respiration in a chamber in which the animal can move around (*Mortola & Frappell, 1998*). This approach to investigation of the respiratory function in mice owes its success to its decisive advantages in terms of practical implementation (quick and easy) and its specific features (no artefacts linked to anaesthetic and invasive manipulations). In addition, it enables prolonged and/or repeated measurements over time and provides a bronchoconstriction index (known as *enhanced pause* or *Penh*). However, this technique has a number of defects : the variability between respiratory cycles is high (*Hamelmann et al, 1997*), the current volumes measured cannot be quantitatively compared (*Enhorning et al, 1998*) and the interpretation of Penh as a measure of airway resistance to airflow is the subject of intensive debate (*Gelfand & Irvin, 1998; Mitzner & Tankersley, 1998*).

Recently, adaptation of double-chamber plethysmography (DCP) to the laboratory mouse was proven to yield stable and reliable pulmonary function values (*Flandre et al, 2003*). This technique combines the advantages of single-chamber plethysmography with true quantitative flow/volume measurements and a calculated resistance the interpretation of which in terms of bronchoconstriction is not disputed. We have successfully implemented DCP to screen the resistance/susceptibility patterns to Sendai virus and to respiratory syncytial virus displayed by a panel of mouse inbred strains (*Faisca et al, 2005 ; Bui Tran Anh et al, 2006*). The structure/function correlations that were established in both studies have shown that the calculated resistance was indeed able to detect tracheobronchic inflammation and to quantify its severity. Conversely, extension of the pathological process to the most distal parts of the respiratory system did not result in further alteration of resistance, which suggests that the calculated resistance mostly reflects the constraints acting on airflow in the large airways. In other words, the resistance value yielded by DCP can be quantitatively similar in mice with tracheobronchitis and in mice with both tracheobronchitis and diffuse pneumonitis. In this context, we hypothesized that a more exhaustive functional picture could be obtained non-invasively by combining DCP with carbon monoxide (CO) uptake measurements. The purpose of this work was to assess the feasibility of CO uptake measurements in mice, in particular by defining conditions under which reproducibility can be maximized. Differences linked to strain, somatic growth, and sex were then examined, yielding reference values in growing male and female conscious and healthy BALB/cBy, SJL/J, C57BL/6, C3H/HeN, DBA/2 and 129/Sv mice. Finally, DCP and CO-uptake measurements were proven to be exquisitely complementary in assessing the functional impact of Sendai virus pneumonia in the laboratory mouse.

Materials and Methods

Animals

Twenty specific pathogen free ~6-wk old male and female (1:1) mice from each of six different inbred strains (BALB/cBy, DBA/2, 129Sv, SJL, C3H/HeN and C57BL/6) were obtained from Charles River Laboratories. These strains had been chosen deliberately because they originated from different lineages, as deduced from genealogical and phylogenetic data (Atchley and Fitch, 1991). The animals

were kept under standard housing conditions (22°C, light/dark cycle 12/12 hours), fed a commercial diet, and given water *ad libitum*. In order to enable them to gradually become accustomed to the experimental environment, the mice were placed in the equipment described below for 15 minutes every day starting 3 days before the first measurement. After the final measurement had been taken when the mice were 13-wk old, BALB/cBy, DBA/2 and 129Sv mice were enrolled in the Sendai virus (SeV) study and the other mice were euthanized by an intraperitoneal overdose of sodium pentobarbital. All the serological analyses carried out at post mortem proved negative for the most common murine pathogenic agents. Housing, data collection and euthanasia procedures complied with the NIH guidelines and the experimental protocol had been approved by the Bioethics Committee of the University.

Design

The study consisted in three steps. First, within- and between-day reproducibilities of CO-uptake measurements were assessed in 10 healthy BALB/c female mice. Within-day reproducibilities consisted in either 7 consecutive measurements over a 1 hour interval (short-term) or 2 measurements twelve hours apart (long-term). Between-day reproducibility was assessed by comparing values measured daily for 8 consecutive days. Secondly, the effect of somatic growth, strain and sex was investigated by measuring CO-uptake in twenty healthy 6- to 13-wk old mice of each of the 6 aforementioned inbred strains for eight consecutive weeks. Finally, twenty mice from each of SeV-resistant (BALB/cBy), -susceptible (DBA/2) and -highly susceptible (129Sv) strains were inoculated with the virus (Faisca *et al*, 2005), and their survival rate, respiratory function and histology was followed for 15 days.

Carbon monoxide uptake

The single chamber Carbon Monoxide Uptake Monitor (Columbus Instruments, model #9811, Columbus, OH) and associated infra-red gas analyzer (CO sensor, model #04062-R2, Columbus Instruments, OH) and dedicated software (CO-uptake and breathing, v1.0, Columbus Instruments, Columbus, OH) non-invasively measures diffusive function of the lung epithelium, by measuring rate of carbon monoxide uptake and respiration rate (Fig. 1B). The principle of measurement is based on the contribution of *Depledge et al* (1981) where the CO level is measured in the animal chamber after exposing the animal for a short period of time (60 sec.) to a gas mixture (78% nitrogen and 20.7% oxygen) with a low level of carbon monoxide (0.28%).

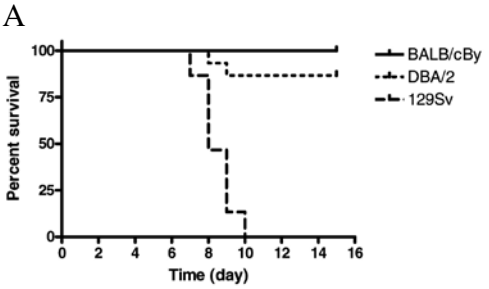

Figure 1. A. Survival of BALB/cBy, DBA/2 and 129Sv mice after Sendai virus challenge. The inoculation procedure consisted of slowly instilling 50 µl of a viral suspension containing ~103 PFUs into the nostrils of the anesthetized mouse.

Figure 1. B. Overview of the equipment, with 2 mice being measured.

Respiratory pattern/mechanics values and lung histology

Besides daily measurement of CO-uptake, respiratory pattern/mechanics values were measured with

the two-chambered, whole body plethysmograph devised by Buxco (model no. PLY-3351), using the practical procedures, quality controls, raw data processing, and respiratory flow curve analysis that were validated previously (*Flandre et al, 2003*). Seven days post-inoculation (pi), five mice per strain were overdosed with pentobarbital sodium and were exsanguinated by cutting the renal artery. Their lungs were inflated with with 4% formalin under a pressure of ~3 kPa for 24 h and then processed, along with pieces of nasal turbinates/trachea, as for routine histopathological examination.

Statistical analysis

Assessment of reproducibility provided three sets of data measured in BALB/c mice over a period of 8 days. One-way ANOVA corrected for repeated measurements was used to refute/establish reproducibility between trials. The 120 mice enrolled in the studies dedicated to the effect of somatic growth, strain and sex provided 8 sets of data measured at one-week intervals when the mice were between 6 and 13 weeks old. Three-way ANOVA corrected for repeated measurements was used to establish the statistical significance of differences in terms of age, sex and strain groups. If significant differences among trials/groups were obtained by using the ANOVA, Student's t tests on least square means were used to differentiate the differences between groups. Within each infected strain, consecutive means of functional values were analyzed by performing the general linear model procedure. Between-days comparisons were then made by using Dunnett's *post hoc* test. Comparisons that yielded P values of <0.05 were considered statistically significant. All statistical analyses were performed with SAS-STAT (SAS Inst. Corp., 1989).

Results

Healthy mice

By repeating all the stages of the investigative procedure (capture, insertion in the chamber, closing of the chamber, injection of the CO bolus and 1-min measurement), the mice were able to gradually grow accustomed to the conditions of the experiment. The number and intensity of the excitation phases fell sharply from the second session onward. At the same time, the regularity of the respiratory rate gradually improved. From the third session onward, all signs of discomfort and anxiety had disappeared. Repeating CO-uptake measurements at short-term intervals resulted in a steep decrease of the values obtained (Table 1).

Table 1. Effect of successive measurements on CO-uptake values in the healthy mouse (BALB/c females)

Time	CO^{up} (µl/min)	sCO^{up} (µl/min.g)
T0	32,5 ± 5,6 [a]	1,8 ± 0,3 [a]
T0 + 7 min.	14,2 ± 3,9 [bd]	0,8 ± 0,2 [bd]
T0 + 15 min.	10,9 ± 1,4 [bc]	0,6 ± 0,1 [bc]
T0 + 20 min.	8,8 ± 0,8 [c]	0,5 ± 0,1 [c]
T0 + 30 min.	9,6 ± 2,1 [c]	0,5 ± 0,1 [c]
T0 + 45 min.	14,7 ± 2,0 [d]	0,8 ± 0,1 [d]
T0 + 60 min.	15,6 ± 1,3 [d]	0,9 ±0,1 [d]

CO^{up} and sCO^{up}, CO-uptake and specific CO-uptake. Values are means ± SD. Means with different letters are significantly different ($P < 0,05$).

Table 2. Intra-day reproducibility of CO-uptake values in the healthy mouse (BALB/c females)

Time	CO^{up} (µl/min)	sCO^{up} (µl/min.g)
9:00, am	32,7 ± 5,4 [a]	1,8 ± 0,3 [a]
9:00, pm	30,4 ± 4,1 [a]	1,7 ± 0,2 [a]

CO^{up} and sCO^{up}, CO-uptake and specific CO-uptake. Values are means ± SD. Means were not significantly different ($P > 0.05$).

Conversely, successive measurements made at 12- (Table 2) or 24-hour (Table 3) intervals were not significantly different, thus excluding any circadian rythmicity and validating the procedure for experi-

Table 3. Between-day reproducibility of CO-uptake values in the healthy mouse (BALB/c females)

Day	CO^{up} (µl/min)	sCO^{up} (µl/min.g)
I	29,1 ± 8,2 [a]	1,3 ± 0,8 [a]
I + 1	29,6 ± 6,0 [a]	1,8 ± 0,4 [a]
I + 2	30,7 ± 4,7 [a]	1,8 ± 0,3 [a]
I + 3	28,1 ± 3,6 [a]	1,7 ± 0,2 [a]
I + 6	28,6 ± 6,1 [a]	1,7 ± 0,3 [a]
I + 8	28,1 ± 4,7 [a]	1,6 ±0,3 [a]
I + 9	29,3 ± 6,6 [a]	1,7 ±0,4 [a]
I + 10	31,8 ± 5,6 [a]	1,8 ±0,3 [a]

CO^{up} and sCO^{up}, CO-uptake and specific CO-uptake. Values are means ± SD. Means were not significantly different ($P > 0.05$).

mental designs in which daily measurements are needed. Generally speaking, the statistical analyses demonstrated that somatic growth, sex, and strain had a significant effect on the CO uptake of healthy mice. Males extracted more CO than females, but these latter had significantly better extraction capacities per unit of body weight (Table 6).

Table 4. Strain-specific CO-uptake values among six inbred laboratory mouse lines

Strain	CO^{up} (µl/min)	sCO^{up} (µl/min.g)
129Sv	38,2 ± 8,5 [d]	1,6 ± 0,3 [b]
BALB/cBy	35,8 ± 6,6 [c]	1,5 ± 0,3 [b]
C3H/HeN	31,6 ± 5,0 [ab]	1,4 ± 0,2 [a]
C57BL/6J	33,2 ± 6,6 [b]	1,6 ± 0,3 [bc]
DBA/2	35,9 ± 6,0 [c]	1,7 ± 0,3 [c]
SJL/J	30,2 ± 7,5 [a]	1,4 ± 0,3 [a]

CO^{up} and sCO^{up}, CO-uptake and specific CO-uptake. Values are strain-specific least square means ± SD. Means with different letters are significantly different ($P < 0,05$).

The age distribution profile of the CO-uptake absolute values obtained from growing mice displayed a quasi-unimodal shape centered on 9 weeks, whereas relative values (per g body weight) showed a quasi-linear decrease between 6 and 13-wk old mice (Table 5). Absolute CO-uptake values were very variable among strains, but three categories were clearly delineated when relative values were

Table 5. Effect of somatic growth on CO-uptake values in the laboratory mouse

Age	CO^{up} (µl/min)	sCO^{up} (µl/min.g)
6 weeks	32,5 ± 6,7 [a]	1,6 ± 0,3 [a]
7 weeks	34,6 ± 7,8 [ab]	1,6 ± 0,4 [a]
8 weeks	34,8 ± 8,1 [ab]	1,6 ± 0,3 [a]
9 weeks	35,8 ± 7,7 [b]	1,5 ± 0,3 [ab]
10 weeks	33,7 ± 7,5 [ab]	1,4 ± 0,3 [abc]
11 weeks	34,4 ± 7,0 [ab]	1,5 ± 0,3 [ab]
12 weeks	34,6 ± 6,8 [ab]	1,5 ± 0,3 [abc]
13 weeks	32,8 ± 6,4 [a]	1,4 ± 0,2 [c]

CO^{up} and sCO^{up}, CO-uptake and specific CO-uptake. Values are age-specific least square means ± SD. Means with different letters are significantly different ($P < 0,05$).

Table 6. Effect of gender on CO-uptake values in the laboratory mouse

Sex	CO^{up} (µl/min)	sCO^{up} (µl/min.g)
Female	31,4 ± 5,6 [a]	1,6 ± 0,3 [a]
Male	36,8 ± 7,7 [b]	1,4 ± 0,3 [b]

CO^{up} and sCO^{up}, CO-uptake and specific CO-uptake. Values are sex-specific least square means ± SD. Means with different letters are significantly different ($P < 0,05$).

calculated, with low (SJL, C3H/HeN), medium (BALB/c, 129Sv, C57BL/6) and high (DBA/2) CO extractors (Table 4).

Diseased mice

Percent survival (Fig. 1A), double chamber plethys-

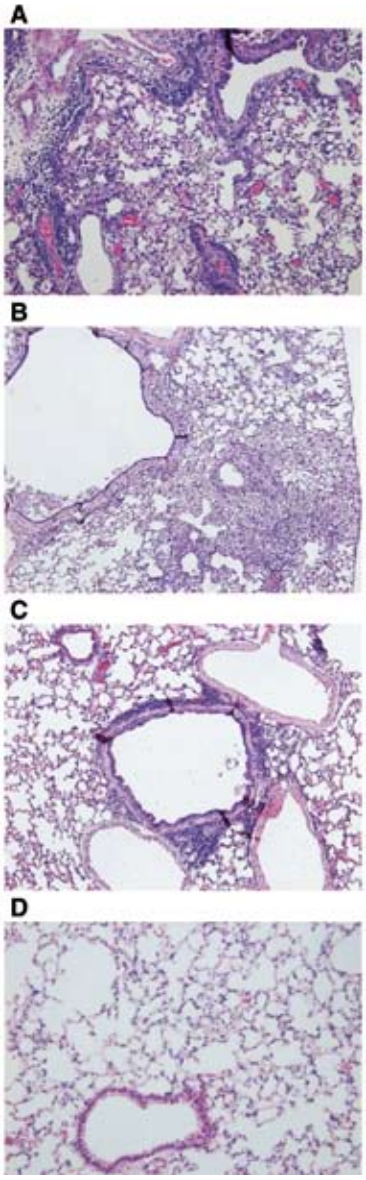

Figure 2. Effect of the mouse strain on the lung histology. H&E-stained sections of the lung from a BALB/cBy (A), a DBA/2 (B) and a 129Sv (C) mouse showing typical histological alterations observed on day 7 after infection (original magnification 40x). A noninfected control lung is shown (D). See text for detailed description.

mographic and CO-uptake values (means ± SD) during SeV-associated disease are presented (Table 7) along with day 7 pi typical lung photomicrographs of each strain (Fig. 2). None of the BALB/c, two of the DBA/2 (on days 8 and 9 pi) and all 129Sv (on days 7, 8 and 9 pi) inoculated mice died spontaneously during the experiment. As far as the BW (body weight) was concerned, two trends were distinguishable. The first (129Sv and DBA/2) was a gradual decrease in BW from day 2 pi (DBA/2) or day 4 pi (129Sv), which peaked in 129Sv on day 7 pi with a weight loss of 20%. The second (BALB/c) was stable BW with a slight decrease of around 5% at day 7. Regarding the respiratory pattern, each strain reacted in a specific manner to infection. In 129Sv, the MV (minute volume) remained stable throughout the experiment, but breathing was faster and shallower on and after day 3 pi. In DBA/2, MV evolution was bell-shaped : increase from day 4 to day 6, and return to normal on day 7 pi. The MV increase was achieved through faster and shallower breathing, and the decrease through lowering of RR (respiratory rate) without restoration of the initial TV (tidal volume). In BALB/c, the MV level remained stable throughout the experiment, via a stable RR/TV combination. As far as respiratory mechanics were concerned, and in particular Sraw (specific airway resistance), two categories became apparent. In 129Sv and DBA/2, Sraw increased on day 3 pi and then stabilised at approximately 175-200% of its initial value. In BALB/c, there was no detectable change in the Sraw. CO-uptake values also revealed two distinct groups : DBA/2 and BALB/c on the one hand, in which CO-extraction remained constant throughout the experiment, and 129Sv on the other, in which it significantly dropped on day 7 pi ($P<0.05$).

Nasal turbinates and tracheae displayed very subtle changes, consisting of degeneration of individual epithelial cells and infiltration of some neutrophils in the *lamina propria*. Three strain-specific histological profiles of the lungs were clearly identified on the basis of quantitative and qualitative criteria. There was little difference between the first histo-

Table 7. Effect of the mouse strain on respiratory functional values after Sendai virus infection.

Value	Strain	Time						
		Day 1		*Day 2*	*Day 3*	*Day 4*	*Day 5*	*Day 6*	*Day 7*
		bi	pi	pi	pi	pi	pi	pi	pi
RR	BALB/cBy	253±2 ^{a}	246±32 ^{a}	262±22 ^{a}	261±18 ^{b}	287±18 ^{c}	271±22 ^{c}	282±57 ^{c}	277±62 ^{c}
	DBA/2	224±18 ^{a}	219±13 ^{a}	221±15 ^{a}	368±61 ^{b}	410±44 ^{c}	394±31 ^{c}	375±27 ^{bc}	353±41 ^{b}
	129Sv	221±22 ^{a}	205±13 ^{a}	217±10 ^{a}	280±27 ^{b}	335±32 ^{c}	328±37 ^{c}	348±36 ^{c}	338±54 ^{c}
MV	BALB/cBy	50±7 ^{a}	46±8 ^{a}	45±5 ^{a}	47±4 ^{a}	52±9 ^{a}	51±8 ^{a}	51±8 ^{a}	51±10 ^{a}
	DBA/2	53±9 ^{a}	48±3 ^{a}	47±7 ^{a}	59±17 ^{a}	68±10 ^{b}	64±6 ^{b}	68±12 ^{b}	55±9 ^{a}
	129Sv	58±8 ^{a}	49±7 ^{a}	53±6 ^{a}	62±3 ^{a}	61±9 ^{a}	63±6 ^{a}	58±8 ^{a}	59±16 ^{a}
sRaw	BALB/cBy	1.18±0.4 ^{a}	1.13±0.4 ^{a}	0.97±0.3 ^{a}	1.22±0.4 ^{a}	1.30±0.4 ^{a}	1.40±0.3 ^{a}	1.12±0.3 ^{a}	1.19±0.3 ^{a}
	DBA/2	1.08±0.3 ^{a}	0.92±0.2 ^{a}	0.98±0.3 ^{a}	1.80±0.7 ^{b}	2.14±0.6 ^{bc}	2.25±0.6 ^{c}	1.68±0.4 ^{b}	1.63±0.4 ^{b}
	129Sv	1.09±0.6 ^{a}	1.00±0.3 ^{a}	1.18±0.3 ^{a}	1.38±0.3 ^{ab}	1.54±0.3 ^{b}	1.49±0.4 ^{b}	1.48±0.4 ^{b}	1.70±0.3 ^{b}
CO-uptake	BALB/cBy	29.9±7.1 ^{a}	31.3±4.2 ^{a}	34.3±4.9 ^{a}	31.1±5.5 ^{a}	30.9±6.7 ^{a}	29.8±2.6 ^{a}	31.5±3.3 ^{a}	31.3±5.5 ^{a}
	DBA/2	32.1±4.7 ^{a}	34.6±7.7 ^{a}	34.2±3.5 ^{a}	29.7±4.8 ^{a}	30.4±5.0 ^{a}	29.2±7.6 ^{a}	29.9±4.7 ^{a}	31.4±5.1 ^{a}
	129Sv	30.8±6.5 ^{a}	29.9±4.3 ^{a}	31.4±5.1 ^{a}	30.1±4.1 ^{a}	29.6±5.3 ^{a}	27.7±3.3 ^{a}	29.5±4.5 ^{a}	26.8±5.2 ^{b}

Values are means ± SD for female mice. Units are breath/min (RR), ml/min (MV), cmH_2O/s (sRaw) and µl/min (CO-uptake). RR, respiratory rate; MV, minute volume; sRaw, specific airway resistance; bi, before inoculation; pi, post-inoculation. Within each strain, means with different letters are significantly different ($P < 0.05$).

logical profile (BALB/cBy) and the normal morphology of murine lungs. The airways almost never contained exudate, their epithelium appeared generally intact, with the exception of a few isolated losses of ciliation and a few areas of epithelial hyperplasia, slight but definite. The *lamina propria* only contained a few non-coalescent foci of infiltration by mononucleated cells. All alveolar spaces were empty, with the exception of a few macrophages, the density of which was comparable to that observable in a healthy lung. In the interstitium, cell density appeared slightly elevated, multifocally. The histopathological diagnosis most compatible with these observations was *slight mononuclear broncho-bronchiolitis*.

The second histological lung profile was that displayed by DBA/2 mice. Airway lumina and alveolar spaces systematically contained a similar exudate. This was a mixture of cell debris, epithelial cells, pleiomorphic lymphoid cells, neutrophils and morphologically altered macrophages (cytoplasmic vacuolation, pycnosis, caryorhexis). The epithelial lining always exhibited large areas of deciliation alternating with degeneration, necrosis, desquamation and marked hyperplasia with uneven thicknesses and cell arrangements. The *lamina propria* was infiltrated by numerous monocytoid or lymphoid cells with round nuclei, which generated so-called "peribronchial/olar and perivascular cuffing" images. Most of the alveolar spaces were optically empty, but groups of alveoli were systematically observed which contained the exudate described above. In general, these alveolar clusters were adjacent to an airway and tended to merge, giving the impression of invading the more peripheral spaces gradually. Overall, the number of alveoli concerned filled more than one-third of any lung section area. The lung interstitium, when examined away from the areas of alveolitis, was diffusely infiltrated by cells with round nuclei of the monocytoid or lymphoid type. The density of the alveolar macrophages was elevated. The histopathological diagnoses most compatible with these observations were *severe necrotising and purulent broncho-bronchiolitis* and

multifocal granulocytic alveolar pneumonia.
The third histological lung profile concerned strain 129Sv. Its specificity mainly related to the extent (diffuse) and type (mononuclear) of distal lesions. The alveolar spaces never contained the exudate described above, and therefore no neutrophils, but contained an extremely high number of macrophages, of which a fraction exhibited regressive alterations (cytoplasmic vacuolation, pycnosis, caryorhexis). Certain areas were noteworthy for the presence of groups of alveoli completely filled with closely packed macrophages. Moreover, the interstitium was very severely infiltrated by cells with round nuclei in a diffuse manner. The histopathological diagnoses most compatible with these observations were *severe bronchial/olar epithelial hyperplasia* and *diffuse monocytoid interstitial pneumonia*.

Discussion

Judging from the calm and indifference of the animals on the one hand and the stability of the respiratory rate on the other, we concluded that the standardized protocol used for the acclimatization of the mice made it possible to keep the impact of the psychological constraints linked to the manual handling and the discovery of a new environment to a minimum. Obviously, successive measurements of CO-uptake over short periods of time yielded progressively lower values. This phenomenon is compatible with the slow washout kinetics of carboxyhaemoglobin which resulted in a progressive saturation of haemoglobin (*Bruce & Bruce, 2006*). When the interval between two consecutive measurements was increased to 12 hours, the CO-uptake values became reproducible, even if the second values often tended to be lower than the first. Daily measurements yielded highly reproducible values.

Effect of body weight

As body weight values are strongly dependent on sex, strain, and somatic growth in mice, wrong interpretations could be made if absolute CO-uptake values were considered to detect body weight-independent effects. However, as the relationship between the mouse body weight and CO-uptake was proven to be linear (*Depledge et al, 1981; Franko & Sharplin, 1993*), a body weight-independent CO-uptake value can be obtained in any mouse by simply dividing the absolute value measured by the corresponding body weight.

Effect of sex

As regards the CO-uptake values, to our knowledge, no significant differences between sexes have ever been reported. We found that female mice extract more CO than males per unit of BW. This difference could theoretically result from better alveolar ventilation and/or perfusion, more diffusion-prone alveolo-capillary membrane or higher blood haemoglobin content. Indeed, weight-specific total lung capacity is larger in the female mouse (*Reinhard et al, 2002*) and rat (*Pinkerton et al, 1982*) and the specific capillary volume as well (*Pinkerton et al, 1982*). Moreover, the female rat possesses larger specific alveolar (1.9 vs. 1.3 mm^2/g) and capillary (1,8 vs. 1.3 mm^2/g) surface areas than males and the alveolo-capillary interface is thicker in the latter (*Pinkerton et al, 1982*). Finally, in the vast majority of mouse strains that have contributed to the mouse phenome database (http://phenome.jax.org/), males were shown to have lower haematocrit and total haemoglobin values.

Effect of strain

Very few mouse CO-uptake values are available in the literature: 1.48 µl/min.g (*Depledge et al, 1982*) and 1.69 µl/min.g (*Franko & Sharplin, 1993*) in CBA and 1.54µl/min.g in C57BL/6 animals (*Franko & Sharplin, 1993*). The C57BL/6-specific least square mean obtained here amounts to 1.57 µl/min.g which is very close to the published value. The ranking of strains in decreasing order of CO-uptake capacity we have obtained (DBA/2 > BALB/c, 129Sv and C57BL/6 > SJL and C3H/HeN) obviously does not parallel that based on blood haemoglobin content (BALB/c > C57BL/6, 129Sv and C3H/HeN > DBA/2 and SJL) (http://phenome.jax.org/) which excludes a causal role. Comparative measurements

of the alveolo-capillary interface thickness between strains are not available, but between-strain specific total lung capacities were recently published : C3H/HeN > BALB/c > C57BL/6 > 129Sv (*Reinhard et al, 2002*). The strain C3H/HeN thus seems to combine the larger lungs and the worst CO-extraction ability which is, at first sight, contradictory. However, C3H-derived substrains were proven to have fewer alveoli and much larger alveolar spaces relative to the C57BL/6J animals (*Soutiere et al, 2004*), which could be interpreted as being consistent with the increased lung volumes previously described, the rationale being that larger alveoli are linked to increased lung distensibility, and, thus, indirectly to lung volumes (*Haber et al, 1983*). Although we can only speculate on the impact of this structural factor on C3H/HeN lung function, it is compatible with a reduced alveolar surface area in C3H/HeN compared to other strains, which would explain their lower CO-uptake capacity.

Effect of somatic growth

To our knowledge, the effect of somatic growth on CO-uptake values has not been reported before. We found that the young mouse extracts more CO than the older mouse, per unit of BW. Again, this result is compatible with a series of data previously reported. First, the mouse blood haemoglobin content decreases with age (http://phenome.jax.org/). Second, studies conducted in rats have shown that total lung capacity relative to body weight is always greater in the younger than in the older animals, thus indicating disproportionately larger lung volumes in younger animals (*Johanson & Pierce, 1973 ; Mauderly, 1982; Sahebjami, 1991*). Finally, somatic growth is accompanied by a progressive thickening of the alveolo-capillary interface in BALB/c animals (*Kawakami et al, 1984*).

Benefits for disease assessment

Specific access to respiratory system function was achieved by monitoring plethysmographic and CO-uptake values. The breathing pattern (RR, TV, MV), respiratory mechanics (sRaw) and CO-uptake values of BALB/c mice in response to inoculation of SeV remained unchanged throughout the experiment, which shows that the respiratory disease remained totally asymptomatic. DBA/2 and 129Sv differed from BALB/c in that they developed compensatory hyperpnoea via faster breathing and hyperpnoea. Specific airway resistance (sRaw) increased in a parallel fashion in both strains, which points to a diminished cross-section area of the airways (*Pennock et al, 1979 ; Flandre et al, 2003*).

To the best of our knowledge, the partitioning of sRaw between the different segments of the mouse respiratory tree is not available yet, which renders reliable interpretation in terms of location of the source of the problem very speculative. However, it was recently shown that nasal obstruction alone can substantially increase sRaw in mice (*Martin et al, 1988 ; Mitzner et al, 2001*). Because (*i*) turbinate histology did not differ between BALB/c, DBA/2 and 129Sv, (*ii*) tracheal and bronchial histological alterations were clearly discriminant between BALB/c on the one hand and the two other strains on the other and (*iii*) the mouse breathing pattern response typical of nasal irritation (braking of the beginning of expiration) or narrowing was never observed (*Gomes et al, 2000, Seifert & Mortola, 2002*), it is tempting to conclude that the increased sRaw in DBA/2 and 129Sv, results from tracheobronchial alterations rather than from superior nasal congestion and/or thickening of the mucosa. Despite roughly similar alterations in respiratory ventilation and mechanics values, DBA/2 and 129Sv animals dramatically differed with respect to CO-uptake, with a significant drop in the latter, which suggests that additional dysfunctions occurred among 129Sv mice, probably a combination of ventilation/perfusion mismatch, intrapulmonary blood shunting and/or impairment of gas diffusion. This specific impairment of CO extraction was paralleled by the development of a diffuse interstitial-type of pneumonia in 129Sv, in which the alveolo-capillary interface was dramatically thickened throughout the lungs, thus impairing diffusion of gases. Moreover, the drop in CO-uptake occurred 1-2 days before

mass mortality was recorded among 129/Sv mice. Conversely, among DBA/2 mice, SeV inoculation caused an alveolar-type of pneumonia, multifocal rather than diffuse, in which the interstitium was not so prominently infiltrated and which caused only marginal mortality.

Taken together, the data therefore suggest that combining double-chamber plethysmography with CO-uptake measurements provide a more exhaustive way to investigate the physiopathology and the evolution of respiratory diseases in the laboratory mouse. In particular, severe plethysmographic profiles like those shared by DBA/2 and 129Sv could be subdivided further into possibly-recovering (DBA/2) and life-threatening (129Sv) categories. Moreover, the data clearly suggest that an experimental pneumonia in the laboratory mouse will be fatal in the next 24-48 hours whenever a drop in CO extraction occurs. This criterion could therefore be used to objectively define the point at which euthanasia is appropriate.

In conclusion, the results presented here lead us to suggest that, when applied to the laboratory mouse, CO-uptake measurement yields a stable and reliable pulmonary function value which gives an early and specific indication of the development of gas exchange- and life-threatening lung damage. This technology is totally non-invasive and the procedure is quick and easy, which makes it possible to examine a large number of animals at the same time or the same animals at several consecutive times, provided a 12-hour interval between successive measurements is allowed. The study also suggests that, whenever this technology is implemented, it is important to use strain-, sex- and age-matched animals. CO-uptake data also provide an objective criterion to define the point at which an experimental disease fatality becomes predictable and euthanasia should therefore be decided.

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