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Plethysmography for the assessment of pneumococcal pneumonia and passive immunotherapy in a mouse model

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ABSTRACT: The increasing prevalence of resistance to antibiotics of *Streptococcus pneumoniae*, the main causative agent of community-acquired bacterial pneumonia, necessitates the development of both new therapeutic strategies and noninvasive methods in order to evaluate their efficacy.

The efficacy of passive immunotherapy with human intravenous immunoglobulin (IVIG) or solvent alone, administered intranasally or intravenously, was evaluated in a mouse model of acute pneumonia. Lung bacterial load was also evaluated, using a classical but invasive method, as was respiratory function (minute ventilation, respiratory frequency and tidal volume) using plethysmography, a simple noninvasive method commonly used in inhalation toxicology, but not previously used to assess respiratory infection.

Forty-eight hours after infectious challenge, the lung bacterial load was significantly lower in IVIG-treated mice than in untreated mice. At the same time, minute ventilation was significantly lower than reference values for untreated mice (36±3 *versus* 57±8 mL·min^{-1}, $p<0.01$, and 31±2 *versus* 50±5 mL·min^{-1}, $p<0.01$ for intranasal and intravenous administration of solvent, respectively) but not in mice treated with IVIG by either route of administration.

Plethysmography therefore appears to be a simple and reliable test for the follow-up of acute respiratory infection.

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Streptococcus pneumoniae remains the leading cause of bacterial pneumonia [1]. The severity of the disease depends on the virulence phenotype of the strain and the immune status of the host. Immunocompromised hosts, such as patients with human immunodeficiency virus, ventilated patients, premature newborns, and the elderly, are particularly susceptible to invasive pneumococcal pneumonia [2–4]. The increasing prevalence of strains resistant to one or several antibiotics necessitates the development of new therapeutic strategies against *S. pneumoniae* [5, 6]. Immunotherapy is a possible alternative or adjunctive strategy for treating *S. pneumoniae* pneumonia [7, 8]. In a mouse model of invasive pneumonia, evaluation of the therapeutic efficacy of human plasma-derived intravenous immunoglobulin (IVIG) administered either intravenously or intranasally was attempted. The therapeutic efficacy of IVIG was evaluated by survival experiments and by following the kinetics of the bacterial load in the lungs from 4 h to 72 h after infectious challenge. It was hypothesized that in mice suffering from severe pneumonia leading to death within 8–10 days, it would be possible to detect major impairment of respiratory function by measurement of respiratory variables, such as minute ventilation, respiratory frequency and tidal volume. Unlike bacterial counts in tissue samples from killed mice, this method of investigation is noninvasive and can be repeated several times in the same animal. In this study, an integrated system for monitoring breathing pattern based on plethysmography was used to evaluate daily for 5 days, the respiratory function of conscious mice infected with *S. pneumoniae* and treated or not treated with IVIG intravenously or intranasally. The values recorded were compared to bacterial counts and the results of histological examination.

Materials and methods

Mice

Six-week-old female BALB/c mice, weighing 18–20g at the time of infectious challenge, were obtained from Charles River (Saint Aubin-lès-Elbeuf, France). The protocols for the animal experiments were approved by the Committee on Animal Care and Use of the institution.

Integrated system of breathing pattern monitoring

Mice were placed in a modified system of Batelle tubes such that it was possible to monitor the flow-breathing pattern of eight restrained animals.

A calibrated pneumotachograph (Fleisch no. 0000, Richmond, VA, USA) and a differential pressure transducer (DP-45, Validyne, Northridge, CA, USA) were attached to the top part of each plethysmograph. The signal from the differential pressure transducer for each animal was amplified by a Gould Carrier Amplifier (Model 13-4615-35, Gould Electronique, Ballainvilliers, France). It was digitized using an analogue to digital converter set, on a Dell Optiplex computer (Dell Computer Corporation, CA, USA) equipped with an input/output AS2 card at a sampling rate of 250 Hz. HEM 2.1 software (Notocord, Croissy sur Seine, France) was used to display respiratory flow, and to calculate respiratory minute ventilation, tidal volume, and respiratory frequency. Data files were imported into a worksheet (Excel 5.0, Microsoft corporation, CA, USA) and using a macro, the mean±SD of all the variables, for the time specified, were processed automatically. It was checked that this program worked properly by extracting data by hand from the tracings and comparing it with the computer output for each type of pattern. Less than 5% difference was noted.

Bacteria

S. pneumoniae strain 4241 serotype 3 was provided by J.J. Pocidalo (Hopital Bichat, Paris, France). It was stored at -80°C in brain-heart infusion (BHI) broth (Difco Laboratories, Detroit, Michigan) with 30% glycerol. Bacteria were grown in BHI overnight at 37°C. A subculture in the exponential growth phase was obtained by incubating 100 μL of the overnight culture in 10 mL of BHI for 6 h at 37°C. This bacterial inoculum was diluted as required in phosphate buffered saline (PBS) pH 7.4 based on optical density at 650 nm. Colony forming-units (cfu) were counted by plating 100 μL aliquots of 1 in 10 dilutions on trypticase soy agar supplemented with 5% horse blood (Biomérieux, Marcy l'Etoile, France).

Infectious challenge and treatment

Mice were anaesthetized by intraperitoneal injection of 50 $mg \cdot kg^{-1}$ of sodium pentobarbital and infected intranasally with 50 μL of bacterial suspension containing 5 $\log_{10}$ cfu. Four groups of eight mice were infected; one group was treated intranasally with IVIG 24 h before infection, one group was treated intravenously with IVIG 3 h after infection and two groups were treated with PBS, one intranasally and the other intravenously. Reference measurements for respiratory variables were made the day before infection. Respiratory function was evaluated daily from the first to the fifth day after infection. All measurements were made over 20 min, after a 10 min adaptation period. Five days after infectious challenge, the surviving mice were sacrificed for histological studies. Lung bacterial load was determined in four other groups of mice, 1 h after infectious challenge, to evaluate initial burden and 48 and 72 h after infectious challenge.

Passive immunotherapy with intravenous immunoglobulin

Human IVIG (Tégéline®, lot 50060432) was purchased from the Laboratoire Français du Fractionnement et des Biotechnologies (Les Ulis, France). IVIG composed of 97% Immunoglobulin (Ig)-G, comprising 58.8% IgG_1, 34.1% IgG_2, 5.4% IgG_3, 1.7% IgG_4, was reconstituted in sterile water according to the instructions of the manufacturer, and stored at -80°C. IVIG at a concentration of 50 $mg \cdot mL^{-1}$ was diluted with PBS immediately before administration to mice.

Histological studies

Mice were sacrificed by intraperitoneal injection of 300 $mg \cdot kg^{-1}$ of sodium pentobarbital (Sanofi Santé Animale, Libourne, France) and a catheter connected to a container of fixative was inserted into the trachea *via* a ventral incision in the neck. At the same time the mice were exsanguinated by severing the abdominal aorta. The thorax was opened, and the lungs were immediately inflated *in situ via* the tracheal cannula with 4% formaldehyde in cacodylate buffer (pH 7.2) at a pressure of 25 cmH_2O. The lungs were then removed and immersed in formaldehyde (4%) for 8 days. A midsagittal slice was taken from each lung and three samples of the lower third of the trachea were collected. These samples were further processed for embedding in paraffin. Sections (5 μm) were cut and stained with haematoxylin and eosin. Tissue sections were examined with a light-microscope and photographed with a photomicroscope system.

Determination of bacterial load

Lungs were dissected aseptically (the trachea and the main bronchi were discarded) and homogenized in 1 mL of PBS using teflon pestles in glass tubes. The bacterial load of lung homogenates was determined from cfu counts.

Calculations and statistical analysis

Data are expressed as mean±SEM. Results were analysed by one-way analysis of variance (ANOVA) and Fisher's test, and p-values <0.05 were considered significant.

Results

Minute ventilation data obtained 24 h before infectious challenge did not differ significantly for the four groups of mice (64±6 and 58±5 $mL \cdot min^{-1}$ for mice treated with IVIG intranasally and intravenously respectively, 57±8 and 50±5 $mL \cdot min^{-1}$ for the untreated mice (PBS intranasally and intravenously, respectively)). The lung bacterial load, measured 1 h after infectious challenge, did not differ significantly between the four groups of mice. Twenty-four hours after

infectious challenge, no significant decrease in minute ventilation was observed in any group. Forty-eight hours after infectious challenge, the lung bacterial load in IVIG-treated mice was significantly lower than that in untreated mice (2.26±0.26 *versus* 5.72±0.11 $\log_{10}$ cfu, $p<0.01$ and 3.19±0.19 *versus* 4.8±0.28 $\log_{10}$ cfu, $p<0.01$ after intranasal and intravenous administration respectively; fig. 1). After 48 h, minute ventilation was significantly lower than reference values in untreated mice (36±3 *versus* 57±8 mL·min^{-1}, $p<0.01$ and 31±2 *versus* 50±5 mL·min^{-1}, $p<0.01$, in PBS intranasally and intravenously administered, respectively) but not in treated mice, for both routes of IVIG administration (figure 1). The decrease in minute ventilation observed in untreated mice was mainly due to a decrease in tidal volume, with no significant change in respiratory frequency (figs. 2 and 3). Untreated mice began to die 4 days after infectious challenge and on the following day, only 3 mice were alive (38%) in each of the two groups of untreated mice, whereas all 8 survived (100%) in the two groups of IVIG-treated mice. Five days after infectious challenge, minute ventilation was 10±2 and 6±2 mL·min^{-1} in the surviving mice of the two untreated groups (PBS intranasally and intravenously, respectively). At the same time, minute ventilation was significantly lower than reference measures in mice treated intranasally with IVIG (42±2 *versus* 64±6 mL·min^{-1}, $p<0.05$) whereas no significant decrease was observed in mice treated intravenously with IVIG (52±3 *versus* 58±5 mL·min^{-1}, nonsignificant). Morphological examination of the lungs of surviving untreated mice showed an intense inflammatory response with inflammatory cell recruitment, interstitial oedema and modification of alveolar structures (fig. 4). Moderate interstitial oedema was observed in mice treated intranasally with IVIG.

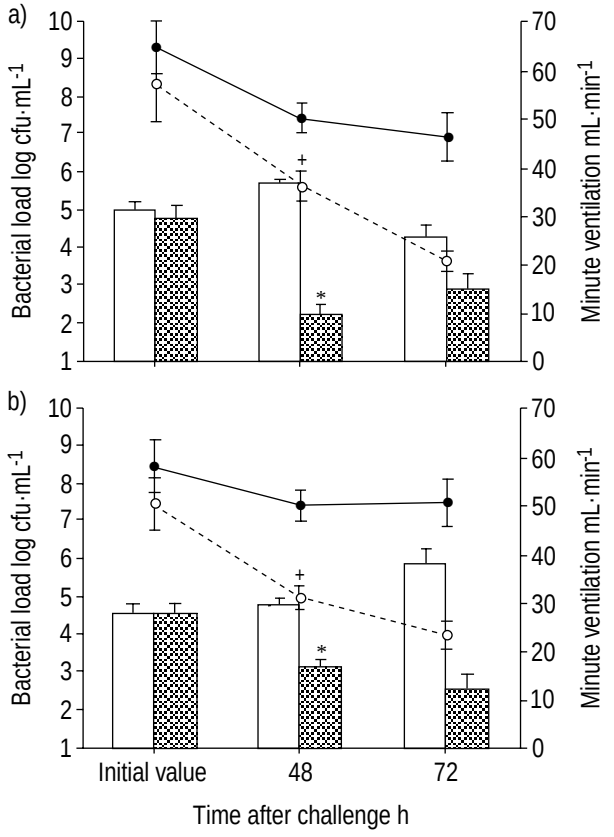

Fig. 1. – Lung bacterial load 1, 48 and 72 h after infectious challenge (histograms, each bar represents the mean of four measurements) and minute ventilation 24 h before and 48 and 72 h after infectious challenge (curves, each point is the mean of eight measurements) after a) intranasal or b) intravenous administration of intravenous immunoglobulin (IVIG) (●, ▩) or phosphate buffered saline (PBS) (○, □). Forty-eight hours after infectious challenge, a significant difference in lung bacterial load (*: $p<0.01$) was observed between IVIG-treated mice and untreated (PBS) mice for both routes of administration. At the same time, a significant decrease in minute ventilation relative to initial values was observed in untreated mice ($^{+}$: $p<0.01$), whereas no significant difference was observed in IVIG-treated mice.

Discussion

This study shows that plethysmography is a simple and sensitive means of monitoring *S. pneumoniae* pneumonia in mice, and of evaluating the efficacy of passive immunotherapy on the pneumonic process.

Methods classically used to evaluate the efficacy of new treatments, required in the face of the increasing resistance of pneumonia infections to antibiotics [9], are

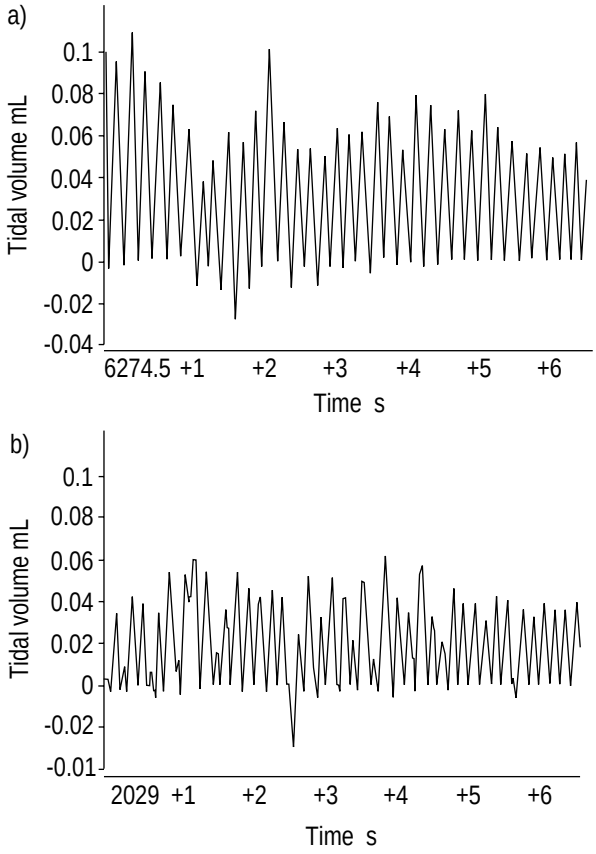

Fig. 2. – Examples of ventilation traces for untreated mice a) 24 h before and b) 48 h after infectious challenge. The decrease in minute ventilation appears to be due to a decrease in tidal volume.

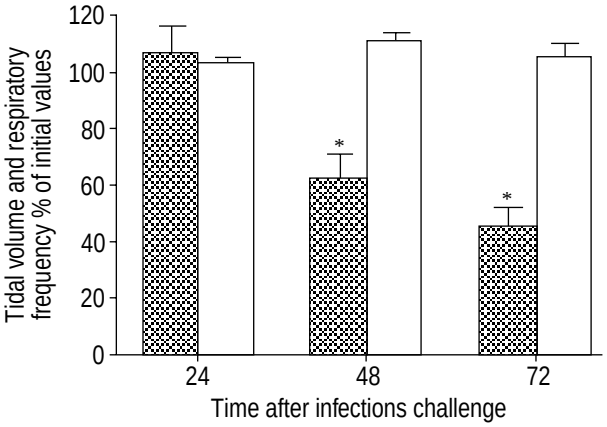

Fig. 3. – Tidal volume (▩) and respiratory frequency (□) in untreated mice 24, 48 and 72 h after infectious challenge, expressed as a percentage of initial values measured 24 h before infectious challenge. Each bar represents the mean±SEM of 8 measurements. *: $p<0.05$ *versus* initial values.

based on assessments of survival rate, body weight and clinical observation of respiratory dysfunction, such as dyspnoea, in animal models [10, 11]. The noninvasive diagnostic methods available are more limited for animal models than in humans, in whom radiograph evaluation or blood gas measurement may be used [12]. The more sensitive and specific methods for animal models, such as bacterial counts from infected tissues, are very invasive and require the sacrifice of the animal [10]. Thus many animals are required to evaluate the progression of an infection over several days so as to provide statistically significant data. To reduce the number of animals required, alternative methods must be found for the evaluation of treatment efficacy.

Monitoring the effects of pneumonia on respiratory function is a potential means of evaluating treatments. Thus oxygen uptake, as evaluated by $\dot{V}O_{2,max}$, has been used as a quantitative index of cardiopulmonary function impairment [13]. This method was proposed following infection in a model of *S. pneumoniae* pneumonia in mice and was found to be noninvasive, reproducible and very sensitive [13]. $\dot{V}O_{2,max}$ was found to have fallen significantly by day 2 of infection, about the same time as the animals began to die. $\dot{V}O_{2,max}$ measurement is based on gas-exchange and is an overall evaluation of cardiopulmonary function. The decrease in $\dot{V}O_{2,max}$ correlates with the oxygen consumption of excised tissues and with the compliance measured on isolated lungs. This demonstrates that there are possible indirect methods for the follow-up of infection, other than monitoring oxygen consumption, which represent an overall evaluation of cardiopulmonary function, based on mechanical variables of lung function.

The ability of freshly excised lungs to expand has also been evaluated in rats infected with *Mycoplasma pulmonis* [14]. Infected lungs were more difficult to inflate with air, mainly due to an increase in surface forces indicating disruption of lung surfactant. Impairment of ventilation has also been suggested to be the cause of hypoxaemia in viral pneumonia [15]. In view of these previous studies, it seemed likely that in our model of pneumonia, which is fatal within 8–10 days, there would almost certainly be substantial and detectable lung dysfunction. As a noninvasive test was required, the measurement of minute ventilation, tidal volume and respiratory frequency by plethysmography appeared to be a potential method for assessing the

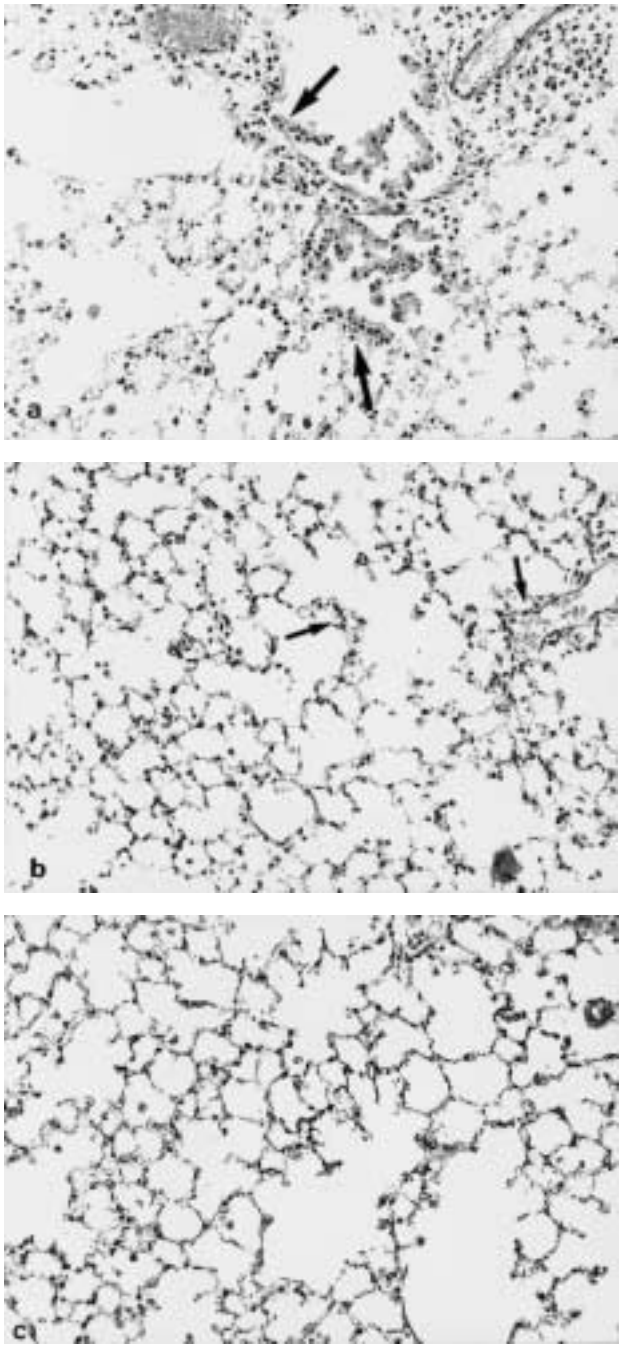

Fig. 4. – Light micrographs of lung sections stained with haematoxylin and eosin from; a) untreated mice; b) mice treated intranasally with intravenous immunoglobulin (IVIG) and c) mice treated intravenously with IVIG, 5 days after infectious challenge. Inflammatory cell recruitment, substantial interstitial oedema and changes in alveolar structures were observed in untreated mice (large arrow) and mild interstitial oedema was observed in mice treated intranasally with IVIG (small arrow); no lesion was observed in mice treated intravenously with IVIG. (original magnification ×40).

effects of pneumonia on respiratory function alone. This noninvasive method is reproducible and can be used repeatedly for the follow-up of pneumonia in an individual animal. It is a very simple method and suitable for use in conscious animals, avoiding the respiratory effects of anaesthesia [16]. To the author's knowledge, this method, commonly used in inhalation toxicology, has not been used before to assess pulmonary function in an animal model of infection. The present study has demonstrated that in infected but untreated mice, minute ventilation decreased early in the course of infection. A decrease of $>35\%$ was detected on day 2 whereas in treated mice, no significant decrease in minute ventilation was observed at this stage, for either route of administration of the antibodies. In the present model, a significant decrease in lung bacterial load was observed in treated mice, 2 days after infectious challenge. Thus, the measurement of minute ventilation by plethysmography appears to be as sensitive as determination of lung bacterial load, one of the most sensitive methods known for testing therapeutic efficacy. The decrease in minute ventilation observed on day 2 in untreated mice was due to a decrease in tidal volume, with no significant change in respiratory frequency. Thus respiratory frequency which is very easy to measure in mice, cannot be recommended for monitoring the infection. The decrease in tidal volume, due to a restrictive syndrome, is consistent with the decrease in compliance observed in other models of respiratory infection [14]. A progressive decrease in minute ventilation was observed in untreated mice as the infection progressed, but the animals did not begin to die until day 4. This demonstrates that survival is not a reliable method for monitoring infection.

Histological examination, a gold-standard for the diagnosis of pneumonia [12], showed typical lesions in untreated mice, similar to those found in human disease [13]. In mice treated intravenously with IVIG, no significant decrease in minute ventilation was observed until the fifth day after infectious challenge (*i.e.,* the day on which they were sacrificed). On day 5, a significant but not fatal decrease in minute ventilation was detected in mice treated intranasally with IVIG (fig. 1). This suggests that intranasal administration of IVIG is less effective than intravenous administration. This may result from IVIG having a nonuniform distribution in the lungs if administered intranasally and its shorter persistance in the lung than in blood (46–50 h in lungs and >7 days in blood) (unpublished data). The mild pulmonary dysfunction is consistent with the slight interstitial oedema observed in some parts of the lungs of mice of this group. However, whereas there is a substantial decrease in minute ventilation in severe infections, a more sensitive method may be required for subclinical infections (*i.e.*, those compatible with survival). Similarly, this method was used in a model of the development of pneumonia, and it cannot be assumed that the findings would be the same when studying the effect of a treatment in established pneumonia.

In conclusion, several noninvasive diagnostic methods are available for human pneumonia, that are at least as sensitive as assessment of mechanical characteristics of lung function [17–19]. However, no such methods are available in animal models of pneumonia, and plethysmography could be used routinely for screening the efficacy of potential new treatments on the pneumonic process. This efficacy could then be confirmed using more specific but highly invasive procedures such as lung bacterial load.

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