Use of a Photodensitometric Technique to Quantify Microscopic Lung Lesions in Mice: Antiviral Activity Against Swine Influenza Virus

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During studies of swine influenza virus (A/NJ/76) infection, a technique was devised to quantify the pulmonary lesions in infected mice treated at different time intervals with antiviral chemotherapeutic agents. The technique is based on the premise that as the severity of microscopic change increases, the optical density of lung sections also increases because of edema and increased cell numbers in infected lungs. Seven days after intranasal instillation of the virus, mice were killed and the lungs were perfused with 2% glutaraldehyde at constant pressure. Lungs were processed in a routine manner, sectioned at standard levels, and stained with hematoxylin and eosin. By using standard photomicrography equipment, multiple optical density measurements were made of lung sections in a carefully controlled systematic manner, and a mean optical density was determined for each lung. The optical density of lungs of mice treated before and after infection with amantadine, rimantadine, or ribavirin was significantly reduced compared with that of the lungs of infected, untreated controls. If treatment was delayed until 15 h after infection, amantadine and ribavirin were effective in reducing pulmonary optical density, but rimantadine was without effect. These findings correlated well with mean lung weight of each group; however, the sensitivity of the optical density technique was greater. Subjective scoring of microscopic lesions revealed differences only between infected and uninfected controls. The densitometric method offers promise as a reliable means of objectively quantifying the pulmonary response to a variety of infectious, toxic, and therapeutic agents.

Animal models for evaluation of potential antiviral compounds have frequently been developed by using human disease pathogens adapted to produce high mortality. The efficacy of the compounds is thus measured by the survival rate of treated, infected animals compared with that of untreated controls. These model systems are unnatural and unrealistic when the purpose intended for the drug is to treat diseases characterized by high morbidity and low mortality (8). During studies of swine influenza (influenza A/NJ/76) (G. H. Scott, manuscript in preparation), it was observed that the mouse-adapted virus had low mortality for adult mice and thus resembled natural influenza infections of humans (6). It was necessary to seek another end point to measure the effectiveness of antiviral compounds on the respiratory disease in mice as well as to evaluate the microscopic changes occurring in the lungs of infected mice.

Numerous systems, many not completely objective, have been proposed to grade morpholog-

ical changes occurring in the lungs of animals infected with various respiratory disease-producing agents. The majority of the reliable procedures are morphometric wherein changes in the various components of the lung, such as thickness of the alveolar septum, changes in capillary and alveolar surface area, and percent occlusion of alveolar spaces by exudate, for example, can be quantified consistently (3, 10). However, these procedures are time consuming and, thus, inefficient for examining numerous specimens.

More commonly, various subjective criteria are established to assess the severity of disease. These usually are edema, inflammatory cell infiltration, and degenerative or proliferative changes in pulmonary tissues. Based upon these criteria, lesions are scored on a numerical scale (usually 0 to 4). These methods are not only biased and imprecise, but are usually not consistent among microscopists. Other methods used include comparison of lung weight and lung specific gravity (2). However, these meth-

ods do not allow for quantitative estimation of microscopic changes.

A technique, described for studying emphysema in pigs and rats, was based on the postulate that emphysematous lung sections are less optically dense than sections of normal lung and that differences could be quantified by a densitometer (4). A similar procedure was used to determine the degree of erythrocyte aggregation *in vitro* (1). It seems likely that pulmonary disease characterized by edema and cellular infiltration into alveolar spaces and the interstitium should increase optical density. If this premise is correct, the effect of drugs, toxins, or chemicals on the pulmonary response can be quantitatively assessed and compared in an unbiased, completely objective manner. This report describes how the technique was utilized to compare the efficacy of antiviral chemotherapeutic agents in mice infected with swine influenza virus.

MATERIALS AND METHODS

Virus. Influenza virus, A/NJ/8/76 ($H_{sw}1N1$), was passed six times in embryonated eggs; adaptation to mice was accomplished in nine serial passages by intranasal instillation of supernatant from a suspension of homogenized lungs from mice infected 3 to 4 days previously. After passage 9, allantoic cavities of 10-day-old embryonated eggs were inoculated with supernatant from infected lungs. After incubation for 48 h at 35°C, infected allantoic fluid was harvested and clarified by centrifugation at $1,200 \times g$ at 4°C for 15 min. Antibiotics were added to the clarified fluid to achieve a final concentration of 250 U of penicillin per ml and 100 μg of streptomycin per ml; samples of the suspension were stored frozen at -60°C. The titer of the infected allantoic fluid was $10^{7.7}$ median egg infectious doses per ml.

Experimental design. Eight-week-old (25 g) outbred, female Swiss mice [Cr1:COBS CD-1 (ICR) BR] obtained from the Sendai-free colony of Charles River Laboratories were divided into eight groups, each containing 10 mice. Three groups were treated prophylactically with amantadine, rimantadine, or ribavirin in drinking water at a concentration of 0.25 mg/ml (approximately 60 mg/kg per mouse per day) 24 h before intranasal instillation of allantoic fluid containing $10^{6.1}$ median egg infectious doses of influenza virus in 0.05 ml. Three other groups were treated therapeutically with each of the drugs beginning 15 h after virus inoculation. In all groups, treatment was then continued for 7 days after inoculation of the virus. Two control groups of mice were utilized: one was infected but not treated and the other was neither infected nor treated.

Necropsy procedures. Seven days after infection, all mice were killed by cervical dislocation. In half the mice from each group, lungs were dissected from surrounding tissue and weighed to the nearest milligram. In the remaining mice, the cutaneous tissue of the neck was dissected free from the tra-

chea and the thorax was opened. A 21-gauge hypodermic needle attached to a flask containing 2% glutaraldehyde, pH 7.2, was inserted into the trachea, and the lungs were perfused *in situ* for 30 s. The pressure of the perfusate was 20 cm of water. To maintain pulmonary distension, a small vascular clamp was placed on the trachea. Thoracic viscera were then dissected free and suspended in 2% glutaraldehyde for 24 to 72 h. Perfused lungs with the heart and thymus attached were subsequently placed in 10% buffered neutral formalin just before tissue processing and embedded whole in paraffin. Paraffin blocks were sectioned horizontally through the lung lobes, taking care not to vary section thickness, and three 6- μm sections were taken at 1.5-mm intervals (Fig. 1). The sections were deparaffinized and stained with hematoxylin and eosin (7). Staining was controlled by carefully maintaining pH and concentration of the solutions and by processing slides simultaneously or in rapid succession through the same solutions.

Densitometry. Slides with sections of lung were placed on the mechanical stage of a Zeiss Ultraphot II (Carl Zeiss, Oberkochen/Württemberg, Germany), and voltage was adjusted to approximately 15; a filter with absorption maxima at 525, 580, and 645 nm was placed between the light source and specimen, and the image was projected onto a viewing screen through the $\times 2.5$ objective. Total magnification was $\times 27$. Twenty to 35 measurements were made on each section directly from the same location on the viewing screen, using a photodensitometer (model 501 M, Photovolt Corp., New York, N.Y.). Optical densities were measured through 0.5-mm-diameter holes spaced 0.85 mm apart in a rectangle of brass foil, which was placed on the microscope slide (Fig. 2). The photovolt meter was adjusted to zero optical density at a place on the slide where there was no tissue. The embedded and sectioned

FIG. 1. Schematic lateral view of mouse lung showing the level of sections on which optical density measurements were made.

FIG. 2. Perforated brass cover on microscope slide through which light was transmitted for optical density measurements. Individual densitometry readings were made through each perforation.

material included such tissues as heart, thymus, large airways, and pleura in each section. If any of these tissues or artifacts such as tissue folds or tears were observed at a perforation on the microscope viewing screen, the field was eliminated from the sample. In this manner, only alveoli and small bronchioles were measured. The above procedure was repeated with each of the three lung sections (20 to 35 readings per section), and the mean optical density of each lung was calculated. To assess reliability of the technique, the perforated brass cover was placed on one randomly chosen lung section 10 separate times and 10 optical densities were obtained. A grand mean, standard deviation, and coefficient of variation were calculated.

Histopathological evaluation. Lungs were scored blind by two different pathologists to determine degree of severity of lesions. The criteria used were (i) pulmonary edema, (ii) inflammatory cell infiltration, and (iii) alveolar septal thickening. Each investigator individually scored the pulmonary lesions on a scale of 0 to 4.

Statistics. Data on lung weight and optical density were analyzed by one-way analysis of variance, using the least significant difference test to determine differences between groups. A linear-regression analysis was used to determine correlation of mean optical density and mean lung weight of each group (9). The individual histopathological scores of degree of lung severity were each analyzed by the Kruskal-Wallis test for nonparametric data (5).

RESULTS

Lesions of swine influenza in untreated, infected controls consisted of multifocal areas of bronchiolitis surrounded by thickening of alveoli, alveolar edema, and minimal to moderate inflammatory cell infiltration. In contrast, lungs of uninfected control mice were free of lesions. These morphological changes were reflected by an almost threefold increase in pulmonary optical density of the infected control group compared with the uninfected controls (Table 1, Fig. 3). Reproducibility of the technique was demonstrated by repeating the procedure 10 times on one lung section. A mean optical density of 0.116 with a standard deviation of ± 0.007 was obtained, and the coefficient of variation was 6.14%.

In general, with the exception of mice treated with rimantadine 15 h after infection, the mean optical density of the lungs of treated mice was midway between the values of uninfected and infected controls and differed significantly from that of the latter ($P < 0.01$). By analyzing lung weights of the various groups, there was significant reduction of lung weight only for mice given rimantadine before virus inoculation and mice given ribavirin 15 h subsequent to infection compared with infected, untreated controls ($P < 0.01$). However, the trend was

TABLE 1. Prophylactic and therapeutic effects of amantadine, rimantadine, or ribavirin on lung optical density and weight in influenza A/New Jersey-infected mice

Group	Mean value $\pm$ standard error	
	Optical density ($\times 10^{-3}$)	Lung wt (mg)
Prophylaxis and therapy		
Amantadine	69 $\pm$ 4 ^{a,b}	310 $\pm$ 14 ^b
Rimantadine	68 $\pm$ 5 ^{a,b}	272 $\pm$ 17 ^{a,b}
Ribavirin	71 $\pm$ 8 ^{a,b}	332 $\pm$ 42 ^b
Therapy		
Amantadine	63 $\pm$ 2 ^a	324 $\pm$ 24 ^b
Rimantadine	84 $\pm$ 11 ^b	352 $\pm$ 17 ^b
Ribavirin	56 $\pm$ 3 ^a	266 $\pm$ 19 ^{a,b}
Controls		
Infected, untreated	98 $\pm$ 11 ^c	390 $\pm$ 36 ^c
Uninfected	38 $\pm$ 1 ^c	142 $\pm$ 2 ^c

^a Differs significantly from infected, untreated control ($P < 0.01$).

^b Differs significantly from uninfected control ($P < 0.01$).

^c Controls differ significantly from each other ($P < 0.01$).

the same as that observed by densitometry, with the lung weights of infected, treated animals falling between values observed for both control groups. The linear regression analysis of the means of pulmonary optical density against means of lung weight gave a coefficient of correlation of 0.92.

Statistical analysis of the microscopic lung scores by both pathologists revealed differences between control groups only ($P < 0.05$ and $P < 0.15$). A similar trend was also found between the lungs of uninfected controls and mice pretreated with ribavirin ($P < 0.15$). Differences were not observed among other groups (Fig. 4). The foregoing data reflect the capability of the densitometric method to detect slight differences in lung consolidation compared with weight and subjective scoring.

DISCUSSION

Of the three methods used to evaluate lung changes, the photodensitometric technique was the most sensitive. Lung weights gave similar data but of questionable significance, whereas significant differences were not seen between any of the groups except the controls with subjective microscopic scoring. Reasons for the increased sensitivity of the optical density technique over lung weight are that: (i) optical density measurements were independent of lung size; (ii) the variability of optical density measurements within groups was less than

FIG. 3. Effect of antiviral agents on the optical density (left) and weight of lungs (right) of mice infected with swine influenza. Open bars, Prophylaxis and therapy; closed bars, therapy. * $P < 0.01$ versus infected controls.

FIG. 4. Subjective scores of microscopic lesions from influenza-infected mice. These data were derived separately by two investigators, W.C.H. (●) and G.S.D. (○).

that observed with lung weight; and (iii) structures not contributing to the pathological process, such as blood, large airways, and tissue artifacts, were selectively eliminated by viewing each field before obtaining an optical density measurement.

Compared with morphometric procedures, such as the point-counting and line intercept methods described by Weibel (10), the densitometry technique offers to the microscopist the advantage of surveying large numbers of specimens in a relatively short time (approximately 20 min for three sections from each lung) with a statistically reliable number of observations. Moreover, this procedure utilizes standard pho-

tomicrography equipment already found in most pathology laboratories. With a minimal amount of training, densitometry can be performed by a technician. On the other hand, morphometry allows one to proceed further and to examine the various factors that contribute to the pathological process (e.g., changes in alveolar septal thickness, capillary thickness, exudate changes, etc.). However, these methods require considerably more time to perform compared with densitometry and can be done in conjunction with the latter if necessary.

With densitometry and most other microscopic techniques for quantifying pulmonary changes, the following criteria must be met to ensure reliability of the data. (i) Lungs must be perfused with an appropriate fixative at an adequate pressure to ensure even inflation of alveoli. A pressure of 20 cm of water above the trachea is suitable (10). At this pressure, the alveolar volume is approximately 50% of the total volume of the pulmonary parenchyma of mouse lungs (personal observation). Reduced pressure causes reduced alveolar volume and, thus, artifactually increases the optical density of the tissue specimen. (ii) Embedding procedures must be considered. Standard paraffin techniques allow for expediency and for the use of a variety of stains compared with techniques employing plastic media. Tissue shrinkage occurs with this method, but by using automated paraffin-embedding procedures, the same changes occur in all lung samples and thus do not interfere with comparative studies utilizing optical density or morphometric techniques. Automated procedures were used in this study. (iii) Sections must be cut at the same thickness, since increased thickness will result in increased optical density. (iv) Staining must be controlled by processing as many sections as possible simultaneously or in rapid succession through the same staining solutions. (v) Light filtration in the microscope influences the sensitivity of the technique and must be considered. Lungs from influenza-infected mice were sampled on day 7 when the pathological features were characterized mainly by mild exudation, such as edema, with only minimal cellular infiltration. With hematoxylin and eosin stains, these lungs were more eosinophilic (red) than basophilic (blue). The latter color would have been expected with extensive inflammatory cell infiltration or proliferation. Because of the increased eosinophilia in the lungs of infected mice, greater changes in optical density were produced by absorbing the red end of the spectrum from the light transmitted through the tissue specimen.

We feel that this technique offers promise as

a means of evaluating the response of lungs to a variety of infectious and toxic agents and will provide an objective, unbiased measure of the therapeutic value of various drugs for treating various respiratory diseases. Meaningful results were certainly obtained by densitometry in this study, whereas the lung weight technique and subjective scoring methods gave data that were, at best, only marginally significant.

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