Comparative Medicine
Copyright 2004
by the American Association for Laboratory Animal Science

Vol 54, No 4
August 2004
Pages 393-396

Comparative Study of Lung Cytologic Features in Normal Rhesus (*Macaca mulatta*), Cynomolgus (*Macaca fasicularis*), and African Green (*Chlorocebus aethiops*) Nonhuman Primates by Use of Bronchoscopy

Mallory K. Tate, DVM,[1] Pedro J. Rico, DVM,[1] and Chad J. Roy, PhD[2,*]

Invasive bronchoscopy and bronchoaveolar lavage (BAL) fluid collection represents an important tool in studies of the respiratory system of nonhuman primates. Bronchoscopy and BAL fluid collection was performed on groups of rhesus (*Macaca mulatta*) and cynomolgus (*Macaca fasicularis*) macaques and African green monkeys (*Chlorocebus aethiops*), and the resulting comparative lavage cytologic features are described. Analysis of the BAL fluid did not reveal significant differences among species with respect to total cells recovered or differential cellular composition. This description of the method used to lavage the nonhuman primates and the resulting lung cytologic findings provide important comparative data for three species commonly used in biomedical research.

Bronchoaveolar lavage (BAL) is a useful, safe method for sampling cellular and biochemical components from the lung. Examination of BAL fluid has been used in animals and humans for assessing disease progression and response to therapy in lung infections (8). Use of BAL enables the clinician or investigator to diagnose an infection at an early stage when there are no other signs of disease such as coughing or exudates in the trachea. When BAL fluid is microscopically examined after centrifugation, the leukocytes in the fluid can aid in determining the presence or absence of an infectious process in the pulmonary tree. The infectious disease process can be visualized by determining characteristic changes such as inflammation, color changes, and exudative changes (froth) in the respiratory system (11, 13).

In the study reported here, we performed bronchoscopy to obtain BAL fluid samples from three of the most commonly studied species of nonhuman primates in biomedical research (1, 5, 6). Knowing the similarities and differences among species can be beneficial in decisions of model selection for aerosol or respiratory disease studies. Bronchial lavage sample concentrations of 150 to 200 $\times$ 10^3 cells/ml have been documented to vary by species (4, 11, 14). Rhesus macaques are in high demand, and the increased acquisition cost makes it advantageous to use other species when possible (5, 6). Use of alternate nonhuman primate species as animal models and the possibility of obtaining the same results would be a great advantage to our scientific community. Also, comparative cytologic data can provide additional baseline data for total cell numbers, white blood cell differential counts, and species comparisons. Visualization of normal bronchi is essential for determining the degree of inflammation in aerosol exposure or respiratory tract disease.

Received: 3/26/04. Revision requested: 4/12/04. Accepted: 4/27/04.
Divisions of [1]Veterinary Medicine and [2]Toxinology and Aerobiology, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland 20702-5011.
[*]Corresponding author.

Materials and Methods

Animals. Fifteen individuals from each of the three species of nonhuman primates currently housed at United States Army Medical Research Institute of Infectious Diseases (USAMRIID) were studied. The cynomolgus macaques were obtained from Charles River BRF (Houston, Tex.), and the country of origin was Mauritius. The cynomolgus macaques were between 6 and 7 years old, with a body weight range of 6.5 to 9.5 kg. The rhesus macaques were captive bred; the country of origin is unknown. The rhesus macaques were between 6 and 9 years old, with body weight range of 7.2 to 10 kg. The African green monkeys were obtained from Primate Products, Inc. (Miami, Fla.), and the country of origin was St. Kitts Island. The African green monkeys were between 5 and 8 years old, with body weight range of 5.5 to 7.8 kg.

During the study period, the monkeys were individually housed according to species in different rooms in 4.3 ft^2 (24 in. $\times$ 26 in. $\times$ 34 in.) stainless-steel squeeze cages. Environmental conditions were maintained at 18 to 29°C and 30 to 70% relative humidity with 10 to 15 fresh air changes/h. The rooms were on a 12:12-h light:dark cycle (lights on at 6 a.m.). Water was provided ad libitum via an automatic watering system, and monkeys were fed commercially prepared monkey biscuits (8714 Harlan Teklad, Madison, Wis.) supplemented daily with fresh fruits or vegetables. Monkeys were provided with behavioral enrichment toys and treats daily. All food was removed 12 h before anesthesia induction. The facility is maintained according to standards recommended in the *Guide for Care and Use of Laboratory Animals*, and is AAALAC-approved. Before the procedure, all ani-

mals were determined to be healthy on the basis of general appearance, activity, results of viral disease testing (measles virus, Herpes papionis [SA8], Simian Immunodeficiency Virus [SIV], Simian Rotavirus [SA11], Simian Retrovirus [SRV], Simian T-cell leukemia virus [STLV-1] and cercopithecine herpesvirus [CHV] 1) and tuberculosis skin testing, and absence of blood biochemical and complete blood count abnormalities. All procedures were approved by the USAMRIID, Laboratory Animal Care and Use Committee, under separate protocols.

Bronchoscopy. A pediatric endoscope (FB15BS; Pentax USA, Orangeburg, N.Y.) equipped with a Pentax camera (PSV400) was used to visualize the respiratory tract and to obtain BAL fluid samples. A color monitor (Sony 14M2M PVM-DU) (Sony Electronics Inc., Park Ridge, N.J.) was used to visualize the trachea and the bronchus, with illumination provided by the Pentax light source (LIT-150PC). Pictures of the lavage site in the lung were taken using the digital camera attached to the eyepiece of the endoscope.

Lavage procedure. The nonhuman primates were anesthetized with a tiletamine-zolazepam combination (Telazol; Fort Dodge Animal Health, Fort Dodge, Iowa) at a dosage of 5 mg/kg of body weight (6, 9, 10). Alternatively, a 10:1 mixture of ketamine (Ketamine HCl inj. USP, Phoenix Pharmaceutical, Inc., St. Joseph, Mo.) and acepromazine (Fort Dodge Animal Health, Fort Dodge, Iowa) at a dosage of 9 mg of ketamine and 0.1 mg acepromazine/kg was used for anesthesia (12). At our facility, Telazol is the anesthetic of choice for procedures performed on macaques and ketamine/acepromazine is used for African Green monkeys. We do not use Telazol in African green monkeys as it has been observed at our facility that they tend to develop excess postanesthesia hypothermia after frequent use of this anesthetic.

Anesthetic was administered by intramuscular injection in the caudal thigh muscles. Once anesthetized, the nonhuman primate was positioned in sternal recumbency on a heated surgical table and was covered with a heated pad. A plastic dental wedge was used to hold the mouth open. A laryngoscope was used to visualize the epiglottis, then Marcaine 0.25% (Abbott Labs, Chicago, Ill.) mixed with K-Y Jelly (Johnson & Johnson Medical, Inc., Skillman, N.J.) was administered topically. The tongue was gently extended outward after the local anesthetic was allowed to take effect. The endoscope was inserted into the larynx, past the vocalis muscle, and into the trachea. The tip of the scope was then extended past the primary bronchial bifurcation to the second-generation bifurcation of the left primary bronchus into the upper lobar bronchi (Fig. 1). Once the position for the lavage sample was confirmed via visualization with the digital camera, warmed 1X phosphate-buffered saline (PBS; Baxter, Skokie, Ill.) was pushed through the sampling tube by use of a 10-ml syringe. The PBS lavage fluid was visualized through the digital image, immediately suctioned using a medical aspirator (Schuco Vac 130 Aspirator, Allied Healthcare Products, Inc., St. Louis, Mo.) into a sterile collection cup, and kept on wet ice until processed for cytologic examination. After sampling was completed, the bronchoscope was carefully removed, lung auscultation was performed to assess lung function, and the nonhuman primate was placed back in the cage and continuously monitored until full recovery from anesthesia.

Sample analysis. The collected BAL fluid sample was analyzed for total and differential cell counts. The fluid samples were centrifuged for 5 min at 451.6 ×*g*, and the supernatant was decanted and stored at 80°C. The cell pellet was re-suspended in 50 ml of Hanks balanced salt solution (HBSS) without magnesium or calcium (Cellgro, Mediatech, Charlottesville, Va.). A blood capillary diluter with 25 ml of 5% acetic acid (Unopette, Becton-Dickinson, Franklin Lakes, N.J.) was used for red blood cell hemolysis. Total cells were counted using an automated cell counter (Coulter Counter 3, Beckman Coulter, Inc., Miami, Fla.).

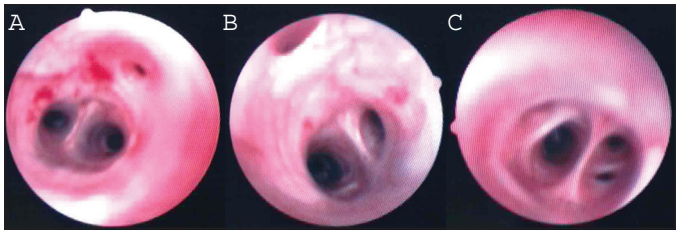

Figure 1. Photographs taken during bronchoscopy of the secondary lobar bifurcation of the bronchi in: (A) African green monkey, with hyperemia due to trauma induced by the bronchoscope, (B) rhesus macaque, and (C) cynomolgus macaque.

After total cell processing, an additional 300 ml of HBSS was added, and the samples were vortexed for 10 sec. Centrifuge slide wells were assembled, and 50 ml of Hyclone fetal bovine serum (Cellgro, Mediatech) was added, followed by 50 ml of the re-suspended sample. The slide assemblies were centrifuged for 4 min at 112.9 ×*g*. The samples were then blot-dried and stained for differential analysis using a Diff-quick stain kit (Dade Co., Ft. Lauderdale, Fla.). Slides were dried, coverslipped, and examined at 400× magnification under light microscopy. Differentiation among macrophages, lymphocytes, neutrophils, eosinophils, and basophils was performed on 200 total cells for each sample on at least five representative microscopic fields. The results of the differential cell counts were expressed as a percentage composition.

Statistical analysis. Total and differential cell counts were tabulated, and descriptive statistics (arithmetic mean ± SD) were calculated. Interspecies differences in total cells were analyzed using one-way analysis of variance, and particular respiratory cell types were analyzed using Kruskal-Wallis non-parametric tests. A P value of < 0.05 was considered significant.

Results

Lavage fluid recovery. The ability to successfully perform bronchoscopy and recover BAL fluid varied among species. Due to the size of the rhesus and cynomolgus macaques, the bronchoscope passed through the larynx and into the upper bronchus without any complications. The respiratory passages of the African green monkeys were slightly narrower compared with those of macaques of similar body weight, and the difficulty of passing the bronchoscope increased as size of the animal decreased. This resulted in mild abrasions, mostly on the upper bronchi, in four of the 15 African green monkeys (Fig. 1A). A gentler approach was needed with this species when it became apparent that the bronchi were generally smaller than those of the rhesus or cynomolgus macaques. Pictures in Fig. 1 give a comparative view of nonhuman primates of the same size. The ketamine/acepromazine combination used for general anesthesia caused hypersalivation in the African green monkeys. This response complicated initial insertion of the bronchoscope because of the impaired visualiza-

Table 1. Total and differential cell analysis of bronchoalveolar lavage fluid from three species of nonhuman primates

Species	Total count[a] ($\times 10^5$/ml)	Macrophages[a,b] (%)	Lymphocytes (%)	Neutrophils (%)	Eosinopils (%)
Rhesus	10.2 ± 1.48	98.20 ± 1.61	1.13 ± 1.41	0.60 ± 0.91	0.07 ± 0.26
Cynomolgus	9.6 ± 2.95	98.33 ± 1.14	0.61 ± 0.78	0.78 ± 0.88	0.17 ± 0.38
African green	8.4 ± 1.79	98.26 ± 1.24	0.84 ± 0.90	0.68 ± 0.75	0.16 ± 0.37

[a]Values represent mean ± SD for each species (n = 15).
[b]Differential cell analysis presented as a percentage of 200 cells in at least five representative fields.

tion of the larynx. Sample recovery from the lungs was generally equivalent among species, with over 80% (8 ml) of the original amount recovered from most animals.

Cellular analysis. The cellular composition of the lavage fluid did not vary among species (Table 1). There were no significant differences among species for lymphocytes, neutrophils, eosinophils, or alveolar macrophages. Also, there were no significant differences among species for BAL fluid sample concentrations. The average total cell count ranged from 8.4 to 10.2 $\times 10^5$ cells/ml. The predominant cell type identified in the lavage fluid samples was the quiescent alveolar macrophage, comprising over 98% of the cells collected in all three species. Lymphocytes, polymorphonuclear cells (neutrophils), and eosinophils comprised the remaining 2% of the cells. Basophils were not identified during the analysis.

Discussion

Results of this study provide updated methods for performing BAL on nonhuman primates due to the decreased volume compared with that of previous methods, where as much as 400 ml of fluid was used. Also, this method is fast and less traumatic to the nonhuman primate than are previously used methods (8, 10, 11) due to the use of the medical aspirator on the scope. All of the nonhuman primates recovered without appreciable complications. The resulting descriptive respiratory cytologic features provide important comparative data for three types of nonhuman primates (rhesus, cynomolgus, and African green monkeys) commonly used in biomedical research. The capability of performing single and repeated lavages to obtain BAL fluid without causing adverse effects to the nonhuman primate offers distinct advantages, such as assessing bronchial disease or progression at any given period during toxicologic or immunologic studies (4, 8, 11). Analytical measurement of BAL fluid for cellular metabolism markers, production of mucosal antibodies, cytokines, enzymatic changes, and phagocytic activities can provide insight into disease processes as well as protective effects provided by mucosal administration of vaccines and therapeutics.

Although past studies have indicated that up to 400 ml was used as the lavage volume (8), the amount we used was sufficient to perform total and differential cell analysis. The amount of lavage fluid recovered from all three species provided additional volume that could have been used for other assays.

The cytologic results were somewhat different than what has been reported in past studies. The total cellular population in the lavage fluid samples ranged from 8.4×10^5 (African green monkeys) to 10.02×10^5 (rhesus) cells/ml. All cell counts in this study were higher than the range of 5.0×10^4 to 2.0×10^5 cells/ml reported for rhesus and African green monkeys in previous studies (2, 3, 11, 15). It is not known whether the age or sex of the nonhuman primates (specifically the rhesus) used in this study affected the total counts, compared with those obtained in previous studies.

The differential cell analysis in the three species was remarkably similar. The overwhelming majority (> 98%) of cells counted in the lavage fluid samples were alveolar macrophages. The high percentage of macrophages observed in this study is in contrast to past studies of nonhuman primate lavage fluid cytology (4, 8, 11, 14). Most reports indicated that alveolar macrophages comprised approximately 80 to 85% of the total respiratory tract cells (2, 3, 14); however, previous studies have indicated ranges as low as 53 to 70% of such cells in rhesus macaques. Lavage in cynomolgus macaques returned the highest percentages of alveolar macrophages, comprising 89 to 94% of the total cells observed (11).

Lymphocytes (or lymphoblasts), neutrophils, and specialized cells such as eosinophils and basophils were sparse in this study. Although the low numbers of lymphocytes and neutrophils were not necessarily unexpected, the lack of at least a low prevalence of eosinophils and/or basophils in the colony was unexpected. The African green monkeys were wild caught before placement in the colony. Although all nonhuman primates are treated with antiparasitics subsequent to arrival at our facility, it is expected that some animals would harbor subclinical infections that are not necessarily responsive to treatment. Subclinical parasitic infection with *Pneumonyssus simicola* (lung mites) was a common problem in facilities that housed wild-caught nonhuman primates (11, 13). Previous studies of lavage fluid taken from wild-caught nonhuman primates provide evidence of this; eosinophil populations ranging from 15 to 36% indicated high incidence of parasitism within a rhesus macaque colony (8), and eosinophil hyperresponsiveness was observed in cynomolgus macaques with respiratory sensitivity to *Ascaris suum* extract (7). The differences observed in this study, compared with previous studies, may be due to an aggressive preventive care policy toward parasitic treatment and decrease in the use of wild-caught nonhuman primates, which could account for the remarkably low prevalence of these types of specialized lymphocytic cells.

The results of this study indicate that differences in respiratory system cellular composition are minimal among these three species. There are many factors that may have accounted for the remarkable similarity. All nonhuman primates in the colony at our institute receive identical preventive care. In addition, the lavage fluid samples were batch analyzed over a period of four days in an attempt to minimize subtle differences in the analysis due to reagent preparation or lavage sampling technique. Two technicians blinded to species and sample analyzed all differentials included in this study. Bias toward a particular shape or coloration could have developed over the course of the analysis, possibly skewing the results to a particular cell type.

On the basis of similarity in lung cytologic features, the African green monkey can be used as a model for BAL studies replacing the more expensive rhesus and cynomolgus macaques and minimizing exposure to CHV-1. The advantages of using African green monkeys in BAL studies are cost effectiveness, smaller size, safe handling, and ease of procurement (5). The

only disadvantage of the African green monkey is the size of the bronchi in comparison with those in the other species. The results of this study provide basic comparative cytologic data that can be used as a reference for future comparative studies using these three types of nonhuman primates.

Acknowledgments

We thank Brittany Goodenow for performing cytologic examination, Sarah Norris for performing statistical review, and the veterinary technicians and caretakers of the Nonhuman Primate Section, Veterinary Medicine Division, USAMRIID. The research described herein was sponsored by the Medical Biological Defense Research Program, U.S. Army Medical Research and Materiel Command, USAMRIID project number 02-4-NN-049. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility where this research was conducted is fully approved by the Association for Assessment and Accreditation of Laboratory Animal Care, International. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.

References

1. **Adams, R.** 2002. Techniques of experimentation, p. 1023-1024. *In* J. Fox, L. Anderson, F. Loew, and F. Quimby (ed.), Laboratory animal medicine, 2nd ed. Academic Press, San Diego, Calif.
2. **Caufour, P., R. Le Grand, A. Chèret, O. Neildez, F. Theodoro, B. Boson, B. Vaslin, and D. Dormont.** 1999. Secretion of β-chemokines by bronchoalveolar lavage cells during primary infection of macaques inoculated with attenuated nef-deleted or pathogenic simian immunodeficiency virus strain mac251. J. Gen. Virol. **80:**767-776.
3. **Chèret, A., R. Le Grand, P. Caufour, O. Neildez, F. Matheux, F. Theodoro, B. Vaslin, and D. Dormont.** 1999. RANTES, IFN-γ, CCR5 mRNA expression in peripheral blood, lymph node, and bronchoalveolar lavage mononuclear cells during primary simian immunodeficiency virus infection of macaques. Virology **255:**285-293.
4. **Drozdowicz, C. and T. Bowman.** 1988. Bronchoaveolar lavage for recovery of pulmonary macrophages in normal macaques (*Macaca nemestrina* and *Macaca radiata*). Lab. Anim. Sci. [Abstr.] **38:**520.
5. **Ervin, F. and R. Palmour.** 2003. Primates for 21st century biomedicine: the St. Kitts vervet (*Chlorocebus aethiops*, SK), p. 50-53. *In* National Research Council, international perspectives, the future of nonhuman primate resources. National Academies Press, Washington, D.C.
6. **Fortman, J., T. Hewett, and B. Bennet.** 2001. The laboratory nonhuman primate, p. 111-131. CRC Press, Boca Raton, Fla.
7. **Gundel, R., C. Wegner, C. Torcellini, C. Clarke, G. Gleich, and L. Letts.** 1990. Relationship between bronchoalveolar lavage (BAL) eosinophil-derived proteins and the onset and recovery of airway hyperresponsiveness. J. Allerg. Clin. Immunol. [Abstr.] **85:**282.
8. **Haley, P., B. Muggenburg, A. Rebar, G. Shopp, and D. Bice.** 1989. Bronchoalveolar lavage cytology in cynomolgus primates and identification of cytologic alterations following sequential saline lavage. Vet. Pathol. **26:**265-273.
9. **Hawk, T. and S. Leary.** 1999. Formulary for laboratory animals, 2nd ed., p. 43 and 54. Iowa State University Press, Ames, Iowa.
10. **Henderson, R. and J. Lowrey.** 1983. Effect of anesthetic agents on lavage fluid parameters used as indicators of pulmonary injury. Lab. Anim. Sci. **33:**60-62.
11. **Kastelo, M., A. Emmert, and R. Kishimoto.** 1979. Recovery of alveolar macrophages from rhesus and cynomolgus macaques by lung lavage. J. Vet. Sci. **40:**271-273.
12. **Lopèz, K., P. Gibbs, and D. Reed.** 2002. A comparison of body temperature changes due to administration of ketamine-acepromazine and tiletamine-zolazepam anesthetics in cynomolgus macaques. Contemp. Top. Lab. Anim. Sci. **41:**47-50.
13. **Osborn, K. and L. Lowenstine.** 1998. Respiratory diseases, p. 263-283. *In* B. T. Bennet, C. R. Abee, and R. Henrickson (ed.), Nonhuman primates in biomedical research, diseases. Academic Press, San Diego, Calif.
14. **Reynolds, H.** 1987. Bronchoaveolar lavage. Am. Rev. Respir. Dis. **135:**250-263.
15. **Weiss, W., T. Murphy, and M. Lynch.** 2003. Inhalation efficacy of RFI-641 in an African green primate model of RSV infection. J. Med. Primatol. **32:**82-88.