In Vitro Interaction of Alveolar Macrophages and Pneumocytes with Feline Respiratory Viruses

JOHN M. LANGLOSS,[1] EDWARD A. HOOVER,[1] * DONALD E. KAHN,[2] AND ALEXIS J. KNIAZEFF[2]

Department of Veterinary Pathobiology, The Ohio State University, Columbus, Ohio 43210,[1] and Department of Pathology, School of Medicine, University of California, La Jolla, California 92093[2]

Feline alveolar macrophages and feline pneumocytes were inoculated in vitro with low multiplicities of either feline calicivirus or feline viral rhinotracheitis virus. Pneumocytes were permissive for both viruses. High titers were attained, and characteristic cytopathic effects developed. Alveolar macrophages were permissive for feline viral rhinotracheitis virus, although the cycle of replication was delayed. Infection of macrophages with feline calicivirus resulted in the production of viral antigens and cytopathic effects; however, viral particles were not detected by electron microscopy, and viral infectivity titers rose only slightly and then fell to undetectable levels by 96 h. The differences in viral susceptibility between these two peripheral pulmonary cell populations that were demonstrated in vitro probably contribute to the differences in pathogenesis of viral rhinotracheitis and calicivirus infections in cats.

Most viruses responsible for respiratory diseases enter the respiratory tract via inhalation, but they localize and produce disease at different levels of the respiratory tract. Although the reasons for tissue tropisms, whether nasal, pharyngeal, tracheobronchial, or alveolar, are not completely understood, such factors as mass mean diameter of the aerosol, temperature sensitivity of the virus, specific and nonspecific defense mechanisms, and susceptibility of various respiratory cell populations are among the recognized variables that determine the ultimate distribution of lesions induced by the respiratory viruses (1, 9, 22, 23).

Localization of cytocidal viral pathogens in the peripheral air exchange tissues is especially important because damage to these tissues leads to rapid loss of respiratory function due to both direct viral damage and the ensuing acute exudative response (15). Acute deaths that occur during human epidemics of influenza have been attributed to viral localization in distal air exchange tissues (7).

The objective of the current study was to examine the interaction of isolated alveolar macrophages and pneumocytes with two common viral respiratory pathogens of cats, feline calicivirus (FCV), a member of *Picornaviridae*, and feline rhinotracheitis virus (FVRV), a typical herpesvirus (4, 13). The pneumocytes examined were the type II alveolar lining cells, which in vivo are the major source of pulmonary surfactant and are the progenitors of the type I, or membranous, pneumocyte (16). Because FVRV causes upper respiratory disease principally (5)

and FCV induces lower respiratory disease (11, 12, 14), our objective was to determine the relationship, if any, between the tropism of these viruses for alveolar cell populations and their propensity to produce alveolar injury in vivo.

MATERIALS AND METHODS

Viruses. The viruses used in the study were FCV strain FPV-255 (13) and FVRV isolate C-27 (4).

Alveolar macrophages. Alveolar macrophages were collected under sterile conditions from the lungs of specific pathogen-free cats that were 3 to 6 months of age. The cats were from a caesarean-derived breeding colony (21) that has been maintained free of feline viruses since 1966. The cats were anesthetized with ketamine HCl and exsanguinated. The tracheae were cannulated at the thoracic inlet, and the lungs were washed in situ five times with 50-ml volumes of saline containing 0.5 U of heparin, 400 U of penicillin, and 200 mg of streptomycin per ml. Cells were separated from wash fluid by centrifugation at $200 \times g$ for 15 min, washed thoroughly in Eagle minimal essential medium with Hanks balanced salt solution containing 10% heat-inactivated fetal calf serum, 1% sodium bicarbonate, and 0.05 mg of gentamicin (Schering Laboratories, Bloomfield, N.J.) per ml (growth medium), and suspended to a concentration of 5.6×10^6 cells per ml. More than 98% of the cells were alveolar macrophages by light microscopic criteria. Volumes of 5 ml of the cell suspension were seeded in 25-cm² plastic flasks, and 1 ml was seeded in each well of Lab-Tek slide chambers (four wells per slide; Lab-Tek Products, Div. Miles Laboratories, Inc., Westmont, Ill.). After a 2-h incubation to allow cell attachment, the cultures were washed with three changes of growth medium. Monolayers were infected 24 h after seeding. Total and viable cells (as determined by trypan blue exclu-

sion) of two replicate flasks were determined. At 24 h there were 6.6×10^5 cells per flask with ≥98% viability; at the terminus, flasks contained 6.0×10^5 cells with >75% viability.

Alveolar pneumocytes. The isolation, morphology, and cultural characteristics of the feline pneumocytes used in this study have been described (16). The cells represented passage 34 of a clone (AK-D) of epithelial cells isolated from the lungs of 55-day-old feline fetuses (normal gestation, 60 days). The epithelioid morphology of the cloned cells and characteristic cytoplasmic lamellar inclusions were the major criteria used to establish that the cells represented type II alveolar pneumocytes (16). Cells were grown to confluency at 37°C in 25-cm² plastic flasks and on Leighton tube cover slips with Eagle minimal essential medium plus Hanks balanced salt solution containing 6% heat-inactivated fetal calf serum, 1% sodium bicarbonate, and antibiotics (200 U of penicillin and 100 mg of streptomycin per ml). The average number of viable cells per flask (1.23×10^5) was determined by enumerating the cells in three flasks.

Infection of cell cultures. Monolayers were infected with a dilution of FCV or FVRV suspensions calculated to produce a low multiplicity of infectivity. Flasks were inoculated with 0.5 ml, and cover slips were inoculated with 0.1 ml of virus suspension. Pneumocyte cultures were inoculated either with an FCV suspension containing $10^{3.75}$ 50% tissue culture infective doses ($TCID_{50}$) per ml (0.02 $TCID_{50}$/cell) or with an FVRV suspension containing $10^{2.75}$ $TCID_{50}$/ml (0.002 $TCID_{50}$/cell). Alveolar macrophage cultures were inoculated either with an FCV suspension containing $10^{3.75}$ $TCID_{50}$/ml (0.004 $TCID_{50}$/cell) or with an FVRV suspension containing 10^2 $TCID_{50}$/ml (0.001 $TCID_{50}$/cell).

Immunofluorescence procedure. Cell monolayers on cover slips and slides were fixed in acetone and stained by direct immunofluorescence for either FVRV or FCV, using feline hyperimmune antisera conjugated with fluorescein isothiocyanate (10, 14). Monolayers were counterstained with rhodamine and examined with a Zeiss ultraviolet microscope.

Cytopathology. Cell monolayers on cover slips and slides were fixed in Bouin's fluid and stained with Giemsa stain and/or with hematoxylin and eosin for examination by light microscopy.

Electron microscopy. Adherent and nonadherent cells were collected from the culture flasks and were pelleted by centrifugation at $150 \times g$ for 15 min, fixed in 3% glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Philips 300 electron microscope.

Virus assay. Cell cultures to be assayed for infectious virus were frozen and thawed three times. The sample were clarified at $150 \times g$ for 15 min and titrated by previously described methods (14). The end points were expressed as the $TCID_{50}$, as determined by the method of Spearman-Kärber (8).

RESULTS

Cytopathology and pneumocytes infected with FCV. The cytopathic effects (CPE) of FCV on pneumocyte monolayers were similar to those observed by others using permissive feline cell lines (13, 18). At 12 h cells in focal areas of the monolayer were rounded and shrunken, with pyknosis and intense cytoplasmic staining. By 48 h the monolayer was destroyed completely. Fine structural lesions included newly formed, membrane-lined vesicles and increased numbers of membrane-associated ribosomes in the cytoplasm (Fig. 1). The cytoplasm became extremely electron dense late in the infectious cycle. Most of the degenerating cells contained cytoplasmic viral particles arranged in crystalline arrays typical of FCV.

Cytopathology of pneumocytes infected with FVRV. The CPE of FVRV were detected in pneumocyte monolayers by 12 h, and by 72 h the monolayers were completely destroyed. Intranuclear inclusions and polykaryocytes were readily apparent in hematoxylin and eosin-stained cover slips. These features are characteristic of the type of CPE encountered with FVRV infection of feline cell cultures (3, 18). Fine structural alterations in the nucleus were evident by 12 h, when herpesvirus nucleocapsids were detected in association with chromatin clumping and focal reduplication of the nuclear membrane. Enveloped virus particles were present in the cytoplasm at 12 h but were more prevalent at 24 h (Fig. 2). Cytoplasmic alterations were minimal until late in the infectious cycle, when mitochondrial degeneration and vacuolar changes were evident.

Cytopathology of alveolar macrophages infected with FCV. The CPE were not evident until 48 h, when the number of cells adherent to the hematoxylin and eosin-stained slides was reduced to approximately 50% of controls. The cytoplasm of many of the remaining cells either was expanded and had reduced staining affinity or was condensed and had increased eosinophilia. The nuclei of affected cells were karyolytic or pyknotic. Phagocytosed remnants of necrotic cells were often observed within cytoplasmic vacuoles of less affected macrophages. By 96 h the entire monolayer was destroyed. Fine structural changes at 48 h included separation of nuclear membranes, formation of intracytoplasmic membrane-bound vesicles, and an increase in ribosomal structures (Fig. 3). Viral particles were not observed.

Cytopathology of alveolar macrophages infected with FVRV. A reduction of ~50% in adherent cells, formation of polykaryocytes, and intranuclear inclusions were all evident by 48 h in FVRV-infected, alveolar macrophage monolayers. By 96 h all cells had detached from the glass surface. Ultrastructural examination indicated complete replication of the virus. Most nuclei contained nucleocapsids, and enveloped
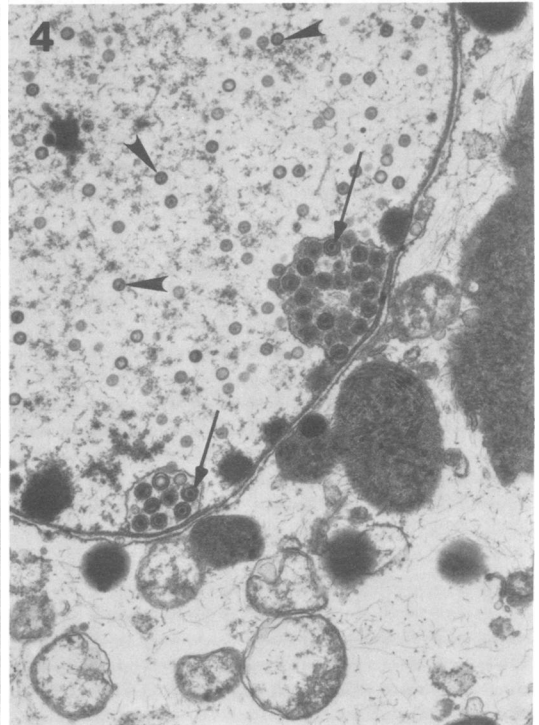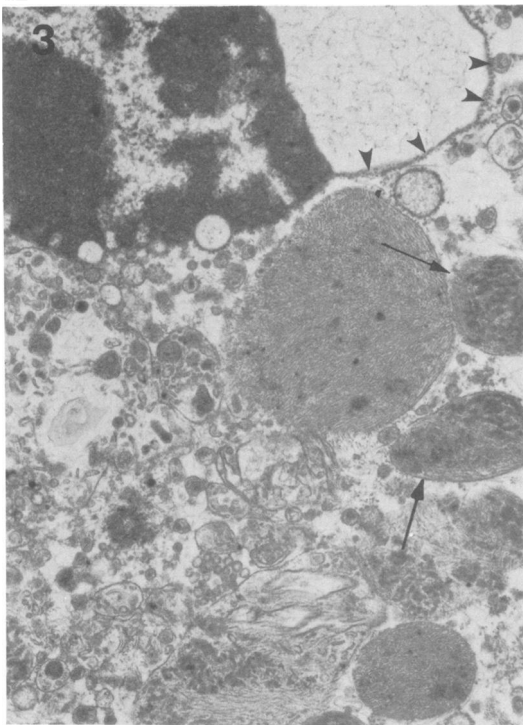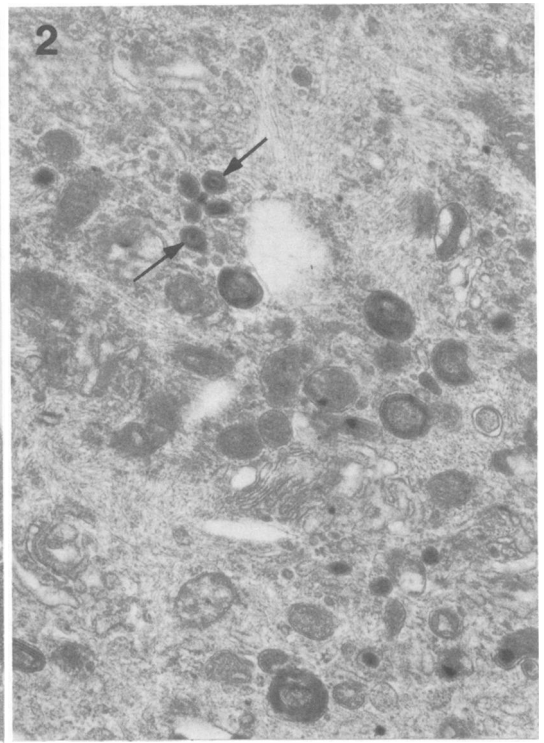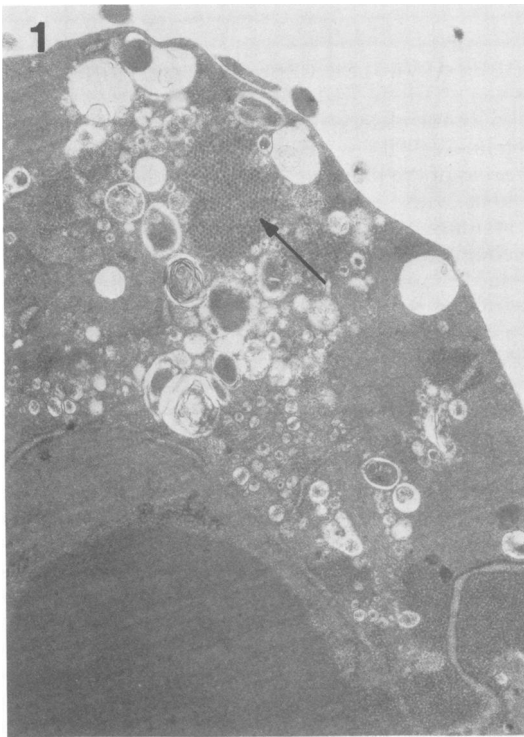
FIG. 1. *Electron micrograph of FCV-infected pneumocyte with characteristic changes, including formation of membranous vesicles, increase in ribosomes, chromatin clumping, and separation of nuclear membranes. Crystalline array of FCV particles is present in the cytoplasm (arrow).* ×16,500.

FIG. 2. *Electron micrograph of FVRV-infected pneumocyte containing mature enveloped herpesvirus in the cytoplasm (arrows).* ×18,300.

FIG. 3. *Electron micrograph of FCV-infected alveolar macrophage showing both nuclear and cytoplasmic changes. The nuclear membranes are widely separated, and ribosomes are adherent to the outer membrane (arrowheads). Note the dilated vesicles and the characteristic residual bodies (arrows) of feline alveolar macrophages.* ×16,000.

FIG. 4. *Electron micrograph showing FVRV nucleocapsids (arrows) in the nucleoplasm and mature enveloped virion (arrowheads) associated with the nuclear membranes of alveolar macrophage. Clumping of chromatin and reduplication of nuclear membranes are also present.* ×16,200.

virus particles were often present in the cytoplasm (Fig. 4). The most consistent cytological lesion associated with FVRV infection was clumping and margination of chromatin together with reduplication and separation of the nuclear membranes.

Detection of viral antigen by immunofluorescence microscopy. The production of viral antigens within infected alveolar cells was determined by staining the monolayers with the appropriate fluorescein-labeled monospecific antiserum after CPE had developed. Pneumocytes and alveolar macrophages infected with FVRV had both cytoplasmic and nuclear fluorescence when stained with the anti-FVRV conjugate. Diffuse cytoplasmic fluorescence was detected in FCV-infected pneumocytes stained with anti-FCV conjugate (Fig. 5). In alveolar macrophages, however, FCV antigen occurred only as multiple fine particles dispersed throughout the cytoplasm of degenerating cells that remained attached at 48 and 72 h (Fig. 6).

Growth of virus in pneumocytes and alveolar macrophages. Both pneumocyte and alveolar macrophage cultures were highly per-missive for FVRV (Fig. 7 and 8). Final viral infectivity titers were similar, although the rate of viral production in alveolar macrophages was delayed compared with that for pneumocytes. The results were substantially different for FCV-infected cultures. Although FCV replicated rapidly to high titers in alveolar pneumocytes (Fig. 9), the virus failed to replicate efficiently in alveolar macrophages (Fig. 10).

DISCUSSION

The in vitro interaction of FCV with alveolar cells resulted in productive infection of pneumocytes and abortive infection of alveolar macrophages. These observations are consistent with earlier studies of FCV infection in cats. Experimental exposure to aerosols of virulent FCV consistently resulted in acute viral injury to distal air exchange tissues (11, 12, 14). Electron and immunofluorescence microscopy indicated that the injury was largely due to rapid cytolytic infection of alveolar pneumocytes (17; J. M. Langloss, E. A. Hoover, and D. E. Kahn, submitted for publication). Occasionally, viral antigen was found in the cytoplasm of cells that
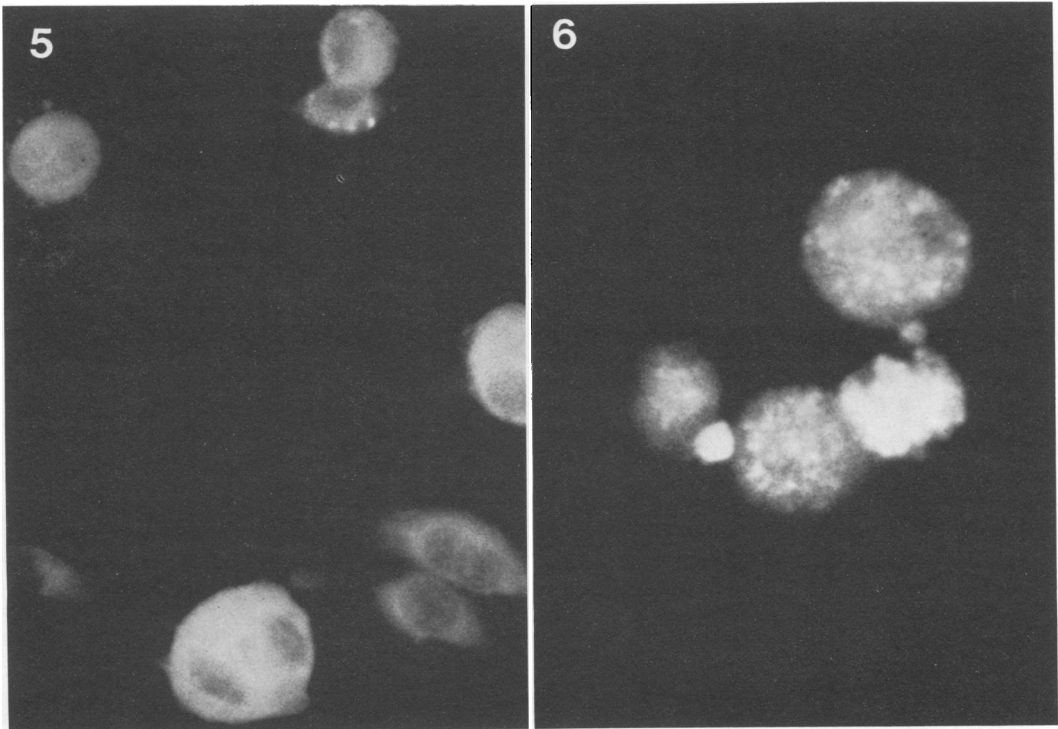

FIG. 5. *Fluorescence micrograph showing that fluorescent FCV antigen is distributed diffusely in the cytoplasm of degenerating pneumocytes. ×1,800.*

FIG. 6. *Fluorescence micrograph of FCV-infected macrophages showing that the particulate fluorescent antigen is dispersed throughout the cytoplasm of several degenerating cells. ×1,700.*
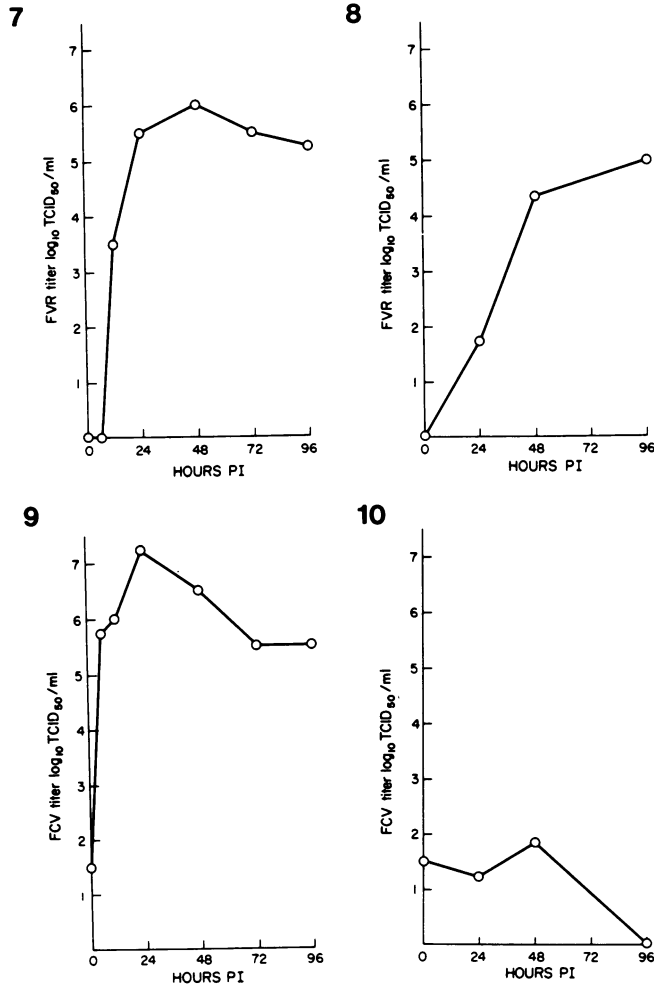
7

8

9

10

FIG. 7. *Sequential infectivity titers of FVRV-infected pneumocyte cell cultures, indicating a highly productive infection with maximal titer at 48 h. PI, Postinfection.*

FIG. 8. *FVRV infectivity titers rose rapidly in alveolar macrophage cultures, reaching maximal titers at 96 h. Each point represents the geometric mean of duplicate cultures.*

FIG. 9. *Sequential infectivity titers of FCV-infected pneumocytes cultures, showing a rapid rise to high titers with a peak of infectious virus production at 24 h.*

FIG. 10. *FCV infection of alveolar macrophages resulted in only a minimal rise in titers at 48 h; by 96 h the cultures were completely cleared of detectable infectious virus. Each point represents the geometric mean of duplicate cultures.*

appeared to be alveolar macrophages; however, virus particles were not found when these cells were examined ultrastructurally. The development of CPE and viral antigens in alveolar macrophages without production of infectious virus indicates that the FCV infection probably is aborted late in the replicative cycle, i.e., during viral assembly.

Evidence that alveolar macrophages have antiviral properties was obtained by Probert et al. (20), who found that calf alveolar macrophages had innate, nonspecific antiviral activity when added to parainfluenzavirus-3-infected kidney cell cultures. However, this activity was abrogated when the macrophages became productively infected by the virus (20). In vivo, the inhibitory effects of virus infection on macrophage antiviral function may be counterbalanced by the rapid turnover of macrophages in alveolar lesions. Clearance of FCV from the lung coincided with the recruitment of large numbers of alveolar macrophages into the lesion (17; Langloss et al., submitted for publication).

FVRV infection of alveolar macrophages and

pneumocytes in vitro indicates that these cells are highly permissive for this virus. Although it is possible that the susceptibility of the alveolar cells in vitro is enhanced by cultivation procedures (22), our findings suggest that the lack of pneumonic lesions in vivo is not due to inherent resistance of the alveolar cell population. That alveolar cells are susceptible to FVRV infection in vivo also is supported by the occasional occurrence of necrotizing alveolar lesions in FVRV-infected cats (19). The failure of FVRV to produce alveolar lesions, even after exposure to aerosols that insure deposition of the virus throughout the respiratory tract (unpublished data), suggests that nonspecific inhibitors of FVRV may be present in lung tissues (1, 2).

LITERATURE CITED

1. **Allison, A. C.** 1974. On the role of mononuclear phagocytes in immunity against viruses. Prog. Med. Virol. **18**:15–31.
2. **Burrows, R.** 1972. Early stages of virus infection: studies in vivo and in vitro. Symp. Soc. Gen. Microbiol. **22**:303–332.
3. **Crandell, R. A., and E. W. Despeaux.** 1959. Cytopathology of feline renal cells. Proc. Soc. Exp. Biol. Med. **101**:494–497.
4. **Crandell, R. A., and R. D. Maurer.** 1958. Isolation of a feline virus associated with intranuclear inclusion bodies. Proc. Soc. Exp. Biol. Med. **97**:487–490.
5. **Crandell, R. A., W. H. Niemann, J. R. Ganaway, and F. D. Maurer.** 1961. Experimental feline viral rhinotracheitis in the cat. J. Am. Vet. Med. Assoc. **138**:191–196.
6. **Fastier, L. B.** 1957. A new feline virus isolated in tissue culture. Am. J. Vet. Res. **18**:382–389.
7. **Finckh, E. S., and L. Bader.** 1974. Pulmonary damage from Hong Kong influenza. Aust. N.Z.J. Med. **3**:16–22.
8. **Finney, D. J.** 1964. Statistical methods in biological assay, 2nd ed., p. 524–531. Hafner Publishing Co., New York.
9. **Green, G. M., G. J. Jakab, R. B. Low, and G. S. Davis.** 1977. Defense mechanisms of the respiratory membrane. Annu. Rev. Respir. Dis. **115**:479–514.
10. **Hoover, E. A., and R. A. Griesemer.** 1971. Bone lesions produced by feline herpesvirus. Lab. Invest. **25**:457–464.
11. **Hoover, E. A., and D. E. Kahn.** 1973. Lesions produced by feline picornavirus of different virulence in pathogen-free cats. Vet. Pathol. **10**:307–322.
12. **Hoover, E. A., and D. E. Kahn.** 1975. Experimentally induced feline calicivirus infection: clinical signs and lesions. J. Am. Vet. Med. Assoc. **166**:463–468.
13. **Kahn, D. E., and J. H. Gillespie.** 1970. Feline viruses. X. Characterization of a newly-isolated picornavirus causing interstitial pneumonia and ulcerative stomatitis in the domestic cat. Cornell Vet. **60**:669–683.
14. **Kahn, D. E., and J. H. Gillespie.** 1971. Pathogenesis of picornavirus infection in the cat. Am. J. Vet. Res. **32**:521–531.
15. **Katzenstein, A. A., C. M. Bloor, and A. A. Leibow.** 1976. Diffuse alveolar damage—the role of oxygen, shock and related factors. Am. J. Pathol. **85**:210–228.
16. **Kniazeff, A. J., G. D. Stoner, L. Terry, R. M. Wagner, and R. D. Hoppenstand.** 1976. Characteristics of epithelial cells cultured from feline lung. Lab. Invest. **34**:495–500.
17. **Langloss, J. M., E. A. Hoover, and D. E. Kahn.** 1977. Diffuse alveolar damage in cats induced by nitrogen dioxide or feline calicivirus. Am. J. Pathol. **89**:637–648.
18. **Lee, K. M., A. J. Kniazeff, C. G. Fabricant, and J. H. Gillespie.** 1969. Utilization of various cell culture systems for propagation of certain feline viruses and canine herpesvirus. Cornell Vet. **59**:539–547.
19. **Love, D. N.** 1971. Feline herpesvirus associated with interstitial pneumonia in a kitten. Vet. Rec. **89**:178–181.
20. **Probert, M., E. J. Stott, and L. H. Thomas.** 1977. Interactions between calf alveolar macrophages and parainfluenza-3 virus. Infect. Immun. **15**:576–585.
21. **Rohovsky, M. W., and R. A. Griesemer.** 1966. The germfree cat. Lab. Anim. Care **16**:53–59.
22. **Smith, H.** 1972. Mechanisms of virus pathogenicity. Bacteriol. Rev. **36**:291–310.
23. **Tyrrell, D. A. J., and R. Parsons.** 1960. Some virus isolations from common colds. III. Cytopathic effects in tissue cultures. Lancet **i**:239–242.