ISSN 110-2047. ALEX. J. VET.SCIENCE. 2002 , VOL 18 No.1 : 115-126

SEQUENTIAL LUNG LESIONS WITH SPECIAL REFERENCE TO ANTIGEN DISTRIBUTION IN EQUINE HERPESVIRUS TYPE 1-INFECTED BALB/C MICE

Shawky A. Moustafa [1] ken Maeda [2] and Toshiharu Hayashim [3]

[1] Department of Pathology Faculty of Veterinary Medicine Zagazig University Benha branch Egypt Laboratories of Veterinary Microbiology [2] and Veterinary Pathology

[3] Faculty of Agriculture Yamaguchi University 1677-1 Yoshida Yamaguchi 753-8515 Japan.

ABSTRACT: The intranasal infection of 55 male BALB/c mice with equine herpesvirus type 1 (EHV-1) was used to study the histopathological changes and the distribution of viral antigens by the indirect immunoperoxidase and routine hematoxylin and eosin in the pulmonary tissues compared with 22 control. The animals were scarificed and necropsied 12 , 24 and 36 hours post-infection (P.I.), beside 2 , 3, 4, 5, 6, 7,10 and 15 days P.I. The lungs were fixed in neurtal buffered formalin. Five microns thick paraffin sections were prepared, stained with immunoperoxidase beside hematoxylin and eosin and examined microscopically.

Specific histopathological lesions were detected in the lung tissues, 24 hours post-infection (P.I.). Necrotic changes and eosinophilic intranuclear inclusion bodies were observed in the lining epithelium of the bronchioles and the alveolar pneumocytes. The maximum number of the inclusion bodies was seen at 2 days P.I. while they were absent at 5 days P.I. Moreover bronchiolitis and adjacent alveolitis beside focal thickening of interalveolar septa together with acute and chronic inflammatory cells were detected, 2 days P.I. The indirect immunoperoxidase technique showed that the majority of EHV-1 antigen positive cells was detected in the bronchiolar epithelium at 36 hours and 2 days P.I. A few antigen was also detected in the alveolar pneumocytes indicating that these cells are also a target for EHV-1. EHV-1 antigen was

mainly detected in the nucleus and cytoplasm of the bronchiolar epithelial cells. Some bronchial epithelial cells exhibited a positive reaction on the plasma membrane.

INTRODUCTION

Equine herpesvirus type 1(EHV-1) is a major cause of respiratory disease abortion prenatal mortality and occasionally neurological signs in horses (Allen and Bryans 1986; Crabb and Studdert 1995). The highly contagious respiratory transmission of EHV-1 resulted in disastrous outbreaks of the disease in domestic horse populations with a significant economic impact on the equine industry. A murine model of respiratory EHV-1 disease which closely mimicked many of the features of natural host was established in various strains of mice (Awan et al.1990). Most commonly BALB/c mice have been used since other strains of mice were found to be less susceptible to infection (Awan et al. 1990; Walker et al. 1998b).

The infectious virus could be readily isolated from the nasal turbinate trachea lungs olfactory bulbs brain and eyes of the EHV-1-infected mice(Awan et al..1990 Inazu et al. 1993 Csellner et al.1995 Baxi et al.1996 Marshall and Field1997). In addition immunocytochemical studies demonstrated viral antigen in lung and nasal tissues (Baxi et al. 1996). However detailed and sequential histopathologic studies of lung lesions after infection were not well known. Therefore this study was carried out to demonstrate the histopathological changes and distribution of antigens in the pulmonary tissues of BALB/c mice experimentally infected with EHV-1.

MATERIALS AND METHODS

Experimental procedure

Seventy-seven male BALB/c mice (Japan Charles River Co. Japan) were used in this study. Fifty-five animals were intranasally infected with approximately 5X106 plaque-forming units of EHV-1 strain 89C25 (Matsumura et al; 1998) under anaesthesia by intraperitoneal injection of a mixture of ketamine (Sanko Japan) and xylazine (Bayer Germany). The other twenty-two mice were used as a control and intranasally inoculated with Dulbecco's modified Eagle's medium under the effect of the same anaesthesia. Groups of 5 animals from the infected mice and 2 animals from the control mice were scarificed at 12 24 36 hours and 2 3 4 5 6 710 and 15 days post infection (p.i.). After necropsy the lungs were fixed in neutral buffered formalin

(pH7.4). Five micron thick paraffin sections were prepared and stained with hematoxylin and eosin.

Indirect immunoperoxidase technique

Paraffin sections were used for indirect immunoperoxidase staining. The endogenous peroxidase was eliminated with 0.5% H2O2 in methanol and then tissues were incubated with the serum from EHV-1 -infected horse (dilution to 1:10) as a primary antibody for 40 minutes. After washing by phosphate-buffered saline (PBS; pH7.4) the sections were incubated with biotin-conjugated anti-horse immunogloblins as a secondary antibody for 30 minutes. Peroxidase activity was detected by staining in a chromogenic substrate solution for 10 minutes (ZymedCaliforniaUSA). The sections were washed in distilled water and slides were counter-stained with hematoxylin. All reactions were carried out at room temperature. Specificity of the reaction was confirmed by EHV-1 -infected horse sera absorbed with formalin-fixed EHV-1 -infected MDBK cells resulting in negative reaction. Also the reaction without a primary antibody served as a negative control.

Evaluation of histopathology and immunoperoxidase for antigen detection

Histopathological changes specific for herpesvirus and immunoperoxid-ase results of antigen detection were evaluated (Scored: 0 = no lesion or no antigen, 1= mild or few, 2= moderate or medium, 3= severe or frequent, 4= very severe or more frequent) and each point represented the mean.

RESULTS

Histopathology

The peribronchiolar arterioles and interalveolar capillaries of the lung were dilated and filled with blood. Slight neutrophilic infiltration was seen in the interalveolar septa at 12 hours P.I. The lining epithelium of the bronchioles particularly the ciliated epithelial cells revealed intranuclear inclusion bodies. These inclusion bodies were small eosinophilic and surrounded by a halo although in some cases they were large enough to replace most of the internal structure of the nucleus (Fig. 1). Margination of the chromatin was also associated with these inclusions; but, enlargement ot the nucleus was rarely detected. Necrotic changes were also seen in the bronchiolar epithelium particularly karyorrhexis of the nuclear chromatin. Moderate leukocytic cellular infiltrations mainly neutrophils. were noticed

around some bronchioles. Moreover slight thickenings of the interalveolar septa with neutrophilic infiltration were also detected at 24 hours P.I.

At 36 hours and 2 days P.I. the inclusion bodies reached their maximum number in the bronchiolar epithelial cells (Fig. 11). Moreover eosinophilic intranuclear inclusion bodies were seen in the alveolar epithelial cells (Fig.2). Necrotic epithelial cells and cellular debris were frequently observed in the lumen of the bronchioles with peribronchiolar infiltration of neutrophils and some lymphocytes (Fig. 3). The interalveolar septa were thickened by infiltration of neutrophils with some lymphocytes. Moreover the alveolar epithelial cells showed focal necrosis and some alveoli were consolidated with leukocytic infiltration.

At 3 and 4 days P.I. severe bronchiolitis was encountered. The bronchioles showed necrosis and desquamation of the lining epithelium with congested blood vessels and peribronchiolar leukocytic infiltration mostly with lymphocytes and some neutrophils (Fig.4). Hyperplasia of the bronchiolar epithelium with some mitotic figures suggesting regenerativ process was observed in many cases. In some cases the lumens of the bronchioles were completely obliterated with desquamated and hyperplastic epithelial cells mixed with inflammatory cells (Fig. 5). The inclusion bodies were still detected in the bronchiolar epithelium but fewer in number. Focal alveolitis and thickening of the interalveolar septa were found. Moreover perivascular lymphocytic aggregations were detected.

At 5 and 6 days P.I. the bronchiolar histopathological lesions were less severe than at 4 days P.I.The epithelial cells showed mild hyperplastic and necrotic changes with peribronchiolar lymphocytes. Meanwhile the intranuclear inclusion bodies were not detected in the bronchioler epithelium.

Perivascular mononuclear cell infiltration was seen. Moreover thickening of the interalveolar septa and consolidation of some alveoli with mononuclear cell Infiltration were still visible (Fig.6)

At 7 and 10 days P.I. the peribronchial and perivascular mononuclear cell infilteration was reduced. Mild hyperplastic changes were still observed in the epithelium of bronchioles but some bronchioles appeared normal. Slight thickening of the interalveolar septa with mononuclear cell infiltration was seen in a few cases.

At 15 days P.I. large areas of the lungs appeared normal where a

few mononuclear cells were distributed sporadically around bronchioles and blood vessels and in the interalveolar septa. No histopathological lesions were detected in the control group.

Indirect immunoperoxidase staining

The lining epithelium of the bronchioles showed positive reaction as early as 12 hours (Fig. 11). EHV-1 antigens were commonly detected in the nucleus and cytoplasm of the bronchiolar epithelial cells. Many cells revealed only nuclear reaction and few cells showed a slight cytoplasmic reaction (Fig.7). Moreover the cell mebrane of some ciliated epithelial cells exhibited a positive reaction. Cells staining positive for EHV-1 were frequently observed in the lumens of the bronchioles and in some cases they completely obliterated the lumens of small bronchioles (Fig. 8). The majority of these antigen-positive cells was seen at 36 hours and 2 days P.I. (Fig.9). Meanwhile few positive cells were still detected until 6 days P.I. Moreover some of the alveolar epithelial cells were stained positive for viral antigen (Fig. 10). No positive staining was observed in any of the uninfected control group.

DISCUSSION

The present study revealed that after intranasal inoculation of mice with EHV-1 specific histopathological changes were detected in the lungs as early as 24 hours P.I. Necrotic changes and eosinophilic intranuclear inclusion bodies were noticed in the lining epithelial cells of the bronchioles. The maximum number of the inclusion bodies was detected 2 days P.I. whereas they could not be detected at the 5th day P.I. These results are consistent with the result of indirect immunoperoxidase staining where the EHV-1 antigens were demonstrated in the bronchioler epithelial cells at 12 hours P.I.and peaked at 36 hours and 2 days P.I. These findings indicate rapid dissemination of EHV-1 throughout the respiratory tract. Similar results were previously reported in horses (Kydd et al. 1994; Sutton et al. 1998) and in mice (Awan et al. 1990; Baxi et al. 1996; Csellner et al. 1998). Since the number of antigen-positive cells were in compliance with the appearance of inclusion bodies the indirect immunoperoxidase could be considered a sensitive test for the detection of the time suitable for the appearance of viral inclusion bodies particularly of herpesvirus infection. Of particular interest was the detection of intranuclear inclusion bodies in the

pneumocytes. In addition the intranuclear inclusion bodies in the alveolar epithelium were detected. In a previous electron-microscopic study in mice where the virus replication was detected in the ciliated epithelial cells of the bronchiole and types I and II pneumocytes (Awan et al. 1990). These observations together with our present results could give an explanation for the early necrotic changes detected in the bronchioler and alveolar epithelium (Awan et al. 1990; Field & Awan1990; Csellner et al.1995; Van Woensel et al.1995; Bartles et al..1998; Walker et al.1998a).

The present study revealed bronchiolitis and focal thickening of the interalveolar septa due to mixed cellular infiltration which were advanced at 3 and 4 days P.I. Infiltration of neutrophils preceded the lymphocyte infiltration as reported previously in horses (Allen and Bryans1986; Kydd et al.1994) and mice Walker et al.1999). The recruitment of neutropils in the early stage of EHV-1 infection suggests the removal of necrotic bronchioler and alveolar epithelium . We think the former hypothesis may be more likely than the latter though farther study is needed to clarify this point. The severity of the bronchiole lesions that were reduced at 5 and 6 days P.I. is in harmony with our immunoperoxidase results since few antigen-positive cells were still detected at this time but its number was few. It was reported that the virus clearance from mouse lungs following primary EHV-1 infection takes from 5 to 12 days P.I. depending on the administered dose and infective viral strain (Awan et al. 1990; Azmi and Field 1993; Inazu et al. 1993; Siater et. al. 1993; Tewari et al. 1994; Alber et al. 1995; Csellner et al. 1995; Walker et al. 1998).

EHV-1 antigens were commonly detected inside the nuclei of the bronchioler epithelial cells which are the main site of virus replication. Interestingly some cells revealed both nuclear and cytoplasmic reaction indicating damage of the nuclear membrane with dissemination of viral particles into the cytoplasm or a stage of protein synthesis preparatory to invasion of the nucleus. Also antigens on epithelial cell surface may be due to attachment of extracellular free virus. Pathogenesis in such virus-epithelial cell interaction awaits further experimental studies.

ACKNOWLEDGEMENTS

We thank Dr. Matsumura (Japan Racing Association Japan) for kindly providing the EHV-1 89c25 strain and anti-EHV-1 horse serum.

REFERENCES

Alber D.G., Greensill J., Killington R.A and Stokes A,(1995): Role of T-cells virus neutralizing antibodies and complement-mediated antibody lysis in the immune response against equine herpesvirus type-I (EHV-1) infection of C3H (H-2k) and BALB/C (H-2d) mice. Res Vet Sci 59, 205-213.

Allen G.P., and Bryans J.T., (1986): Molecular epizootiology, pathogenesis, and prophylaxis of equine herpesvirus-1 injections. Prog Vet Microbiol. Immunol. 2, 78-144.

Awan A.R., Chong Y.C.,and Field N.J., (1990): The pathogenesis of equine herpesvirus type 1 in the mouse: a new model for studying host responses to the infection. J. Gen Virol 71, 1131-1140.

Azmi M., andField H.J., (1993): Interactions between equine herpesvirus type 1 and equine herpesvirus type 4: T cell responses in a murine infection model. J. Gen Virol 74, 2339-2345.

Bartels T., Steinbach F., Hahn G., Ludwig H., and Borchers K., (1998): In situ study on the pathogenesis and immune reaction of equine herpesvirus type 1 (EHV-1) infections in mice. Immunology 93, 329-324.

Baxi M.K., Borchers K., Bartels T., Schellenbach A., Baxi S., and Field H.J., (1996): Molecular studies of the acute infection latency and reactivation of equine herpesvirus-1 (EHV-1) in the mouse model. Virus Res. 40, 33-45.

Colle C. F. 3rd, Tarbet E.B., Grafton W.D., Jennings. S.R., and O'Callaghan D.J., (1996): Equine herpesvirus-1 strain KyA a candidate vaccine strain reduces viral titers in mice challenged with a pathogenic strain RacL. Virus Res 43,111-124.

Crabb B.S., and Studdert M.J., (1995): Equine herpesviruses 4 (equine rhino-preumonitis virus) and 1 (equine abortion virus). Adv. Virus Res 45, 153-190.

Csellner H., Walker C., Love D.N., and Whalley. J.M., (1998): An equine herpesvirus 1 mutant with a lacZ insertion between open reading frames 62 and 63 is replication competent and causes disease in the murine respiratory model. Arch Virol 143, 2215-31.

Csellner H., Whalley J.M., andLove D.N., (1995): Equine herpesvirus 1 HVS2SA isolated from an aborted foetus produces disease in BALB/c mice. Aust Vet J. 72, 68-69.

Field H.J., and Awan AR., (1990): Effective chemotherapy of equine herpesvirus I by

phosphonylmethoxyalkyl derivatives of adenine demonstrated in a novel murine model for the disease. Antimicrob Agents Chemother. 34, 709-717.

Inazu M., Tsuha 0., Kirisawa R., Kawakami Y., and Iwai H., (1993): Equid herpesvirus infection in mice. J. Vet Med Sci 55,119-121

Kydd J.H., Smith K.C., Hannant D., Livesay G.J., and Mumford J.A., (1994): Distribution of equid herpesvirus-l (EHV-1) in the respiratory tract of ponies: implications for vaccination strategies. Equine Vet J 26, 466-469.

Marshall K.R., and Field H.J., (1997): Demonstration of equine herpesvirus-l neuronal latency in murine olfactory bulbs using a novel combined in situ PCR and protein synthesis method. Virology 229, 279-282.

Marshall K.R., SunY., Brown S.M., and Field H.J., (1997): An equine herpesvirus-1 gene 71 deletant is attenuated and elicits a protective immune response in mice. Virology 23,120-27.

Matsumura T., Kondo T., Sugita. S., Damiani A.M ,O' Challaghan. Đ.J. , and Imagawa. H., (1998): An equine herpesvirus type 1 recombinant with a deletion in the gE and gl genes is avirulent in young horses. Virology 242, 68-79.

Schultheiss P.C., Collins. J.K., and Carman J., (1993): Use of an immunoperoxidase technique to detect equine herpesvirus-l antigen in formalin-fixed paraffin-embedded equine fetal tissues. J Vet Diagn invest 5, 12-15.

Siater J.D., Gibson J.S., and Field H.J., (1993): Pathogenicity of a thymidine kinase deficient mutant of equine herpesvirus 1 in mice and specific pathogen-free foals. J Gen Virol 74, 819-828.

Sutton. G.A., Viel L., Carman P.S., and Boag B.L., (1998): Pathogenesis and clinical signs of equine herpesvirus-1 in experimentally infected ponies in vivo. Can J Vet Res 62, 49-55.

Tewari D., Whalley .J.M., Love D.N., and Field H.J. ,(1994): Characterization of immune responses to baculovirus-expressed equine herpesvirus type 1 glycoproteins D and H in a murine model. J Gen Virol 75;1735-1741.

Van Woensel P.A., Goovaerts D., Markx D., andVisser N., (1995): A mouse model for tes-ting the pathogenicity of equine herpesvirus-l strains. J Virol Methods 54, 39-49

Walker C., Love D.N., and Whalley J.M., (1998 a): Comparison of the pathogenesis of acute equine herpesvirus 1 (EHV-1) infection in the horse and the

mouse model: a review. Vet Microbiol 68, 3-13.

Walker C., Packiarajah P., Gilkerson J.R., Love D.N., and Whalley J.M., (1998b): Primary and challenge infection of mice with equine herpesvirus 1 strain HSV25A. Virus Res 57, 151-162.

Walker C., Perotti. V.M., Love D.N., and Whalley. J.M., (1999): Infection with equine herpesvirusl (EHV-1) strain HVS25A in pregnant mice. J Comp Pathol 120, 15-27.

Whitwell K.E., Gower. S.M., and Smith. K.C., (1992): An immunoperoxidase method applied to the diagnosis of equine herpesvirus abortion using conventional and rapid microwave techniques. Equine Vet J 24,10-12.

List of figures

Fig.1. An eosinophilic intranuclear inclusion body in the bronchiolar epithelial cell at 2 days p.i. HE. x400.

Fig.2. An eosinophilic intranuclear inclusion body in the alveolar epithelium at 2 days P.I. H&E. xl 000.

Fig.3. Bronchiole showing necrotic epithelial cells and cellular debris in the lumen with peribroncbiolar neutrophil infiltration at 2 days P.I. H&E., x400

Fig.4. Desquamation of the bronchiolar epithelium and severe peribronchiolar infiltration with lymphocytes and neutrophils at 3 days P.I. H&E., X 200.

Fig.5. Complete obliteration of the lumen of bronchioles by desquamated and hyperplastic epithelial cells mixed with inflammatory cells. H&E., x200.

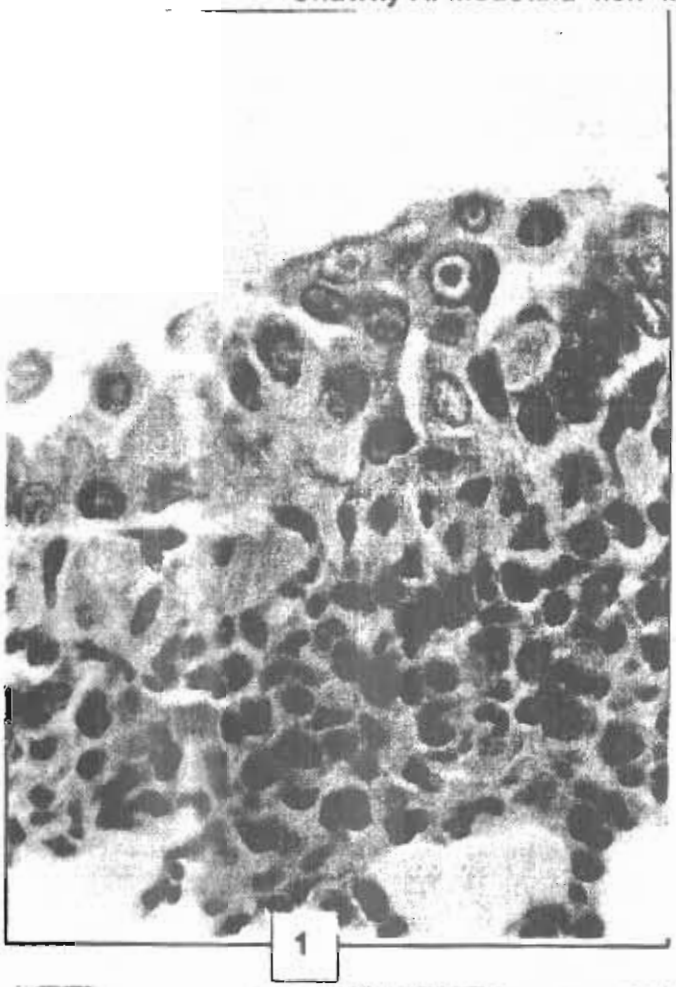
Fig.6. Thickening of the interalveolar septa and consolidation of some alveoli with lymphocytic infiltration at 5 days P.I.H&E., x200.

Fig.7 Bronchiolar epithelial cells showing positive staining for EHV-I antigens with a distinct red pigmentation of the nucleus and cytoplasm. Indirect immuno-peroxidase at 2 days P.I. Indirect immunoperoxidase. x400

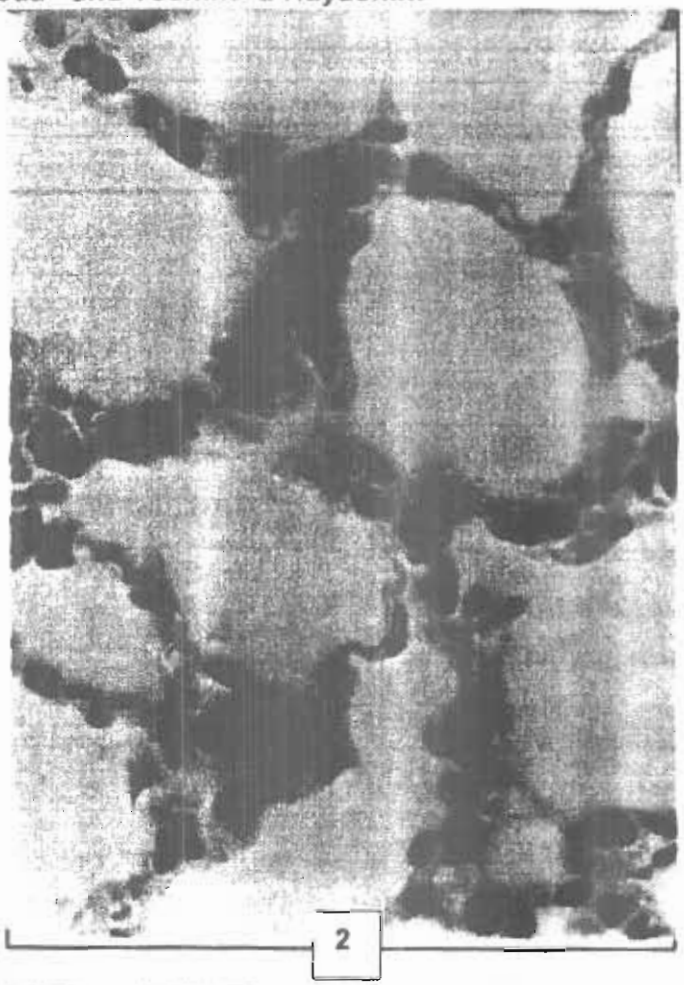
Fig.8 EHV-1 antigen-positive cells completely obstruct the lumen of a small bronchiole at 2 days P.I.Indirect immunoperoxidase. x400

Fig.9 Bronchioles showing a large number of the lining epithelial cells positive for EHV-1 antigen at 2 days P.I. Indirect immunoperoxidase. x200

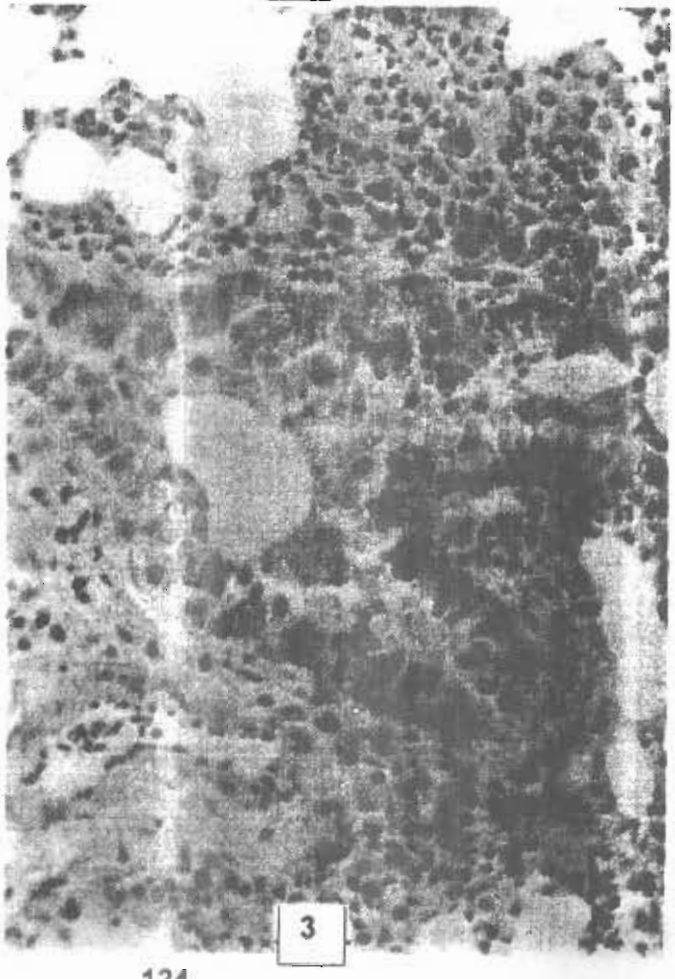
Fig.10 Two alveolar epithelial cells are positive for viral antigen at 2 days P.I. Indirect immunoperoxidase. x400



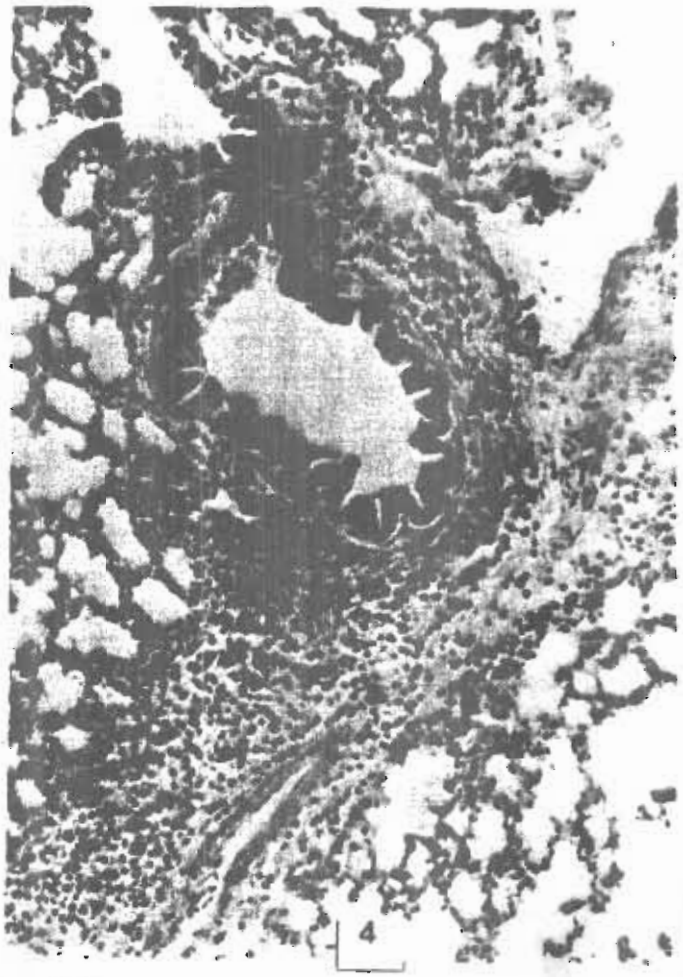
1



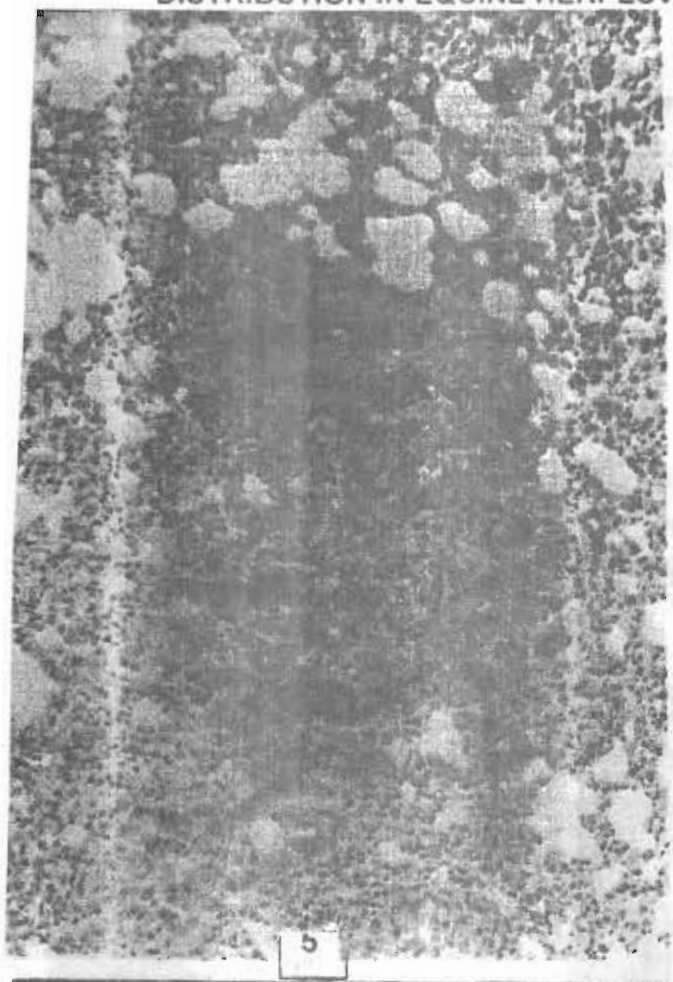
2

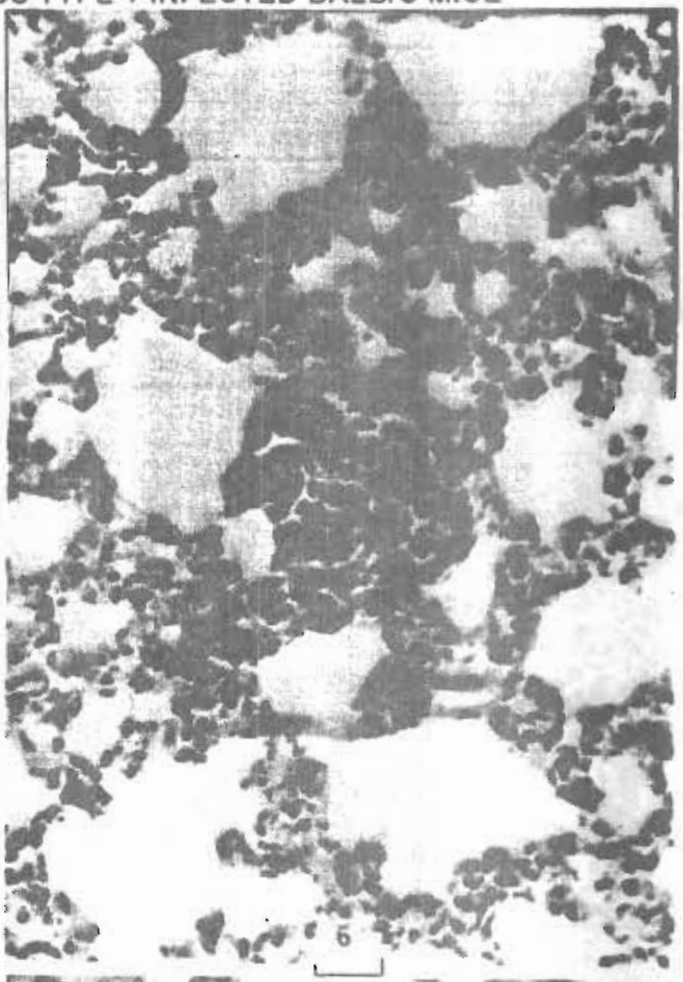


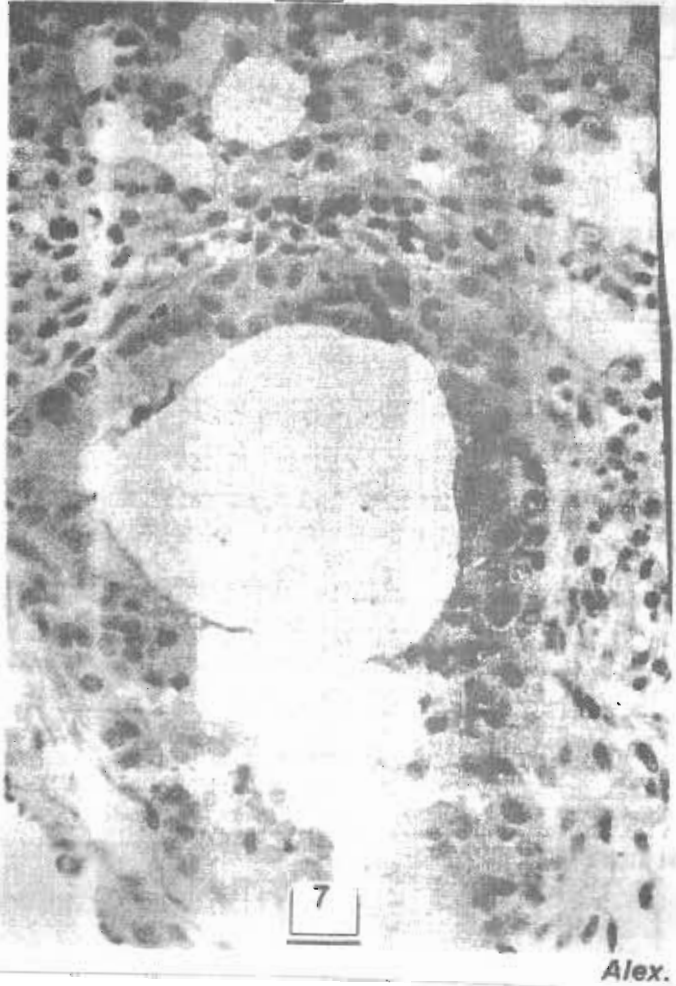
3

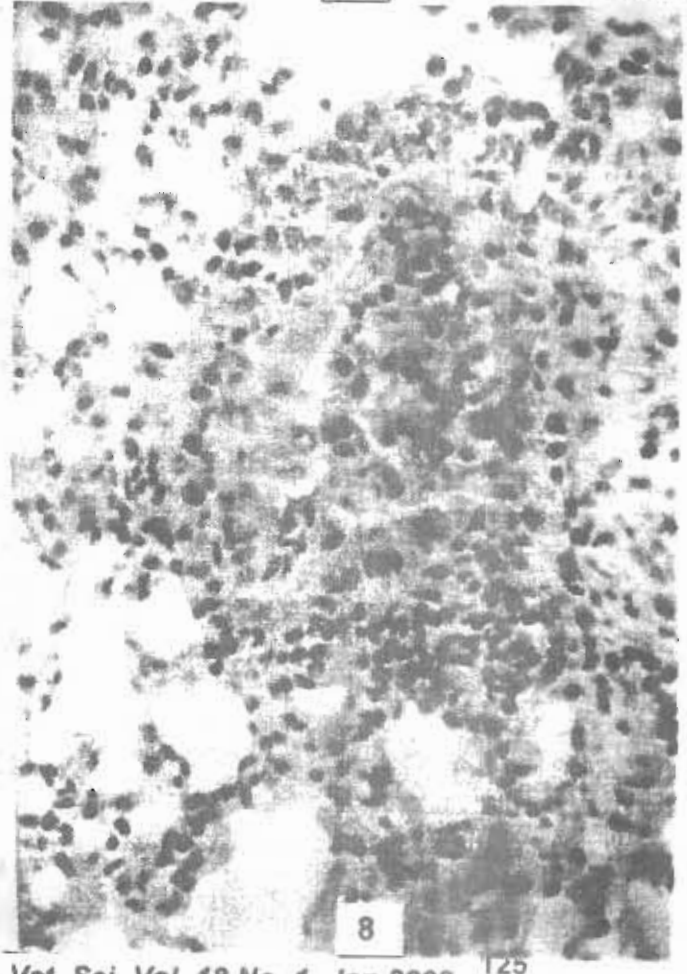


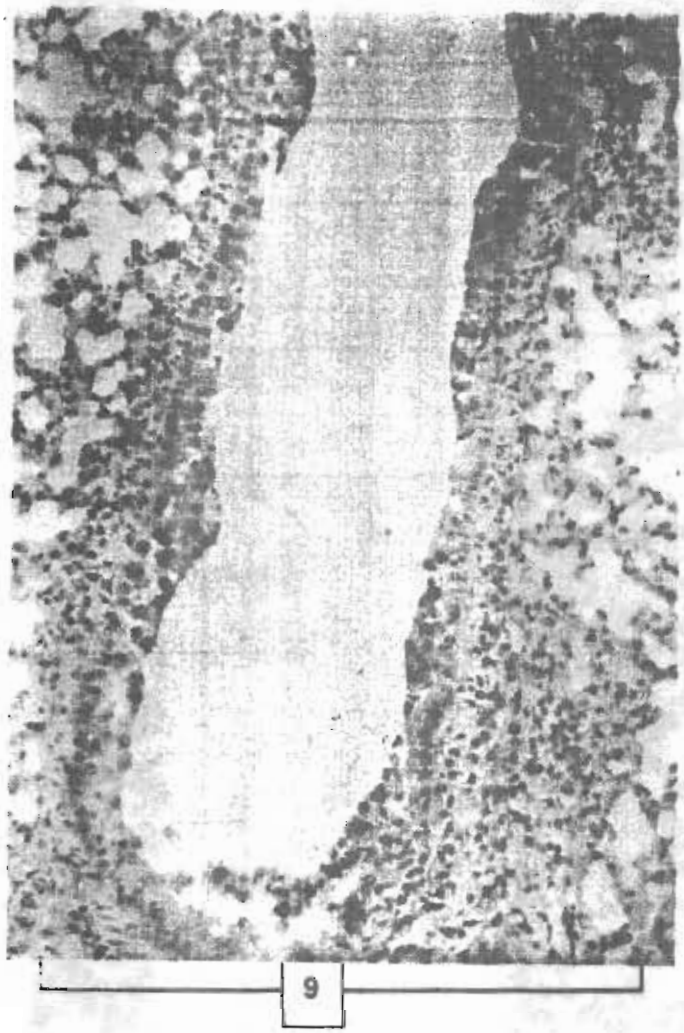
4


5

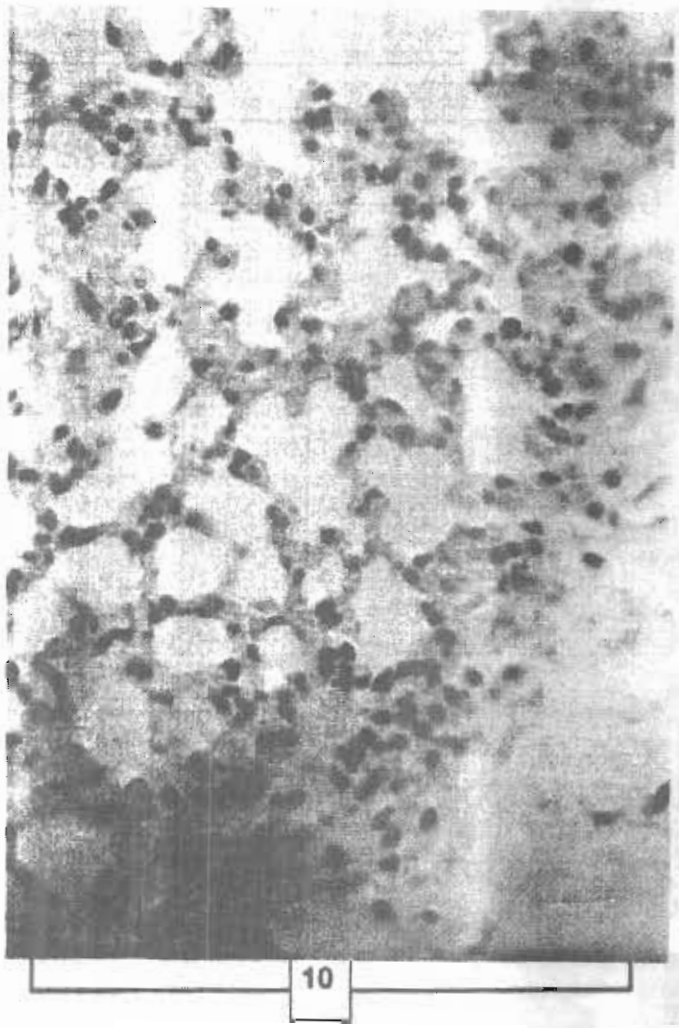

6


7


8



9



10

Fig.11 :Mean histological scores in the lungs of mice following EHV-1 infection