

AN OUTBREAK OF ABORTION ASSOCIATED WITH EQUINE RHINOPNEUMONITIS VIRAL INFECTION IN IRAN.

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Introduction.

The previous studies on existence and distribution of equine rhinopneumonitis virus (ERV) infection in soliped animals in Iran suggested that the infection was widespread among equine population throughout the country(6,7). The present communication reports an outbreak of abortion in mares due to ERV and describes the results of tests undertaken to characterize the isolated virus.

Materials and Methods.

Source of materials. – In January 1972 an outbreak of abortion was reported among mares at Army Animal Husbandry and Remount Section in Varamin. An 8½ month aborted foetus was submitted to Razi Institute for laboratory diagnosis. The foetus was posted and small pieces from the lung, liver, heart, and spleen tissues were obtained and stored both at – 70°C., for virus isolation, and in 10% formalin saline solution, for histopathological examination.

Virus isolation. – A 10% suspension of each of the specimens in a ELY medium (Earle's balanced salt solution, lactalbumin hydrolysate, and yeast extract) containing 200 units of penicillin, 200/μg. of streptomycin per milliliter, was made. The suspension was centrifuged at 10,000 r.p.m. for 30 minutes and supernatant fluid was used for virus isolation by inoculating hamster and primary calf kidney cell culture.

One day-old and 1–3 day-old hamsters were inoculated with 0.5 ml (intraperitoneally) and 0.025 ml. (intracerebrally), of the inoculum, respectively (4,8). The animals were closely observed for 10 days. The liver of intraperitoneally inoculated hamsters and the brain of those inoculated intracerebrally, after producing illness or developing nervous disorder, were harvested for further passages.

For isolation of virus in primary calf kidney cell cultures, 0.2 ml. of inoculum was inoculated into each of 4 cell culture tubes. The inoculated cells were examined microscopically everyday for the appearance of cytopathic effect (CPE). Further passages were made in the usual manner, using primary or secondary monolayers of calf, horse, dog, rabbit, or pig kidney cell cultures.

Histological studies. – Fixed tissues were processed by paraffin embedding method. The embedded tissues were cut 5-micron in thickness by microtom. The prepared sections were stained with Harris haematoxylin and eosin and examined under the microscope.

Cell cultures. – Primary monolayer cell cultures were prepared by trypsinization method of Dulbecco and Vogt as modified by Youngner (11). Cells were grown in a ELY medium containing 10% inactivated calf serum, 100 units of penicillin, and 100/ μ g. of streptomycin/ml.

Secondary cultures of cells were prepared by adding 0.25% trypsin solution to monolayers of cell cultures and growing the obtained cell suspension in the culture growth medium.

Equine rhinopneumonitis virus. Equine rhinopneumonitis virus, strain L., (2) at its 8th passage level in bovine kidney cells was supplied by Dr. G.F.de Boer, from The Central Veterinary Institute, Amsterdam.

Antiserum preparation. – Rabbits were used for the production of antiserum against the virus strains. The animals received 10 intravenous injections of 10 ml. of cell culture propagated virus. The serum was collected 10 days after the last inoculation and kept at -20°C . until required.

Neutralization tests. – 0.3 ml. of each of serial 10 fold dilutions of the virus was mixed with an equal volume of inactivated antiserum. For the control, normal rabbit serum was mixed with the virus dilutions. The virus-serum mixtures were incubated for 2 hours. Then each mixture was tested for virus infectivity by inoculating 4 cell culture tubes per mixture. The extent of neutralization was expressed as neutralization Log-Index which is the difference between the virus titres of virus serum mixtures and the control series.

Ether sensitivity test. – To the virus suspension diethyl ether was added at the rate of 20 per cent(1). The mixture was held in a screw-capped bottle. The bottle was kept at 4°C . for 18 hours and then the ether was removed by pouring the treated virus suspension into an uncovered sterile Petri dish and leaving at room temperature for 15 minutes. Stock virus suspension diluted with ELY instead of ether was handled in a similar manner and served as control.

The infectivity of both ether treated virus suspension and the control was titrated in horse kidney cell culture.

Chloroform sensitivity test. – To 4 ml. of stock virus suspension in a

screw-capped centrifuge tube 0.2 ml. of chloroform was added. The resulting emulsion was gently mixed for 10 to 15 minutes and then was centrifuged at 500 r.p.m. for 5 minutes. The clear part of the supernatant was removed and tested for infectivity as before. 4 ml. of virus suspension, without chloroform was similarly treated and used as control (5).

Sodium desoxycholate sensitivity test. – Stock virus suspension, after being centrifuged at 10,000 r.p.m. for 60 minutes, was mixed with equal volume of a 1 in 500 solution of sodium desoxycholate in PBS containing 0.75% bovine albumine, The mixture was shaken and then incubated at 37°C. for 60 minutes. Virus suspension with equal volume of PBS with 0.75% bovine albumine, instead of sodium desoxycholate solution, was treated in a similar manner and was used as control. Infectivity titration of the treated virus suspension and the control were performed as above(9).

Cytological changes. – Cells grown on coverslip in Leighton tubes, were infected, and then, at appropriate intervals the coverslips were fixed with Carnoy fixative and stained with Harris haematoxylin and eosin stains before being studied microscopically for cytologic changes.

Growth curves. – Sufficient cell culture tubes were infected each with 0.2 ml. of the virus. An adsorption period of 2 hours at 37°C. was allowed and then cell sheet of each tube was washed 3 times each time with 2ml. of ELY to remove free virus. 1.5 ml. of maintenance medium was added to each tube and they were reincubated. At varying intervals pooled fluid of 4 cell culture tubes was centrifuged at 3000 r.p.m. for 15 minutes and the supernatant was tested for virus content. The infectivity titration was performed in cell culture tubes.

Results.

Pathological changes. Severe subcutaneous and intramuscular gelatinous oedema was noted in mandibular space and pharyngeal region. The oedema was extended down in the front part of the neck, thorax, and the chest of the aborted foetus.

Lungs were moderately congested. Some subpleural petechial and echymatic haemorrhages were present in diaphragmatic lobes of the lungs. An excessive amount of clear yellow straw coloured fluid was found in the pleural cavity.

Liver was enlarged, swollen and congested. Numerous pinpoint subcapsular grayish necrotic foci were seen in the liver. No significant gross pathological changes were detected in any other organ.

Histological sections of the liver revealed severe congestion. Central

lobular veins and sinusoids were filled with red blood cells. There were some focal haemorrhages in liver paranchyma. Numerous focal disseminated necrotic foci were present in liver tissues. The foci were sharply demarkated and consisted of coagulative type of necrosis with mononuclear cells infiltration (Fig. 1). Hepatic cells which surrounding the necrotic foci contained small eosinophilic intranuclear inclusion bodies. Most of the nuclei containing inclusion bodies were in normal size and chromatin margination were not present, but in some cells large eosinophilic inclusion bodies with distinct clear halo and nuclear chromatin margination was noted (Fig. 2).

Lungs were showing some degree of congestion with severe intralobular and alveolar oedema. There were focal disseminated necrotic area which were surrounded by healthy lung tissues. The necrosis was of coagulative type with abundant nuclei debris. Bronchiols were compact and filled with mass of necrotic cells and debris. Most of the bronchial epithelial cells were eroded and sloughed into the lumen. Fairly large eosinophilic intranuclear inclusion bodies were seen in the bronchial epithelial cells. The inclusions in lung cells, however, were larger and more distinct than those ones in liver cells (Fig. 3).

Isolation and properties of the virus. – 36 to 48 hours after inoculation of primary calf kidney cell cultures with lung, liver, and heart tissues from aborted foetus, a cytopathic changes was observed which was reproduced more evidently in further serial passages in the same cell culture.

The virus, subsequently named Varamin strain, was also isolated following inoculation of the same materials in suckling hamsters. Hamsters inoculated intracerebrally developed a fatal encephalitis 4 days after inoculation. The mortality among hamsters inoculated intraperitoneally began on 7th day following inoculation.

The isolated virus was passaged serially in primary calf kidney cell cultures and was used at the 5th passage level for identification of its properties.

The isolate grew readily in cell monolayers of horse, calf, sheep, rabbit, dog, and pig kidney producing similar cytologic changes in infected cells. The first noticeable changes consisting of appearance of rounded and refractile cells on different foci of the cell sheet, was noted 24 to 48 hours following inoculation. The infection spread rapidly and affected the whole cell sheet which resulted to the complete destruction of the cell monolayer within 1 or 2 days (Fig. 4). The rounded cells have a great tendency to aggregate and pile up like bunches of grapes. The virus produced some syncytia and various stage of cytoplasmic and nuclear degeneration in infected cells. Acidophilic intranuclear inclusion bodies, typical of herpes virus infection, were also observed in infected cells (Fig. 5,6).

The growth curves of the virus, at its 5th passage level in calf kidney cells, was determined in calf kidney and horse kidney cell cultures (Fig. 7). The

growth of the virus in both cells were essentially identical to each other. There was a 7 hour latent period before newly formed infective virus appeared in the medium, and the maximum titer of $10^{6.5}$ TCID₅₀/ml. Occured 32 hours after infection.

The virus was found highly sensitive to ether, chloroform, and sodium desoxycholate (Table 1.) Antigenic comparison of the new isolate and equine rhinopneumonitis virus strain L., showed that the two strains were identical, antiserum produced in rabbit against each strain neutralized both strains to a similar extent (Table 2).

Discussion.

Equine rhinopneumonitis virus has been reported, as the cause of a mild or acute respiratory disease and also as the cause of abortion in equine animals, from all continents.

In Iran, a serological survey on 573 horses, 34 donkeys, and 114 mules indicated that the virus infection is extensively disseminated among equine population throughout the country (7). Isolation of the virus from kidney culture of a still-borne foal in extremis (6), confirmed the above finding and emphasized the need of an investigation on the occurrence of the abortion associated with equine rhinopneumonitis among Iranian mares.

An outbreak of abortion offered a good opportunity for such investigation. The abortion was diagnosed to be produced as the result of rhinopneumonitis virus infection both by observing the specific lesions in the aborted foetus and by virus isolation.

The histopathologic changes in foetal tissues resembled those found typical of rhinopneumonitis infection (3,10). Isolated virus was also identified as equine rhinopneumonitis virus on the bases of its cultural properties, lability to organic solvents, and antigenic similarity to a known strain of equine rhinopneumonitis virus.

Calf kidney cell culture and suckling hamsters were shown to be suitable host systems for virus isolation. Intracerebral inoculation was found a better route of infection in hamster, as it was first reported by Kawakami et al. (8).

Summary.

This is the first outbreak of equine viral abortion ever been reported among Iranian mares. The diagnosis was supported by the necroscopy findings in the aborted foetus and by the virus isolation.

The virus, designated Varamin strain, was identified as equine rhinopneumonitis virus on the bases of its cytopathogenicity to different cell cultures, lability to lipid solvents, and its antigenic similarity to a known strain of equine rhinopneumonitis virus.

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References.

- 1) Andrewes, C.H., and Horstmann, D.M. : The susceptibility of viruses to ethyl ether. *J. Gen. Microbiol.*, 3, (1949): 290-297.
- 2) De Boer, G.F. : Equine rhinopneumonitis virus in Dutch horses. *Proc. 1st. int. Equine Infec. Dis. Stresa*, (1966): 101-111.
- 3) Dimock, W.W., and Edwards, P.R.: The differential diagnosis of equine abortion with special reference to hitherto undescribed form of epizootic abortion of mares. *Cornell Vet.* 26, (1936): 231-240.
- 4) Doll, E.R., Richards, M.G., and Wallace, M.E. : Adaptation of equine abortion virus to suckling Syrian hamster. *Cornell Vet.* 43, (1953) : 551-558.
- 5) Feldman, H.A., and Wang, S.S. : Susceptibility of various viruses to chloroform. *Proc. Soc. Expl. Biol. and Med.*, 106, (1961): 736-738.
- 6) Hazrati, A. : First isolation of equine rhinopneumonitis virus in Iran. *Arch. Inst. Razi*, 25, (1973): 9-10.
- 7) Hazrati, A., and Dayhim, F. : Serological survey on infection with equine rhinopneumonitis virus among soliped animals in Iran. *Veterinary Rec.* 93, (1973): 70-72.
- 8) Kawakami, Y., Nakano, K., Tokui, T., Inui, S., and Murase, N. : Experiments of adaptation to hamsters of equine rhinopneumonitis virus by intracerebral inoculation. *Bull. Nat. Inst. Anim. Hlth.* 61, (1970): 24-28.
- 9) Theiler, M. : Action of sodium desoxycholate on arthropod borne viruses. *Proc. Soc. Expl. Biol. & Med.* 96, (1957): 380-382.
- 10) Westerfield, C., and Dimock, W.W. : The pathology of equine virus abortion. *J. Am. Vet. Med. Ass.* 109, (1946): 101-111.
- 11) Youngner, J.S. : Monolayer tissue culture. 1. Preparation and standardization of suspensions of trypsin-dispersed monkey kidney cells. *Proc. Soc. Expl. Med. N.Y.* 85, (1954): 202-206.

Table 1. Sensitivity of Varamin strain (Equine rhinopneumonitis virus) to lipid solvents.

	-Log. virus suspension dilution.					
	0	1	2	3	4	5
Control virus suspension	+	+	+	+	+	±
Virus treated with ether	0	0	0	0	0	0
Virus treated with chloroform	0	0	0	0	0	0
Virus treated with sodium desoxycholate	0	0	0	0	0	0

+ = Virus positive.

0 = Virus negative.

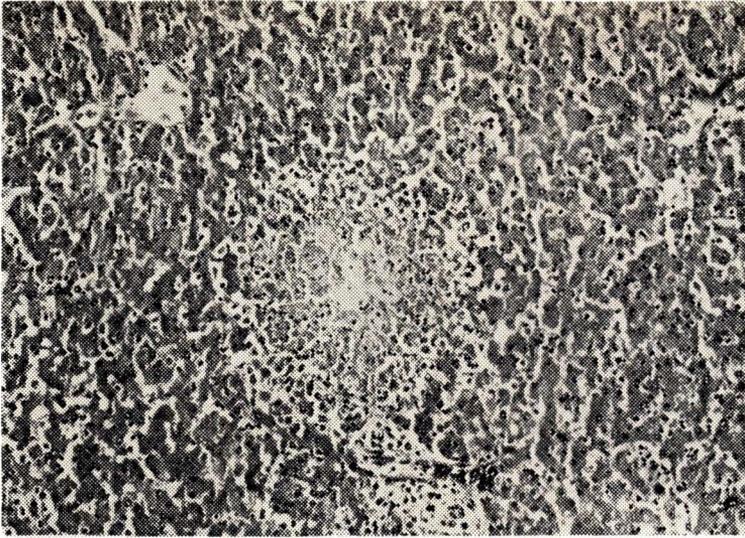
Table 2. Cross neutralization tests between strain Varamin and strain L. of equine rhinopneumonitis virus.

	-Log. virus suspension dilution					
	0	1	2	3	4	5
Varamin strain + Normal serum	+	+	+	+	+	+
Varamin strain + Varamin antiserum	0	0	0	0	0	0
Varamin strain + Strain L. antiserum	0	0	0	0	0	0
Strain L. + Normal serum	+	+	+	+	+	+
Strain L. + Strain L. antiserum	0	0	0	0	0	0
Strain L. + Varamin antiserum	0	0	0	0	0	0

+ = Virus positive.

0 = Virus negative.

1



2

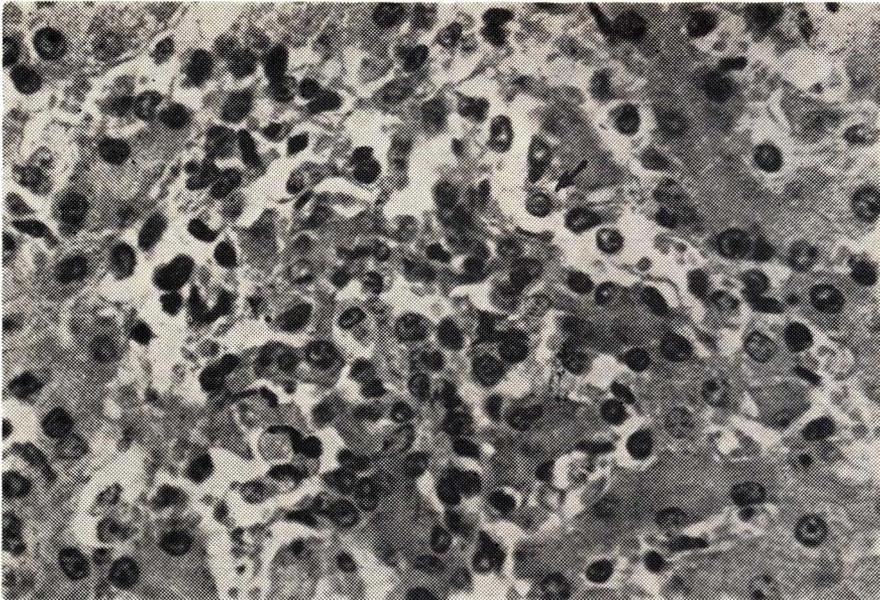
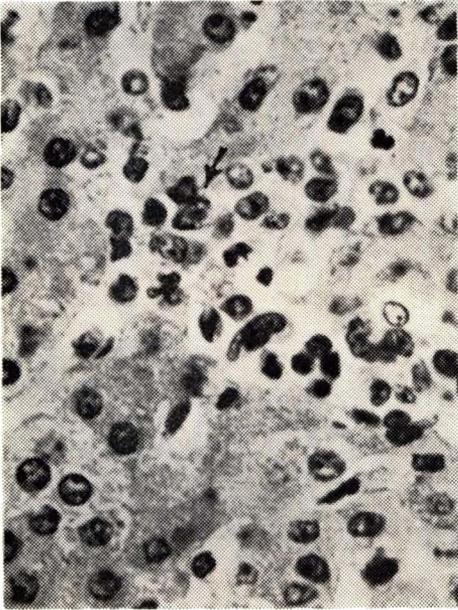
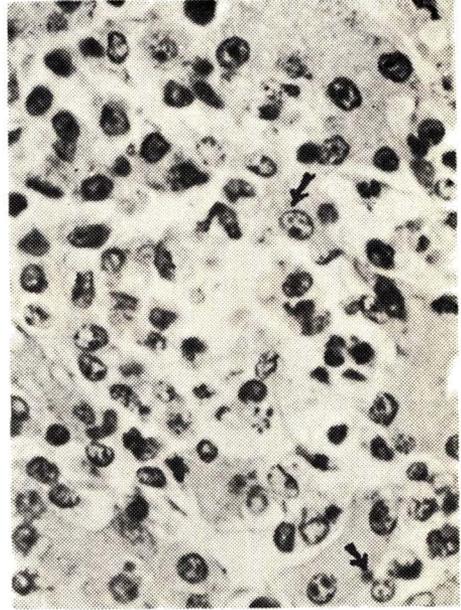


Fig. 1. Liver sections from the aborted foetus showing necrotic foci (1) Note the cellular aggregation and small intranuclear inclusion bodies (2).



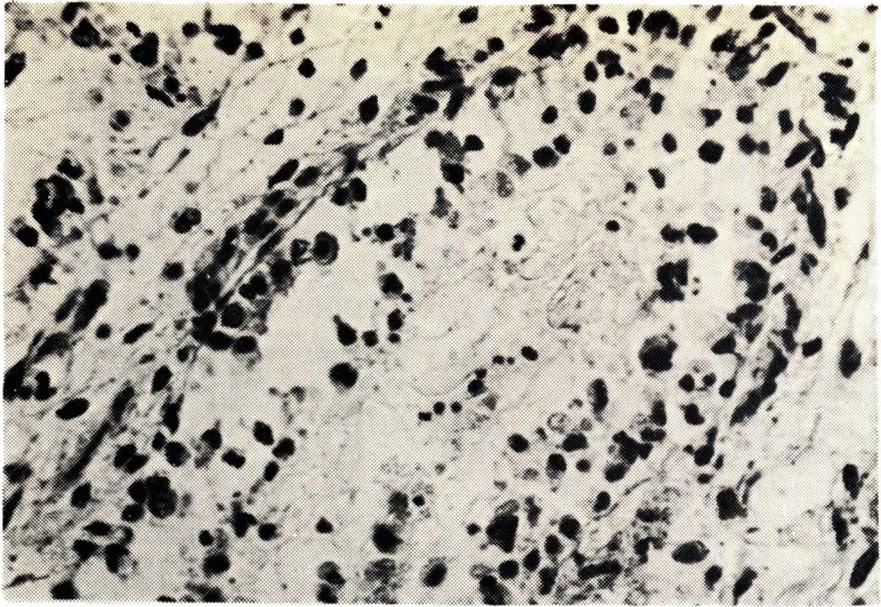
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Fig. 2. Liver sections from the aborted foetus showing small and large intranuclear inclusion bodies (1) with nuclear chromatin margination in hepatic cells (2).

1



2

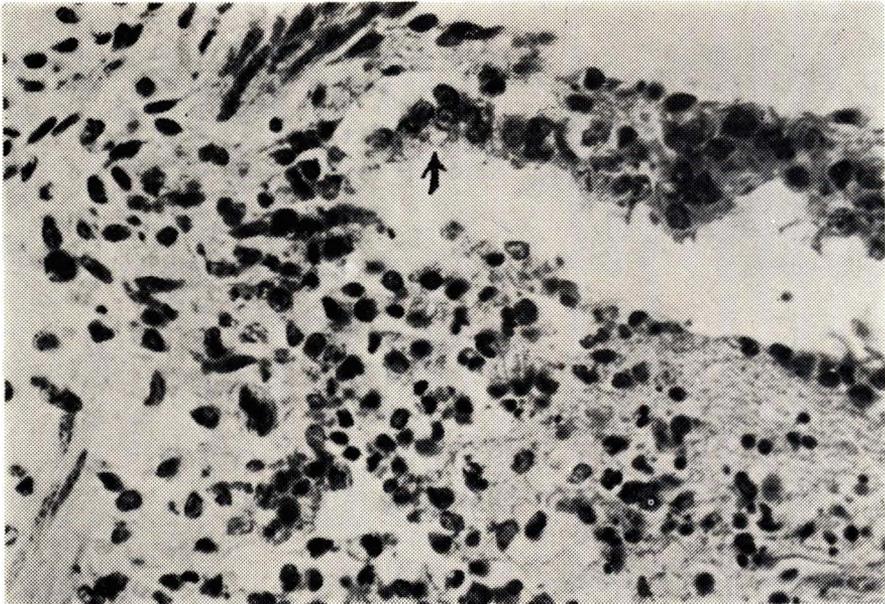


Fig. 3. Lung sections from the aborted foetus showing necrosis of epithelial cells, (1) presence of necrotic cells and debris in bronchial lumen and intranuclear inclusion bodies in respiratory epithelium (2).

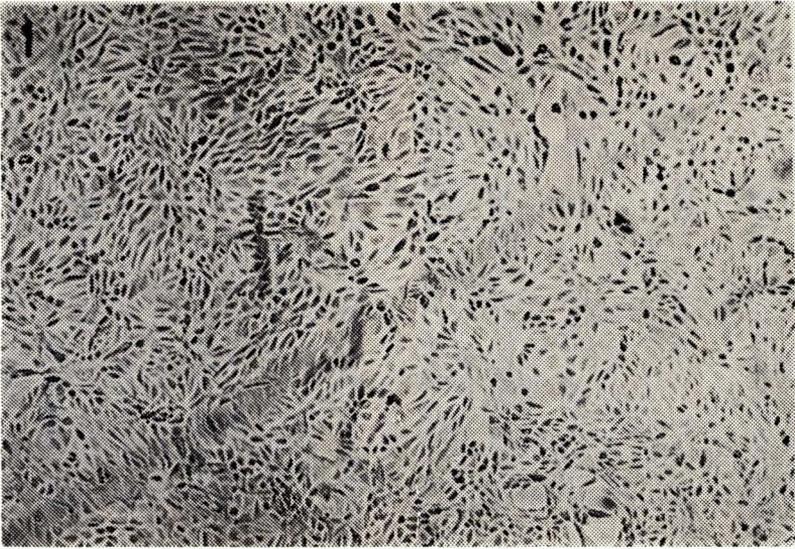


Fig. 4. Normal culture of horse kidney cells (1) and the cells (2) infected with strain Varamin of ERV 48 hours previously.

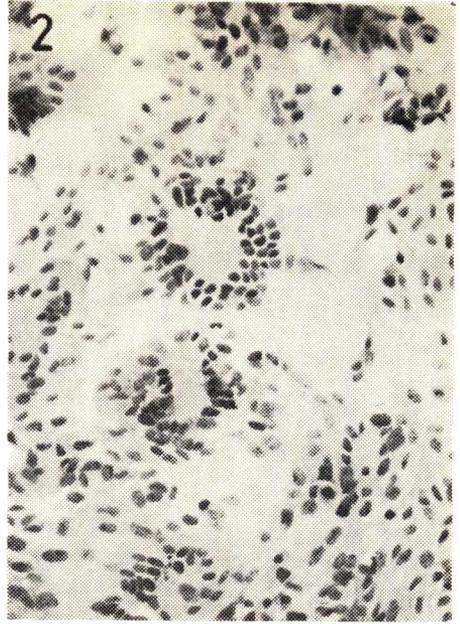
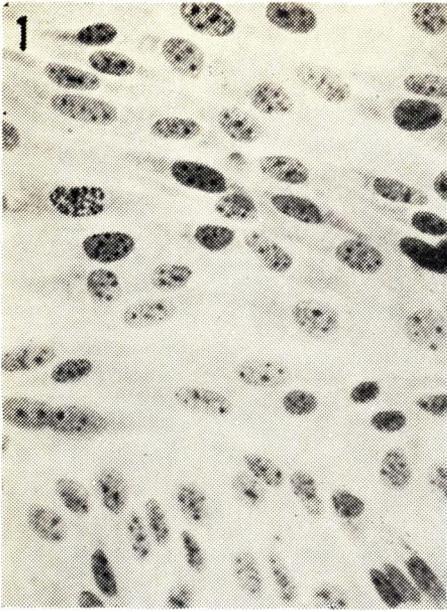


Fig. 5. Normal horse kidney cells culture (1) and cells infected with Varamin strain of ERV showing some syncytia formation (2,3) and intranuclear inclusion bodies (3,4).

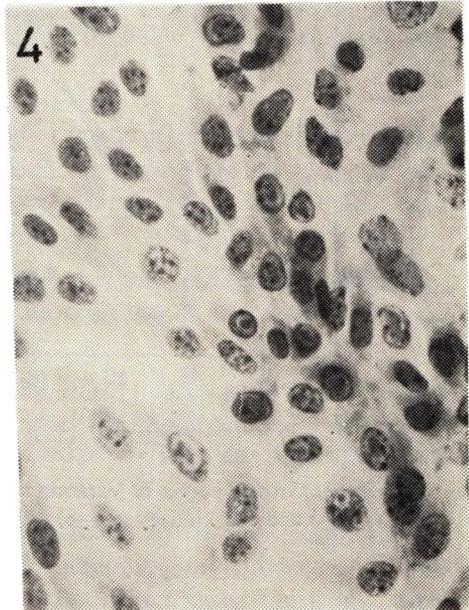
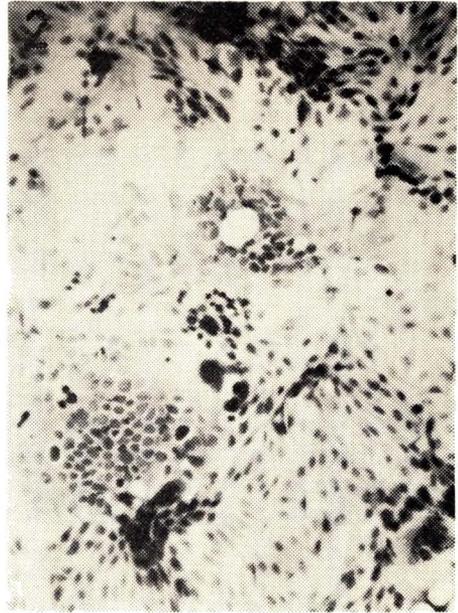


Fig. 6. Normal calf kidney cell culture (1) and cells infected with Varamin strain of ERV showing some syncytia formation (2,3) and intranuclear inclusion bodies (3,4).

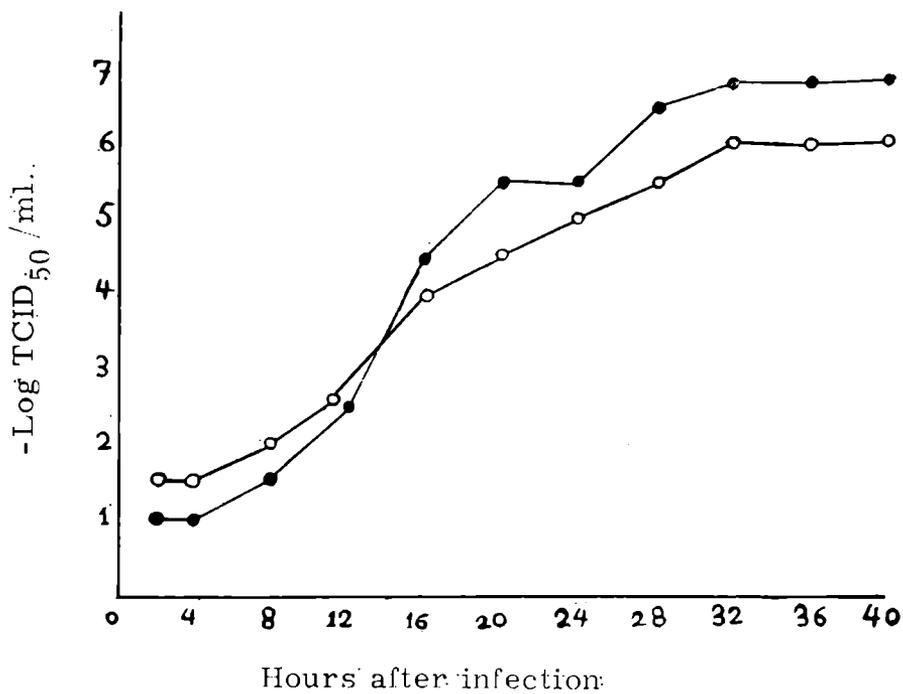


Fig. 7. Growth curves of Varamin strains of equine rhinopneumonitis virus in horse kidney (\bullet — \bullet) and calf kidney (\circ — \circ) cell cultures.