MULTIPLICATION OF MYXOMA VIRUS IN EPITHELIAL CELL CULTURE OF RABBIT KIDNEY

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The study of the development of virus, utilizing the tissue culture technique, permitted to follow the various phases of particle formation from adsorption to maturation. This simple system presents less factors interferring in the mechanism, permitting, with even higher evidence, the observation of alterations caused by intracellular parasitism, different from the ones caused by tissue ageing.

Sanarelli (13) was the first author to show that the rabbit myxoma has a virus as its ethiological agent, which is presently classified as a member of the Pox group (6).

The verification of virus presence in the cell, as a morphologic entity, was done by Aragão (1) and has been confirmed by several other studies; (12, 10, 7). The first photos of partially purified virus, at the electron microscope, were done by von Borries et al. (3). Its presence in the conjunctive cell, denominated myxomatous, was evidenced by Epstein et al. (5); those documentations, however, are not convincing, since the particles do not show constant shape and size.

Some aspects on virus infection in conjunctive and epithelial tissue of the domestic rabbit (*Oryctolagus* sp.) and of the wild one (*Sylvilagus* sp.) were described in a more recent paper (15).

Several authors succeeded in the multiplication of the virus in rabbit tissue culture, as well as in pleural cavity cells, (2), testicle, (16), and more recently, in epithelial tissue of kidney (4). The methods used in our laboratory to study the evolutional cycle of vaccine virus (14), permitted to follow some phases of the virus development at the electron microscope.

The purpose of this work is to show, at the electron microscope, the cellular alterations through culture infections by virus of low and high passages.

Materials and methods

Cell cultures — Casein-hydrolysate medium, (8, 9), has been used with the following modifications:

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a) Lactalbumin hydrolysate (enzymatic) instead of Caseyn hydrolysate.

b) 0.07% final of lactalbumin hydrolysate instead of 0.05%.

c) no alpha-thocopherol.

The medium for culture growth contained 18 to 20% inactivated calf serum. The inoculated cultures sometimes received no more than 5% serum, but in general, during growth and after inoculation, the culture medium was enriched with 18 to 20% calf serum, adding 200 U.I. Penicillin and 50 μg Streptomycin.

Primary rabbit (60 to 70 days old) kidney cell cultures were obtained by 0.20% trypsin dispersion in phosphate buffer solution at room temperature and moderate stirring during 4-5 hours. After filtration through 8 layers of cheese cloth, the cell suspension was centrifuged (20 min at 45 g) and the sediment washed in serum-free medium. The cells were centrifuged and suspended in culture medium enriched with 18% calf serum and antibiotics. After incubating for 48 hours at 37°C the medium was discarded, new one was added and generally the cultures became confluent in three to four days. A previous washing was done with warmed PBS to remove destroyed cells. Since the growth medium with the same percentage of serum and antibiotics was to be used also for maintenance of the inoculated cultures, no washing was necessary to remove serum excess.

While the cultures did not show a cytopathic effect the medium was changed every 2 or 3 days. Harvesting of the supernatant of the inoculated tubes was done after stirring with glass pearls, to liberate cells not yet detached, when the cytopathic effect and infection of the whole layer appeared.

Only primary cultures of rabbit kidney cells were used to obtain passages but replication cultures were used sometimes for virus titration.

Virus — The sample was originally isolated by P. M. Freire from the Instituto Biológico de São Paulo, during an outbreak in a rabbit colony at Itaquaquecetuba, São Paulo. It has had two rabbit passages. The resulting tumours were maintained in glycerin solution at -20°C. A fragment of these tumours was used as inoculum for rabbits *Oryctolagus*, after being triturated with sand and buffer.

Two virus samples obtained from experimental tumours were used in this work:

Sample E — has been inoculated in tubes and bottles, dilutions of 1:10 till 1:1.000. The supernatants of the infected tubes and bottles were harvested after 7 days, and when inoculated in rabbits proved to be non-infectious.

Sample D — has been inoculated in tubes and bottles, incubated for 31 days with periodical changes of medium.

The supernatant harvested on the 31st day from several tubes proved infectious when inoculated in rabbits. The same occurred with the supernatants harvested from bottles on either the 7th, 13th and 20th day. The virus harvested from the tubes on the 31st day, constituting the first passage of myxoma virus in rabbit kidney cells, was used as inoculum for the second passage of the virus in the same cell system. For the subsequent passages always the pure supernatant has been used as inoculum.

As the myxoma virus is of slow adaptation to cultures, it has been kept in contact with the cells during variable periods of time in the incubator at 37°C.

The first change of medium was done only after 96 hours. In the continuance of the passages, the adaptation time virus-to-cell at the incubator was given up and the first change was done after 48 hours.

An immune-serum was prepared in rabbit by one endovenous inoculation of 1.0 ml of suspension of virus of high passage (38th). The bleeding was done by cardiac-puncture 15 to 20 days after inoculation and the obtained serum was inactivated, distributed to flasks and stored at —20°C.

Electron microscopy — The electron micrographs showed infected cells collected 20 to 48 hours after contact with myxoma virus of the 10th, 33rd and 38th passage in tissue culture. Only the virus of the latter induced evident cytopathic effect.

After elimination of the medium, the infected cultures were trypsinized for 5 minutes at 37°C with a 0.20% trypsin solution (Difco 1:250) in phosphate buffer (pH 7.0), to detach the tissue from tube walls. The cell suspensions obtained were centrifuged for 10 minutes at 60 g and the sediment was resuspended in osmic acid at 1% in subtosan. After fixation for 15 to 20 minutes the cells were dehydrated in alcoholic series and included in a 3:7 mixture of methyl-butyl meta-chrylate. Every change of medium from fixation to final inclusion was done after successive centrifugation and decantation.

Ultrathin sections were obtained through a Porter-Blum microtome and examined with a Siemens ÜM 100 b and a Elmiskop 1 microscopes, magnifying x 1.300 to x 20.000. The electromicrographs were amplified photographically.

Results and discussion

In the passages from 1 to 12 the virus did not induce any cytopathic effect in the cultures, but the samples collected from the replaced media, showed always a positive lethal result when inoculated in rabbits.

From the 13th passage on, a morphologic alteration of the infected cells started 40 to 50 hours after inoculation, diminishing the time necessary to appear this cytopathic effect until, in the 67th passage, 50% of the culture showed the effect even before the 20th hour after infection.

There also appeared a change in the pathogenic properties of the virus used in this experiment. In the low passages no cytopathic effect was induced by the virus, but when inoculated in rabbits *Oryctolagus*, it proved to be lethal within 10 to 11 days. In the high passages, the virus caused great cytopathic effect showing, however, only antigenic properties.

Rabbits inoculated several times with the virus of the 38th passage, did not show any myxomatous symptoms when inoculated afterwards with virus of low passages. The activity of the virus was determined then by inoculations in monolayer cell cultures. The virus dilutions were done with the same culture medium and 8 to 10 confluent cell cultures received each 0.1 ml of the virus dilution. 0.9 ml of culture medium with calf serum and antibiotics were added to complete the volume to 1.0 ml. After an incubation for 48 hours at 37°C the reading of the infected tubes was done and the $TCID_{50}$ (Reed-Müench method) resulted in titer of $10^{9.19}$ / 0.1 ml (38th passage). Mc Kercher & Saito (11) obtained a similar result working with the same material and conditions, with a titer of $10^{3.71}$ in the 21st passage and $10^{6.5}$ in the 40th passage.

The myxoma virus obtained in the 38th passage, diluted from 1:10 to 1:100 million, was neutralized with nondiluted immune-serum (mixture of equal parts). Since nothing was known about the activity of the immune-serum, no particular attention was given to inoculate known quantities of virus. It has been observed thus that the serum which neutralized the virus of that passage, proved to be immune.

Electron microscopic observations of the culture cells infected with first passages virus demonstrated nuclear rarefaction as the principal alteration, similar to that observed in epithelial cells of the rabbit *Oryctolagus*, inoculated with pathogenic virus, obtained through passages in animals, or low passages in cultures. Apparently, the cytoplasm still contains imature virus particles, mitochondria and endoplasmic reticulum are present (Figs. 1 and 2). The cultures infected with virus of high passages, antigenic and not pathogenic, present cells with numerous electron dense particles, displayed at the periphery, and corpuscles of low density. Mitochondria and endoplasmic reticulum are present. This aspect corresponds to a still intermediate phase of virus development in cell (Fig. 3). In a more advanced phase of infection the cell components tend to disappear and practically only dense and agglomerated particles may be observed (Fig. 4). In other cells, besides those particles, we found numerous corpuscles, delimited by the membrane and the central region of less electron density (Fig. 5). Remarkable is the fact that in both cases the nucleus did not show rarefaction, only alterations of morphology and disposition.

Probably a relation exists between vacuolated nucleus and pathogenic virus and non-vacuolated nucleus with non-pathogenic but antigenic virus.

This vacuolation is a consequence of the infection of the cells by the loss of nuclear material. The newly formed virus is a structurally complete particle and shows pathogenicity which can be demonstrated by the inoculation of rabbits *Oryctolagus;* tissue cultures infected with this virus do not show any cytopathic effect.

To verify the relation between vacuolation and formation of complete virus particles it would be necessary that the cells incorporate radioactive substances, constituent of their desoxyribonucleic acid or nuclear proteins, infecting them afterwards with the virus, and then doing an autoradiograph of the corpuscles formed in these cells.

Referring to the virus of high passages, incomplete particles also are formed, showing, however, antigenic activity. The synthesis of the virus structural components proceeds intensely, causing a cytopathic effect in a short period of time. In several animals, at the site of inoculation with the 38th passage virus, a small response appeared persisting from 8 to 10 days, with posterior desquamation. This response may be caused by some complete virus that develop in the cultures or by particles of the inoculum of low passages that did not penetrate into the cells and did not follow the successive passages.

Summary

The myxoma virus, isolated from cutaneous tumours of the rabbit *Oryctolagus,* was partially purified and inoculated in epithelial tissue cultures of rabbit kidney. Ultrathin sections from cultures infected with this virus, when observed with the electron microscope, demonstrated frequently a vacuolated nucleus, signifying a loss of nuclear material, evidently related to the infection with the virus of low passage. Nuclear vacuolation has also been observed with frequency in epithelial

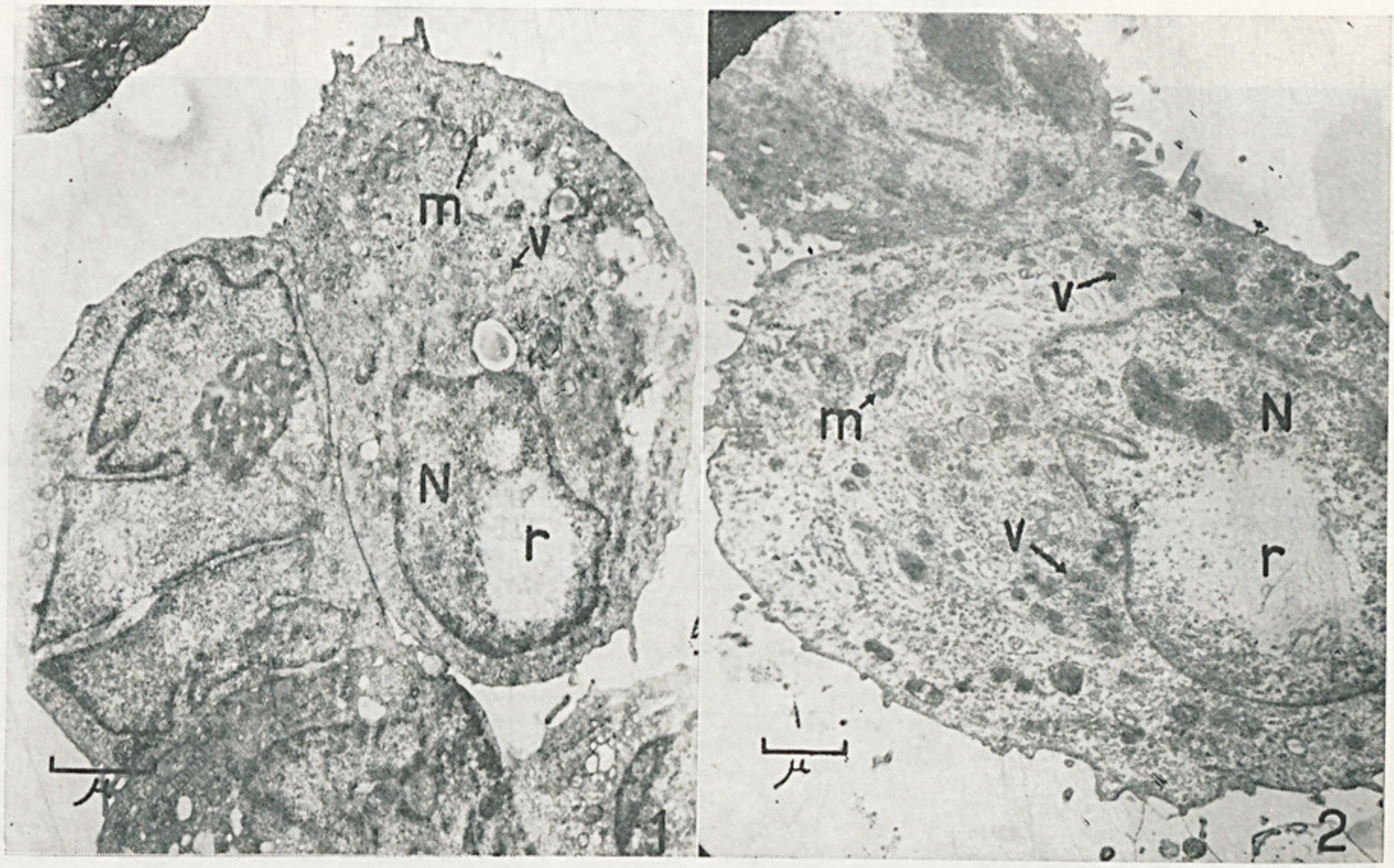
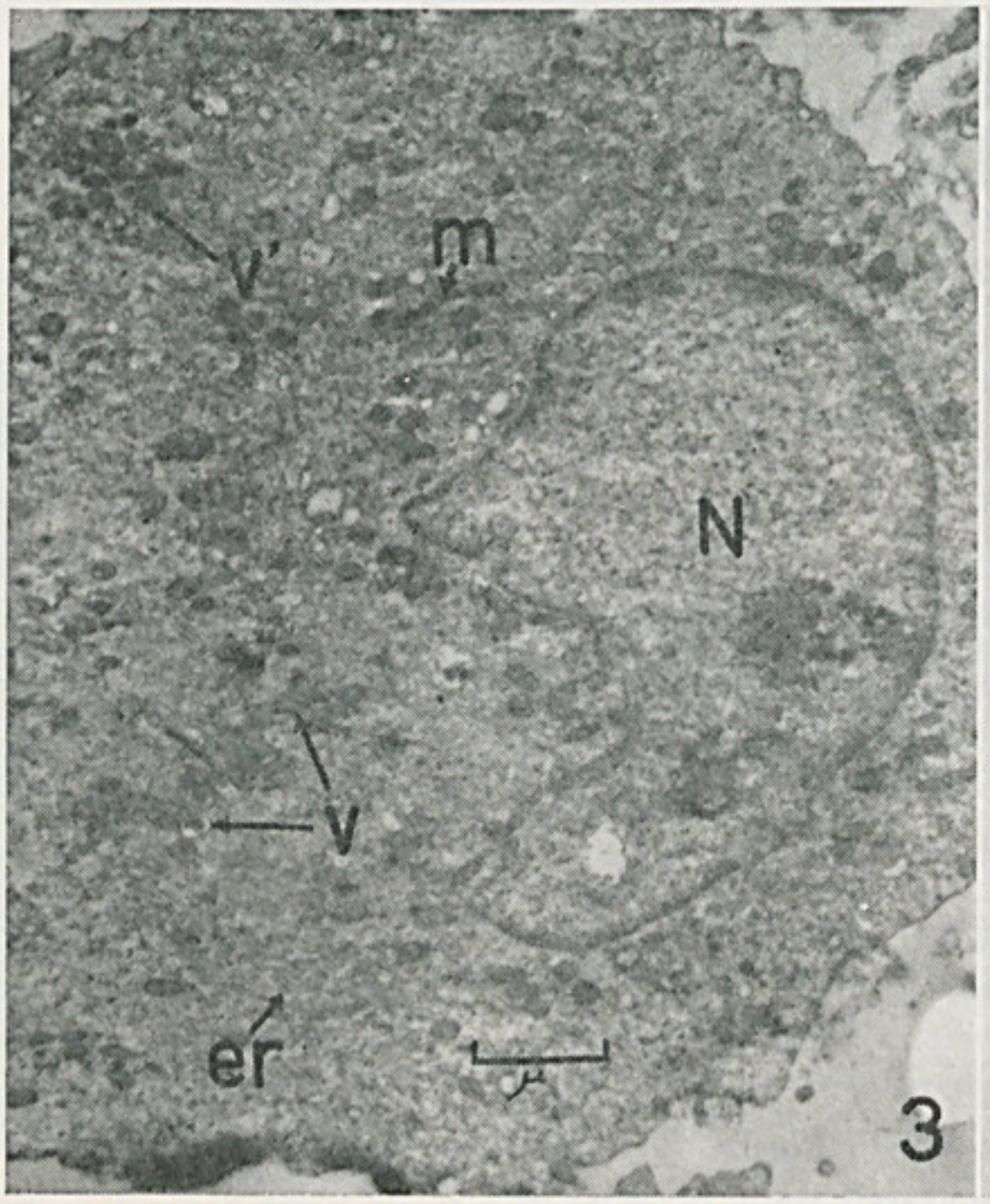


Fig. 1 — Cell infected with virus of 10th passage; N — nucleus with vacuolation r; V — immature virus particles; m — mitochondria. Methyl-butyl methacrylate embedding. Magnification: x 24.000.

Fig. 2 — Cell infected with virus of low passage (10th) showing: N — nucleus; r — vacuolation; V — immature virus particles; m — mitochondria. Methyl-butyl methacrylate embedding. Magnification: x 21.500.



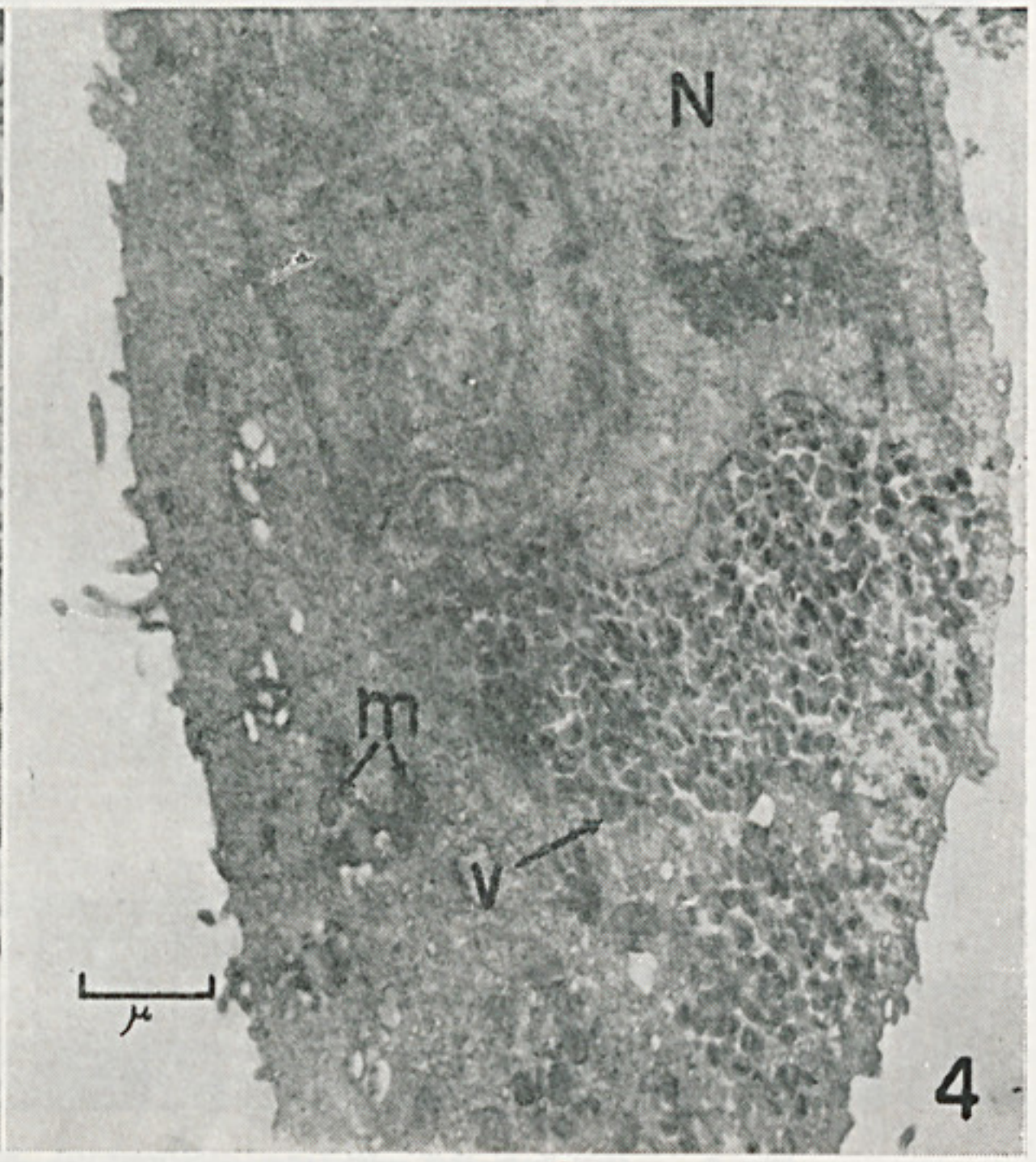


Fig. 3 — Cell infected with virus of high passage (33rd); N — nucleus without vacuolation; V — immature virus particles; V' — viruses in a more advanced phase of development; m — mitochondria. Methyl-butyl methacrylate embedding. Magnification: x 21.500.

Fig. 4 — Cell infected with virus of 33rd passage; N — nucleus without vacuolation; V — virus in an advanced phase of development; m — mitochondria in low number. Methyl-butyl methacrylate embedding. Magnification: x 21.500.

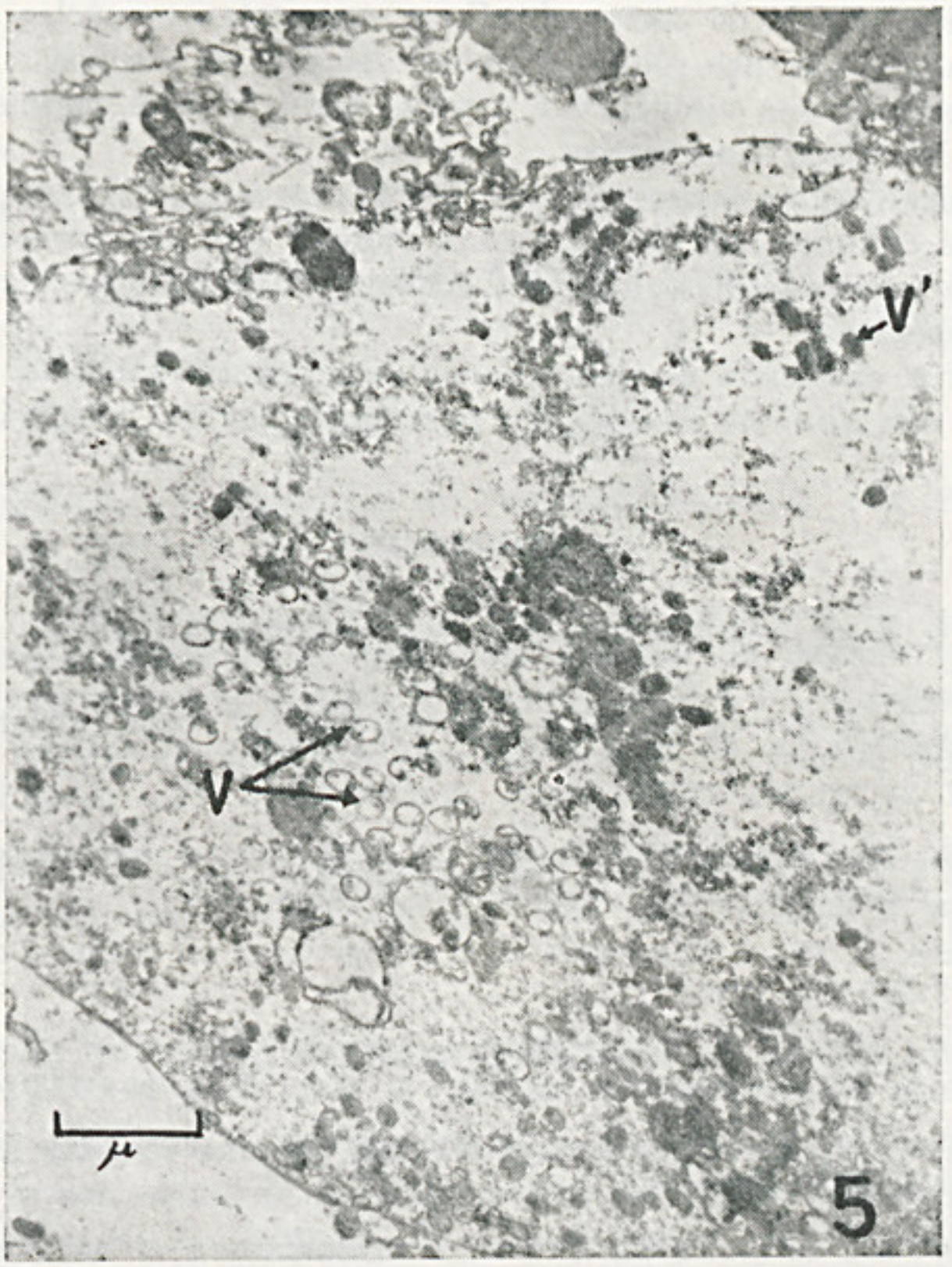


Fig. 5 — Cell infected with virus of 33rd passage; V — virus particles of low electron density, apparently limited only by membranes; V' — electron dense virus particles. Methyl-butyl methacrylate embedding. Magnification: x 24.000.

cells of rabbit skin. Up to the 12th passage, no cytopathic effect occurred, but the virus maintained its pathogenic characteristics when inoculated in rabbits.

The viruses of high passages (33rd and 38th) induced evident cytopathic effect 48 hours after inoculation. They have no pathogenic properties when inoculated in rabbits, but are antigenic. An immune-serum was prepared from those animals and its activity demonstrated by virus neutralization, preventing a cytopathic effect in tissue cultures. Cultures infected with 33rd and 38th passage virus showed cells with numerous particles, however without nuclear vacuolation. It is possible that there exists a connection between vacuolated nucleus and pathogenic virus, and non-vacuolated nucleus with non-pathogenic but antigenic virus.

Resumo

Multiplicação do virus do myxoma em cultura de célula epitelial de rim de coelho

O virus do myxoma, obtido de tumores cutâneos do coelho *Oryctolagus*, foi parcialmente purificado e inoculado em culturas de células epiteliais de rim de coelho. As células das culturas infectadas com êsse vírus, examinadas ao micros-

cópico eletrônico através de cortes ultrafinos, apresentavam núcleo rarefeito, efeito êsse, também observado nas células epiteliais da pele de coelhos. Até a 12.ª passagem não ocorreu efeito citopático e o vírus manteve suas características patogênicas quando inoculado em coelhos.

O vírus de altas passagens (33.ª e 38.ª) provocou efeito citopático evidente nas células das culturas com êle infectadas, embora elas não apresentassem núcleos com rarefação. Quando inoculado em coelhos, o vírus apresentava apenas propriedades antigênicas.

É possível a existência de uma relação entre núcleo rarefeito e vírus patogênico e, núcleo não rarefeito e vírus não patogênico, porém, antigênico.

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