East African Veterinary Research Organization, Muguga, P. O. Box 32, Kikuyu, Kenya

The Propagation and Growth Characteristics of Rinderpest Virus in HeLa Cells

By

B. Liess* and **W. Plowright**

With 10 Figures

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The host-cell range of culture-passaged rinderpest virus of the strain Kabete "0" (RBOK) was briefly described by *Plowright* (1962). In particular, it was reported that infectivity persisted in primary human amnion cells for 42—66 days but that no cytopathic effects were observed; HeLa and MK2 cells (*Westwood, Macpherson* and *Titmus*, 1957) did not show virus specific changes detectable in unstained preparations.

The accumulating evidence for a close relationship between rinderpest, measles and distemper viruses (*Imagawa, Goret* and *Adams*, 1960; *Warren*, 1960) suggested that a further effort should be made to adapt rinderpest virus to a cell system which was also readily susceptible to measles virus. If successful, this would make possible comparative studies on cytopathology, haemadsorption and haemagglutination by the two agents.

This communication describes the growth and cytopathogenicity of the Kabete "0" strain of rinderpest virus in monolayers of HeLa cells.

Materials and Methods

Virus

This consisted of fluid harvested on the 7th day from primary calf kidney cultures infected at seeding with the 95th serial passage of the strain RBOK. At the stated passage level the virus was completely non-pathogenic for E. African cattle. It was titrated in cells of the same type in the manner described by *Plowright* and *Ferris* (1959 and found to contain $10^{5.7}$ TCD_{50} per ml. The titre was calculated by the method of *Thompson* (1948) from final observations on the 12th day after inoculation.

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Cell cultures

HeLa cells were of an uncloned strain, received from the Virus Reference Laboratory, Colindale (London). They were used after 47 to 69 passages in this laboratory, the growth medium consisting of Hanks' saline with 0.5% lactalbumen hydrolysate and 0.1% yeast extract, supplemented by 10% normal, adult ox serum, 10% tryptose phosphate broth and an additional 1.5% of 5% (w/v) $NaHCO_3$. For maintenance the same medium was used or the ox serum was reduced to 2%. Antibiotics (penicillin, streptomycin and nystatin) were employed in the usual concentrations.

Following publication of the paper by *Moura* (1962), describing the effect of lamb serum on the cytopathic effects of measles virus in HeLa cells, comparative experiments were carried out with rinderpest virus. In these unheated, normal lamb serum was substituted for ox serum at the same concentrations as those detailed above.

Stock cultures were grown in medical flat bottles, subculture being carried out by detaching the cells with a mixture of Versene (0.02%) and trypsin (0.01%) in Ca: Mg-free saline and resuspending them in 3 times the original volume of growth medium. For virus studies cultures were established in Petri dishes, usually containing coverslips (*Ferris* and *Plowright*, 1958), medical flat bottles of 20 ozs. capacity and Pyrex tubes, sometimes with coverslips. The two latter types of vessel were incubated for 3 days at 36.5° C in a stationary position and then transferred to rollers rotating at 8 r. p. h. at the same temperature. For prolonged incubation complete medium changes were effected every 2—3 days.

Calf kidney monolayers were prepared as already described (*Plowright* and *Ferris*, 1959).

Virus Titrations

For the detection and titration of infectivity in HeLa cells, 10-fold dilutions of virus were inoculated into 5 tubes each of calf kidney cells. The latter were at least 4 days old when used and transferred immediately to roller drums. Complete medium changes were carried out every 2 days and microscopic observations were continued until the 10th or 11th days post-inoculation. All titres were calculated by the method of *Thompson* (1948).

Growth Curve

Pyrex tubes (160 × 15 mm) were seeded with 10^6 HeLa cells in 1 ml. of growth medium, and incubated for 24 hours, after which time a confluent cell sheet had formed. The medium was then discarded and substituted by 1 ml. of undiluted, infected fluid from the 12th virus passage in HeLa cultures. An aliquot of this was frozen at −70° C for later titration. The culture tubes were rolled for 5 hours at 37° C, following which the medium was discarded and the monolayers washed once with 2 ml. of P. B. S. (*Dulbecco* and *Vogt*, 1954). Finally fresh medium was added (1 ml.) and the tubes were returned to the roller. At each time interval, shown in Fig. 8, 3 tubes were harvested as follows:

a) *Free virus* — pooled fluids were centrifuged at 2000 r. p. m. for 5 minutes and the supernatant was frozen at −70° C for later titration.

b) *Cell-associated virus* — 3 monolayers were washed once with 2 ml. each of 0.02% Versene in Ca: Mg-free saline and then detached by 5 minutes treatment at 37° C. with 1 ml. of the same solution. After dispersal the cells were pooled, deposited at 1000 r. p. m. for 5 minutes and resuspended in

3 ml. of maintenance medium. This suspension was treated for 5 minutes in an ultrasonic bath* and then clarified by centrifugation for 5 minutes at 3000 r. p. m. The supernatant was regarded as cell-associated virus and stored at $-70°$ C until titration. Free virus treated by ultrasonics under these conditions was found to have a half-life of about 7 minutes (*Plowright*, 1962).

The medium in the remaining tubes was renewed completely on the 3rd, 6th and 9th days post-inoculation.

Haemadsorption

Tube cultures of HeLa cells infected 8 days previously with measles virus (strain Edmonston, 9th HeLa passage) or 15 days previously with rinderpest virus (10th HeLa passage) were tested for their capacity to adsorb erythrocytes from 2% (w/v) suspensions in 0.85% NaCl. The technique employed was similar to that used by *Kohn* (1962) for measles virus in KB cells. Erythrocytes from the following species were tested viz. *Cercopithecus aethiops* (Vervet), *Papio* sp. (Baboon), rabbit, guinea pig, dog, sheep and ox. Three tubes were used for virus and red cell type; adsorption took place for ½ hour at 37° C, after which the cell sheets were washed twice with P. B. S. Occasionally cultures were fixed and stained by the M. G. G. technique.

Virus Neutralisation

Identification of the cytopathic agent in HeLa cells was by neutralisation tests, employing convalescent rinderpest antisera and virus of the 17th passage in HeLa cells. Virus or virus/serum mixtures were inoculated into tubes together with calf kidney cell suspensions in growth medium (*Plowright*, 1962b). Final readings of cytopathic endpoints were made on the 12th day after seeding.

May-Grünwald-Giemsa (M. G. G.) Staining

Giemsa stain was obtained in powder form**. Coverslip cultures or cells growing in Leighton tubes were fixed in Carnoy's fluid for 5 minutes only, stained by a method essentially similar to that of *Jacobson* and *Webb* (1952) and mounted in a neutral medium***.

Detection of Intracellular Rinderpest Antigen with Fluorescent Antibody

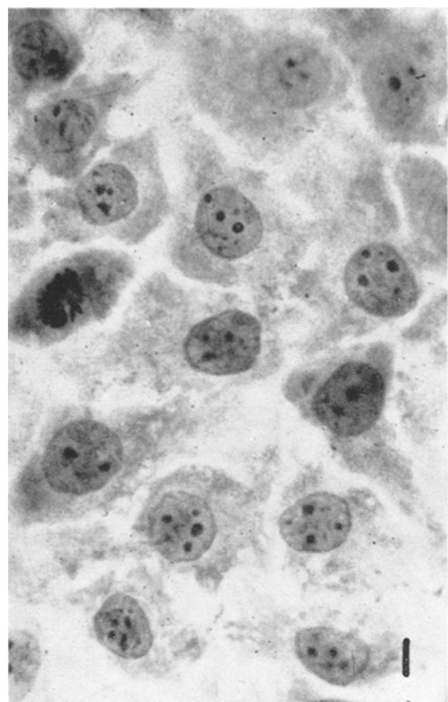
The indirect method of *Weller* and *Coons* (1954) was employed after fixation for 1 minute in acetone previously cooled to $-70°$ C. Hyperimmune serum against bovine gamma-globulin (fraction II) was prepared in rabbits and the rabbit anti-bovine was conjugated with fluorescein isothio-cyanate by the technique of *Marshall, Eveland* and *Smith* (1958). Bovine hyperimmune serum (10% v/v) was used for primary treatment of the fixed cultures. Details of the fluorescence microscopy techniques will be given in a subsequent communication.

Results

Establishment of the Virus in HeLa Cells

For the first passage a suspension of HeLa cells was mixed with the virus preparation (see Materials and Methods), to give an apparent multiplicity of 0.7, and then seeded into Petri dishes with coverslips. Some of

* Dawe Instruments Ltd., London. Type 1150A; 500W; 41.23 kc/s.
** Matheson, Coleman and Bell, Cincinnati, Ohio.
*** G. T. Gurr, Ltd., London.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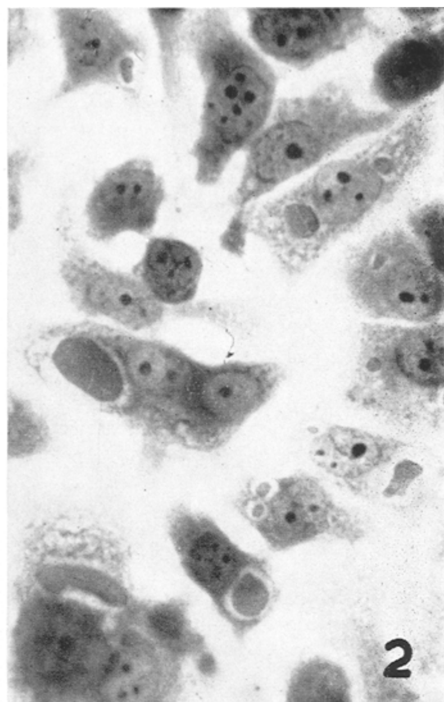
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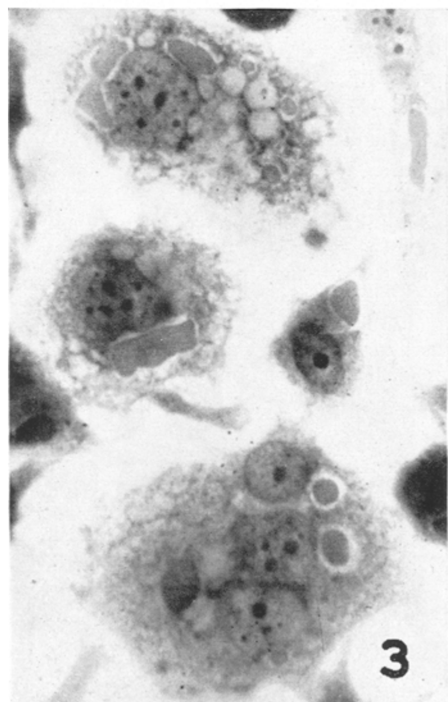
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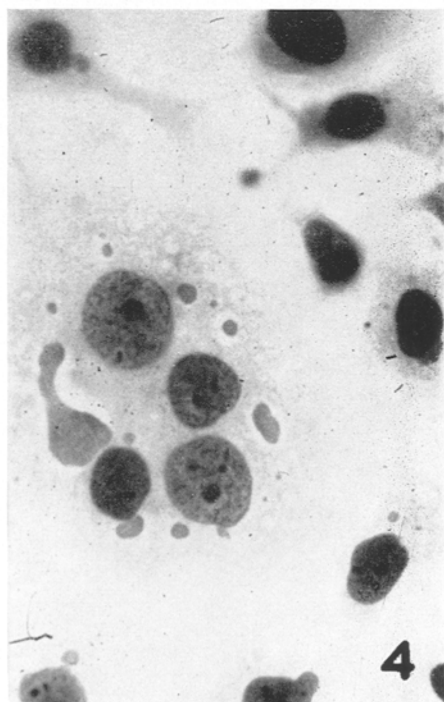
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the latter were fixed on the 3rd and 5th days and stained by the M. G. G. technique. All infected cultures showed a thinning of the cell sheet in comparison with controls and, even at 3 days, contained a large number of spindle-shaped, stellate or rounded cells. A proportion of the stellate elements contained more than one nucleus and, in some cases, cytoplasmic inclusions of a weakly basophilic or neutrophilic staining reaction, delineated by a clear halo. On the 5th day the number of abnormal cells and

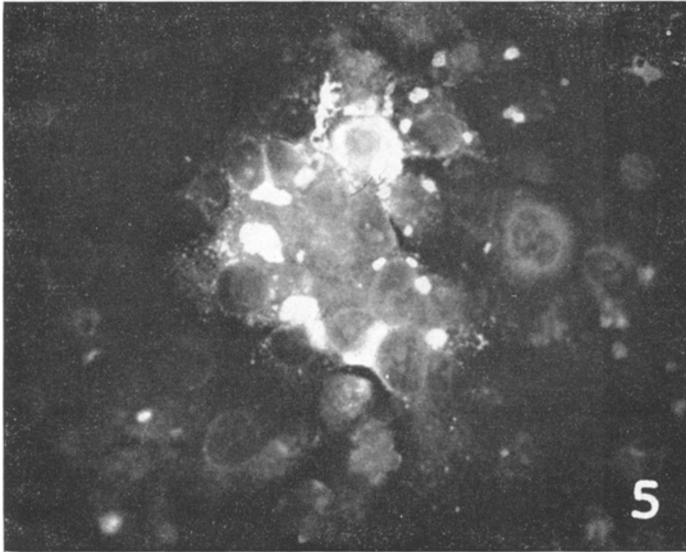

Fig. 5. Second passage of the Kabete "0" strain of rinderpest virus in HeLa cells. A syncytium with 8 to 10 nuclei and several brightly fluorescent areas in the cytoplasm. Note several small fluorescent spots in the adjacent cells and some weak, diffuse cytoplasmic fluorescence in a binucleate cell on the right. Indirect fluorescent-antibody technique. (×400.)

cytoplasmic inclusions had increased, while the latter showed a tendency to become acidophilic (see Plate 1). Fluid harvested at this time had a titre of $10^{4.4}$ TCD_{50}/ml. and was used to initiate the 2nd passage.

The 2nd passage was also carried out in Petri dishes with coverslips, which were harvested at 2, 3, 5 and 7 days post-seeding. Examined by the fluorescent antibody technique these showed steadily-increasing numbers of foci consisting of small syncytia or groups of 3 to 10 single cells

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Plate 1. HeLa cells infected with rinderpest virus of the strain Kabete "0" – 1st HeLa passage, 5 days post-inoculation. May-Grünwald-Giemsa staining. (×400.)

Fig. 1. Uninfected control culture.

Fig. 2. Cells containing one or two nuclei and showing distinct cytoplasmic inclusion bodies surrounded by clear halos.

Fig. 3. Cells with one or several nuclei, exhibiting cytoplasmic vacuolation and inclusion bodies of a neutrophilic staining reaction.

Fig. 4. Monolayer stained after 4 hours incubation in distilled water. Cytoplasmic inclusion bodies weakly basophilic. Cytoplasmic basophilia reduced.

with discrete areas of cytoplasmic fluorescence, similar to those in the early stages of infection of calf kidney cells (*Liess* — to be published) (see Fig. 5). Comparable preparations stained by the M. G. G. technique showed no virus-specific changes until the 5th to 7th days when a few single cells or small syncytia were found to contain cytoplasmic inclusions.

The 3rd to 6th virus passages were effected by detaching the cells of infected cultures with versene/trypsin solution and mixing 1 part of them

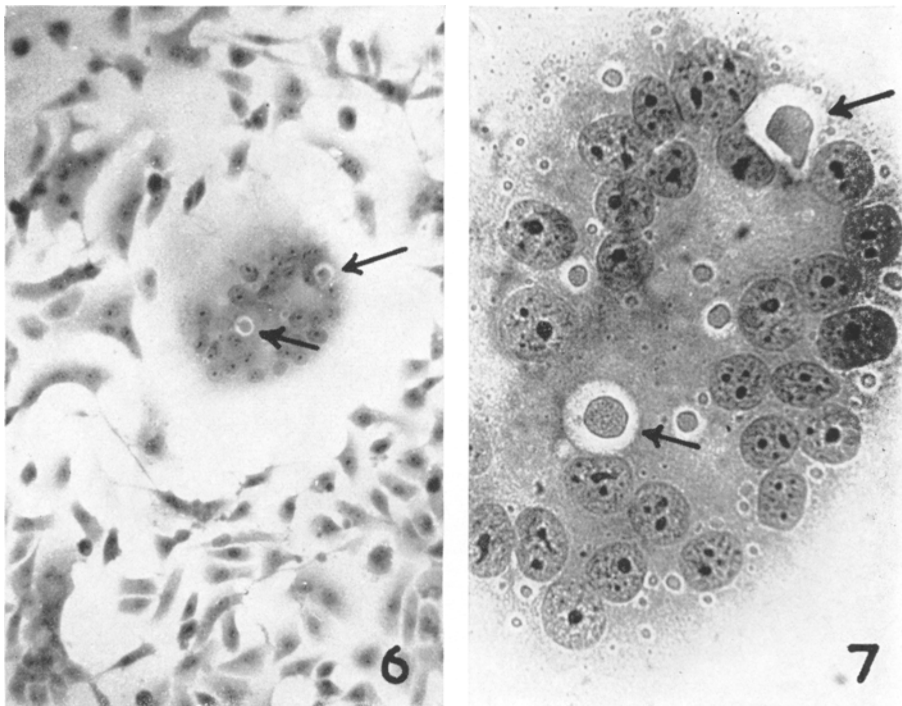


Fig. 6. Rinderpest-infected HeLa cells, 3 days post-infection. A rounded syncytium shows several cytoplasmic inclusion bodies (arrowed), clearly defined by a broad halo. Note also stellate cells with long processes. Haematoxylin-eosin staining (× 125).

Fig. 7. Same syncytium as in Fig. 6. Numerous inclusion bodies of different sizes are scattered in the cytoplasm; some of them have a granular substructure. H. and E. staining (× 600).

with 1 or 2 parts of clean cells. The resulting suspension was usually seeded into tubes, which were rolled from the 3rd to the 6th or 8th days. For the 7th to the 14th passages cell-free fluid was employed to infect clean cell suspensions, at the time of their seeding into roller tubes or bottles. Thereafter infected cultures were frozen at —70° C. and thawed rapidly to release intracellular virus into the fluid.

Cytopathic changes were first detected at 3 days in unstained cultures of the 3rd passage. They took the form of increasing numbers of small

rounded foci with refractile or granular cells and small syncytia, which occasionally became vacuolated. With ageing of infected cultures the syncytia frequently assumed a granular amoeboid appearance, while all types of affected cells tended to detach from the glass. Nevertheless, a significant clearing of the glass surface was never observed, at least up to the 14th passage. This was apparently due to the tendency for apparently-

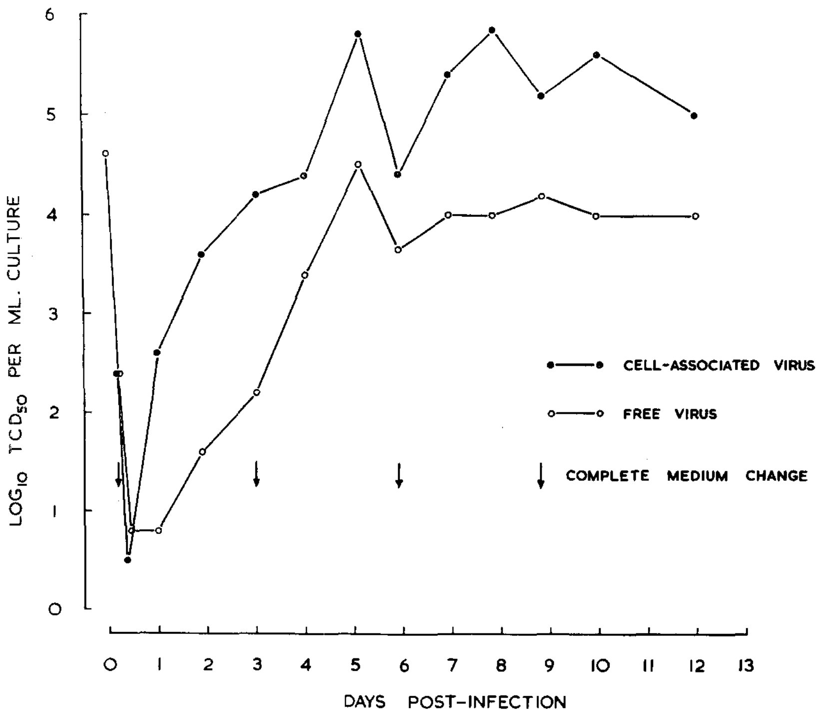
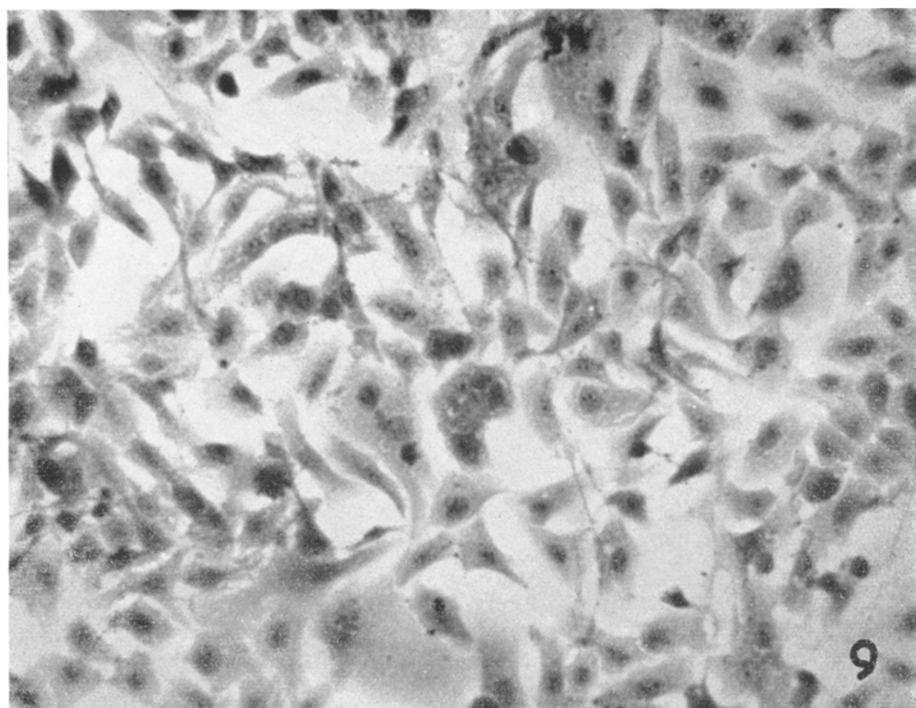


Fig. 8. Growth of rinderpest virus* in HeLa cells.

normal cells to grow into and obscure the cytopathic foci. In the latter case only the shrunken remnants of syncytia could be detected. The most extensive cytopathic effect was seen when the size of the inoculum was increased by freezing and thawing of infected whole cultures. Maintenance medium containing only 2% ox serum caused an apparent increase in the cytopathic effect, which was probably due to a reduction on the overgrowth and piling-up of the cells.

The Growth of Virus in HeLa Cells

As shown in Fig. 8 the input virus was reduced to about 1% of the original figure within 5 hours of seeding. At 10 hours a small amount of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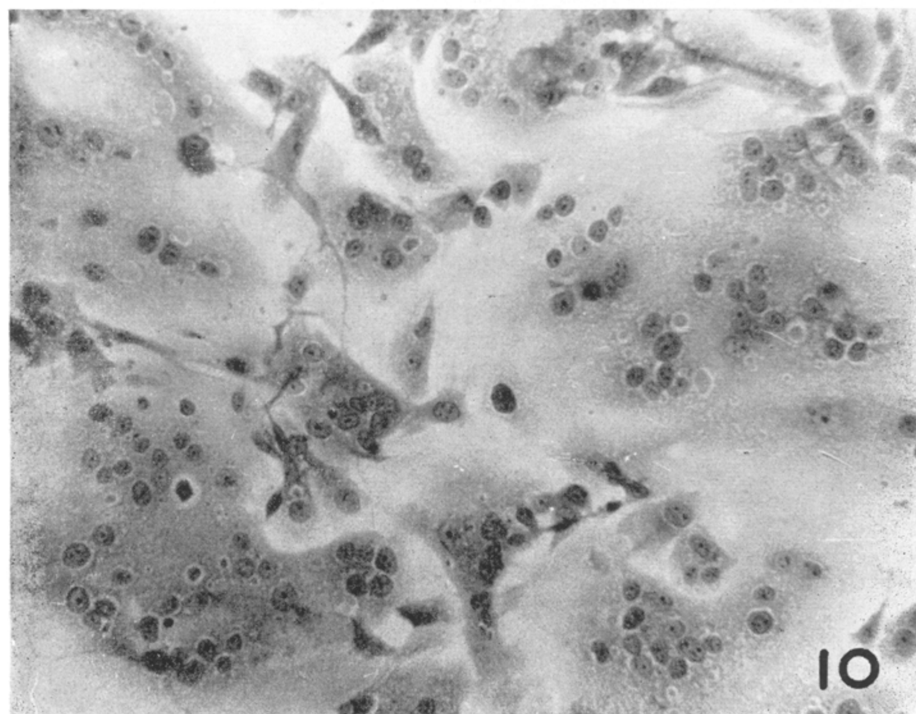
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free virus was present in the fluid but this probably reflected the inadequacy of the washing process. Intracellular virus increased logarithmically from some time after the 10th hour to a peak at the 5th day of $10^{5.8}$ TCD_{50} per tube. Some free virus was probably liberated by the 24th hour to account for the maintenance of titre as compared with the 10th hour; in the absence of virus release a decline due to heat inactivation would have been expected. Intracellular virus was invariably 1.0 to 2.0 $\log_{10}$ units higher than free virus, the mean difference being 1.4 units.

Table 1. The Effect of 10% Ox or Lamb Serum on the Growth of Rinderpest Virus in HeLa Cells

Cells used for titration	Inoculum	Titre* per ml. for virus grown with	
		10% Ox serum	10% Lamb serum
Primary calf kidney	Free virus	5.4	4.9
	Cell-associated + free virus	6.5	5.6
HeLa	Cell-associated + free virus	5.6	5.2

* $\log_{10}$ TCD_{50}/ml.

The Effect of Lamb Serum on the Cytopathogenicity and Growth of Virus

The substitution of 10% lamb serum for 10% ox serum, in the growth and maintenance media, immediately increased the speed and extent of the cytopathic effect, resulting in the production of more and larger syncytia (see Fig. 9 and 10) and increased numbers of stellate cells with anastomosing processes, especially if the cultures were rolled. Lamb serum did not, however, increase virus yields on the 6th day; as was shown by the results of titrations given in Table 1.

Haemadsorption (Results)

Measles-infected cultures showed, as expected, adsorption of erythrocytes of the 2 monkey species but not with those of other animals. Extensive haemadsorption occurred to syncytia (*Kohn*, 1962) and to other morphologically-altered cells. Rinderpest-infected cultures did not adsorb any of the erythrocytes which were tested, although they showed extensive cytopathic changes, inducing syncytium formation.

Fig. 9 and 10. Rinderpest-infected HeLa cells grown with normal ox or normal lamb serum. Roller-tube cultures 4 days after infection with the same dose of virus. May-Grünwald-Giemsa staining (×125).

Fig. 9. Culture grown with 10% normal ox serum. Only a few, small, multinucleated cells, but many stellate cells are present. Numerous, small, cytoplasmic inclusion bodies.

Fig. 10. Culture grown with 10% normal lamb serum and showing large, confluent syncytia.

Neutralisation

The standard rinderpest-immune serum (7463) had a neutralising titre of $10^{-2.0}$ against $10^{2.6}$ TCD_{50} of HeLa-propagated rinderpest virus; against $10^{1.6}$ TCD_{50} of virus the titre was $10^{-3.0}$. The first figure was closely comparable to those obtained for the titre of this serum against $10^{2.2}$ to $10^{2.6}$ TCD_{50} of calf kidney-propagated rinderpest virus ($10^{-1.8}$ to $10^{-2.4}$, mean $10^{-2.1}$ in seven separate experiments). In four experiments the $\log_{10}$ SN_{50} titre of serum 7463 against HeLa propagated measles virus ($10^{1.6}$ to $10^{2.2}$ TCD_{50}) varied from a trace to $10^{-0.8}$, i. e. about 100-fold lower than against rinderpest virus.

Discussion

The particular strain of rinderpest virus employed in this study was propagated without great difficulty in HeLa cells and specific cytoplasmic inclusions were detected even in the first passage. Two virulent strains of virus, in the form of fluids from early bovine kidney passages or, in one case, of leucocytes from infected cattle blood, did not produce cytopathic effects in HeLa cells and the investigation was not carried further. The failure to detect any virus-specific cytologic changes in HeLa cultures of the second rinderpest passage may have been due to the relatively low multiplicity of infection (0.1); at this stage the indirect, fluorescent-antibody technique was of considerable value in following virus multiplication.

Measles virus, after 23 passages in human kidney cells, was reported by *Black* et al. (1956) to produce no virus-specific changes in the first two HeLa-cell passages but characteristic cytopathic effects were seen later. Adaptation of measles virus to growth into HeLa cultures with the production of typical cytopathic changes, was also reported by *Adams* et al. (1956) and *Adams* and *Imagawa* (1957). Attempts to use HeLa cultures for the primary isolation of measles virus were unsuccessful, according to *Bech* and *von Magnus* (1958).

The cytopathology of rinderpest virus in HeLa monolayers resembled that produced in calf kidney cells by the same strain of virus (*Plowright* and *Ferris*, 1959), at least in so far as the production of "stellate" cells and syncytia was concerned. The early cytoplasmic inclusions were definitely weakly basophilic to neutrophilic in HeLa cells, but this was after 5 minutes fixation in Carnoy's fluid and M. G. G. staining; *Plowright* and *Ferris* (1959) found these bodies to be eosinophilic, but they used Bouin fixation and haematoxylin-eosin staining. No intranuclear inclusions as described for calf kidney monolayers (*Plowright* and *Ferris*, 1959; *Plowright*, 1962), were seen in HeLa cells.

The predominance of cell-associated over free virus in rinderpest-infected HeLa cultures is similar to that recorded by *Black* (1959) for meas-

les virus growing in Hep-2 cells and by *Bussell* and *Karzon* (1962) for distemper virus in chick-embryo tissue cultures; the maximal titres attained were also comparable for the three viruses. According to the experiment described by *Plowright* (1962), the figures for intracellular virus in rinderpest-infected calf kidney cells were always the same or lower than those for free virus. But in this case it is possibly of significance that the cells were detached from the glass with a mixture of versene (0.02%) and trypsin (0.01%). The latter is now known to have a destructive effect on the infectivity of measles virus (*de Maeyer* and *Enders*, 1961; *Ruckle-Enders*, 1962), and it is possible that infective rinderpest virus at the cell surface may be susceptible to inactivation by trypsin.

The difference in the haemadsorbing ability of measles- and rinderpest-infected cultures offers a simple means of differentiating these two agents.

Summary

After 95 passages in primary calf kidney cultures rinderpest virus proliferated readily in HeLa cells and produced characteristic cytopathic effects. A growth-curve experiment showed that the titre of intracellular virus was consistently 10—100 fold greater than that of free virus, thus conforming to the pattern usually described for the agents of measles and canine distemper. Whereas HeLa cultures infected with measles virus readily adsorbed erythrocytes of *Cercopithecus aethiops* (Vervet) and *Papio* sp. (Baboon), no haemadsorption was noted in rinderpest-infected cultures. The latter also failed to adsorb erythrocytes of rabbits, guinea pigs, sheep, ox and dog.

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