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Role of Interferon in Replication of Virulent and Attenuated Strains of Measles Virus (*)

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SUMMARY

Small concentrations of actinomycin D increased the yields of both virulent and attenuated strains of measles virus in cultures of the stable simian BSC-I cell line by as much as five- to tenfold. The EDMONSTON virulent strain of measles virus did not induce detectable levels of interferon in these cells but interferon was induced by the SCHWARZ vaccine (attenuated)strain of virus over a wide range of virus input. Actinomycin D suppressed the induction of interferon in BSC-I cells. Interferon produced in BSC-I cells by the attenuated virus was able to protect both these cells and Vero (another stable line of green monkey kidney) cells against vesicular stomatitis virus.

Plaques produced by the virulent and attenuated virus in Vero cells were approximately the same size, in contrast to variations observed in BSC-I cells. This was correlated with the failure of the Vero cells to produce interferon when inoculated with either the virulent or attenuated strain of virus and the failure of actinomycin D to affect yields of infectious virus. The results suggest that the effect of actinomycin D on virus yields and on plaque size in BSC-I cells is a result of inhibition of introduction of interferon.

INTRODUCTION

Pre-treatment of chick-embryo fibroblast monolayers with actinomycin D before infection with the EDMONSTON (virulent) strain of measles virus led to a higher yield of virus than that produced in untreated cells infected with the same virus (Anderson & Atherton, 1964). This finding was confirmed by Matumoto, Arita & Oda (1965), who used the SUGIYAMA strain of measles virus adapted to FL cells. However, Parfanovich *et al.* (1966) were unable to detect any difference in yields of measles virus grown in chick embryo fibroblasts or human cells in the absence or presence of 0.1 to 1 μg./ml. of actinomycin D. The present report describes the replication of virulent and attenuated measles virus strains in the absence and presence of actinomycin D under conditions in which the genotype of the host cell appears to dictate the ability of the virus to induce interferon.

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METHODS

Virus. The virulent EDMONSTON strain of measles virus used in this study has been described in detail (Rapp. 1964). The virus has been passed six times in BSC-1 cells. The SCHWARZ attenuated strain was isolated from a commercial lot of vaccine and was passed four times in BSC-1 cells. All virus stocks were prepared in cells growing in 16 OZ (470 ml.) bottles. When virus-induced cytopathic effects involved 75 to 100% of the cells (2 to 4 days), the cells were disrupted into the medium by two cycles of freezing and thawing. The suspension was clarified by low speed centrifugation and the supernatant fluid dispensed in 1 ml. ampoules. The ampoules were flame-sealed, the virus was quick-frozen and the ampoules stored at —65° until used.

Cells. The BSC-1 cells, a continuous cell line derived from African green monkey kidneys (Hopps *et al.* 1963), were received from Dr R. Dulbecco. The cells were grown in bottle cultures in Eagle's basal medium supplemented with 10% foetal bovine serum, 10% tryptose phosphate broth, antibiotics (100 units penicillin, 100 μg. streptomycin and 50 units mycostatin/ml.) and 0.75 g. sodium bicarbonate/l. When the cells were grown in Petri dishes in an atmosphere of 5% CO_2, the same medium was used except that it contained 2.25 g./l. of sodium bicarbonate. Maintenance medium contained only 5% foetal serum. Human embryonic kidney (HEK) cells were grown in Melnick-Hanks medium supplemented with 10% foetal serum, antibiotics and bicarbonate as described above. These cells were maintained in the same medium containing 2% foetal serum. Cells were grown in 16 × 125 mm. tubes for virus growth studies and in 60 mm. plastic Petri dishes for plaque titrations. The Vero cells used in these experiments were originally established from the kidneys of African green monkeys by Yasumara & Kawatika (see Shishido *et al.* 1967) and were supplied by Dr J. Desmyter. They were grown and maintained as described above for the BSC-1 cells. Desmyter, Melnick & Rawls (1958) have recently shown that this strain of Vero cells is defective in interferon production when challenged with many viruses known to induce interferon.

Growth studies and virus assay. Replicate monolayers containing 3 to 5 × 10^5 HEK or BSC-1 cells in tubes were infected with a known multiplicity of virus. After 90 min. of adsorption all cultures were washed twice with warm tris buffer (pH 7.4) and maintenance medium was added. After various intervals the cultures in two tubes were frozen and thawed twice. The cell debris was removed by low speed centrifugation and the virus dispensed in 1 ml. ampoules, quick-frozen and stored at —65°. All harvests were assayed by the plaque technique under agar in BSC-1 cells as previously described (Rapp. 1964). Two 60 mm. dishes were used for each virus dilution and plaques were read 4 days after inoculation of the cultures. When Vero cells were used in the plaque assay, the second overlay was omitted and plaques were stained on the fourth day with a 1/30,000 dilution of neutral red.

Actinomycin D. Actinomycin D was dissolved in Eagle's basal medium at 37° at a concentration of 1 mg./ml. and stored at —20°. For use, this solution was thawed, diluted as desired in Eagle's basal medium and added to the cell cultures 2 hr before infection of the monolayer. Actinomycin D was also included in the maintenance medium during the period of virus replication.

Interferon production and assay. Measles interferon was prepared in BSC-1 and Vero cells according to the technique suggested by Desmyter *et al.* (1967). Simply, the

fluids of harvests were acidified to pH 2 with concentrated HCl diluted 1/20 (0.7 ml. of diluted acid per 10 ml. of sample) and store at 4° for 48 hr. At this time, the pH was brought to neutrality with N-NaOH. The fluids were then centrifuged twice, two hr each time, at 30,000 rev./min. in a Spinco Model L 2 ultracentrifuge, using an SW30 rotor. The preparations were shown to be free of measles virus by inoculation of five tubes of BSC-I cells. It was also shown that trypsin treatment destroyed the interferon activity of the preparations. For interferon assays, monolayers of BSC-I or Vero cells in Petri dishes were drained and covered with 3.5 ml. of Eagle's basal medium containing 2% foetal serum. Then 0.5 ml. of various dilutions of interferon were added to the cultures. At least two Petri dishes were used for each dilution of interferon. After 18 hr of incubation at 37° in a CO2 incubator, the fluids were removed and the cells were washed once with 4 ml. of Eagle's medium (without serum, pH adjusted to 7.2) and challenged with 70 to 100 p.f.u. of vesicular stomatitis virus suspended in 0.2 ml. of Eagle's basal medium. After adsorption for one hr at 37°, 5 ml. of an overlay containing 1.2% bacto agar, 10% foetal serum, 1/30,000 neutral red and 0.22% bicarbonate were added.

The results of interferon assays were recorded 2 days after incubation of the challenged cultures in a CO2 incubator at 37° as the reciprocal of the highest serial dilution which resulted in approximately 50% reduction of plaque formation by vesicular stomatitis virus as compared with untreated controls. Cultures treated with dilutions of interferon of known potency were included as positive controls.

RESULTS

Effect of actinomycin D on replication of virulent measles virus

BSC-I and HEK cells were infected with the EDMONSTON virulent measles strain at an input multiplicity of about 1 p.f.u./cell. Two hr before infection, indicated doses of actinomycin D (Table 1) were added to the growth medium. Three tube cultures of cells were used for each dose of actinomycin D. The virus was adsorbed at room temperature for 90 min., at which time the cultures were washed twice with warm tris-saline and 1 ml. of maintenance medium containing the same amount of actinomycin D was added to each tube. The cultures were incubated at 37° for 36 hr. Concurrently, confluent monolayers of both types of cells on coverslips were flooded with maintenance medium containing the indicated doses of actinomycin D. Two coverslips were used for each dose of actinomycin D. After 36 hr the cultures were stained with haematoxylin and eosin and the cells examined for cytological changes.

All HEK cell cultures treated with 0.0125 to 0.2 μg./ml. of actinomycin D showed evidence of cell destruction; the control cells not treated with actinomycin D were morphologically normal. Subsequent experiments confirmed these results. The BSC-I cells tolerated 0.1 μg./ml. of actinomycin D for the period of observation but higher concentrations of actinomycin D destroyed the cultures in 24 to 48 hr.

Yields of measles virus 36 hr after inoculation of the cultuers in the presence and absence of actinomycin D are summarized in Table 1. Increasing concentrations of actinomycin up to 0.1 μg./ml. resulted in increased virus yields from BSC-I cells. A higher concentration (0.2 μg./ml.) of the drug was toxic for BSC-I cells and they were unable to support virus growth. A similar pattern of toxicity was observed for

Vero cells. The otxicity of actinomycin D for HEK cells was reflected in low yields of virus (Table 1). Subsequent experiments, therefore, employed only BSC-I and Vero cells.

The previous experiments summarized in Table 1 demonstrated that the optium

Table 1. *Effect of varying concentrations of actinomycin D on production of virulent measles virus*

Actinomycin D μg./ml.	P.f.u.* per ml. × 10 ⁻¹ BSC-1	HEK
None	210	280
0·0125	N.T.	90
0·0250	240	35
0·0500	300	18
0·0750	460	2
0·1000	1000	0·01
0·2000	0·02	N.T.

HEK, Human embryonic kidney cells.
N.T., Not tested.
* 36 hr after inoculation.

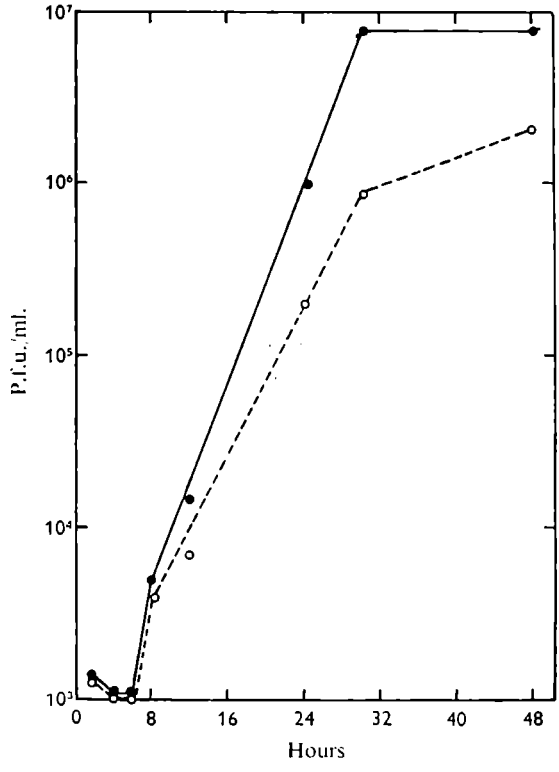

Fig. 1. Replication of EDMONSTON virulent strain of measles virus in BSC-1 cells in absence and presence of actinomycin D. Multiplicity of infection was about 1 p.f.u./cell. ●—●, Actinomycin D 0·1 μg./ml.; ○- -○, no actinomycin.

effect of actinomycin D in BSC-I cells was achieved with 0.1 μg./ml. Growth studies were then performed using this concentration of the drug. Replicate cultures of BSC-I cells in tubes were infected with the virulent strain of measles virus at a multiplicity of I p.f.u./cell. After 90 min. adsorption at room temperature, the cultures were washed twice with warm tris-saline. Drug-free maintenance medium or medium containing 0.1 μl./ml. actinomycin D was then added and the cultures reincubated.

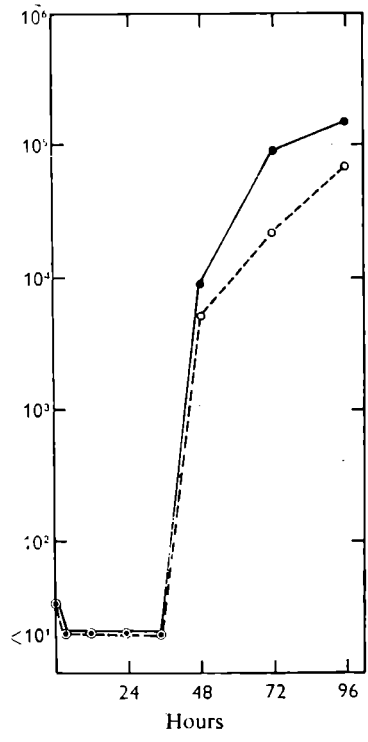

Fig. 3

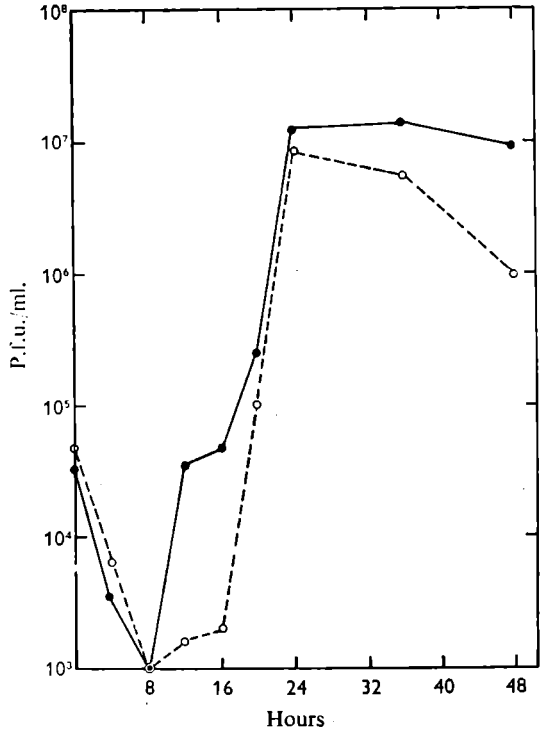

Fig. 2

Fig. 2. Replication of EDMONSTON virulent strain of measles virus in BSC-1 cells in absence and presence of actinomycin D. Multiplicity of infection was about 10 p.f.u./cell.

Fig. 3. Replication of SCHWARZ attenuated strain of measles virus in BSC-1 cells in absence and presence of actinomycin D. Multiplicity of infection was about 0·01 p.f.u./cell. ●—●, Actinomycin D 0·1 μg./ml.; O- -O, no actinomycin.

New virus was detected as early as 8 hr after infection in drug-free and actinomycin D-treated cultures (Fig. 1). At this time a few giant cells were observed in unstained cells, both in the presence and absence of actinomycin D. The maximum titre in cultures treated with actinomycin D was reached 30 hr after infection and represented about 90% more virus than was obtained from control (no drug) cultures at this time. At 48 hr after inoculation the yield from actinomycin D-treated cultures was still about fivefold greater than from untreated cells. However, when a higher multiplicity of inefction was used (10 p.f.u./cell), the difference in yields from treated and untreated

BSC-1 cells was not as marked although there was a decreased latent period in the actinomycin-treated cultures as compared to drug-free cultures (Fig. 2).

Relpication of attenuated measles virus in the presence of actinomycin D

BSC-1 cells in tubes were infected with the SCHWARZ attenuated vaccine strain of measles virus at a multiplicity of infection of 0.02 p.f.u./cell The lower input was used because it was difficult to obtain high-titre stocks of the attenuated virus. Cell monolayers were incubated for two hr before infection with maintenance medium containing 0.1 μg./ml. of actinomycin D and this concentration was maintained thereafter. The controls were treated in the same manner but without the drug. After 90 min. of virus adsorption at 23 to 24° (room temperature), the tubes were incubated at 33° and tested at intervals for total virus production.

In confirmation of our previous finding (Rapp, 1964), 32 to 48 hr were required for the detection of progeny attenuated virus (Fig. 3). The maximum yield of the SCHWARZ virus was not reached until 72 to 96 hr after infection and actinomycin-treated cultures regularly yielded more virus than did untreated cultures. The difference in titre, though not striking, was reproducible.

Replication of virulent and attenuated measles virus in Vero cells

When Vero cells were infected with virulent measles virus, multinucleated giant cells appeared as early as 12 hr after infection at a multiplicity of infection of about 1 p.f.u./cell. Most of the cell monolayer was involved within 24 hr and the entire monolayer was destroyed in 48 hr. When the attenuated SCHWARZ strain was used, giant cells were more localiezd in the first 24 hr; however, 80% of the surface of the monolayer was covered with large giant cells at the end of the second day of infection. The difference in the response of Vero cells to the virulent and attenuated SCHWARZ strains was much less striking than that normally observed in BSC-1 cells.

When actinomycin D, at a concentration of 0.1 μg./ml., was incorporated into the maintenance medium, no significant differences in cytopathic effects were noticed in drug-treated Vero cells and controls. In BSC-1 cells this difference was quite obvious and cells treated with actinomycin exhibited faster cytopathic effects than the control cultures not treated with the drug.

A study of the replication of the SCHWARZ attenuated virus in Vero cells was then undertaken. The experiments were similar to those carried out in BSC-1 cells. The multiplicity of infection was 0.02 p.f.u./cell. Monolayers were treated for two hr before infection with 0.1 uμ./ml. of actinomycin D and this concentration of the drug was maintained in the cultures until they were harvested (Fig. 4). Although the first progeny virus in BSC-1 cells was not detected until 48 hr after infection (Fig. 3), new virus was detected in the Vero cells by 36 hr. Vero cells showed the same response to infection with attenuated measles virus in the presence or absence of actinomycin D (Fig. 4).

Under agar overlay, round plaques developed within 4 days after inoculation of Vero cells with either virulent or attenuated strains of measles virus. The plaques induced by the attenuated virus were the same size as those induced by the virulent

virus. In both cases, plaques of about 2 mm. were observed. The size and the concentration of plaques were reduced when neutral red was incorporated into the overlay. The heterogeneity reported earlier (Rapp, 1964) for plaques induced by virulent measles virus in BSC-I was also observed in Vero cells.

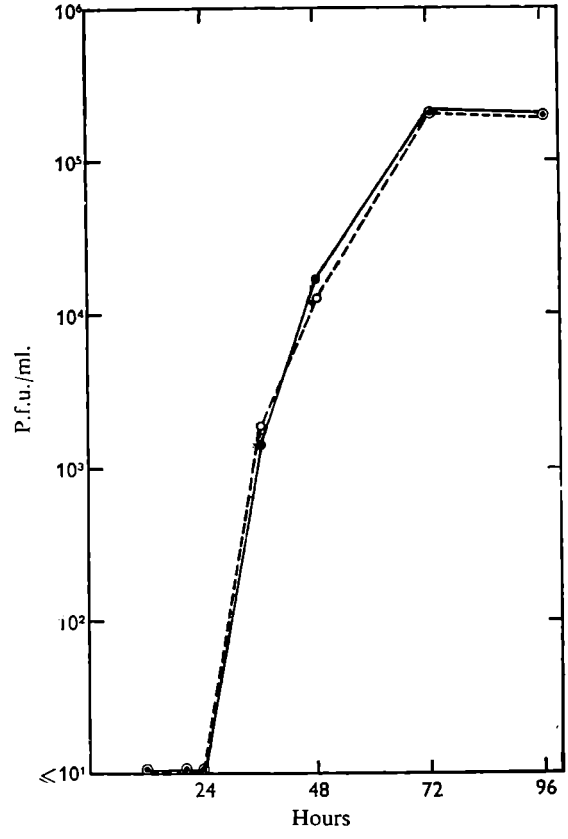

Fig. 4. Replication of SCHWARZ attenuated strain of measles virus in Vero cells in absence and presence of actinomycin D. ●—●, Actinomycin D 0·1 μg./ml.; O- -O, no actinomycin.

Interferon production in BSC-1 cells

The production of interferon was first investigated in BSC-I cells using different multiplicities of infection. The results (Table 2) show that virulent measles virus produced less (non-detectable levels) interferon than did the attenuated virus. The latter induced fairly constant levels of inerferon at multiplicities ranging from 0.001 to 0.1 p.f.u./cell. In subsequent experiments it was shown that BSC-I cells infected with only 0.02 p.f.u./cell produced maximum titres of interferon within 3 to 4 days after infection. Interferon induced in BSC-I by the SCHWARZ strain was then titrated

in BSC-I and Vero cells under identical conditions. It was found that the sensitivity of Vero cells was slightly lower than that of BSC-I, but both cells were protected by the exogenous interferon against vesicular stomatitis virus (see also Table 3).

Production of interferon in BSC-I and Vero cells treated with actinomycin D

BSC-I and Vero cells in bottle cultures were treated with 0.1 μg./ml. of actinomycin D and infected with 0.02 p.f.u. of SCHWARZ measles virus/cell.. Three days after incubation at 33°, the fluids were harvested, acidified and centrifuged as described in Methods. Each sample was titrated in both BSC-I and Vero cells. The results (Table 3) show that while interferon was not produced in Vero cells in the presence or absence of actinomycin D, it was produced in BSC-I cells when the monolayer was not treated with actinomycin D. Results in Table 3 also reveal that interferon produced in BSC-I cells can protect Vero cells against challenge with vesicular stomatitis virus.

Table 2. *Interferon production following inoculation of BSC-I cells with varying multipliciites of virulent and attenuated strains of measles virus*

Virus	Input in p.f.u./cell	Interferon titre*
EDMONSTON, virulent	1	< 4
	0·2	< 4
	0·02	< 4
SCHWARZ, attenuated	0·1	8
	0·02	32
	0·01	16
	0·001	16

* Titre expressed as reciprocal of interferon dilution resulting in 50% reduction in plaques formed by vesicular stomatitis virus.

Table 3. *Comparative production of interferon in BSC-I and Vero cells by attenuated measles virus in presence and absence of actinomycin D*

Virus	Actinomycin D	Origin of interferon	Interferon titre* in BSC-1	Interferon titre* in Vero
SCHWARZ, attenuated	Absent	BSC-1	32	16
SCHWARZ, attenuated	Present†	BSC-1	< 4	< 4
SCHWARZ, attenuated	Absent	Vero	< 4	< 4
SCHWARZ, attenuated	Present†	Vero	< 4	< 4

* Titre expressed as reciprocal of interferon dilution resulting in 50% reduction in plaques by vesicular stomatitis virus.

† 0·1 μg./ml. during virus replication.

DISCUSSION

Ho & Enders (1959) and De Maeyer & Enders (1961) originally described the induction of the synthesis of interferon in cultures infected with measles virus. Enders (1962) and De Maeyer & Enders (1965) later suggested that attenuation of measles virus strains was accompanied by, or was perhaps even due to, the ability of such strains to induce larger amounts of interferon than that induced by virulent strains of the same virus. The results presented in this report clearly support the observations that the SCHWARZ vaccine strain of measles virus is able to induce larger amounts of interferon in BSC-I cells than is the virulent EDMONSTON strain of virus, as Desmyter *et al.* (1967) found *in vivo* in children.

It is of some interest that cultures infected with *either* the virulent or attenuated strain of measles virus yielded larger amounts of virus in BSC-I cells in the presence of actinomycin. D. These results are similar to those previously observed with arboviruses (Heller, 1963; White & Cheyne, 1965) and with Newcastle disease virus (Wheelock, 1963). The ability of actinomycin D to suppress the synthesis of messenger RNA as well as DNA-dependent ribosomal RNA (Reich *et al.* 1962) and the need for mRNA for synthesis of interferon has suggested that the drug depresses interferon synthesis by suppressing synthesis of DNA-dependent RNA. Since it is most probable that the suppression of the synthesis of interferon by actinomycin D was the reason for enhanced virus yields, the observations suggest that a test for higher yields of virus in the presence of actinomycin may be a more sensitive indicator for the production of interferon than is the extraction and assay of interferon from infected cultures. It is possible that the extraction and assay procedures destroy some quantities of interferon or simply that the challenge procedures are not sensitive enough to detect small amounts of the compound.

The ability of BSC-I cells to produce interferon had been previously demonstrated by Russell *et al.* (1966) with dengue virus. The finding by Desmyter *et al.* (1968) that Vero cells fail to produce interferon following exposure to a variety of interferon-inducing viruses suggested the possibility that this cell line would be useful to determine the role of interferon in measles virus replication. The failure of measles virus to induce interferon in this cell system supports the finding of Desmyter *et al.* (1968) with other viruses.

The observations presented in this report can best be explained by assuming that the mechanism of action of actinomycin D in increasing infectious measles virus yields from cells capable of making interferon is due to the suppressive action of actinomycin D on the synthesis of interferon. Although the Vero cells were unable to synthesize interferon, they could be protected by exogenous interferon (made in BSC-I cells) from challenge with vesicular stomatitis virus, a finding that also confirms an observation made by Desmyter *et al.* (1968) with other viruses.

Apparently, plaque size is also affected by interferon production. This is probably the reason that plaques of attenuated measles strains in BSC-I cells (Rapp, 1964) are smaller than those induced by the virulent virus. In Vero cells where interferon is not produced, the size of the plaques induced by virulent and attenuated strains are approximately the same. Thus, the use of plaque morphology to distinguish between virulent and attenuated strains of virus must be correlated with the ability of the cell

to synthesize interferon. In cell systems in which interferon cannot be detected by usual procedures, it may be worthwhile to measure the effects of actinomycin D on virus yields as another factor in the possible synthesis of small amounts of interferon. However, it must be pointed out that interferon does not account for all the observed differences between the growth characteristics of the virulent and attenauted viruses. The attenuated virus still has a 24 hr eclipse phase in Vero cells (as compared to the shorter eclipse of the virulent strain in the presence of actinomycin D in BSC-I cells) and attains lower titres than the virulent virus (compare Fig. 4 with Fig. 1).

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