J. Hyg., Camb. (1978), **81**, 171
Printed in Great Britain

Differential inhibitory effects of 5-bromodeoxyuridine on vaccinia and monkeypox viruses

By T. YOSHII and R. KONO

Central Virus Diagnostic Laboratory, National Institute of Health, Nakato, Musashimurayama-shi, Tokyo 190-12, *Japan*

(*Received* 15 *November* 1977)

SUMMARY

Replication of vaccinia and monkeypox viruses was impeded in the presence of 5-bromodeoxyuridine (BUdR) in RL-33 cells derived from rabbit lung tissue. The different degree of inhibition was found among strains of those viruses, in which it was evident that five strains (CV-1, Lister, IHD, Dairen-I and Ikeda) of vaccinia viruses resulted in more reduced yields of infectious virus than four strains (Sen-19, Orang Utan, Copenhagen and Sierra Leone) of monkeypox viruses. There was also strain variation in BUdR sensitivity within vaccinia viruses but not in the case of monkeypox viruses.

INTRODUCTION

Since the monkeypox virus was first described by Magnus *et al.* (1959), a number of poxviruses which belonged to the variola-vaccinia subgroup were isolated from monkeys, apes and humans suffering from a smallpox-like illness. The isolations of variola-like viruses from monkeys and apes have brought a problem in smallpox eradication programmes, requiring further studies for elucidating the origin of those viruses. Differences in biological characters between vaccinia and monkeypox virus strains are becoming increasingly apparent (Arita & Henderson, 1968; Lourie *et al.* 1972; Marennikova *et al.* 1972; Gispen, Verlinde & Zwart, 1967; Bourke & Dumbell, 1972): they are ceiling temperature for growth, lesions on chorioallantoic membrane, replication and plaques in cell cultures, reaction in rabbits following infection, production of haemagglutinin and so forth. In addition, Gispen & Brand-Saathof (1974) discovered three precipitable specific antigenic substances produced by vaccinia, variola, and monkeypox infections using the immunodiffusion technique.

On the other hand, we noticed the study by Easterbrook & Davern (1963) suggesting a different susceptibility of two vaccinia virus strains, IHD and V-MH to 5-bromodeoxyuridine (BUdR) inhibition. We attempted to extend this line of study to obtain more precise information with regard to different susceptibility between vaccinia and monkeypox viruses, which has not been reported before. The present communication describes the differential inhibitory effects of BUdR on the replication of vaccinia and monkeypox viruses in RL-33 cells.

MATERIALS AND METHODS

Virus

Vaccinia (strains of CV-1, Lister, IHD, Dairen-I and Ikeda) and monkeypox (strains of Sen-19, Orang Utan, Copenhagen and Sierra Leone) viruses used were from the Department of Enteroviruses, National Institute of Health, Tokyo. Virus stocks were prepared by passing in RK-13 cells twice and stored at −70 °C.

Cell culture

RL-33 cells (Yoshii & Kono, 1977) derived from rabbit lung were grown in Eagle's minimum essential medium (MEM) containing 10% inactivated calf serum, 0·11% $NaHCO_3$ and antibiotics (penicillin 250 i.u./ml, streptomycin 100 μg/ml). Subcultivations of the cells were done at a split ratio of 5:1 every 6 to 7 days. Maintenance medium was MEM containing 3% inactivated calf serum, 0·11% $NaHCO_3$ and antibiotics.

Infectivity assay

Plaque assay was carried out as follows: Confluent monolayers of RL-33 cells in 2-oz prescription bottles were washed once with phosphate-buffered saline (PBS), and inoculated with appropriately diluted virus in 0·2 ml amounts. After virus adsorption for 2·5 h at 37 °C, the infected cell cultures were overlaid with 6 ml of agar medium, and incubated at 37 °C. Agar overlay medium consisted of MEM containing 0·8% Bacto-agar (Difco), 0·15% $NaHCO_3$, 2% inactivated calf serum, antibiotics (penicillin 250 i.u./ml, streptomycin 100 μg/ml) and DEAE-dextran (Pharmacia Mw: 2×10^6) at concentrations of 200 μg/ml. The second overlay medium containing 0·006% neutral red was added in 3 ml amounts to the cultures 4 days after virus inoculation. Plaques were counted from day 5 to 6 after inoculation.

Preparation and photodynamic inactivation of NR virus

This procedure for virus inactivation was adopted on one step growth experiment (shown in Fig. 4). The inactivation by white light of poliovirus grown in the presence of neutral red was described (Wilson & Cooper, 1965). Virus grown in neutral red (NR) solution is referred to as 'NR virus'. The NR pox virus was prepared by passing in MEM containing 10 μg/ml of NR in RK-13 cells twice in the dark. The NR virus was employed for photodynamic inactivation of residual virus on infected cells after washing off excess virus at the end of the adsorption period. Irradiations took place at 27 °C, 6 cm below two 15-watt 'white light' fluorescent strips.

Replication of viruses in the presence or absence of BUdR

Confluent monolayers of RL-33 cells grown in 2-oz prescription bottles were washed twice with PBS. Cells were infected with poxvirus at various multiplicities according to the experiments and the virus was allowed to adsorb for 2 h at 37 °C. After adsorption, the infected cell monolayers were washed three times with

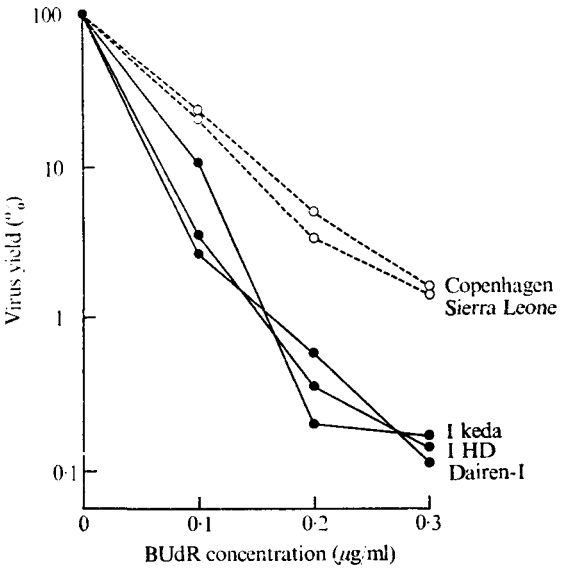

Fig. 1. The effect of BUdR on the replication of vaccinia and monkeypox viruses in RL-33 cells. RL-33 cells were seeded in 2-oz prescription bottles and infected at an input m.o.i. of 0·005 p.f.u./cell. After adsorption for 2 h at 37 °C, the infected cell monolayers were washed 3 times with PBS, 5 ml of maintenance medium containing various concentrations of the drug was added to the bottles and the infected cultures were incubated at 37 °C for 30 h p.i. Depression of virus yield: ●—●, vaccinia viruses; ○---○, monkeypox viruses.

PBS to remove excess virus, and 5 ml amounts of maintenance medium in the presence or absence of various concentrations of BUdR were added immediately. Thereafter, cell cultures were incubated at 37 °C during each experimental period. Cell cultures were disrupted and the supernatant fluid obtained by low speed centrifugation was tested for infectivity assay as described above.

Chemicals

The stock solutions of BUdR (Sigma-chem) were made in PBS and kept at −20 °C. All operations concerning the use of BUdR were performed in the dark.

RESULTS

Effect of BUdR on the multiplication of vaccinia and monkeypox viruses

Figures 1 and 2 show the dose response relation between depression of virus yields at 30 h harvest after inoculation and the concentration of BUdR present in the medium. In the range of 0·2 μg/ml to 0·3 μg/ml of the compound, approximately tenfold or more decrease of virus yields was shown between vaccinia and monkeypox viruses. The results indicate that the members of vaccinia were more sensitive than those of monkeypox to BUdR inhibition. As shown in Fig. 3, it is of interest that strain variation in sensitivity to BUdR was evident among vaccinia viruses in the experiment of 72 h harvest after inoculation as suggested previously (Easterbrook & Davern, 1963). On the one hand, no such strain variation was observed among 4 strains of monkeypox viruses. Complete inhibition of all the

https://doi.org/10.1017/S0022172400024992 Published online by Cambridge University Press

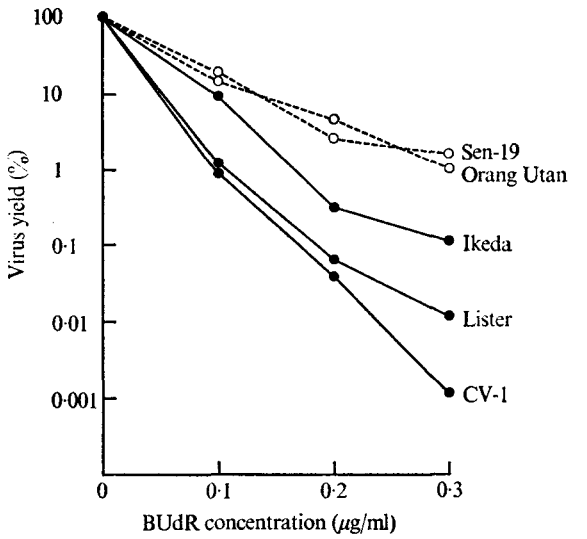

Fig. 2. The effect of BUdR on the replication of vaccinia and monkeypox viruses in RL-33 cells as in Fig. 1.

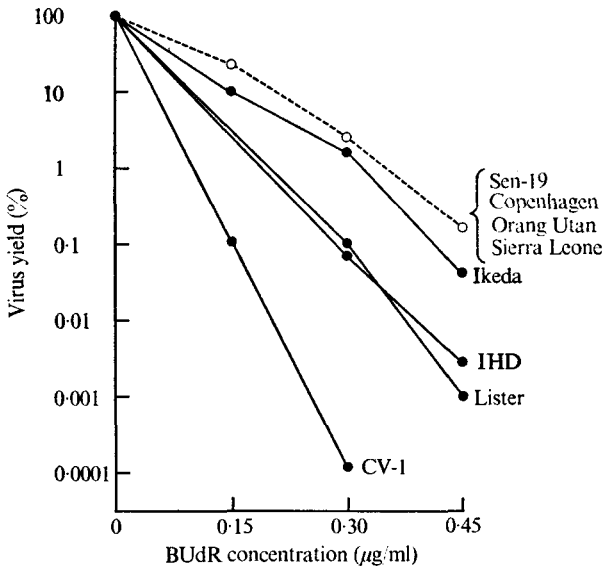

Fig. 3. The effect of BUdR on the replication of vaccinia and monkeypox viruses in RL-33 cells. The procedure was carried out as described in the legend to Fig. 1, but the input m.o.i. was 0·0005 p.f.u./cell and the infected cultures were harvested at 72 h after inoculation. The results with four strains of monkeypox viruses were almost identical inhibition of production of infectious virus.

viruses tested occurred at the concentration of 0·6 μg/ml of the compound in the medium. Furthermore, the experiment of 24 h harvest after incubation (Fig. 4) was performed to exclude the possibility that the results indicated in Figs. 1, 2 and 3 might have been due to the mutants induced by BUdR as a mutagen of DNA virus (Sambrook, Padgett & Tomkins, 1966). Such a possibility was removed by the one-step growth inhibition test by the compound.

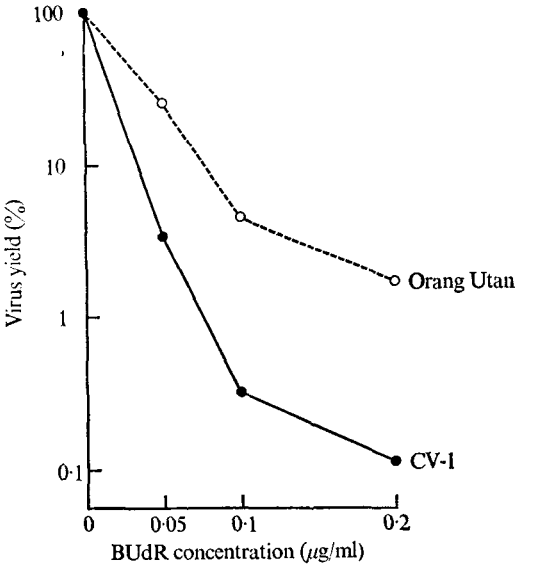

Fig. 4. The effect of BUdR on the replication of vaccinia and monkeypox viruses in RL-33 cells using NR viruses described in the text. The procedure was carried out as in the legend to Fig. 1, but the input m.o.i. was 0·1 and 0·02 p.f.u./cell for CV-1 and Orang Utan strains respectively. Inactivation of residual viruses after virus adsorption was described in the text. Infected cultures were harvested at 24 h after inoculation.

Table 1. *Effect of BUdR on the cytopathic effects by vaccinia and monkeypox viruses in RL-33 cells**

	Development of c.p.e. at 3 days after inoculation								
	Vaccinia					Monkeypox			
Conc. of BUdR (μg/ml)	CV-1	Dairen-I	IHD	Lister	Ikeda	Orang Utan	Sen-19	Copen-hagen	Sierra Leone
0	++++†	++++	++++	+++	++++	++	++	++	++
0·05	+++	+++	++++	++	+++	++	++	++	++
0·10	+	++	++	+	++	+	+	+	+
0·20	−	+	+	+	+	+	+	+	+
0·40	−	−	−	−	−	−	−	−	−
0·60	−	−	−	−	−	−	−	−	−

* Monolayers of RL-33 cells were infected with 500 to 1000 p.f.u. of virus and they were incubated with MEM in the presence or absence of various concentrations of BUdR at 37 °C.

† ++++ indicates extensive destruction of cell monolayer; +, indicates small regions of c.p.e.; −, no c.p.e.

Effect of BUdR on the development of viral cytopathic effects

Inhibition of viral c.p.e. by BUdR was tested in the presence or absence of various concentrations of the compound in 2-oz prescription bottle cultures of RL-33 cells. The inoculum consisted of 500–1000 p.f.u. of virus and the concentrations of BUdR were indicated in Table 1. The different degree of inhibitory effects of the compound on the development of c.p.e. was observed between vaccinia and

https://doi.org/10.1017/S0022172400024992 Published online by Cambridge University Press

Table 2. *Titrations of vaccinia and monkeypox viruses in the presence or absence of BUdR in RL-33 cells*

Virus	Log $TCID_{50}$/ml BUdR 0 μg	Log $TCID_{50}$/ml BUdR 0·3 μg	Log infectivity depression
Vaccinia			
CV-1	5·9	3·9	2·0
IHD	6·9	5·4	1·5
Monkeypox			
Copenhagen	6·7	6·2	0·5
Orang Utan	6·4	6·2	0·2
Sierra Leone	6·2	5·9	0·3

monkeypox viruses at the third day after inoculation (Table 1). Under these conditions, members of vaccinia were more sensitive than those of monkeypox to BUdR inhibition on the appearance of c.p.e. in RL-33 cells. No cytotoxicity of the compound at concentrations employed here was observed.

Titrations of viruses were also carried out in the presence (0·3 μg/ml) or absence of BUdR. Serial tenfold dilutions of the viruses were prepared in MEM, and 0·2 ml amounts of each dilution were inoculated into each of four tube cultures of RL-33 cells. The inoculated cultures were incubated at 37 °C and were observed daily for 6 days for the presence of cytopathic changes. The results obtained are summarized in Table 2. As can be seen, titres of vaccinia were more affected than those of monkeypox by the compound.

DISCUSSION

The aim of this work was to compare the sensitivity of vaccinia and monkeypox viruses to a thymidine analog, 5-bromodeoxyuridine (BUdR) in RL-33 cells. Our findings suggest that the concentration of 0·2 μg/ml to 0·3 μg/ml of the inhibitor in the maintenance medium has different inhibitory effects on the multiplication of vaccinia and monkeypox viruses. This observation adds an additional *in vitro* marker for distinguishing vaccinia and monkeypox viruses. The suggestion of Easterbrook & Davern (1963) is in agreement with ours with regard to different susceptibility to BUdR among vaccinia virus strains. The CV-1 strain was much more sensitive to BUdR inhibition than 4 other strains of vaccinia viruses. On the other hand, there was little difference in that sensitivity among four strains of monkeypox viruses including one of human origin (the Sierra Leone strain). In addition, the plaque diameters produced in RL-33 cells on day 6 after inoculation by four strains of monkeypox virus were between 0·9 and 1·0 mm, whereas those produced by five strains of vaccinia virus were between 1·8 and 2·4 mm. (T. Yoshii, unpublished data).

Although the mechanism by which BUdR inhibits the replication of DNA viruses in varying degrees is not fully understood, this different inhibitory effect described in the present communication among poxviruses is of great practical value. Hence, the introduction of this marker may produce useful information in

searching for the origin of monkeypox viruses. The BUdR marker described here may be a useful tool for differentiation of monkeypox virus from white monkeypox (Gispen & Brand-Saathof, 1972) as well as variola viruses.

REFERENCES

Arita, I. & Henderson, D. A. (1968). Smallpox and monkeypox in non-human primates. *Bulletin of the World Health Organization* **39**, 277–83.

Bourke, A. T. C. & Dumbell, K. R. (1972). An unusual poxvirus from Nigeria. *Bulletin of the World Health Organization* **46**, 621–3.

Easterbrook, K. B. & Davern, C. I. (1963). The effect of 5-bromodeoxyuridine on the multiplication of vaccinia virus. *Virology* **19**, 509–20.

Gispen, R., Verlinde, J. D. & Zwart, P. (1967). Histopathological and virological studies on monkeypox. *Archiv. für die gesamte Virusforschung* **21**, 205–16.

Gispen, R. & Brand-Saathof, B. (1972). 'White' poxvirus strains from monkeys. *Bulletin of the World Health Organization* **46**, 585–92.

Gispen, R. & Brand-Saathof, B. (1974). Three specific antigens produced in vaccinia, variola, and monkeypox infections. *Journal of Infectious Diseases* **129**, 289–95.

Lourie, B., Bingham, P. G., Evans, H. H., Foster, S. O., Nakano, J. H. & Herrmann, K. L. (1972). Human infection with monkeypox virus: laboratory investigation of six cases in West Africa. *Bulletin of the World Health Organization* **46**, 633–9.

Magnus, P. von, Anderson, E. K., Peterson, K. B. & Anderson, A. B. (1959). A pox-like disease in cynomolgus monkeys. *Acta Pathologica et Microbiologica Scandinavica* **46**, 156–76.

Marennikova, S. S., Seluhina, E. M., Mal'ceva, N. N., Cimiskjan, K. L. & Macevic, G. R. (1972). Isolation and properties of the causal agent of a new variola-like disease (monkeypox) in man. *Bulletin of the World Health Organization* **46**, 599–611.

Sambrook, J. F., Padgett, B. L. & Tomkins, J. K. N. (1966). Conditional lethal mutants of rabbitpox virus. I. Isolation of host cell-dependent and temperature-dependent mutants. *Virology* **28**, 592–9.

Wilson, J. N. & Cooper, P. D. (1965). The effect of light on poliovirus grown in neutral red. *Virology* **26**, 1–9.

Yoshii, T. & Kono, R. (1977). Establishment of a rabbit lung cell line (RL-33) and its viral susceptibility. *Japanese Journal of Medical Science and Biology* **30**, 149–57.