THE INFLUENCE OF AGE AND NUMBER OF MADIN-DARBY BOVINE KIDNEY CELLS IN MONOLAYERS ON THE SIZE AND NUMBER OF PLAQUES FORMED BY BOVINE ENTEROVIRUS*

M. A. BARYA AND T. MOLL

Once a complete monolayer of MDBK cells was obtained, the plaque size of bovine enterovirus was apparently unaffected by the number of cells in the monolayer and the age of the cell culture. Similarly, the number of plaques produced by a given virus inoculum was not influenced by the cell concentration in a complete cell sheet. Nevertheless, the plaque count was adversely affected by the nutritive stage of the cell culture. The plaque decreased to about 30 per cent when the cell cultures were fed 48 hours before virus inoculation, instead of 20 to 24 hours.

The virus yield per cell was about 500 PFU when the cells were in the exponential phase of growth compared with only about 100 PFU per cell when the cells were in stationary or decline phase of growth.

INTRODUCTION

Any virus work requires accurate quantitation of infectivity of the virus concerned. Among the various methods of virus assay available, tissue culture constitutes the most modern and sensitive technique. However, there are some problems associated with tissue culture methodology. For instance, there is occasionally inconsistency in the number and size of plaques formed by a certain virus even though experimental conditions are apparently kept constant. This difficulty was experienced during studies on bovine enteroviruses (Barya, 1966).

A study was thus undertaken to determine the effect of age and number of Madin-Darby bovine kidney (MDBK) cells in monolayers on the size and number of plaques formed by bovine enterovirus.

MATERIALS AND METHODS

Bovine enterovirus, designated BEV-1, was isolated from the pooled

---

* Based on a portion of Ph. D. thesis accepted by the Washington State University.

** Department of Microbiology, Faculty of Veterinary Science, West Pakistan Agricultural University, Lyallpur and Department of Veterinary Microbiology, W.S.U., Pullman, Washington, U.S.A.

faecal samples of nine calves, three to six months old, and two pregnant cows (Moll and Davis, 1959). After plaque-purification by the method of Dulbecco and Vogt (1954), the virus stock was prepared according to the method adopted by Barya *et. al.* (1967).

The virus infectivity was quantitated in MDBK cell live cultures according to the method employed by Barya *et. al.* (1967).

An initial number of 3×10^5 MDBK cells in 2-ounce bottles was incubated at 37°C. for 13 days. The medium was replaced with Earle's maintenance medium (EMM) on day 3, 5, 7, 9, 10, 11 and 12. Three bottles were selected at random and each day the average cell count per bottle was determined by trypsin-dispersion and cell counting in a haemocytometer.

Each day, virus BEV-1 was diluted to about 1000 PFU per ml. and 0.2 ml. of the diluted virus titrated by the plaque method in each of three tissue culture bottles. After 48 hours incubation, the infected cells were stained and the plaques counted. The size and number of plaques was determined.

In order to estimate the virus yield per cell, three tissue-culture bottles were infected with 0.2 ml. of a 2×10^5 PFU of BEV-1 from day 2 through 13. The virus inoculum was adsorbed for 1/2 to 1 hour at 37°C. followed by addition of 6 ml. of maintenance medium and the infected bottles were incubated at 37°C. for 30 hours. The virus yield was pooled from three bottles after freeze-thawing three times. The pooled virus samples for each day were stored at 20°C. until assayed.

RESULTS

Under identical experimental conditions, the plaque size was relatively large (3-4 mm) when the cell monolayers were thin and incomplete, the plaque size decreased gradually as the number of cells in the monolayers increased. When a complete cell monolayer was obtained, the diameters of the plaques were approximately 2 mm. (Table 1, Fig. 1.)

The number of plaques formed by a given amount of virus in incomplete monolayers was approximately doubled when a complete cell sheet was inoculated. When a continuous monolayer was obtained, the age and number of cells did not seem to influence the number of plaques formed. However, it was found that the nutritive stage of the cells greatly affected the plaque numbers formed by a given virus. The numbers of plaques were consistently lower when the cells were fed with fresh maintenance medium 48 hours as compared to 24 hours before virus inoculation (Table 1, Fig. 1.)

The virus yield was approximately 200 to 500 PFU per cell for cells in the exponential phase of growth and only approximately 100 PFU per cell when

the cells were in the stationary phase (Table 1, Fig. 2.) The MDBK cells has a generation time of 24 hours (Table 1, Fig. 1.)

TABLE 1.—*Effect of Age of MDBK Cell Cultures and Number of Cells Per Culture on the Virus Yield, Number of Plaques and Plaque Size.*

Days at 37°C.	Virus Yield			Plaques	
	$\log_{10}$ of MDBK Cells per ml.	$\log_{10}$ PFU per ml.	PFU per Cell	Average No. per Bottle	Average size in mm. (48 hrs. of incubation)
0	4.700	..	..	..	..
1	4.670	..	..	..	..
2	4.964	7.653	490	..	..
3[a]	5.258	7.748	310	27	4.2
4	5.566	7.800	172	42	3.6
5[a]	5.790	7.937	140	58	2.8
6	5.896	7.775	76	75	2.7
7[a]	5.798	7.902	113	54	2.6
8	5.926	7.760	68	81	2.4
9[a]	5.801	7.923	133	70	2.3
10	5.877	7.858	96	74	2.3
11[b]	5.818	7.925	114	76	2.3
12	5.860	7.573	52	85	2.0
13[a]	5.965	7.760	62	82	2.1

(*a*) Cell culture fluid exchanged with Earl's maintenance medium containing 5% lamb serum.

(*b*) Cell culture fluid exchanged with maintenance medium containing 7.5% lamb serum.

DISCUSSION

Within a given time of incubation of infected cell monolayers, the size of virus plaques was influenced only up to to a certain extent by the number of cells in the monolayers. Where the monolayers became relatively thick and complete, the plaque size remained more or less constant. The differences in plaque size are most probably due to the cell concentration around the centre of infection. Thus infective virus particles, released from infected cells, spread and infect cells farther from the focus of infection when the cell concentration is low. Young cell cultures, when in the exponential phase of growth, synthe-

size about 500 PFU per cell while only 100 PFU per infected cell are synthesized in the stationary phase of growth. This might also be reflected in a larger plaque size in young cell monolayers.

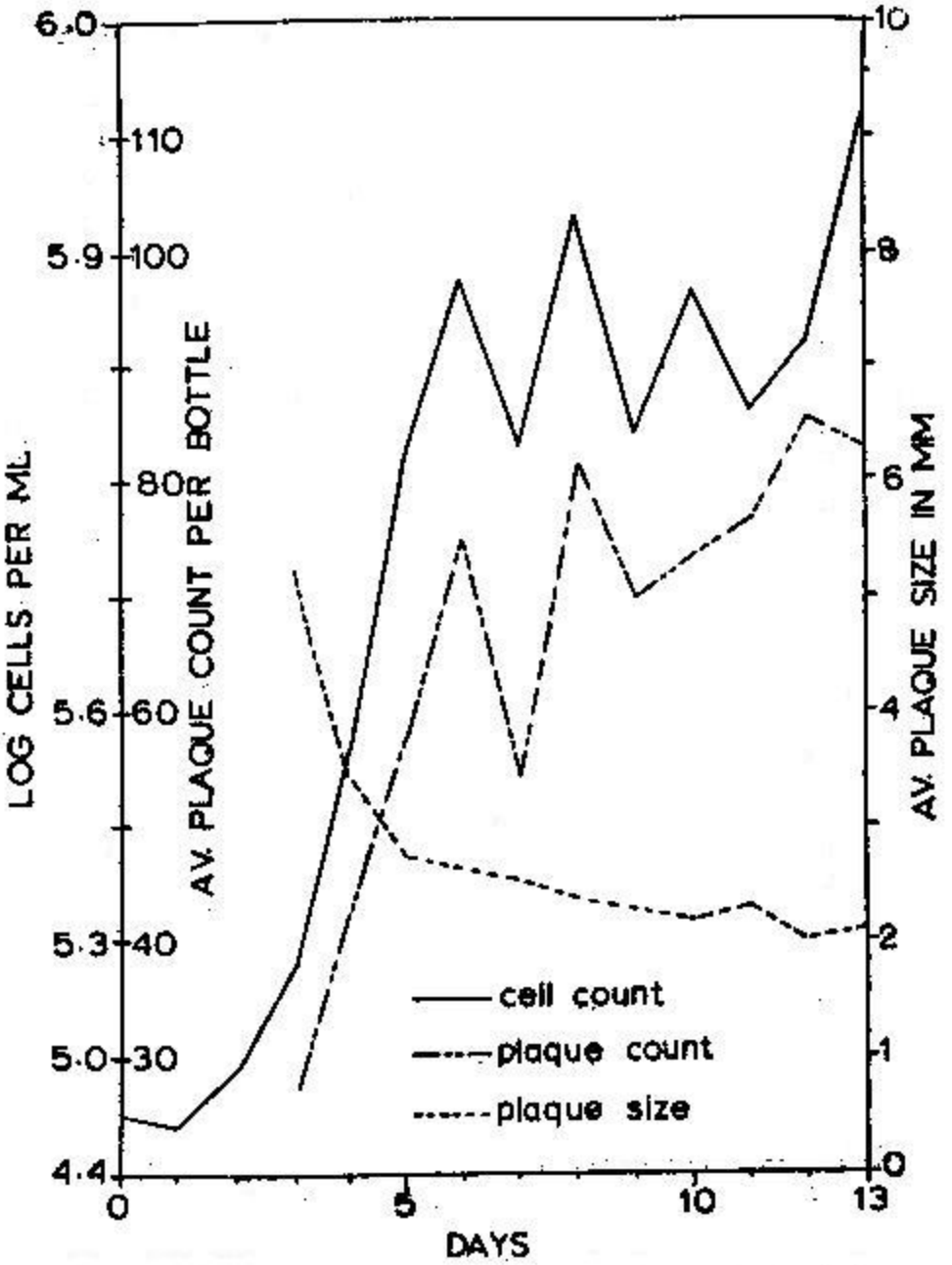

Fig. 1. Effect of age and number of tissue culture cells on number and size of plaques.

Once a complete monolayer was obtained, the number of plaques formed by a given inoculum of virus suspension was apparently not influenced by the number of cells in the monolayers and the age of the cell cultures. The number

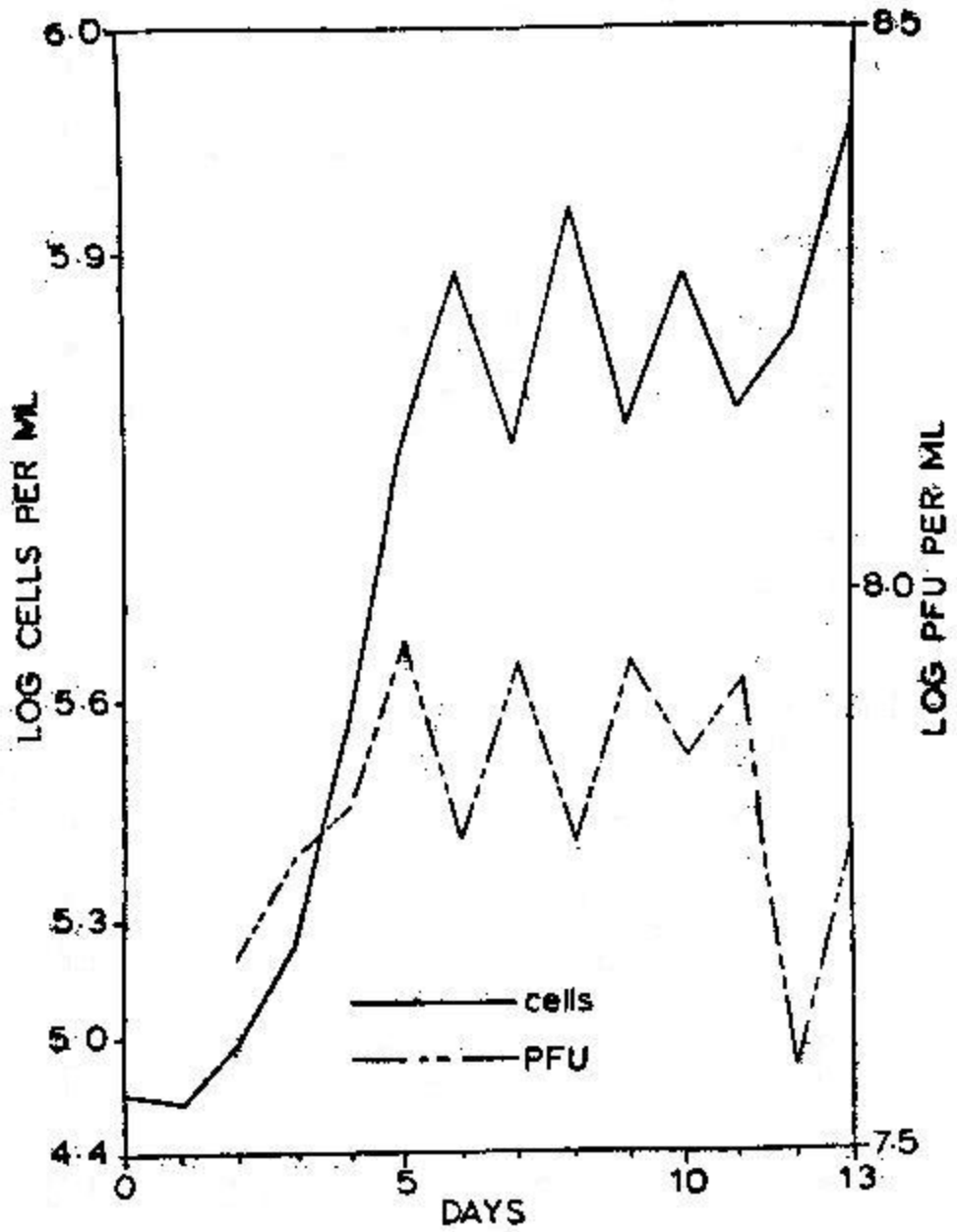

Fig. 2. Effect of age of MDBK cell culture and number of cells in culture on the virus yield when infected with a constant virus dose.

of plaques for a constant inoculum of virus was about one-third of the actual number when the monolayers were incomplete and with empty spaces. This can be explained by the adsorption of virus particles on the glass surface of the empty spaces in the cell sheets. The number of plaques decreased to about 50 per cent when the monolayers were fed with fresh maintenance medium 48 hours before virus inoculation instead of 24 hours. Thus, the number of plaques apparently seemed to be affected by the nutritive stage of the cell cultures. An acid pH of the cell culture medium after 48 hours might also be a contributing factor. However, insufficient concentration of nutrients per cell after 48 hours appears to be a more important factor for a low plaque count. It is likely that inadequate nutrition results in diminished synthesis of cell receptors for virus adsorption. Holland and McLaren (1961) have demonstrated that for the human enteroviruses, susceptible cells have specific receptors in the insoluble lipoproteins of the cell membranes, mainly in the microsome fraction and that non-susceptible cells lack these specific receptors.

These experiments demonstrate that the cells should be used when they have formed a complete monolayer and the medium should be changed about 24 hours before use in order to obtain a relatively accurate and reproducible titration of virus by the plaque method.

The age of cell cultures seems to influence the virus yield per cell. The cells in the exponential phase of growth yield about five times as many PFU per cell as those in the stationary phase. This is probably due to the fact that cells in the exponential phase of growth are more active metabolically than those in the stationary or a decline phase of growth. Optimal concentration of nutrients per cell apparently also was beneficial for the synthesis of relatively high virus yield. This study suggests that 4 to 5 days old cell cultures should be used for optimal virus yield.

LITERATURE CITED

Barya, M. A. 1966. The serologic characteristics of several bovine enteroviruses. Ph.D. Thesis. Washington State University, Pullman, U.S.A.

Barya, M. A., T. Moll, and D. E. Mattson. 1967. Antigenic analysis of bovine enteroviruses through studies of kinetics neutralization. *Amer. Jour. Vet. Res.* **28,** 126: 1283-1294.

Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of lines with Poliomyelitis viruses. *Jour. Exp. Med.* **99**: 167-182.

Holland, J. J., and L. C. McLaren. 1961. The location and nature of enteroviruses receptors in susceptible cells. *Jour. Exp. Med.* 114, 2: 161-171.

Moll, T., and A. D. Davis. 1959. Isolation and characterization of cytopathogenic enteroviruses from cattle with respiratory disease. *Amer. Jour. Vet. Res.* **20**: 27-32.