Potential of Kidney Cell Cultures from Nonhibernating Thirteen-Lined Ground Squirrels (Citellus tridecemlineatus) for Virus Propagation

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Kidney cells were cultured in vitro from nonhibernating thirteen-lined ground squirrels (Citellus tridecemlineatus). These primary ground squirrel kidney cultures were tested for the ability to support the replication of 13 viruses representing nine virus groups. The cultures were shown to be susceptible to every virus tested, either by an increase in infectious virus or by the cytopathic effect produced.

This laboratory has recently been engaged in several studies utilizing depressed metabolic states which were induced by hypothermia or hibernation. Parameters studied have included microbial intestinal flora alterations (Barnes and Burton, in press) and the immune response (6, 7, 17). Utilization of metabolic depression achieved by hypothermia or hibernation seemed to afford potential for virus studies; therefore, a suitable tissue culture system from a mammal capable of hibernation was sought. This report describes such a system and evaluates its application to studies of virus replication in general, and more specifically, to the effects of metabolic depression on the viral replicative process.

The thirteen-lined ground squirrel (Citellus tridecemlineatus) was utilized as the source of kidneys for primary cell culture. These primary cell cultures of nonhibernating or normothermic ground squirrel kidneys were tested for the ability to support the replication of an assortment of viruses representing most of the major groups of animal viruses.

MATERIALS AND METHODS

Cell cultures. Primary kidney cell cultures were obtained by killing adult ground squirrels in groups of three or four and excising the kidneys. The capsule was removed and the kidney was minced. Kidney fragments were trypsinized in a 0.25% solution of trypsin in Hanks balanced salt solution (5). Trypsinization was allowed to proceed for 12 hr with slow agitation at 4 C. The cells were then washed and suspended at a concentration of 1.5 X 10⁶ viable cells per ml in Eagles basal medium (1) containing 10% equine serum plus 100 units of penicillin and 100 μg of streptomycin per ml. The cell suspension (1.5 ml) was placed in Leighton tubes, each containing a cover slip (10 by 22 mm). The cells were incubated at 35 C, and the growth medium was changed twice weekly until monolayers were established.

Virus stocks. The virus stocks utilized in this study and their sources are enumerated in Table 1.

Viral assay. All titrations of picornaviruses, adenoviruses, reoviruses, arbovirus, herpesvirus, and poxvirus were carried out in HeLa cells by utilizing the standard TCID₅₀ titration (13).

Primary African green monkey kidney cells (BBL) were utilized for TCID₅₀ titrations of SV40 virus. Newcastle disease virus was titrated by the TCID₅₀ method in Vero cells. Mumps virus was assayed by hemagglutination (13).

The ID₅₀ end points were calculated by the method of Reed and Muench (10).

Experimental. Confluent, or nearly confluent, monolayers of cultures were tested for ability to support virus replication. Growth medium was removed from the culture, and the monolayer was washed twice with 2.0 ml of medium 199 (9). An appropriate dilution of each virus was made in medium 199, and three cultures were infected with 0.3 ml of each of the virus suspensions. The virus was allowed to adsorb at 35 C for 30 min, and 1.2 ml of warm medium 199 was added. The infected cultures were incubated at 35 C and checked daily for the appearance of cytopathic damage. When such damage appeared, the maintenance fluids from cultures infected with the same virus were removed, pooled and frozen at −70 C until titrations were performed. The cover slips were removed from the Leighton tubes, and the cells were fixed in 10% Formalin and stained with hematoxylin and eosin. Noninfected control cultures from each lot of cultures infected were maintained in a manner identical to the infected cultures. Maintenance fluids

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from the noninfected controls were checked for the production of cytopathic effects (CPE) in HeLa cells and in Vero cells and for the hemagglutination of a 0.5% suspension of chicken erythrocytes in saline. Cells and cover slips from noninfected control cultures were also fixed in Formalin, stained with hematoxylin and eosin, and observed for the presence of cytopathic damage.

RESULTS

Primary ground squirrel kidney cell cultures prepared in the manner described yielded usable cultures in approximately 3 to 5 weeks. The cultures were rarely completely confluent, usually covering 50 to 75% of the surface area of the cover slip. About 60% of the cultures planted yielded usable cultures, the rest failing to provide sufficient cell growth for the infection studies. The cultures contained a mixed cell population of both epithelial and fibroblastic cells (Fig. 1).

No viral agent was discovered in the noninfected control cultures as tested by the spontaneous appearance of CPE in the cultures or production of CPE by the inoculation of HeLa and Vero cells with maintenance fluids of control cultures. In addition, no hemagglutinin activity for chicken erythrocytes was present in control maintenance fluids.

The cultures were susceptible to the groups of viruses tested. Evidence was available for the replication of each viral agent tested except mumps. Titers achieved in the cultures were, for the most part, comparable to those achieved with the virus stocks in other cell systems (Table 2).

The viruses caused characteristic CPE which included “balloon” cells with herpesvirus (Fig. 2), necrosis and rounding of cells with sloughing as produced by coxsackie A-1 virus (Fig. 3), extensive cytoplasmic vacuolization with SV40 (Fig. 4), giant cells and extensive syncytial formation with mumps virus (Fig. 5), and cytoplasmic bridging with Semliki forest virus (Fig. 6). Characteristic CPE were observed with the other viruses tested also.

DISCUSSION

The importance of the application of numerous in vitro cell culture systems to the study of animal virology is well known. The report of Enders et al. in 1949 (2) marked the beginning of an extensive search for suitable sources of tissues for

| Virus               | Source                                | Stock propagated in | Virus group       |
|---------------------|---------------------------------------|---------------------|-------------------|
| Coxsackievirus A-1  | H. A. Wenner, U. of Kansas            | HeLa cells          | Picornavirus      |
|                     | (Tompkins strain)                     | HeLa cells          | Picornavirus      |
| Coxsackievirus B-3  | H. A. Wenner, U. of Kansas            | HeLa cells          | Picornavirus      |
| Echovirus 8 (Bryson | H. A. Wenner, U. of Kansas            | HeLa cells          | Picornavirus      |
| strain)             | Poliovirus 1 (Brunhilde strain)       | HeLa cells          | Picornavirus      |
| Reovirus 1          | H. A. Wenner, U. of Kansas            | HeLa cells          | Reovirus          |
| Newcastle disease   | N.I.H. research                       | Allantoic cavity    | Paramyxovirus     |
| virus               |                                       | chicken embryos     |                   |
| Mumps virus (Jeryl  | Commercial vaccine, Merck Sharp &     | Chicken embryo      | Myxovirus         |
| Lynn strain)        | Dohme, West Point, Pa.                | fibroblasts         |                   |
| Semliki Forest virus| D. P. Durand, U. of Missouri          | Allantoic cavity    | Arbovirus, group A|
| Adenovirus 2        | H. A. Wenner, U. of Kansas            | HeLa cells          | Adenovirus        |
| Adenovirus 12       | H. A. Wenner, U. of Kansas            | HeLa cells          | Adenovirus        |
| Herpesvirus hominis | J. E. Prier, Pa. Dept. of Health      | BSC-1 cells         | Herpesvirus       |
| (strain 1026)       | Commercial smallpox vaccine, Wyeth    | HeLa cells          | Poxvirus          |
| Vaccinia virus      | Laboratories, Marietta, Pa.           |                    |                   |
| Simian virus 40 (SV40)| Fred Rapp, Baylor University         | Primary African     | Papovavirus       |
|                     |                                       | green monkey kidney cells |               |
FIG. 1. Normal primary ground squirrel kidney cell culture. All figures except Fig. 2 taken at X 100 magnification. Cells fixed on cover slips and stained with hematoxylin and eosin.

Fig. 2. Cytopathic effect (CPE) of Herpesvirus hominis (X 250).

Fig. 3. CPE of coxsackievirus A-1.

Fig. 4. CPE of SV* virus.

Fig. 5. CPE of mumps virus.

Fig. 6. CPE of Semliki forest virus.
in vitro cell cultures. Earlier achievements were reviewed by Ross and Syverton (12). Some of the systems which have been characterized and reported recently include marmoset kidney cultures (14), human lymphoblasts and lung fibroblasts (16), fetal baboon primary cultures and cell lines (3), Vero cell line (11), fathead minnow cell line (15), and human placenta cell cultures and cell lines (4). Although the number of culture systems presently available is quite large, the continued development of culture systems suitable for application to virus studies is still important.

Primary ground squirrel kidney cell cultures were found to support the propagation of a large number of viral agents. In most cases the increase in titer alone was sufficient evidence of viral replication. The increase in titer observed with vaccinia virus was not significant, considering the method of titration utilized. However, this rise of titer accompanied by a characteristic production of "microplaques" in the infected cultures was felt to be indicative of virus replication. The mumps virus vaccine utilized was of too low a titer, $5 \times 10^3$ TCID$_{50}$, to give hemagglutination. After passage in primary ground squirrel kidney cell cultures, there was still no observable hemagglutinin activity. The extensive formation of syncytiatal cells upon infection with the mumps virus was indicative of intracellular viral activity, although virus replication was not actually demonstrated.

Perhaps the most unexpected of the viruses which were found to replicate in this system was poliovirus 1. We feel that in light of previous reports of poliovirus replication being confined to primate cell cultures (8), this observation should be confirmed by verifying that the viral agent was actually poliovirus and that the cells in which replication occurred were actually of ground squirrel origin. To date, viral harvest from polio-virus-infected squirrel kidney cultures has been shown to be neutralized by antisera produced in rabbits to attenuated poliovirus 1 (Lsc-2ab) received through the courtesy of Albert Sabin.

Primary ground squirrel kidney cell cultures were found to have several advantages for virus studies. (i) The cells were easily maintained for long periods of time in serum-free medium 199 maintenance medium. (ii) Cytopathic damage produced by viruses replicating in the cells was extensive and usually quite characteristic for the virus causing the damage. (iii) The wide range of viral susceptibility makes these cultures extremely well suited for comparative studies of different viruses in a single culture system. (iv) Our experience showed these cultures to be free of viral contaminants.

 Attempts to pass these cells serially and establish a cell line have, thus far, been unsuccessful.

This evaluation of a new culture system derived from a mammal capable of hibernation indicates potential for application to traditional virus studies, viral propagation, and diagnostic work; and, in addition, this system may be applied to a new type of viral study involving non-drug-induced depressed metabolism.

### Table 2. Results of virus infection of primary ground squirrel kidney cell cultures

| Virus             | Stock virus (TCID$_{50}$/0.2 ml) | Infecting dose (TCID$_{50}$/0.2 ml) | Titer (TCID$_{50}$/0.2 ml) achieved in primary ground squirrel kidney cell cultures$^a$ |
|-------------------|---------------------------------|------------------------------------|----------------------------------------------------------------------------------|
| Poliovirus 1      | $10^{7.0}$ b                    | $10^{4.0}$                         | $10^{6.5}$                                                                         |
| Coxsackievirus A-1| $10^{6.2}$ b                    | $10^{4.2}$                         | $10^{7.3}$                                                                         |
| Coxsackievirus B-3| $10^{6.8}$ b                    | $10^{4.3}$                         | $10^{7.7}$                                                                         |
| Echovirus 8       | $10^{6.8}$ b                    | $10^{4.5}$                         | $10^{7.2}$                                                                         |
| Reovirus 1        | $10^{6.5}$ b                    | $10^{4.5}$                         | $10^{7.0}$                                                                         |
| Semliki Forest virus | $10^{6.2}$ b                 | $10^{4.2}$                         | $10^{5.2}$                                                                         |
| Mumps virus       | Not done$^c$                    | $<2$ HAU                           | $<2$ HAU                                                                           |
| Newcastle disease virus | $10^{5.6}$ d               | $10^{4.5}$                         | $10^{6.3}$                                                                         |
| Adenovirus 2      | $10^{5.2}$ b                    | $10^{4.6}$                         | $10^{5.2}$                                                                         |
| Adenovirus 12     | $10^{5.8}$ b                    | $10^{4.6}$                         | $10^{5.7}$                                                                         |
| SV40              | $10^{5.7}$ e                    | $10^{5.7}$                         | $10^{5.6}$                                                                         |
| Herpesvirus 1026  | $10^{5.8}$ b                    | $10^{5.8}$                         | $10^{5.7}$                                                                         |
| Vaccinia virus    | $10^{6.8}$ b                    | $10^{6.0}$                         | $10^{6.7}$                                                                         |

$^a$ Cytopathic effect observed in all infected cultures.
$^b$ Titration performed in HeLa cell cultures.
$^c$ Titration not performed; vaccine reported to have titer not less than 5,000 TCID$_{50}$.
$^d$ Titration performed in Vero cell cultures.
$^e$ Titration performed in primary AGMK cell cultures.
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