Herpesvirus saimiri.
III. Plaque Formation Under Multi Agar, Methyl Cellulose and Starch Overlays[1] (35456)

M. D. DANIEL, H. RABIN,[2] H. H. BARAHONA, AND L. V. MELÉNDEZ
(Introduced by B. F. Trum)

New England Regional Primate Research Center, Harvard Medical School, Southboro, Massachusetts 01772; and Institute for Comparative Biology, Zoological Society of San Diego, San Diego, California 92112

Herpesvirus saimiri (H. saimiri) is an indigenous virus of squirrel monkeys, *Saimiri sciureus,* which has been shown to produce malignant lymphoma in nonhuman primates (1–5). H. saimiri represents the only virus of primate origin which is known, at the present time, to be oncogenic in primates. Malignant lymphoma has also been produced with H. saimiri in laboratory rabbits (6). Bioassay of H. saimiri has consisted of tube dilution titration in owl monkey kidney cells (1, 2). The desirability for a more rapid and accurate assay system for this important and unique virus prompted us to attempt the development of a plaque assay. A brief mention of the plaque forming ability of H. saimiri was made in an earlier report (2). The present communication describes in detail the plaquing studies done with H. saimiri in various nonhuman primate cell cultures under a variety of conditions.

Materials and Methods. Cell cultures. The following cell types were used in screening for plaque production with H. saimiri: Early and continuous passage owl monkey kidney (OMK); early passage marmoset kidney (MMK); early and continuous passage squirrel monkey kidney (SMK and LLCMK4, respectively); continuous passage African green monkey kidney (Vero and BSC-1); early passage fetal squirrel monkey heart, lung, and intestine (SMHI, SML, and SMI, respectively).

[1] This investigation was supported by NIH, U.S. Public Health Service Grant No. RR 00168-09 and Contract No. PH 43-63-56 from the Special Virus Cancer Program of the National Cancer Institute, NIH, U.S. Public Health Service.

Stock cultures were grown in 250-ml disposable plastic flasks. Early passage cells were maintained at least 2 months before being used to prepare stock virus or for plaque work to reduce the possibility of contamination with latent agents. Plaquing was routinely done in cells grown in 60-mm diameter disposable plastic petri dishes. All incubation was at 37° and cell transfers were achieved by trypsinization using standard procedures.

Tissue culture media. Growth medium for all cultures was minimum essential medium of Eagle supplemented with 10% fetal calf serum. Additionally, Vero and BSC-1 cells were maintained on Eagle's basal medium supplemented with 0.5% calf serum. All media contained 250 units/ml of penicillin, 250 μg/ml of streptomycin, and 2.5 μg/ml of fungizone and all were adjusted to pH 7.2–7.4 with $NaHCO_3$ prior to use.

Three types of solidifying agents were employed for plaque experiments; methyl cellulose at a final concentration of 1 or 1.5%, agar (either Bacto agar, Difco, or Ion agar No. 2, Consolidated Laboratories) at final concentrations of 0.5 and 1.0%, and starch (hydrolyzed starch, Mann) at a final concentration of 10%. In conjunction with solidifying agents, four nutrient media were tested; Temin's modification of Eagle's medium (TE), lactalbumin hydrolysate supplemented with MEM vitamins and amino acids, MEM supplemented with 5% fetal calf serum, and MEM supplemented with nonessential amino acids and 5% fetal calf serum (MEM-NEAA). In certain experiments, protamine sulfate and DEAE-dextran were added to the

overlay medium as indicated in the text.

Visualization of plaques. Three types of staining procedures were tested for plaque visualization; supravital staining with neutral red at a final concentration of 1:7500, staining with 1% crystal violet in 20% ethanol, and fixation with acid alcohol followed by staining with 0.1% amido black. Neutral red proved to be toxic when incorporated in the original overlay. However, successful staining was achieved in circumstances in which several overlays (3–5) were added with neutral red incorporated in the final overlay only.

Virus inoculation and comparative titration. Virus inoculation for plaque production was performed according to standard procedure as previously described (7, 8). For comparative purposes tube dilution assays were performed. These were scored on the basis of cytopathogenic effect (CPE) and titers were calculated by the method of Reed and Muench.

Neutralization tests. Plaque reduction tests were done using constant virus (50 plaques/dish) and varying twofold serum dilutions. Neutralization tests employing tube dilutions were based on inhibition of CPE and were performed with constant serum (1:10) and varying tenfold virus dilutions (9). Antisera to H. saimiri were prepared in squirrel monkeys as previously described (2).

Herpesvirus saimiri. Strain S295C of this virus, which was originally isolated at the New England Regional Primate Research Center (1) was used throughout these studies. Stocks of virus were prepared in OMK, SMK, and SML cells and stored at −70° or below. Essentially the same results were obtained with virus preparations grown in these three cell types.

Results. Plaque formation in different cell types. Plaques developed under all experimental conditions in all cell types tested except Vero, BSC-1, and LLCMK4 cells. Plaque populations in all susceptible cultures were heterogeneous in size with diameters ranging from 0.5 to 2.5 mm at 10 days of incubation. The most sensitive cell type was SMH which had about four times the sensitivity of SML, SMI, OMK, and MMK which all had similar degree of susceptibility. Squirrel monkey kidney cells, while still susceptible, were found to be about four times less so than these latter four cell types. Titers in SMH ranged from 2×10^6 to 6×10^6 pfu/ml.

Effect of nutrient media on plaque formation. The average number of plaques per standard inoculum was similar with all four types of media. However, when used in conjunction with agar, plaques were clear with TE and LH media while they were turbid with MEM and MEM-NEAA. Plaques were essentially all clear with all media when used in conjunction with methyl cellulose. Medium TE gave similar plaque counts when supplemented with either 5 or 10% fetal calf serum, or 5% calf serum.

Effect of solidifying agents and overlay additives on plaque production. Plaques developed with all the solidifying agents tested. The highest counts were routinely obtained with methyl cellulose under which the plaques were clear but small; less than 1.5 mm in diameter. Plaque counts were fairly high under starch but this medium rendered the plaques difficult to visualize. Overlay medium containing either type of agar resulted in fewer plaques than either methyl cellulose or starch. Plaque counts with 1.0% agar were slightly higher ($p = .05$ in 2 out of 3 expts.) than with 0.5% agar but the plaques were smaller; 0.5 to 1.0 mm in diameter in 1.0% agar compared to 1.5 to 2.5 mm in 0.5% agar (Fig. 1). The addition of protamine sulfate to agar overlays raised the frequency of plaques but did not increase their diameter. Much of this information is presented in Table I. Protamine sulfate enhanced plaque formation at a concentration of 200 μg/ml ($p = .05$) and to a greater degree at 400 μg/ml ($p = .01$) when used in agar medium. When added to methyl cellulose overlays, protamine sulfate at 25 and 400 μg/ml had little or no plaque enhancing effect. DEAE-dextran at a concentration of 100 μg/ml in agar overlay enhanced plaque production to a slight but significant degree ($p = .05$). Arginine at a concentration of 300 μg/ml had no significant effect on plaque

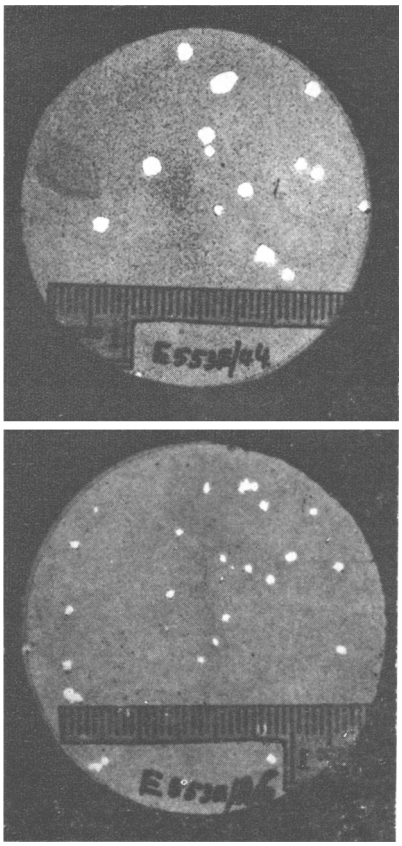

FIG. 1. E553E/44: squirrel monkey lung cells showing H. saimiri plaques overlaid with 0.5% agar and E553E/26: the same overlaid with 1% agar.

formation when added to agar overlay media.

Plaques could be visualized and scored microscopically. The microscopic appearance of plaques was highly characteristic and they could be counted in an unequivocal fashion with the use of stereoscopic dissecting microscope. Using methyl cellulose at 1 to 1.5%, plaques could be scored microscopically at 3 to 4 days; and macroscopically at 6 to 7 days. With 0.5% agar, microscopic and macroscopic scoring of plaques could be done at 4 and 9 days, respectively, but clearest visualization of plaques was obtained at 8 to 12 days. With 1% agar at least 12 to 15 days were required for best visualization of plaques to be achieved.

TABLE I. Effect of Overlay Medium on Plaque Production by Herpesvirus saimiri in Cultures of Squirrel Monkey Lung Cells.

Overlay medium	Mean plaque count
Bactor agar, 0.5%	17
Ion agar, 0.5%	12
Bacto agar, 0.5% + protamine sulfate, 400 μg/ml	43
Starch, 10%	46
Methyl cellulose, 1.0%	60

Proportionality between virus concentration and numbers of plaques. Table II shows the results of two experiments comparing virus concentration with plaque count. As can be seen, there was a direct, linear relationship between the two. In other experiments, plaque counts were shown to be directly proportional to the volume of the inoculum. Adsorption kinetics experiments showed that 60 min was adequate for maximum adsorption.

TABLE II. Kinetics of Plaque Formation by Herpesvirus saimiri in Cultures of Squirrel Monkey Lung Cells.

Virus dilution	Mean plaque count
Squirrel monkey lung grown virus	
1×10^{2}	266.6
1×10^{3}	30.0
1×10^{4}	1.6
Squirrel monkey kidney grown virus	
1×10^{2}	78.3
1×10^{3}	6.6
1×10^{4}	0.3

Plaque reduction. The dosage response relationship shown in the plaque kinetic experiments indicated a cause and effect association between H. saimiri and plaque formation. Results of plaque inhibition tests using a specific antiserum also indicated the same association. This antiserum at a 1:80 final dilution showed a 50% reduction in plaque count. Hyperimmune serum at a final dilution of 1:10 showed a plaque reduction greater than 99%. As a control, inhibition of CPE tests were run in parallel with plaque reduction using the same biological reagents; cells, virus, and antiserum. The results of these tests agreed closely with those obtained by plaque reduction. The sera had a neutraliza-

tion index of 2.0. Selected normal squirrel monkey serum failed to neutralize virus in either type of test.

Discussion. The development of a plaque assay for H. saimiri would enable studies in pathogenesis, transformation, and physical–chemical characterization with this virus to proceed in a quantitative fashion. A further feature of plaque production is the ability to obtain cloned populations of virus for subsequent examination of such interests as pathogenesis and possible vaccine production. To these ends, preliminary studies were conducted to assay the susceptibility of several types of cell cultures of nonhuman primate origin. Several such cell types proved to be susceptible under both liquid and solid media. The most sensitive cell type found was SMH but SML, SMI, and MMK were also very sensitive. Three different solidifying media were compared; agar, starch, and methyl cellulose. While plaques were produced in each case, the highest numbers were found under methyl cellulose. While the plaques under this medium were small, they were highly characteristic, developed rapidly, and could be scored microscopically a few days after incubation. Plaque counts under starch were fairly high but the plaques themselves were difficult to visualize. The main advantage of agar, aside from the fact that plaques were of relatively large diameter when produced under it, was its solidity, enabling single plaques to be picked with assurance for cloning. The toxicity of neutral red, encountered when plaquing for clonal harvests, was overcome by adding neutral red to only the final overlay of a series of overlays added to individual cultures over a period of several days. As is the case with certain other viruses (10), protamine sulfate enhanced plaque formation under agar. Another cationic polymer, DEAE-dextran, did so to a slight, but significant, degree as well.

In the course of these studies, several attempts to obtain stable plaque size populations of virus were made by plaque passaging harvests of individual plaques of distinct size classes. However, heterogeneous plaque size populations were produced on serial plating of virus from these plaques. This suggests that plaque size may well be a highly variable characteristic of H. saimiri in contrast to previous reports with Herpes T and H. simplex and Herpes B (11–14).

It seems clear that the plaques produced were due to virus in the inoculum as they were proportional to both the dilution of the inoculum, where they showed linear one-hit kinetics, and to the volume of the inoculum. This latter point is of interest as it has been previously found that several other viruses, including Herpes virus T. and H. simplex, may show a disproportionality (8). Another suggestion of the direct relationship between H. saimiri and specific plaque formation is the fact that antiserum to H. saimiri reduced the plaque count to a significant degree when used in a standard plaque reduction type of neutralization test. Selected normal squirrel monkey sera from uninfected animals produced no such reduction.

To clone H. saimiri based on plaque size was not possible, however, since no screening was done for antigenicity, density, pathogenicity, and the like, we were not able to determine whether these plaquing procedures were useful in the obtention of viral variants.

Summary. Herpesvirus saimiri produced plaques in cultures of fetal squirrel monkey heart, lung, intestine, and kidney and in owl monkey kidney and marmoset kidney cell cultures. The plaque populations in all cell types were heterogeneous in respect to size. Harvests of single plaques produced heterogeneous plaque populations on serial plating. Plaques were most numerous under methyl cellulose. Under agar, plaques were less numerous but were larger. Protamine sulfate and, to a limited degree, DEAE-dextran stimulated plaque production under agar while arginine had no effect. Plaques also formed under starch but were difficult to visualize. The number of plaques was directly proportional to the virus dilution, was also proportional to the volume of the inoculum, and was reduced by specific antiserum.

The authors express their sincere thanks to Tania Sarris, Daniel Silva, and Dewayne Seagondollar for their able technical assistance. Our thanks are also due to Mr. Charles Bates for photographic assistance and to Mrs. Marjorie Vallee for secretarial help.

1. Meléndez, L. V., Daniel, M. D., Hunt, R. D., and García, F. G., Lab. Anim. Care **18**, 374 (1968).

2. Meléndez, L. V., Daniel, M. D., García, F. G., Fraser, C. E. O., Hunt, R. D., and King, N. W., Lab. Anim. Care **19**, 372 (1969).

3. Meléndez, L. V., Hunt, R. D., Daniel, M. D., García, F. G., and Fraser, C. E. O., Lab. Anim. Care **19**, 378 (1969).

4. Meléndez, L. V., Daniel, M. D., Hunt, R. D., Fraser, C. E. O., García, F. G., King, N. W., and Williamson, M. E., J. Nat. Cancer Inst. **44**, 1175 (1970).

5. Hunt, R. D., Meléndez, L. V., King, N. W., Gilmore, C. E., Daniel, M. D., Williamson, M. E., and Jones, T. C., J. Nat. Cancer Inst. **44**, 447 (1970).

6. Daniel, M. D., Meléndez, L. V., Hunt, R. D., King, N. W., Williamson, M. E., Bacteriol. Proc. **70**, 195 (1970).

7. Daniel, M. D., and Meléndez, L. V., Proc. Soc. Exp. Biol. Med. **127**, 919 (1968).

8. Daniel, M. D., and Meléndez, L. V., Archiv. Gesamte Virusforsch. **25**, 8 (1968).

9. Schmidt, N. J., *in* "Diagnostic Procedures for Viral and Ricketsial Diseases" (E. H. Lennette and N. J. Schmidt, eds.), p. 119. Amer. Pub. Health Ass., New York (1964).

10. Takemoto, K. K., Progr. Med. Vir. **8**, 314 (1966).

11. Daniel, M. D., and Meléndex, L. V., Archiv Gesamte Virusforsch. **25**, 18 (1968).

12. Daniel, M. D., and Meléndez, L. V., Bacteriol. Proc. **69**, 197 (1969).

13. Rapp, F., J. Bacteriol. **86**, 985 (1963).

14. Benda, R., and Cinatl, J., Acta Virol. **10**, 178 (1966).

Received Nov. 23, 1970. P.S.E.B.M., 1971, Vol. 136.