naturally-occurring seasonal forms of this tumor are temperature-related states of the same tumor and suggest that the "virus-free" tumor contains a relatively complete viral genome in masked or latent form.

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Plaque Titration of Herpes Simplex with Antibody-Free Liquid Overlay* (32810)

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Dilute inocula of herpesvirus produce discrete lesions or plaques in tissue culture monolayers. Discrete plaques persist for a limited time in the presence of liquid culture medium which contains neither specific antibody nor materials such as agar, starch, or methyl cellulose designed to prevent viral spread through the culture medium. Eventually virus spreads from primary plaques to induce multiple areas of secondary infection. Between the development of the maximum number of primary plaques and the appearance of secondary lesions there is an interval when plaque counting in this liquid overlay system is a reliable means of titration.

Utilizing the above principles Farnham (1) and Wheeler (2) described plaque assays for herpesvirus in HeLa cultures overlayed with antibody-free liquid medium. Wheeler performed plaque counts on living, unstained cultures with a microscope at 50× magnification. The method has been reliable and extremely useful but counting at 50× has been tedious and time consuming and counts had to be done at a fixed time after infection

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which led to an inflexible laboratory schedule. To overcome these disadvantages we have developed a method for counting plaques at 8× with a dissecting microscope after fixing and staining.

Materials and Methods. HeLa cells were grown in 16 × 125-mm screw-capped culture tubes in slant racks at 35°C. Subcultures were prepared from 7-day old cultures by scraping cells off the tube with a glass rod. Cells were dispersed by being forced several times through a 25-gauge hypodermic needle and then being diluted in growth medium to approximately 100,000/ml. One ml of cell suspension was added to each culture tube which was gassed with 5% carbon dioxide in air to keep the pH at approximately 7.0. Cultures were refed with growth medium on the second and third days and used for viral titrations on the fourth day when solid monolayer sheets were present.

Growth medium consisted of 20% inactivated horse serum, 79.9% Hank's balanced salt solution, 0.1% yeast extract (Yeastolate), phenol red, 100 units of penicillin and 100 μg of streptomycin per ml. A modified Scherer's maintenance medium (HSMS) was employed (3).

Two strains of herpes simplex were used: HF produced plaques composed of small, rounded cells and small giant cells and HPF produced syncytial plaques in HeLa cultures (3).

The HeLa cell cultures were infected with various concentrations of herpesvirus in maintenance medium (HSMS). Groups of three to six cultures were inoculated with 0.1 ml of virus suspension, and incubated at 35°C for 1 hour for adsorption of virus. Then 1 ml of HSMS was added and cultures were reincubated at 35°C. The HSMS was changed at 24 hours. Cultures were examined for plaques by two methods at 48 hours after infection. (i) Microscopic counts at 50× were done on unstained or stained cultures (50× method) (ii) Counts were done with a dissecting microscope at 8× after fixing and staining (8× method). Viral titers were expressed in plaque forming units per ml.

Paragon–formalin was selected as the most satisfactory stain. It was prepared by adding 7 ml of Paragon[1] to 500 ml of 10% formalin. Infected cultures were fixed and stained as follows: Maintenance medium was decanted. One ml of Paragon–formalin was added per culture. After remaining at room temperature overnight, stain was decanted and cultures were rinsed three times with cool tap water and allowed to dry. Uninfected cells stained reddish-purple and infected cells bluish-violet.

Results. Plaque titrations of HF herpesvirus by the 50× method with antibody-free maintenance medium overlay have compared favorably with 100% and 50% infectious end point titrations in tissue cultures and with pock counts on the chorioallantoic membrane (2,3). In order to validate the new plaque count method at 8× it was necessary to compare it with the established 50× method. Eighty-five groups of three to five cultures were inoculated with dilutions of HF or HPF virus which produced 10–150 plaques/culture. Plaque counts were done on each culture by the 50× and 8× methods. Counts done by the 8× method averaged 3.6% higher than 50× with a standard deviation (SD) of 10.9%. The percentage variation between 50× and 8× counts were less when counts per culture were higher: e.g., SD of 11% at 10–40, 8.8% at 40–60, 7.4% at 60–100, and 8.1% at 100–150 plaques/culture. When counts per culture were below 10 (65 groups of three to five cultures) the SD was 23.8%.

Repeated plaque count titrations of single virus suspensions, either HF or HPF, have shown good reproducibility with the 8× and 50× methods. Coefficients of variation (SD/mean × 100) have been under 16% when titrations were performed on samples which induced 15–125 plaques/culture in dilutions up to 10^{-3}. In a typical experiment 48 groups of cultures containing 4 cultures/group were inoculated with virus suspensions containing 5–140 plaques/culture. At 8× the average coefficient of variation for the entire 48 groups was 13.3% and at 50× it was 15.5%. When there were 68–78 plaques/culture the coefficient of variation was 4.5% at 8× and 8.9% at 50×. These figures approach the error when a single tube contain-

[1] Paragon C and C Company, 190 Willow Avenue, Bronx, New York.

TABLE I. Replicate Titrations of the Same Sample by 50× and 8× Methods.

Titration no.	50× Method[a]		8× Method[b]	
	Av no. of plaques in 0.1 ml at 10^{-3} dilution	Titer (plaque forming units/ml)	Av no. of plaques in 0.1 ml at 10^{-3} dilution	Titer (plaque forming units/ml)
1	117	$10^{6.068}$	129	$10^{6.110}$
2	117	$10^{6.068}$	120	$10^{6.079}$
3	119	$10^{6.075}$	111	$10^{6.045}$
4	106	$10^{6.025}$	97	$10^{5.986}$
5	94	$10^{5.973}$	98	$10^{5.991}$
6	143	$10^{6.155}$	133	$10^{6.123}$
7	100	$10^{6.00}$	106	$10^{6.025}$
8	127	$10^{6.103}$	126	$10^{6.100}$
Average	115	$10^{6.060}$	115	$10^{6.060}$

[a] SD of plaque count from mean = 14; coefficient of variation = 12.1%.
[b] SD of plaque count from mean = 13; coefficient of variation = 11.3%.

ing 70 plaques is counted repeatedly, the coefficient of variation at either 8× or 50× being approximately 4%.

Table I shows the results of eight titrations of a single sample. Plaques were counted at 50× in unfixed and unstained cultures which were then counted at 8× after Paragon–formalin treatment. One tenth ml samples were measured with a 1.0 ml pipette to prepare serial 10-fold dilutions. Four cultures were inoculated with virus at each dilution.

There has been a linear relationship (Fig. 1) between dilution of virus samples and

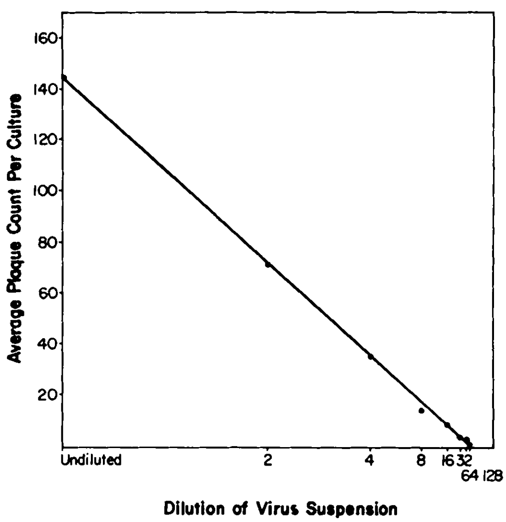

FIG. 1. Linear relationship between serial dilution of virus and plaque counts by 8 × method.

plaque counts with both the 8× and 50× methods, suggesting that 1 plaque is produced by 1 infectious unit of virus under the conditions of these titrations.

Viral titrations by plaque count with maintenance medium (HSMS) overlay depends upon development of the maximum number of primary plaques before visible secondary plaques appear. In order to reconfirm that the maximum number of primary plaques was present at 48 hours one set of cultures was fed with antibody-containing HSMS (made with 10% pooled human serum from donors with high neutralizing titers) and another set was fed with antibody-free HSMS. Antibody-containing medium eliminated significant secondary plaque formation. One day after infection small numbers of plaques were present in both sets indicating incomplete plaque development. Two days after infection plaque counts averaged 116 in antibody-free and 100 in antibody-containing cultures. Three days after infection, plaque counts in antibody-free medium were obviously too high due to secondary plaque formation. Plaque counts in antibody-containing cultures averaged 114. On the fourth day generalized infection was present in antibody-free cultures but antibody-containing cultures averaged 113 discrete plaques. Therefore, the maximum number of plaques was present and secondary plaques had not appeared at 48 hours in antibody-free medium.

The error introduced by plaque overlap in the 8× method appeared to be insignificant despite the small area of the cell sheets. The cell sheet at the end of 4 days incubation has averaged 950 mm^2. After 2 days of incubation the median plaque size produced by HF herpesvirus in HSMS was 0.55 mm. When there were 140 plaques/culture, overlap, as observed by inspection at 8×, was 5.6%; at 78 plaques it was 2.8%; at 30 it was 1.3% and below 15 there was essentially no overlap. When the culture medium contained neutralizing antibody, the median plaque size at 2 days was 0.47 mm, at 3 days 0.65 mm, and at 4 days 0.86 mm. When plaque counts in antibody-containing cultures averaged 120/culture, there was 4% overlap at 2 days, 12% at 3 days, and 19% at 4 days, indicating increased overlap as plaque size increased.

Most accurate counts were made at both 50× and 8× when there were 60–80 plaques/culture at 48 hours after infection. The coefficient of variation at this level was 4.5–9% and plaque overlap was less than 3%.

Discussion. The 8× method is reliable and eliminates tedious, time-consuming plaque counting with a microscope and the necessity of performing counts at a fixed time after infection. It is well suited for routine titrations. Where higher magnification is necessary to distinguish between plaques having different morphologies or where fixing and staining do not fit into the experimental design, the 50× method is required.

Both methods are examples of the use of liquid culture medium overlay in studies involving plaque formation and enumeration. Liquid overlay methods have a number of advantages over methods utilizing solid or antibody-containing overlays. Some of these are: (i) elimination of plaque inhibition by overlay materials such as agar and antiserum, (ii) avoidance of cell exposure to potentially toxic overlay, and (iii) greater flexibility in choice of culture containers, conditions of incubation, and manipulation of cultures which make possibe (a) elimination of carbon dioxide incubators, (b) examination of live and unstained or fixed and stained cells, (c) reduction in contamination with bacteria and fungi, (d) economy of cells, culture medium, and incubator space, (e) examination of plaques with the light microscope at high or low power, fluorescence microscope at high power or dissecting microscope at low power, (f) use of a smaller cell sheet by earlier counting of small plaques, (g) washing of cultures anytime after infection, (h) exposure to antiviral chemicals or other agents at anytime, and (i) easy adaptation to hemagglutination tests, interferon assays, and plaque assays by fluorescent antibody methods.

The necessity to count plaques at a fixed time after infection is sometimes a disadvantage of the liquid overlay method. This can be overcome by incubating cultures at 25°C where plaque development with herpesvirus ceases or by fixing with formalin at the proper time and examining cultures when convenient. Plaque counts with liquid overlay usually must be done while plaques are small; and hence, magnification is often necessary. In some experiments, such as fluorescent antibody studies, high magnifications and small plaque size are desirable (4). Small plaque size allows use of diminutive cell sheets grown in small containers of good optical quality. In other instances it is desirable to avoid tedious plaque examination at high magnification. In these circumstances, fixing and staining may allow plaque examination at low magnification as in our 8× method.

Plaque count methods with antibody-free liquid culture medium overlay have been reliable in titration of adenovirus (4), vaccinia (5–7), ectromelia (7), "cold virus" (8), echo virus (9), and canine distemper (10).

Since 1960 we have frequently incorporated 10 μg of hydrocortisone hemisuccinate/ml of maintenance medium (11). This allows cell sheets to be maintained in a better state so that plaque counts are easier to perform (12). Hydrocortisone does not alter the total count.

Summary. Two reliable methods for plaque titration of herpes simplex in antibody-free liquid culture medium have been described. Some advantages over plaque titrations with solid or antibody-containing overlays are elimination of inhibitory effects of agar and antiserum, greater choice in type of culture vessel which allows for saving of culture materials, technician's time, and incubator space, op-

tional elimination of carbon dioxide incubators, greater range of magnification, and more flexibility in manipulation of cultures.

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The Ratio of Soluble to Insoluble Collagen in Normal Chick Dermis*† (32811)

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Weiss and Matoltsy (1) reported a total absence of wound healing in chick embryos prior to the tenth day of incubation. The epithelial cells of the wound margin, instead of covering the wound surface as they do in older embryos, penetrate the underlying mesenchymal stroma. We thought that the explanation for this behavior of the epithelium should be sought in the embryonic mesenchyme.

The importance of the mesenchyme, e.g., the dermis, for the differentiation of the epidermis and its derivatives during embryogenesis has been well established (2-5). Differentiation and proliferation of epidermal cells in newts was found to be dependent on mesenchymal factors (6). McLoughlin (7) was able to alter specifically the differentiation of embryonic epidermis by combination with mesenchyme from various organs. Epidermal cells cultured without mesenchyme do not differentiate but degenerate, whereas frozen-thawed mesenchyme is able to support epidermal differentiation (8). This property of frozen-thawed mesenchyme, however, is lost after its exposure to trypsin (8). This finding supports the assumption that large molecules are responsible for the inductive action of the mesenchyme. Wessells (9) succeeded in inducing and maintaining cornification of embryonic chick epidermis in the absence of mesenchyme by explanting it on top of a Millipore membrane and supplying it with a suitable concentration of embryo juice. Other substrata failed to support epidermal differentiation. These findings are regarded as evidence for the importance of the physical structure of the substrate for epidermal cornification.

Collagen is one of the most important structural elements of the dermis. Dodson obtained differentiation of epidermis grown on collagen gells. Enquist and Adamson (10) stated that "a lack of healing means absence of collagen synthesis." In view of these data, a correlation between collagen polymerization in granulation tissue and epidermal wound coverage appears plausible. Our investigations, reported in this paper, were concerned with

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