A Rapid Hemadsorption Plaque Assay for Mumps Virus[1] (35483)

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Plaque assays of mumps virus in monolayers of chick, monkey, or human origin have made use of the development of nonstaining foci of infection under agar incorporating neutral red (1–3), or of hemadsorbing foci after removal of a viscous nutrient overlay (4–7). These assays have been allowed to incubate for at least 5 days, and often for more than 7, but the relation between incubation time and plaque number has not been detailed. In connection with current studies on the replication of mumps virus in different organs of the chick embryo, both *in ovo* and *in vitro*, a rapid assay system was needed in which the input virus, virus replicated, and virogenic cells could be accurately quantitated on a comparative basis. The hemadsorption plaque assay on HeLa cell monolayers described here fulfills these criteria.

Materials and Methods. Virus and cells. A strain of mumps virus (Amaris) originally isolated from a pregnant woman with parotitis and passaged 21 times in calf serum–adapted HeLa cells was used. Harvest fluids were subjected to two cycles of low speed centrifugation to remove cells and cell debris, and the supernatant was distributed in aliquots and stored at −90°. Virus dilutions were made in Eagle's basal medium (BME) using Hanks' balanced salt solution (BSS) and containing 5% heat-inactivated fetal bovine serum and 100 units of penicillin and 100 μg of streptomycin/ml (maintenance medium, MM). The virus produced no detectable cytopathic effect (CPE) in chick embryo fibroblasts and minimal CPE in HeLa cells, except for transient syncytia formation following reisolation from organ suspensions of infected chick embryos. The HeLa cells used in the assay were from the same stock in which the virus was passaged.

Assay procedure. Monolayers of HeLa cells were prepared in 30 cm^2 polystyrene flasks (Falcon Plastics, Los Angeles) by plating 10^6 trypsinized cells in 3 ml of BME with 10% fetal bovine serum. The medium was replenished at least once, and at 6–8 days after plating the cell sheet was confluent and ready for use in the assay. After rinsing the cells with Hanks' BSS, 0.3-ml aliquots of the virus dilutions to be assayed were adsorbed in replicate flasks for 2.5–3 hr at 35°, during which time the flasks were gently rolled at 30-min intervals to disperse the medium over the cells. Other experiments (unpublished data) from this laboratory have shown that maximum adsorption occurs within the first 2 hr, but, even then, as much as 50% of the virus could be recovered from the adsorbing medium. After adsorption, the medium was decanted, the cells were gently rinsed with BSS, 3 ml of MM with, or without, 0.6% Difco Bacto agar was added, and the flasks were left undisturbed at 35° for 3–7 days.

After incubation for the desired time, the overlay was decanted, the cells were rinsed with BSS, 3 ml of a 0.1% suspension of washed guinea pig erythrocytes in BSS were added, and the flasks were placed at 4°. The hemadsorption plaques were counted after at least 3 hr at 4°, and remained distinct for 2-3 days at this temperature. Unattached erythrocytes were effectively dislodged from the cell sheet with gentle shaking and inversion of the flasks for plaque counting, rather than by rinsing. The plaques were visible macroscopically under transmitted light, and were often confirmed by microscopic examination. Fixation of plaques with cold absolute methanol, ethanol, or 10% formalin after rinsing the cells with cold BSS was not found to be advantageous, because small plaques became less distinct and the yellow appearance

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of the HeLa cells made microscopic confirmation of plaques difficult.

Calculation of virus titers. Numbers of plaques from as many dilutions as possible were summed, and a weighted mean and its 95% confidence limits were calculated by the method of Lorenz (8).

Results. Effect of overlay. Numbers of plaques in replicate flasks under liquid vs semisolid agar overlay were within the 95% confidence limits given by Lorenz (8). The agar therefore had no inhibitory effect on plaque formation. Under liquid overlay, some secondary plaques radiating from primary foci were often present by 5–6 days, but rarely by 4 days after virus inoculation. Under semisolid agar, secondary plaques were first present by 6–7 days of incubation after inoculation. Primary and secondary plaques were distinguished by their spatial relationships and size. Secondary plaques radiated, often unilaterally, from densely hemadsorbing primary foci and, in the absence of excessive crowding of primaries, were readily distinguishable also on the basis of their small size.

Effect of incubation time on plaque size and number. Between 3 and 7 days after virus inoculation, there was an increase in plaque size which was accompanied by an increase in numbers of primary plaques up to 5 days. Plaques could first be counted with ease at 3 days after infection of the monolayers, and were 0.06–0.2 mm in diameter. Mean plaque diameters increased to around 0.2 mm by 5 days and to 0.6 mm by 7 days under semisolid agar overlay. Some variation in range and mean plaque diameters was encountered between experiments. Numbers of plaques in replicate flasks of similar virus dilution increased between 3 and 5 days after infection, while between 5 and 6 days no significant increase was observed. A slight increase in plaque numbers at 7 days under agar overlay was considered to be due to the presence of large secondary plaques which were inadvertently counted along with the primaries. In Fig. 1 are plotted the results from 3 titrations in which plaques were counted in replicate flasks at 3, 4, 5, and 6 days after virus inoculation, and in one of these series also at 7 days. The 95% confidence limits of the total plaque counts from 2

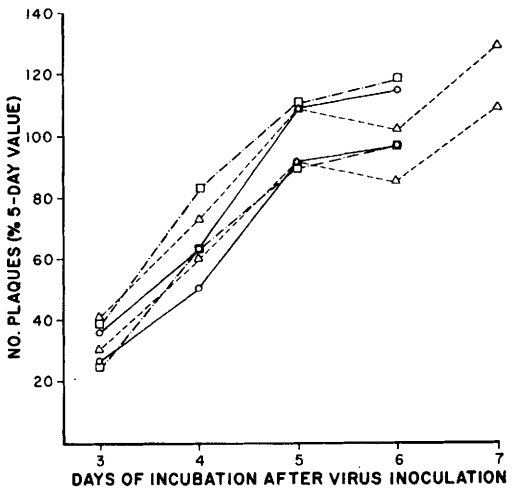

FIG. 1. Relationship between plaque numbers and incubation time: 95% confidence limits of total plaque counts are expressed as percentage of the respective 5-day plaque count in three separate titrations.

to 3 flasks from each of 3 or 4 dilutions are expressed as a percentage of the 5-day counts for each series, since the titers were not equal in each. The 5- and 6-day values were statistically indistinguishable, so the number of plaques developing by 5 days was assumed to be close to 100% under the assay conditions employed. From seven experiments in which plaques were counted in replicate flasks at 3, 4, and 5 days, and an additional five experiments in which they were counted only at 4 and 5 days, the numbers of plaques at 3 days as a percentage of the 5-day count ranged from 20 to 40% (mean 33.5%), while the numbers at 4 days were 57–77% (mean 69.2%) those counted at 5 days. The size distribution of plaques on successive days was consistent with enlargement of the first formed plaques and appearance, at least until 5 days, of new plaques in the smaller size groups.

Replicate flasks gave excellent agreement in plaque counts in most cases (Table I). Independent dilution series from the same vial of stock virus also gave differences within the calculated 95% confidence limits (8). Different batches of HeLa cells did, on occasion, give as much as 2–3-fold differences in plaque counts for the same stock virus. These differences appeared to relate to the age of

TABLE I. Numbers of Hemadsorbing Plaques in Replicate Flasks at 4 Days of Incubation.

Relative virus concentration		
1	4	16
214[a]	63[a]	16[a]
224	65	16
230	66	17
241[a]	69[a]	18[a]

[a] Pairs of flasks with most widely divergent plaque numbers at each dilution: all fall within the 95% confidence limits given by Lorenz (8).

the cells in culture prior to infection, cells older than 8 days giving lower counts and smaller plaques than those 6–8 days old.

Relation between number of plaques and virus dilution. Serial 2-, 4-, and 5-fold dilutions, usually from an initial 10^{-3} dilution, were most commonly employed in titrations of stock virus, since approximate virus titers were already known from ID_{50} titrations in tubes. For virus suspensions of unknown titer, serial 10-fold dilutions were employed. Plotting the mean number of plaques from replicate flasks against the dilutions gave a proportionality between plaque count and relative virus concentration which was linear (Fig. 2). This is considered evidence that each plaque has resulted from one infectious unit. Such a linear relationship existed on each day, from the third through the seventh, after virus inoculation.

Application of hemadsorption plaque assay to infectious center assay of infected cells. The plaque assay described above was readily adapted to determining percentages of virogenic cells derived, for example, from cell culture monolayers or organs of infected animals. Serial dilutions in MM of cells dispersed with trypsin (or trypsin–EDTA) were plated in 3-ml amounts on HeLa monolayers without an adsorption period, and hemadsorbed at 4 days. Addition of 3 ml of semisolid nutrient overlay to a 0.3-ml volume of suspended cells in the assay flasks resulted in a 2-day delay in plaque formation compared with similarly prepared flasks with a liquid overlay. Presumably the suspended cells had been trapped away from the assay monolayer when an agar overlay was used, and for this reason a liquid overlay is routinely used.

We have found it desirable, when possible, to disperse infected monolayers 8–12 hr after infection. Prior to 8 hr, trypsinization released uneclipsed virus from the cells into the medium, while beginning at about 16 hr, trypsin treatment hastened release of progeny virus, which would normally not be liberated into the medium until 20–24 hr. Free virus in the medium after trypsinization of cells already liberating virus was eliminated from the assay by repeatedly rinsing the cells with MM. In three experiments using HeLa cells harvested 8–12 hr after infection, the number of virogenic cells found was 40–60% of the expected value calculated from the adsorption multiplicity.

Discussion. This hemadsorption plaque assay in HeLa cells represents a useful method for rapid comparative titrations of free mumps virus and virogenic cells. Although the definitive number of plaques is only reached after 5 days of incubation, readings for comparative titrations can be made at 3 or 4 days. We routinely employ a 4-day read-

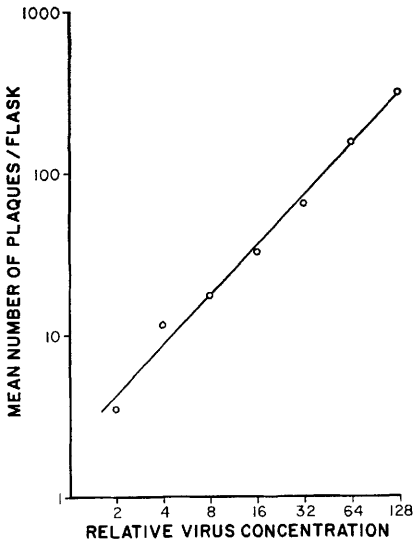

FIG. 2. Relationship between mean number of mumps virus hemadsorption plaques per flask after 4 days' incubation and relative virus concentration. Duplicate HeLa flasks were inoculated with 0.3 ml of serial twofold dilutions of appropriately diluted stock virus. After 2.5-hr adsorption, virus was decanted, the cell sheet was rinsed, and 3 ml of 0.6% agar in MM was added as overlay.

ing under liquid overlay. The frequent presence of secondary plaques in flasks incubated under liquid overlay for longer than 4 days necessitates use of a semisolid agar overlay for 5 day readings, a procedure which is cumbersome and suffers from the disadvantage that the agar is sometimes difficult to remove completely from the assay cells and could interfere with hemadsorption. On the other hand, readings are more difficult to make at 3 days after inoculation than at 4 days because the plaques are smaller and sometimes indistinct.

Since even after 2–3-hr adsorption as much as 50% of the virus fails to adsorb the HeLa cells, titers calculated from readings on the fifth day of incubation are not to be regarded as representing the actual number of infectious units in the assay sample, but only relative to the method of assay. Added to this is the consideration, if readings are made at 4 days after infection, that only about 70% of the final number of plaques have appeared. In none of the mumps virus plaque assays previously described (1–7) have the factors affecting plaque counts been discussed.

The relatively slow development of secondary plaques under liquid overlay suggested that plaque formation may be occurring predominantly through intracellular cell to cell transfer of progeny virus, as in vaccinia (9–12) and herpes (13), rather than through an antibody sensitive extracellular phase. However, experiments in this laboratory (unpublished data) designed to follow plaque formation after a variable period under mumps antiserum have shown that viral spread is inhibited in the presence of antiserum but resumes once the antiserum has been removed. These results are consistent with a budding mode of viral spread as has been visualized in electron micrographs of mumps virus replication (14). Another possible explanation for the slow development of secondary plaques may reside in a combination of the slow replication of this strain of mumps virus (20–24 hr), and low virus yields per infected cell [suggested from the low virus titers relative to input virus and low yields from freeze–thaw lysates (unpublished data)]. Low virus yields have been shown, theoretically at least, to have a profound influence on plaque development and morphology (15, 16). The explanation for apparent age-related differences in plaque size and number between different batches of HeLa cells may also reside in lower virus yields from cells which had been in culture longer, but future experiments must clarify this hypothesis.

Summary. A hemadsorption plaque assay in HeLa cells for mumps virus and virus-infected cells is described. Slow development of secondary plaques allows use of a liquid overlay for readings made after 3 or 4 days of incubation. Since the definitive number of plaques developing under the conditions of assay do not develop for 5 days, earlier readings are especially useful for comparative titrations, and readings at 4 days are easier to make than at 3 days.

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