Experience with an Image-Analyzing Computer in Virus Plaque Measurements

WINFRIED SCHEIRER
Arzneimittelforschung GmbH, Laskegasse 5-11, A-1121 Vienna, Austria

Due to the extremely fast and reliable plaque-number determinations, the image-analyzing computer is most useful for large screening programs involving plaque-reduction tests.

An Imanco Quantimet 720 image-analyzing computer was used for counting virus plaques in different test systems for about 1 year. The results were compared with those of manual plaque counting. This article describes the apparatus and shows its possibilities for use in virology.

MATERIALS AND METHODS

Test systems employed: (i) mouse L-929 cells challenged with vesicular stomatitis virus, strain Indiana (VSV). L-929 cells (Flow Laboratories, Irvine, Scotland) were seeded into 35-mm plastic petri dishes (C. A. Greiner & Söhne, Nürtlingen, Western Germany) at 2.8 × 10^4 cells per dish in 1.6 ml of Eagle minimal essential medium (MEM, GIBCO) completed with 10% of inactivated calf serum. Interferon (2) dilutions were added immediately thereafter in 0.2 ml of Eagle MEM, and dishes were incubated for 24 h at 37°C in a CO_2-incubator containing a humid atmosphere of 95% air and 5% CO_2. A confluent monolayer was formed after that time. The medium was removed and about 100 to 200 plaque-forming units (PFU) of VSV were added in 0.8 ml of Eagle MEM supplemented with 2% inactivated calf serum (ECS-2). After an infection period of 1 h at 37°C, the inoculum was removed, and 1.2 ml of 0.8% Methocel (MC4000CP, Fluka, Buchs, Switzerland) in ECS-2 was added. Plaques were formed after incubation for 48 h and stained with 0.2% gentian violet (Fig. 1, sample A).

(ii) Chicken embryo fibroblasts (CEF) challenged with cowpox virus, strain Brighton (CP). The method of Lindenmann and Gifford (3) was used, with slight modifications (1). Two- or 3-day-old confluent monolayers of CEF in 5-cm plastic petri dishes (NUNC, Roskilde, Denmark), grown in Parker medium 199 supplemented with 10% inactivated calf serum, were covered with 2 ml of medium 199, 0.3 ml of interferon dilution, and 0.2 ml of a sonically treated CP suspension (about 50–200 PFU in HANKs balanced salt solution containing 0.5% gelatin). The dishes were incubated in a CO_2-incubator and cells were stained after 48 h with 0.2% gentian violet, when plaques were clearly established (Fig. 1, sample B).

(iii) Human phimosis cells challenged with VSV. Foreskins from children up to 4 years of age were trypsinized and cell cultures were made by standard methods. Cells were used at passage levels 4 to 10. The growth medium was Eagle MEM completed with 10% fetal calf serum. Confluent monolayers grown in 35-mm plastic petri dishes were treated with 1 ml of interferon dilutions in ECS-2 for 24 h in a CO_2-incubator. After removal of interferon, infection was carried out for 1 h with about 20 to 50 PFU of VSV in 1 ml of Eagle MEM. The medium was layered onto the cells (0.8% Methocel in Eagle MEM). Plaques were visible after 48 h of incubation and cells were then stained with 0.3% gentian violet (Fig. 1, sample C).

Function of the image-analyzing computer. With the Imanco Quantimet 720 analyzing computer (Fig. 2), as used by us, an object is picked up by a television camera and electronically resolved into 500,000 picture spots. These are scanned row by row for difference in gray density. The gray difference, which is necessary to detect the plaques against the stained cell monolayer, must be determined for each test series. The plaques detected by the instrument can

![Fig. 1. Cell monolayers in petri dishes stained with gentian violet. A, Mouse L-929 cells challenged with vesicular stomatitis virus (dish diameter, 35 mm); B, chicken embryo fibroblasts challenged with cowpox virus (50 mm); C, human phimosis cells challenged with vesicular stomatitis virus (35 mm).](http://aem.asm.org/)
also be discriminated by size, as measured in picture spots. The results can be printed out either as the number of plaques as discriminated by a certain gray difference, as the total area of plaques, or as the sum of all plaque diameters. Further features are: (i) electronic reduction of resolution, which is needed for granulated areas as usually formed in the CEF/CP test, and (ii) print-out of the average of 16 measurements, which reduces the statistical error of a single determination.

We used a dia-slide-projector type Leitz-Prado for illumination of petri dishes, in which the optical system was fitted with two milk-glass screens for a more uniform lightening of samples. The sample holder was fitted to a slide changer. To screen off uneven areas on the periphery of monolayers, the sample holder was also fitted with variable frames. We always took the average of 16 measurements, which needed approximately 1.5 s.

RESULTS

System L-929/VSV. This system was without problems in computer counting. Measurements obtained by hand counting corresponded well with computer results (Fig. 3 and Table 1).

**Table 1. Mouse L-929 cells challenged with vesicular stomatitis virus**

| Mouse interferon dilution | Hand counted | Computer counted |
|---------------------------|--------------|------------------|
| 1:5                       | 9            | 13               |
| 1:10                      | 27           | 23               |
| 1:20                      | 87           | 81               |
| 1:40                      | 108          | 106              |
| 1:80                      | 144          | 132              |
| 1:160                     | 149          | 143              |
| 1:320                     | 140          | 132              |
| 1:640                     | 146          | 147              |

**Fig. 2.** Image-analyzing computer assembly, consisting of computer, dia-slide projector, and television camera. Also shown is a research microscope with a television camera holding supply, which can alternatively be used with the computer.

**Fig. 3.** Inhibition of plaque formation by pretreatment of L-929 cells with mouse interferon. Challenge virus was vesicular stomatitis. Symbols: O, computer counted; *, hand counted.

**Systein CEF/CP.** Since CEF monolayers usually stain in a somewhat uneven pattern, due to specific cell growth characteristics of fibroblast-type cells, difficulties arose in detection of very small plaques. To pick up plaques in this system, the electronical resolution had to be decreased for optimal results. Comparison to hand-counting values again show good agreement. If the computer was set to detect optimal plaque numbers in controls (no plaque inhibition), a slight overestimation of plaque numbers usually occurred in an inhibition range above 90% (Fig. 4 and Table 2).

**Fig. 4.** Inhibition of plaque formation by pretreatment of chicken embryo fibroblasts with chicken interferon. Challenge virus was cowpox virus. Symbols, O, computer counted; *, hand counted.

**Table 2. Chicken embryo fibroblasts challenged with cowpox virus**

| Chicken interferon dilution | Hand counted | Computer counted |
|-----------------------------|--------------|------------------|
| 1:2                         | 1            | 5                |
| 1:4                         | 4            | 5                |
| 1:8                         | 12           | 10               |
| 1:16                        | 18           | 16               |
| 1:32                        | 37           | 34               |
| 1:64                        | 46           | 42               |
| 1:128                       | 55           | 50               |
| 1:256                       | 55           | 45               |
System human phimosis cells/VSV. In this system we were not able to obtain useful results. This was caused by the slight and inhomogeneous staining, which is characteristic for this cell line. Differences in staining density between plaques and cellular background are only very slight and are sometimes even smaller than the staining variations observed between different petri dishes within one series.

DISCUSSION

Damages of parts of the monolayer, caused either by mechanical damage or by imperfect cell growth, generally caused no difficulties because plaque numbers decrease proportionally to the smaller monolayer area. The damage was registered only as one count, provided it consisted of a connected area. This type of registration is a consequence of the full feature setting, which calculates areas only once, even if the measuring beam is interrupted by cellular material. The counting of very small cellular damages, imitating small plaques, can also be prevented by the size discriminators. To obtain good results, the cell monolayer must be as uniform as possible, and a well-standardized staining procedure should be used, which must secure an even staining density for all petri dishes. Under these conditions, an image-analyzing computer is a very helpful aid for evaluation of plaque tests in which plaques exhibit a good contrast against the cellular background. The results are highly reproducible, and time savings are large compared to manual evaluation. For example, a test with 300 dishes requires between half a day and 1 day when counted by hand, depending on the number of plaques per dish. With the analyzing computer, the time needed is about 1 h, independent of plaque number. In most assays, inhibition of virus multiplication is calculated from the 50% inhibition, at which plaque numbers of untreated controls are reduced to 50% by a given inhibitor dilution. In this inhibition region, the results of hand counting and computer analysis agree very well.

The image-analyzing computer is, therefore, most helpful for large screening programs, involving plaque-reduction tests, because of the extremely fast and reliable plaque-number determinations.

LITERATURE CITED

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