# Cloning of Dengue Virus Type 2 by the Direct Fluorescent Antibody Technique (37529)

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Selection and purification of virus clones is an essential step in research involving virtually every phase of virology. This process generally involves selection of a well-isolated virus plaque, and extracting the progeny virus from the semisolid medium above the plaque. However, certain viruses do not readily produce plaques, or may not produce them on an acceptable cell. The latter problem occurs particularly in the development of live, attenuated vaccines, where the use of continuous or transformed cells is precluded by safety considerations.

Dengue virus type 2 (DEN-2), TH 36 isolate, failed to produce detectable plaques on a variety of primary and diploid cell systems. For this reason, a method of cloning based on fluorescent antibody methods was developed. This technique is theoretically applicable to virtually any virus–cell system, since it only requires production of virus-specific antigen in the infected cell.

*Materials and Methods.* The preparation of primary hamster kidney cell cultures (HK CC) (1), lactalbumin hydrolysate medium (LAH) in Hanks balanced salt solution (HB SS) (1), and hyperimmune ascitic fluids (2, 3) have been previously reported. Ammonium sulfate fractionated anti-DEN-2 ascitic fluids were conjugated with fluorescein isothiocyanate as described (4), and further purified by chromatography on Sephadex G-25 (4, 5). Lab-Tek (Westmont, Ill.) 8-well Chamber/Slides containing about $1 \times 10^4$ cells/well (64 $mm^2$) were inoculated with 0.1 ml of virus suspension. Following 1 hr incubation at 33.5°, 0.2 ml medium was added. After 4 days at 33.5° fluids were removed, diluted 1:2 in LAH medium and frozen at —70°. Wells were washed with HBSS and cells fixed in acetone (—20°). Fixed cells were rehydrated in 0.01 *M* Sorenson's phosphate-buffered saline (SPBS, pH 7.2), and stained with 0.05 ml of a 1:16 dilution of conjugated antibody in SPBS at 37° for 1 hr. After thorough rinsing with SPBS, cells were scanned with an ultraviolet microscope, designed as previously reported (6).

DEN-2 virus, after 2 serial suckling mouse brain passages and 10 serial passages at 28° in HKCC, was used as a virus inoculum. This virus pool was calculated to contain $10^{5.7}$ fluorescent focus-forming units (FFU)/0.1 ml by the above method. Tenfold dilutions of this virus pool produced 10-fold reductions in FFU titer. For cloning work, wells were infected with 0.1 ml of a $10^{-5.3}$ or $10^{-5.7}$ dilution of this stock. Primary foci generally consisted of 30-100 stained cells in a group which appeared bright apple-green under the ultraviolet microscope against a dark background of nonstaining noninfected cells. Occasional single cells or very small groups of less than 10 cells which fluoresced were considered to constitute secondary foci of infection by progeny virus released from the primary focus. These generally stained to a lesser degree than primary foci. Uninfected controls never demonstrated virus-specific fluorescence upon fixation and staining with conjugated anti-DEN-2 ascitic fluid.

*Results and Discussion.* Table I shows results of three representative experiments. By the $\chi^2$ frequency test, there was no significant

TABLE I. Frequency of Focus Formation by Terminally-Diluted Aliquots[a] of DEN-2 on HKCC at 33.5°.

| | Experiment number | | | | | |
|---|---|---|---|---|---|---|
| | 1 | | 2 | | 3 | |
| No. foci/well | Obs.[b] | Exp.[c] | Obs. | Exp. | Obs. | Exp. |
| 0 | 38 | 39.1 | 23 | 21.0 | 14 | 12.7 |
| 1 | 20 | 18.6 | 17 | 23.4 | 24 | 20.3 |
| 2 | 5 | 4.4 | 16 | 11.9 | 10 | 16.3 |
| 3 | 0 | 0.7 | 4 | 4.2 | 5 | 8.7 |
| 4 | 0 | 0.1 | 1 | 1.1 | 8 | 3.5 |
| ≧5 | 0 | 0.01 | 0 | 0.2 | 2 | 1.5 |
| λ[d] | 0.476 | | 1.066 | | 1.603 | |

[a] Input multiplicity estimated at 1 FFU/well.

[b] Observed frequency of wells showing given number of foci.

[c] Expected frequency of wells showing given number of foci ($n$), based on Poisson distribution, $P_n = (\lambda^n)\ (e^{-\lambda})/n!$, obtained by multiplying $P_n$ by the total number of observed foci per experiment.

[d] $\lambda$ = Total number of foci/total number of wells in each experiment.

difference between the observed frequencies and those predicted by the Poisson distribution. Along with the effect of dilution of FFU titer, this strongly suggests that a primary focus is caused by one infectious DEN-2 particle. Those cells containing one and only one primary focus were considered to have contained a clone. Fluids of these wells containing a clone were estimated to contain $10^{1.0}$–$10^{2.5}$ FFU/0.1 ml of progeny virus upon subsequent titration. Clearly, DEN-2 had replicated and released infectious progeny, in addition to producing viral antigens.

The wide applicability of this method deserves elaboration. The same techniques and reagents have been applied to DEN-2 in a number of other cell systems, virtually without modification (Dr. R. W. Atchison, personal communication). Because of the broad cross-reactivity of dengue virus types, one hyperimmune reagent will suffice to detect cells infected by any dengue virus (4, 6, 7). In addition, the FFU method is quicker than plaque assays (2–4 days *vs* 1–5 weeks for DEN-2), and may have a wider operational temperature range (unpublished observations). Titrations of infectivity obtained by the direct FFU method for our dengue preparation ($10^{5.7}$/0.1 ml) are similar to those obtained by plaque assay on LLC-$MK_2$ cells ($10^{5.6}$/0.1 ml) and by intracerebral injection of suckling mice ($10^{5.1}$/0.01 ml). Most importantly, this is a device by which viruses can be cloned in noncytopathic and noncytocidal virus–cell systems.

*Summary.* Dengue virus type 2 has been cloned in hamster kidney cells, a noncytopathic, noncytocidal virus–cell system, utilizing a direct fluorescent antibody method. A clone is considered to have arisen from the infection of a susceptible cell by a single infectious particle of DEN-2.

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