A Simple and Sensitive Method for Assay of Chick Interferon (35123)

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In the course of a study on the mechanism of interferon (IF) production in chick embryo (CE) cells, it was necessary to assay many IF samples at one time. Performance of the assay by the ordinary plaque reduction test (1) required too much material and labor, and therefore we attempted to simplify the technique. An agar diffusion method devised by Porterfield (2) was first tried with modifications, but the periphery of the plaque inhibition zone was quite irregular and the sensitivity of the test was far below that of the plaque reduction test, as pointed out by that author (3). A new, simple method was then worked out, which was as sensitive as the plaque reduction method. Details of experiments concerning the new test are reported below.

Materials and Methods. Viruses. The giant-plaque-forming u mutant of Sindbis virus, proved to be sensitive to IF (4), was used mainly. It was maintained by the cloning passage in CE cells stated earlier (5); *i.e.*, at each passage a giant plaque formed with a limiting dilution of the previous passage was rinsed and used as the seed for passage. A clone of small-plaque (sp) virus was separated by incubating plaque dishes of the parent Sindbis virus at 40° and isolating a small plaque; it formed small plaques at 40°, perhaps due to growth inhibition at this temperature, but formed plaques of the usual size at 37°. It was maintained in the manner of the standard passage (5). Vesicular stomatitis virus (VSV) of the Indiana serotype was supplied through the courtesy of Dr. N. Ishida, Tohoku University, Sendai, and also maintained by passage in CE cells.

CE cells and plaque assay. Preparation of primary CE cell monolayer cultures was described earlier (6). Growth and maintenance media contained inactivated calf serum at 10 and 2%, respectively. The plaque assay was described previously (4) and virus titers are expressed as plaque-forming units (PFU). For Sindbis virus mutants u and sp and for VSV, the interval between the first and second (neutral red) overlays was 1, 3, and 2 days, respectively. All fluid and agar media used contained 0.0025 *M* Tris buffer of pH 7.2, and incubation of monolayer dishes was made in a humidified 37° incubator.

IF samples. Fluids harvested from CE cell cultures infected with the parent Sindbis virus were dialyzed against KCl–HCl buffer at pH 2.5 and then against buffered saline of pH 7.2, and heated at 56° for 30 min. Four lots were prepared, and one lot possessing the highest IF titer was used in most of the present experiments.

Well inoculation. Monolayer cultures of CE cells in 90-mm dishes were washed once with PBS (7), and 9 metal cups routinely used for antibiotic assay were placed on the cell sheet of each dish, with one cup in the center. Ten ml of agar overlay was added immediately, to prevent excessive evaporation of moisture from the cell monolayer. After solidification of the agar medium, the cups were removed to form wells. Fluid in the wells, when present, was aspirated with a Pasteur pipette. Inoculation of IF or virus was performed as described in the text. The dishes were never turned during the subsequent incubation.

IF assay by the plaque reduction method. An IF sample was serially diluted by 2-fold increments and added in 2-ml amounts to three 60-mm dishes of CE cells per dilution. After 1 day's incubation at 37°, the fluids were removed and about 100 PFU of challenge virus was given to all dishes. Control cultures were treated in the same manner, except that maintenance medium was used in

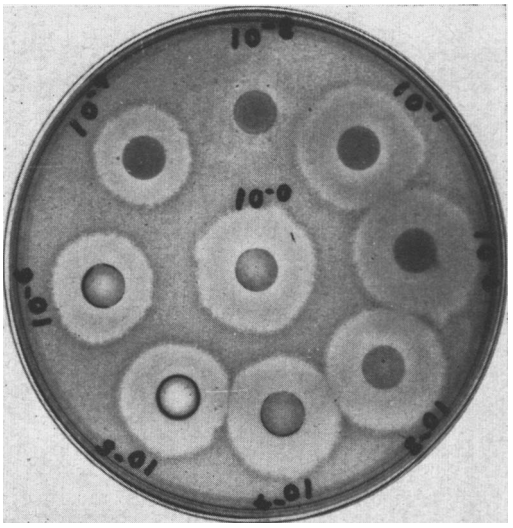

FIG. 1. Formation of plaques around wells inoculated with different dilutions of a seed of Sindbis u virus.

place of IF. The end point was the highest dilution which reduced the plaque number to less than 50% of the control value.

DEAE-dextran. A 1% aqueous solution was autoclaved and added to the overlay medium at the desired concentration.

Results. A preliminary test was performed to see how the virus would diffuse through the agar overlay after the well inoculation. A seed of Sindbis u virus was serially diluted and added into wells in 0.02-ml amounts, using a micropipette. After 3 days' incubation at 37°, a second overlay consisting of 4 ml of 1% agar containing 1:10,000 neutral red was added, and the dish was placed at 20° for 1 day before reading. As illustrated in Fig. 1, the 10^{-0} through 10^{-7} dilutions formed plaques of similar sizes whereas the 10^{-8} dilution formed none.

A more detailed test using 2 dishes (18 wells) per dilution showed that when virus was present in the inoculum it resulted in formation of a plaque whose size was fairly constant regardless of the concentration of virus added (Table I).

In the next experiment, wells were first treated with IF and then challenged with virus. Effects of two different methods of IF treatment were compared. In method I, IF serially diluted with maintenance medium was inoculated in the amount of 0.02 ml/well using a micropipette. The central well received the same amount of maintenance medium as control. After 1 day's incubation at 37°, a constant dose of Sindbis u virus, 0.02 ml of a 10^{-1} diluted seed, was added into the wells, using a micropipette. Method II differed from method I in that each well was filled with 0.08 ml of IF and next day was aspirated with a Pasteur pipette just prior to virus inoculation. With both methods, a clear inhibition of plaque formation was observed around wells treated with higher concentrations of IF. An example of such a test is presented in Fig. 2. When the above two methods were compared in a simultaneous test, method II showed a higher sensitivity (Table II). In the following experiments, therefore, method II was employed exclusively.

Since the well measured 8 mm in diameter, the plaque diameter minus 8 mm was called "normalized plaque diameter," and it was decided to take as the end point the highest dilution of IF which reduced the normalized plaque diameter to less than half that of control.

The next experiment examined the influence of different concentrations of challenge virus. Four pairs of monolayer cell cultures were treated with IF by the above method II, and challenged with different dilutions of Sindbis u virus. As revealed in Table III, a 1000-fold fluctuation in the seed strength did not influence the IF titers obtained. This fact permitted the use of a syringe and needle

TABLE I. Plaque Formation Around Wells Inoculated with Different Concentrations of Sindbis u Virus.

Dilution of seed virus	No. of plaque-positive wells/ no. of total wells inoculated	Plaque diam[a] (mm)		
		Mean	Max	Min
10^{-1}	18/18	20.8	23.3	15.2
10^{-3}	18/18	20.3	22.8	19.2
10^{-4}	18/18	20.8	22.2	18.9
10^{-5}	17/18	19.5	23.5	17.0
10^{-6}	15/18	18.8	20.8	16.0
10^{-7}	2/18	16.5	19.0	14.0

[a] Plaque-negative wells are omitted.

TABLE II. Comparison between Two Different Methods of IF Treatment in the Well Inoculation Assay.

IF treatment	Dish no.	Diam of the plaque formed around the well treated with the indicated dilution of IF (mm)								
		4×	8×	16×	32×	64×	128×	256×	512×	Control[c]
Method I[a]	1	11	11	13	22	23	20	21	21	22
	2	12	12	12	13	21	20	21	21	21
Method II[b]	1	8	8	11	11	16	21	20	21	21
	2	8	11	11	11	15	20	20	21	22

[a] Each well received 0.02 ml of IF and next day was challenged with 2.2×10^7 PFU of Sindbis u virus.

[b] Each well was filled with 0.08 ml of IF, which was aspirated off next day just prior to virus inoculation. The challenge virus dose was the same as in method I.

[c] Maintenance medium.

in place of micropipettes at the step of challenge. In the experiments hereafter described, the challenge virus was inoculated to wells dropwise from a syringe so that each well received approximately 0.02 ml.

The reproducibility of the test was then studied. Four IF samples were assayed by the well inoculation method in octuple tests. Parallel assays by the ordinary plaque reduction test were carried out using Sindbis u and VSV as the challenge virus. It was found that the new test was fairly reproducible, the standard deviation of titers being about ± 1 $\log_2$. Sindbis u virus was not inferior to VSV in the sensitivity to IF, and the titers determined by the two methods were comparable (Table IV).

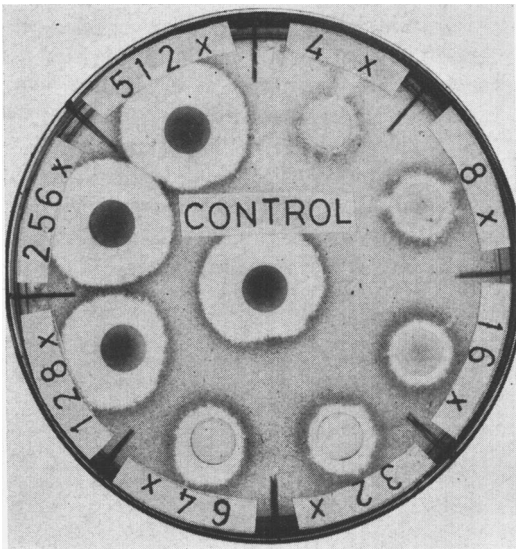

FIG. 2. An example of the IF assay by the well inoculation method showing an end point of 1:64.

It was then determined whether the parent Sindbis virus could substitute for the u mutant in the present test. In this case, however, the plaques formed did not show such a smooth periphery as those of the u mutant, either in the presence or in the absence of DEAE-dextran. Then an sp clone newly isolated was tested. When the overlay contained DEAE-dextran at 100μg/ml, it formed plaques as large as the u mutant. When 3 pairs of monolayer cell cultures were treated with IF and challenged with Sindbis sp and u viruses in the presence or absence of DEAE-dextran, the same end points were obtained (Table V).

TABLE III. Effect of Varying the Challenge Virus Dose upon the Titer of IF Determined by the Well Inoculation Method.

Dilution of seed virus used for challenge	Virus in the challenge inoculum (PFU)	Titers of IF determined in duplicate test (reciprocal of dilution)
10^{-1}	88,000	16, 16
10^{-2}	8800	4, 32
10^{-3}	880	16, 64
10^{-4}	88	16, 32

Discussion. The present method for IF assay has greatly simplified the technique. One monolayer dish suffices to titrate each IF sample, and reading the results is not time

TABLE IV. Examination for the Reproducibility and Sensitivity of the IF Assay by the Well Inoculation Method.

IF sample	Well inoculation: Challenge dose of Sindbis u virus[a] (PFU)	Well inoculation: Reciprocal of dilution: Individual titers in octuple tests	Well inoculation: Reciprocal of dilution: Geometric mean	Well inoculation: SD (log_2)	Plaque reduction (reciprocal of dilution): Titer with Sindbis u virus	Plaque reduction (reciprocal of dilution): Titer with VSV
1	5.3×10^5	16, 16, 16, 32, 32, 64, 64, 64	32	±0.93	64	8
2		8, 8, 16, 16, 16, 16, 32, 64	17.3	±1.03	8	4
3	4.8×10^6	8, 8, 8, 8, 8, 16, 16, 32	11.2	±0.76	32	32
4		2, 2, 2, 4, 4, 4, 8, 8	3.7	±0.84	4	4

[a] The virus titers are approximate values because the challenge virus was inoculated dropwise from a syringe and needle.

consuming. The sensitivity of the test was also comparable to that of the commonly practiced 50% plaque reduction test (1). Furthermore, the quantity of an IF sample needed for the test is small. The reproducibility of the end point may be satisfactory, the standard deviation being ±2-fold. A similar range of fluctuation of end points is usually encountered in the plaque reduction test. Theoretically, the reproducibility can be enhanced by adopting multiple tests and determining a geometric mean of end points obtained. Duplicate or triplicate tests may be performed with ease by the present method.

The greatest advantage in the present method is that the challenge virus can be used without a preliminary titration, because a wide fluctuation in the challenge virus strength did not influence the IF titer obtained. The reason for this may be that the inhibition of plaque formation is mainly effected by IF diffusing through agar and does not depend so much upon the suppression of viral growth in the well. It is even more advantageous that many IF-treated monolayer dishes can be challenged using one syringe and needle.

Application of the well inoculation method to the assay of IF of other species might be possible if a virus is available which is sensitive to IF and which forms sufficiently large plaques in cells of respective species, like Sindbis u virus. This mutant had been obtained as an agar-inhibitor-resistant daughter variant of an IF-sensitive sp clone (5). In this connection, it seems important that an sp clone of Sindbis virus could substitute for the u mutant when DEAE-dextran was incorporated in the overlay. This fact may be noteworthy when searching for a suitable vi-

TABLE V. IF Assay by the Well Inoculation Method Using Sindbis sp and u Viruses and DEAE-Dextran-Containing Overlay.

Challenge virus	Challenge virus dose[a] (PFU)	Concentration of DEAE-dextran in overlay (μg/ml)	IF titers obtained in duplicate test (reciprocal of dilution)
sp	1.9×10^6	100	64, 64
u	2.2×10^7	0	64, 64
	2.2×10^7	100	64, 64

[a] See the footnote of Table IV.

rus to be used in the assay of IF of other species.

Summary. A new method for the assay of chick interferon (IF) was devised. Serial dilutions of IF were inoculated into wells made in the agar overlay covering chick embryo cells, and after 1 day's incubation at 37° the wells received a constant dose of the u mutant of Sindbis virus. Three days later a large plaque whose size was almost independent of the challenge virus dose appeared around the control well. This plaque formation was inhibited by the IF pretreatment. Thus, one monolayer dish, 90 mm in diameter, sufficed to titrate each IF sample. The highest IF dilution which reduced the normalized plaque diameter (the plaque diameter minus the cup diameter) to less than half that of control was comparable to the end point determined by the ordinary 50% plaque reduction test. The standard deviation of end points was approximately ±2-fold. A 1000-fold fluctuation in the challenge virus dose did not influence the IF titer determined. When a small-plaque clone of Sindbis virus was used and DEAE-dextran incorporated in the agar overlay, the same result was obtained.

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