Use of Disposable Micro Tissue Culture Plates for Antiviral and Interferon Induction Studies

ROBERT W. SIDWELL AND JOHN H. HUFFMAN

Department of Virology, ICN Nucleic Acid Research Institute, Irvine, California 92664

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A reproducible test system requiring small amounts of test compound was developed for evaluating antiviral and interferon-inducing activity. In the antiviral experiments, KB cells were grown in disposable polystyrene microplates covered with a standard domestic plastic wrap. Viruses used in the system were types 1 and 2 herpes simplex virus, vaccinia virus, type 3 adenovirus, myxoma virus, pseudorabies virus, type 3 parainfluenza virus, types 1A and 13 rhinovirus, vesicular stomatitis virus, coxsackievirus B, and type 2 poliovirus. Inhibition of viral cytopathogenic effect was the primary criterion of evaluation of antiviral activity. Reduction in cell and supernatant fluid virus titers was used as a secondary means of evaluation. The microplate system was adaptable for determining prophylactic, therapeutic, and inactivating effects against viruses. Mouse L-929 cells were used for the interferon induction studies, with vesicular stomatitis virus utilized as the indicator of interferon activity. Known active compounds evaluated in this microplate system had activity similar to that seen in macro in vitro systems.

It is a common occurrence in laboratories synthesizing new drugs to produce initially only small quantities of the material, therefore creating a need for low cost methods by which these small quantities can be evaluated for biological activity. This problem has been particularly apparent for antiviral and interferon-inducing substances.

Investigators interested in viral chemotherapy have described a number of in vitro procedures. Rightsel et al. (14) described the use of macro vinyl plastic panels for virus assays; these have since been successfully employed for primary antiviral studies (1, 20). Drug-impregnated paper discs in virus plaque suppression tests have also been used (4, 13), as well as the more conventional virus titer reduction tests with glass tubes (3, 21). Recently Miller et al. (12) described a novel semiautomated procedure with inhibition of labeled thymidine or uridine uptake into viral nucleic acid as a criterion for evaluation of antiviral and interferon-inducing activity. Disposable microplates were used in a portion of the study. Although each procedure has been successfully employed in antiviral screening studies, disadvantages exist because of the relatively large amounts of test material needed, difficulty in differentiation between antiviral effects and toxic effects, diffusion inhibition (in the case of the paper disc method and possibly the label uptake method) resulting from insoluble test compounds, or the relatively high cost involved in materials and equipment.

In the present report, we describe a new antiviral evaluation procedure, with micro tissue culture plates, which offers certain advantages over the other systems currently in use. An adaptation of this procedure for evaluating potential interferon inducers is also reported.

MATERIALS AND METHODS

Micro tissue culture plates. Disposable plastic micro plates containing 96 flat-bottomed circular cups were used. These plates, obtained sterile in sealed plastic bags, were the Micro-Test II plate (Falcon Plastics, Division of BioQuest, Oxnard, Calif.), the flex vinyl Microtiter plate (Cook Engineering Co., Alexandria, Va.), and the Disposo-Tray Microplate (Linbro Chemical Co., New Haven, Conn.). Plate sealants studied included Micro-Test II pressure-sensitive film, Disposo-Tray adhesive-backed clear acetate, Microtiter pressure-sensitive tape, and Saran Wrap (Dow Chemical Co., Midland, Mich.).

Cell culture. Cell lines used were KB (human carcinoma of the nasopharynx), L-929 (mouse fibroblast), and RK-13 (rabbit kidney). The cells were trypsinized by a standard method (16), and 0.2 ml of Eagle's minimum essential medium (MEM; Grand Island Biological Co., San Francisco, Calif.) containing $1.8 \times 10^6$ cells/ml was added to each panel cup. The growth medium was supplemented with 10% heat-inactivated fetal calf serum, 100 $\mu$g of streptomycin per ml, and 100 units of penicillin per ml and was buffered with 0.22% NaHCO$_3$. The panel was sealed and incubated at 37°C for 18 hr to establish a monolayer for antiviral experiments.

Viruses. The deoxyribonucleic acid (DNA) viruses used were: type 1 herpes simplex virus (HSV), strain...
HF; type 2 HSV, strain MS; vaccinia virus (VV), strain Lederle CA; pseudorabies virus (PRV), RK17C24 derivative of Aujeszky strain; type 3 adenovirus (AV), strain GB; and myxoma virus (MV), strain Sanarelli. The ribonucleic acid (RNA) viruses used were: type 1A rhinovirus (RV), strain 2060; type 13 RV, strain 353; type 3 parainfluenza virus (HA-1), strain C243; type 1 Coxsackievirus B (CV), strain Connecticut 5; type 2 poliovirus (PV), strain MEF-1; and vesicular stomatitis virus (VSV), strain Indiana.

The type 1 HSV, VV, MV, HA-1, RV, CV, and PV were obtained from F. M. Schabel, Jr. (Southern Research Institute, Birmingham, Ala.); the PRV was received from A. S. Kaplan (Albert Einstein Medical Center, Philadelphia, Pa.); the AV was provided by D. A. Fuccillo (National Institute of Neurological Disease and Stroke, Bethesda, Md.); the type 2 HSV and the VSV were obtained from the American Type Culture Collection (Rockville, Md.). The viruses were used in a concentration of from 10 to 320 cell culture 50% infectious doses (CCID50)/0.2 ml, with RK-13 cells used for PRV and MV and KB cells used in experiments with all the other viruses.

Viral chemotherapy experiment. An 18-hr monolayer of cells in the plastic panels was used. The panel cups received 0.1 ml of virus suspended in MEM supplemented with streptomycin, penicillin, 5% inactivated fetal calf serum, and 0.25% NaHCO3. Within 10 min after addition of the virus, 0.1 ml of test compound in medium was added to the appropriate cups. Test compounds were diluted in one-half log10 dilutions, with 1,000 μg/ml being the maximum concentration used. The plates were then resealed, incubated for 3 days, and examined microscopically for cytopathic effect (CPE). Included in each panel were toxicity controls (containing test compound and medium only), virus controls (containing virus and medium only), and cell controls (containing medium only).

A layout of a typical panel in which four compounds were evaluated against one virus is shown in Fig. 1. All additions of virus, medium, and test compound were done with standard 1.0- or 2.0-ml pipettes. Sterile paper toweling was used to blot medium remaining on the panel surface and to cover the panel during addition of medium, compound, or virus.

Viral CPE was determined with the 40× magnification of an inverted microscope and was graded on a standard scale of 0 (normal cells) to 4 (virtually complete destruction of the cell layer). Reduction of CPE resulting from exposure to test compound was evaluated statistically by a modification of the virus rating (VR) method described by Ehrlich et al. (1). To determine a VR, the sum of the values assigned to the CPE of each cup of the treated, infected cells (T) at each drug level was subtracted from the sum value of the CPE in an equal number of virus control (C) cups. If the test compound was slightly toxic at one or more levels, the total C-T value of those levels was divided by 2. The C-T total of all drug levels was then divided by 10 times the number of test cups used per drug level. In our experience, a VR of > 1.0 is usually indicative of definite antiviral activity, whereas a VR of 0.5 to 0.9 indicates moderate or questionable activity. A VR of 0.1 to 0.5 suggests only slight anti-viral activity which possibly could be attributed to cytoxicity.

Determination of extra- or intracellular virus. Extracellular and intracellular virus titer reductions were determined in secondary experiments carried out to confirm previously seen antiviral activity. Extracellular virus titers were determined by assaying the cell-free supernatant fluid from treated cells in 18-hr monolayers of the appropriate cells in panel cups. Titers of intracellular virus were determined by washing the drug-treated cell monolayers three times with MEM and then adding 0.2 ml of medium to each cup and freezing and thawing the cells three times. The medium was then removed and assayed in the appropriate cell monolayer. Virus was indicated if CPE was demonstrable in the indicator cells within 4 days.

Determination of prophylactic and virucidal activity. Prophylactic activity of test compounds was determined by incubating the chemical with the cell monolayer for 1 hr at 37°C before addition of the virus. The remainder of the procedure was identical to that described above for the primary antiviral screening experiments. Virucidal activity was determined by incubating dilutions of test chemical for 1 hr at 37°C with undiluted stock virus (104 to 106 CCID50/ml) which had previously been clarified by low-speed centrifugation. Serial log10 dilutions of the virus-compound mixture were added to cell monolayers in the plastic panels which were then sealed and incubated at the appropriate temperature for 3 days. Virucidal activity was expressed as the reduction in virus titer observed as compared with virus which had been incubated with medium only but which had otherwise been handled identically to the test material.

Determination of interferon induction. An 18-hr monolayer of L-929 cells was prepared in a manner identical to that of a standard viral chemotherapy experiment, except medium 199 (Grand Island Biological Co.) was used instead of MEM. After 18 hr, the medium was replaced with medium containing test compound in various concentrations. The plates were incubated 3 hr at 37°C, the supernatant fluid was removed, and the cells were washed three times. A 0.2-ml amount of medium was then incubated with the cells at 37°C for 20 to 24 hr, removed, and assayed for interferon. The assay was performed by adding serial twofold dilutions of the supernatant fluid to an
TABLE 1. In vitro effect of 9-β-D-arabinofuranosyl-adenine (ara-A) on herpes simplex virus with the

disposable micro tissue culture plate test system

| Ara-A concn (mg/ml) | Toxicity | CPE inhibited | Extracellular virus titer reduction (logs) | Intracellular virus titer reduction (logs) |
|---------------------|----------|---------------|------------------------------------------|------------------------------------------|
| 1,000               | Toxic    | 100           | 3.0                                      | 3.5                                      |
| 320                 | Slightly toxic | 100 | 2.5                                      | 2.7                                      |
| 100                 | Very slightly toxic | 100 | 1.8                                      | 2.5                                      |
| 32                  | 0        | 82            | 1.0                                      | 1.3                                      |
| 10                  | 0        | 75            | 0.0                                      | 1.5                                      |
| 3.2                 | 0        | 31            | 0.0                                      | 0.5                                      |
| 1.0                 | 0        | 31            | 0.0                                      | 0.5                                      |

* Virus control: CPE, 4+; extracellular virus titer, 10^2-3 CCID_{50}/ml; intracellular virus titer, 10^2-3 CCID_{50}/ml. Virus rating of ara-A herpes simplex virus = 1.1.

18-hr monolayer of L-929 cells. The plates were sealed and incubated for 20 to 24 hr, the supernatant fluid was removed, and the cell sheets were washed once with medium. Vescular stomatitis virus (100 CCID_{50}/0.2 ml) was then added to each cup. Sterile medium was added to toxicity or cell control cups. After a 3-day incubation, the cells were examined microscopically for CPE. Interferon titers were expressed as the maximum dilutions of supernatant fluid which inhibited 50% of the virus. The original, drug-exposed cells, from which the interferon-containing supernatant fluid had been withdrawn, were washed a single time and VSV was titered in them and in normal cells to determine if they were rendered refractory to the virus by exposure to the test compound. Protection was expressed as the per cent reduction of CPE in treated cells compared to normal cells.

RESULTS

Antiviral experiments. An example of an antiviral experiment carried out with the disposable microplate test system is seen in Table 1. In this example, the effect of 9-β-D-arabinofuranosyl-adenine (ara-A) on type 1 HSV was evaluated, both by the usual parameter of CPE inhibition and, in addition, by extra- and intracellular virus titer reduction. Over 700 compounds have been evaluated with the CPE inhibition parameter, with the method yielding what was considered to be quantitative, specific, and reproducible data.

To demonstrate further the quantitative potential of this microplate procedure, ara-A, 1-β-D-arabinofuranosylcytosine (ara-C), and 5-iodo-2'-deoxyuridine were evaluated for both mean cytotoxicity (graded on a similar basis as CPE) and mean viral CPE inhibition scores at nine different concentrations. These scores were plotted against concentration (Fig. 2). All three compounds inhibited viral CPE at apparently less than cytotoxic levels, with the most effective of the materials having the cytotoxicity and the viral CPE inhibition curves intersecting at the lowest point. In the figure, the two ara-C curves meet at a point below the abscissa, indicating this compound had the highest degree of antiserum-virus activity.

Many of the compounds known from published reports as having activity against one or more of the viruses used in this system were tested against these viruses with the microtest system (Table 2).

Prophylactic and virucidal experiments carried out in the microplate system with experimental chemicals were as quantitative and reproducible as the antiviral experiments described above.

Interferon induction experiments. Results of an experiment with poly I:C to induce interferon in cells are shown in Fig. 3 and 4. As shown in Fig. 3, the titer of the induced interferon corresponded directly with the concentration of poly I:C used. The L-929 cells which had been exposed to poly I:C for 3 hr were resistant to VSV challenge (Fig. 4). This resistance was overcome by a massive concentration of virus, and the resistance was erratic or not detectable when low concentrations of poly I:C (1.0 or 0.1 µg/ml) were used.

DISCUSSION

Since the advent of the disposable microplate, numerous reports have been made on their efficacy. In virology, investigators have described their use for infectivity titrations (2, 15), neutralization (17), hemagglutination (5, 18), and complement fixation (19) tests. To our knowledge, this is the first reported use of the system for viral chemotherapy and for interferon induction studies, although reference has been made to the use of microplates for the assay of interferon production from animals and human sources.
Table 2. Comparison of the antiviral activity of known active compounds as determined by the disposable micro tissue culture plate system

| Name                                      | Activity† | Herpes virus 1 | Herpes virus 2 | Vaccinia virus | Adenovirus | Myxoma virus | Pseudorabies virus | Parainfluenza virus 3 | Rhino virus 13 | Polio virus 2 | Coxsackie virus | Vesicular stomatitis virus |
|-------------------------------------------|-----------|----------------|----------------|----------------|-------------|--------------|-------------------|-----------------------|----------------|----------------|----------------|-----------------------------|
| 9-β-D-Arabinofuranosyladenine             | 1.1, STR  | 0.6            | 0.9            | 0.0            | 0.8         | 1.1          | 0.1               | 0.1                   | 0.0            | 0.0           | 0.0            | 0.0                         |
| 1-β-D-Arabinofuranosylcytosine           | 1.3, STR  | 0.8            | 0.8            | 0.0            | 0.6         | 0.4          | 0.1               | 0.1                   | 0.0            | 0.0           | 0.0            | 0.1                         |
| 5-Iodo-2'-deoxyuridine                   | 1.4, STR  | 1.8            | 1.3            | 0.0            | 0.8         | 0.6          | 0.0               | 0.0                   | 0.0            | 0.0           | 0.0            | 0.0                         |
| 1-Adamantanamine·HCl                    | 0.2       | 0.3            | 0.3            | 0.0            | 0.0         | 0.1          | 0.0               | 0.0                   | 0.4            | 0.0           | 0.0            | 0.0                         |
| 2(α-Hydroxybenzyl)benzimidazole          | 0.3       | 0.1            | 0.1            | 0.0            | 0.0         | 0.0          | 0.1               | 0.1                   | 0.4            | 0.0           | 0.2            | 0.0                         |
| N’,N’-anhydrobis[β-hydroxyethyl]biguanide·HCl | 0.1   | 0.0            | 0.1            | 0.1            | 0.2         | 0.0          | 0.1               | 0.1                   | 0.3            | 0.0           | 0.0            | 0.0                         |
| Trifluorothymine-2'-deoxyriboside       | 1.3       | 0.9            | 0.9            | 0.2            | 1.0         | 1.0          | 0.2               | 0.0                   | 0.0            | 0.0           | 0.0            | 0.0                         |
| 2-Methyl-4(5-methyl-5H-az-triazino[5,6-b]indol-3-yl)amino)2-butanol | 0.2 | 0.7            | 0.0            | 0.0            | 0.0         | 0.0          | 0.0               | 0.0                   | 0.7            | 0.0           | 0.2            | 0.0                         |
| Streptothricin                           | 0.2       | 0.1            | 0.0            | 0.0            | 0.0         | 0.0          | 0.0               | 0.0                   | 0.5            | 0.0           | 0.0            | 0.0                         |
| Guanidine·HCl                            | 0.0       | 0.0            | 0.0            | 0.0            | 0.0         | 0.0          | 0.0               | 0.0                   | 0.0            | 0.0           | 0.0            | 0.0                         |

† Activity expressed as virus rating (VR). STR = significant (>1.0 logio) extra- and intracellular virus titer reduction. ITR = insignificant (<1.0 logio) extra- and intracellular virus titer reduction.

Fig. 3. Interferon demonstrated in the supernatant fluid from L-929 cells exposed for 3 hr to poly I:C and then incubated for 24 hr before removal for assay. Data expressed as per cent inhibition of vesicular stomatitis virus CPE in supernatant fluid-exposed L-929 cells. Test carried out in disposable micro tissue culture plate.

Fig. 4. Protection against vesicular stomatitis virus (VSV) challenge induced in L-929 cells by 3 hr of exposure to poly I:C. Data expressed as per cent inhibition of VSV CPE in cells which had been incubated for 24 hr after removal of inducer, washed, and then exposed to various concentrations of virus. Test carried out in disposable micro tissue culture plate.
(22). Micromethods have also been reported for determining bacterial sensitivity to antibiotics (6, 9). We have been using the technique, with occasional modifications, for 2 years and have found it to be reproducible, quantitative, capable of differentiating between antiviral and cytotoxic effects, and, importantly, requiring only small amounts of costly new compounds. The antiviral activity of known compounds as demonstrated in this system was similar to that reported by others and seen by ourselves with the more standard macro techniques.

If all controllable parameters were held constant, the system was effective in demonstrating an identical degree of antiviral activity on repeated tests. Such variations as virus or cell concentrations, time of addition of test compound to the virus-infected cells, incubation temperature, or medium constituents caused moderate fluctuations in the calculated VR. It is therefore important to include a "positive control" as a standard with each chemotherapy experiment. Since the visual scoring of CPE and cytotoxicity was a key factor in these studies, the same person usually did the microscopic examination of the plates.

Of the plates studied, the Micro-Test II plate was considered most suitable. The Microtiter plate was moderately cytotoxic; presoaking in 100% ethyl alcohol followed by multiple rinses in distilled water did not alleviate the problem. Recent preliminary studies with a new Microtiter plate indicated it was not cytotoxic, although the plate lacked raised edges around each cup. We understand a new noncytotoxic Microtiter plate having raised edges is now being produced. The Disposo-Tray Microplate was not cytotoxic but lacked the raised edge. The raised edge was an advantage because it enabled each cup to be more easily sealed. All of the plate sealants studied effectively closed the plates, but the Saran Wrap was ideal because of its ease in removal, its low cost, and its transparency. Contamination resulting from the wrap was never experienced.

The feasibility of this in vitro system for demonstrating the interferon-inducing activity of new compounds is somewhat questionable, since some compounds, such as tilorone hydrochloride and pyran copolymer, which are effective inducers of interferon in animals (7, 8, 10, 11), have not demonstrated this activity in macro in vitro systems (R. F. Krueger, personal communication), nor have we been able to demonstrate their activity in this microplate system. To date, only poly I:C has induced significant amounts of interferon in the microplate system. However, this system does seem to be as sensitive as currently used tube cell culture systems for demonstrating interferon activity.

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