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Hemadsorption Focus Assay for Growth of Influenza and Parainfluenza Viruses in Human Dermal Fibroblasts (42112)

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Abstract. An assay for virus-induced hemadsorption foci in cultures of human dermal fibroblasts is described. The assay allows determination of activity of species-specific as well as nonspecific antiviral agents or biological factors, such as interferons. The assay may be used for abortive (e.g., influenza virus) and productive (e.g., parainfluenza virus) infections of human fibroblasts. © 1985 Society for Experimental Biology and Medicine.

The lack of a readily available assay for growth of influenza and parainfluenza viruses in human cells *in vitro* has hampered studies for potential efficacy in humans of species-specific antiviral agents, biological factors, or cellular responses. We propose an assay for virus-induced hemadsorption by infected cells in culture in order to provide a method for direct assay of such antiviral activities using commonly available human dermal fibroblasts.

Materials and Methods. *Cells and viruses.* Human foreskin fibroblasts (HFF), HEp-2 cells, Madin-Darby canine kidney (MDCK) cells, and LLC-MK2 cells (initially obtained from Flow Laboratories, McLean, Va.) were serially passed in Earle's minimal essential medium (MEM) containing 5 to 10% fetal calf serum (FCS, heat inactivated), with penicillin (100 units/ml), amphotericin B (2.5 μg/ml), and glutamine (0.1 m*M*), buffered to pH 7.2–7.4.

Recombinant strains of influenza A/Alaska/6/77 H3N2 and influenza A/Udorn/307/72 H3N2 were obtained from the National Institutes of Health Reference Reagents Program. Influenza A/California/10/78 H1N1 wild-type virus and cold adapted vaccine virus were kindly provided by Dr. Louis Potash, Flow Laboratories, McLean, Virginia. Influenza A/Texas/1/77 H3N2 was grown in allantoic fluid of 10-day old embryonated hens' eggs and stored at −70°C. Influenza B/Hong Kong/8/73 and parainfluenza type 1 and 3 viruses were clinical isolates which had been passed in MDCK or LLC-MK2 cells and stored at −70°C.

Hemadsorption focus assay. HFF at passages 6 to 11 were seeded into 24-well polystyrene culture plates (Costar, Cambridge, Mass.) and grown to confluency. Monolayers were washed with phosphate buffered saline (PBS), exposed for 1 hr at 37°C in serum-free medium to 0.1 ml of influenza virus ($10^{2.75}$ 50% tissue culture infectious doses, or $TCID_{50}$), or parainfluenza virus ($10^{2.2}$ $TCID_{50}$), washed again, and overlaid with 1 ml of serum-free MEM, and incubated at 37°C in 5% CO_2 atmosphere.

After various time intervals, culture supernatants were aspirated and saved for assays of infectious virus and for determinations of hemagglutinin titer (see below). Guinea pig red blood cells (RBC, 1 ml of a 0.5% v/v suspension in PBS) were added to the cell monolayers for 15 min at 4°C. Nonadsorbed RBC were then removed by aspiration and monolayers were washed twice with PBS at 4°C. The remaining attached RBC were fixed to the monolayers by addition of 1 ml of a 0.5% (v/v) solution of glutaraldehyde in PBS, and incubation for a further 30 min at 4°C. Hemadsorption foci indicated by the RBC were counted using an inverted microscope. In certain experiments, cultures and overlying foci were stained with modified Wright–Giemsa stain (Diff-Quik, Dade Diagnostics, Aguada, Puerto Rico). A plastic grid was

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placed under each well to ensure that an equivalent area in each well was counted.

Assays for infectious virus and hemagglutinin activity in culture supernatants. Aliquots of the supernatant fluids removed just prior to hemadsorption were immediately serially diluted and assayed for infectious virus using either MDCK cells (for influenza viruses) or LLC-MK2 cells (for parainfluenza viruses) (2). Aliquots of the supernatant fluids from cultures challenged with influenza virus were also frozen at −20°C and later tested for the presence of hemagglutinin activity using established methods (1).

Assay for interferon activity. HFF were grown to confluency in 6-well culture plates (Costar). Human recombinant interferon-α_2 (5×10^7 IU/ml, Schering-Plough, Kenilworth, N.J.) was serially diluted at 0.5 $\log_{10}$ intervals using MEM with 10% FCS. Dilutions of interferon, as well as medium alone, were added to duplicate wells (1 ml/well) of washed HFF and the cultures were incubated for a further 24 hr at 37°C. Medium was subsequently removed and the monolayers were infected by addition of 10^3 $TCID_{50}$/ml of influenza A/California/10/78 H1N1 wild-type virus or the cold adapted vaccine virus. Cultures were washed and reincubated at 37°C for 24 hr, then assayed for presence of hemadsorption foci as described above.

Results. Infection with influenza A/Alaska H3N2, A/Texas H3N2, A/Udorn H3N2, influenza B, and parainfluenza types 1 and 3 viruses all produced hemadsorption foci in HFF cultures (Fig. 1). The optimal times to count or fix the foci were 24 hr after infection for influenza A viruses, and 72 hr after infection for parainfluenza virus (Table I). Fixation using glutaraldehyde did not alter the number of foci, and permitted counting at a later time. The number of hemadsorption foci was approximately equal to the infecting dose of virus measured in $TCID_{50}$. The HEp-2 human epithelial cell line, infected with a recombinant strain of influenza A/Alaska H3N2, also demonstrated countable foci at 24 hr (data not shown). Infection of HFF with influenza A produced little or no release of infectious virus or hemagglutinin activity into the supernatant culture fluids (Table I). However, infection with parainfluenza type 3 did result in production and release of infectious virus into the supernatant fluid (Table I). Infection with parainfluenza type 1 was similarly productive (data not shown).

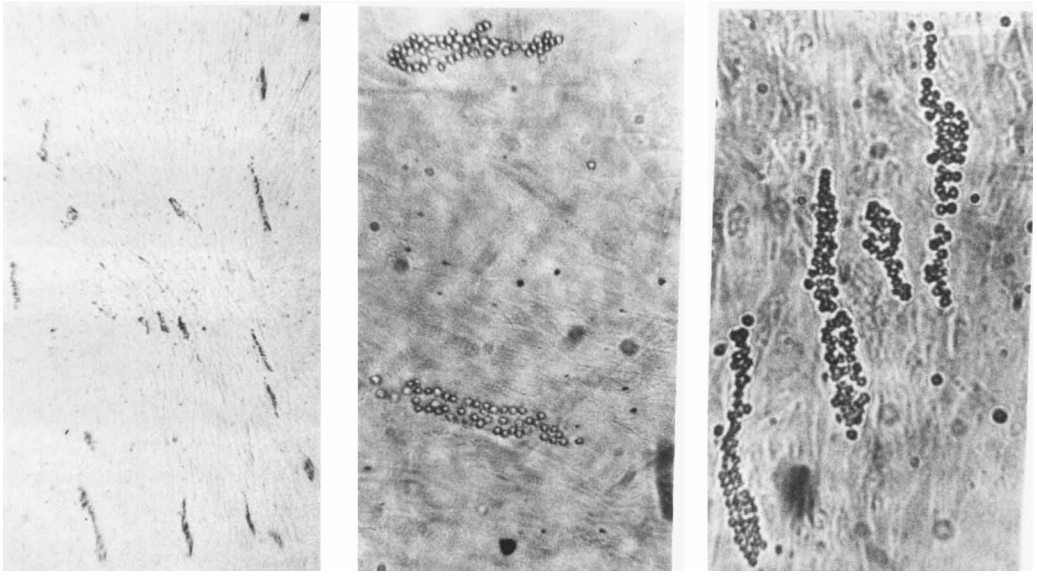

FIG. 1. Hemadsorption foci present in a human foreskin fibroblast cell culture 24 hr after exposure to influenza virus. (*Left*), unstained, original magnification 40×; (*center*), unstained, original magnification 100×; (*right*), stained using modified Wright–Giemsa method, original magnification 100×.

TABLE 1. GROWTH OF INFLUENZA A AND PARAINFLUENZA TYPE 3 VIRUSES IN HUMAN FORESKIN FIBROBLASTS[a]

Time after infection (hr)	Influenza A			Parainfluenza type 3	
	Hemadsorbing foci (mean No. ± SD)	Supernatant fluid virus titer[b]	Supernatant fluid hemagglutinin activity	Hemadsorbing foci (mean No. ± SD)	Supernatant fluid virus titer[c]
1	0	<1	—	0	<1
8	0	<1	—	0	<1
24	159 ± 15	<1	—	0	<1
48	51 ± 5	0.5	+[d]	0	2.3
72	11 ± 3	0.2	+[d]	57 ± 10	4.7
96	N.D.[e]	N.D.	N.D.	92 ± 12	4
120	N.D.	N.D.	N.D.	Innumerable	4.3

[a] Cultures were infected with $10^{2.75}$ $TCID_{50}$ influenza A/Alaska H3N2 or $10^{2.2}$ $TCID_{50}$ parainfluenza type 3.
[b] Titers are expressed as $\log_{10}$ $TCID_{50}$/0.1 ml, assayed using MDCK cells.
[c] Titers are expressed as $\log_{10}$ $TCID_{50}$/0.1 ml, assayed using LLC-MK2 cells.
[d] Positive only in undiluted supernatants.
[e] N.D. = not done.

The sensitivities of influenza A/California wild-type or vaccine viruses to recombinant interferon-α_2 were tested by hemadsorption focus assay using HFF cells, with replicate assays performed on different days (Fig. 2). The dose–response curves obtained on the different days were virtually equivalent. The amount of interferon required to reduce foci by 50% was 0.022 IU/ml for challenges with the influenza A/California wild-type virus (Fig. 2). The influenza A/California cold adapted vaccine virus was sensitive to the interferon to an almost equivalent extent (0.012 IU/ml), also determined using replicate assays on different days (data not shown).

Discussion. The hemadsorption focus assay for replication of influenza and parainfluenza viruses in readily available human dermal fibroblasts allows measurement of the effects of species-specific or species-variable antiviral factors or agents, and may also allow measurement of autologous cell-mediated antiviral responses. The data presented here show that replication or inhibition of virus can be reproducibly demonstrated using this assay. Such methods allow ready quantitation of antiviral effects in abortively as well as productively infected human tissues. Although methods for isolation and quantitation of influenza and parainfluenza viruses by plaque assay are readily available, such standard assays employ continuous canine (MDCK) (12, 13) and monkey (LLC-MK_2) (11) cell lines rather than human cells.

The use of commonly available foreskin fibroblasts, such as described here, allows measurement of activity of other human interferon preparations (for example, those not produced using recombinant DNA techniques) or other biological factors which are not active in cells derived from other species. Because dermal fibroblasts can be obtained

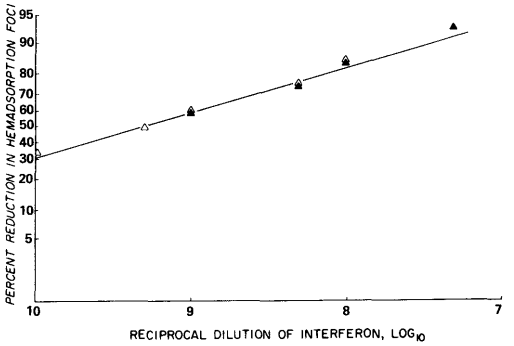

FIG. 2. Interferon-α_2 activity against influenza A/California/10/78 H1N1, measured by reduction of hemadsorption foci on 2 different days (△, ▲, respectively). The amount of interferon that reduced foci by 50% at 24 hr was interpolated to be 0.019 and 0.025 IU/ml in the two assays, based upon a stock interferon titer of 5 × 10^7 IU/ml. Number of foci in 100% value: △, 639; ▲, 858.

easily from the extremities of available leukocyte donors by punch biopsy, such an assay can also be used in studies of cell-mediated antiviral responses, eliminating the confounding features of allogeneic stimulation.

Prior studies with influenza viruses have used mainly cells of nonhuman origin. Influenza viruses do not productively infect most human cells *in vitro* (4, 8, 15) although hemagglutinins are produced (7). A few human cell types or cell lines, or clones thereof, have been shown to support influenza virus replication (10). For example, other investigators have demonstrated persistent and productive influenza virus infections of human embryonic lung and kidney cells (3, 14), and have used an inhibitor-resistant influenza virus strain at high multiplicities of infection, with trypsin maintained in the medium, to develop a highly productive infection of a human fibroblast cell line (9).

Hahon and colleagues (5–7) have described an hemadsorption focus assay with Sendai virus for measurement of interferon activity using the clone 1-5c-4 variant of a human conjunctival cell line, originally described by Wong and Kilbourne (15). While we have also used the hemadsorption focus assay in human continuous cell lines (for example, HEp-2), we believe that the demonstration of its use in dermal fibroblasts offers further advantages: (a) because the assay works for both abortive and productive infections, clinical virus isolates of sufficient titer can be tested without further passage in productive cells; (b) nontransformed cells rather than transformed human cell lines can be used. Unique or accentuated features of an established cell line are therefore avoided, and the results may better reflect *in vivo* events; (c) since fibroblasts are frequently used in research on "aging," the effects of aging on cell responses to viruses and antiviral agents may be measured; (d) cell-mediated antiviral responses against autologous dermal fibroblasts can be measured, avoiding confounding allogeneic stimulation; (e) human dermal fibroblasts are readily obtained and easily maintained; and (f) the hemadsorption foci are much more easily visualized overlying the monolayer of cells with fibroblast morphology, even though the foci of adsorbed red blood cells can also be distinguished when overlying monolayers of round cells, such as HEp-2 cells.

The hemadsorption foci can be stained quickly and easily with a modified Wright–Giemsa stain (Fig. 1), or using an immunoperoxidase (IP) procedure (data not shown), and remain readily distinguishable from the underlying fibroblast monolayer. The ease of reading and sensitivity of the assay are not enhanced by either method of staining, and the latter method adds considerable effort to the development of results. Thus, we have not recommended these additional steps. As described, the hemadsorption focus assay for replication of influenza or parainfluenza viruses in human dermal fibroblasts should be useful for further elucidation of human antiviral defenses.

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