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Use of human monocytes in the evaluation of antiviral drugs: quantitation of HSV-1 cytopathic effects

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Summary

An assay for the evaluation of antiviral and immunomodulator potency was developed using pure populations of cultured human monocytes. The assay involved culturing of human monocytes until they were fully susceptible (15–20 days) to lytic infection with HSV-1. When susceptible cells were cultured with recombinant interferon-α or a synthetic interferon inducer such as polyinosinic:polycytidylic acid prior to infection, a significant enhancement in resistance to the cytopathic effects of HSV-1 was observed. Likewise, a dose dependent reduction in cell lysis was observed when acyclovir was added immediately after virus infection. Monocyte resistance to HSV-1 was determined by the retention of pinocytic activity as determined by the uptake of neutral red dye. Relative pinocytic activity was quantitated using a simple colorimetric procedure. This antiviral assay can be completed in 48 h; is easy to perform, highly sensitive and reproducible.

Human monocyte; Differentiation; Herpes simplex virus type 1; Interferon-α; Poly I:C-LC, acyclovir; Viability index; Drug screening

Introduction

A number of assay systems using either plaque reduction or cytopathic effect measurements and employing a variety of cell types are currently being used to

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establish the potency of antiviral agents. Unfortunately, the data generated in these assays are derived from cells (e.g. VERO, primary rabbit kidney, human embryonic skin or muscle, mouse embryo) which are irrelevant to the pathology that the virus induces in man. In contrast, cells of the mononuclear phagocyte system are of greater relevance for the determination of antiviral efficacy since these cells constitute the first line of cellular defense against most viruses (Morahan et al., 1985).

The use of mononuclear phagocytes as a primary culture system for the evaluation of antiviral agents has been hampered due to (i) inadequate cell yield, (ii) contamination with other cell types, and (iii) lack of adequate long-term maintenance procedures. We have been successful in overcoming these difficulties and have established a methodology for the production of homogeneous populations of in vitro differentiated human blood monocytes which show a time dependent increase in permissiveness to herpes simplex virus type 1 (HSV-1) replication. Moreover, the assay described in this study provides several important advantages over other monocyte/macrophage HSV assays in which the production of infectious virus is required to quantitate drug efficacy (Domke-Opitz et al., 1986; Linnavuori and Hovi, 1983). These include (i) direct measurement of cell integrity (i.e. viability and function), (ii) potential for single cell analysis, (iii) ease of performance, and (iv) rapidity (i.e. 48 h assay). This study illustrates the versatility and usefulness of a new human mononuclear phagocyte antiviral assay for the evaluation of both nucleoside analogs and immunomodulators.

Materials and Methods

Preparation of human mononuclear cells

Mononuclear cell enriched leukocyte concentrates were obtained from normal donors undergoing lymphocytapheresis with the help of a Fenwal C.S. 3000 continuous flow centrifugation unit (Stevenson et al., 1983). Two hundred milliliters of concentrate containing approximately 7.5×10^9 leukocytes were diluted 1:4 with sterile PBS (Dulbecco's w/o calcium and magnesium) and 35 ml layered over 15 ml of Lymphoprep™ (Nycomed A/S Oslo, Norway). After centrifugation for 40 min at 23°C and 1200 rpm ($600 \times g$), the mononuclear cells were collected from the interphase and washed twice in elutriation media [Hanks' Balanced Salt Solution, w/o calcium and magnesium containing 2% human serum albumin (Bern Blood Bank)]. Cells were suspended in elutriation medium and 250 ml (3×10^9 cells) loaded into the separation chamber of a Beckman JE-6B elutriation system which was placed in a Beckman J2-21M induction drive centrifuge. Sample loading was done at 5°C at a flow rate of 14.6 ml/min and a rotor speed of 2500 rpm. Three fractions were collected. Fraction 1 consisted of the first 500 ml of eluate and fraction 2 consisted of the next 150 ml collected at a flow rate of 15.4 ml/min. Fraction 3 (200 ml) was collected at a flow rate of 25 ml/min. Each fraction was centrifuged at $600 \times g$ for 10 min and washed twice in RPMI-1640 containing 1%

human type AB serum. Cell purity was assessed following cytocentrifugation (Cytospin 2, Shandon Instruments, Sewickley, PA). Morphological criteria together with cytochemical staining were used to determine monocyte purity. Fraction 3 contained greater than 97% monocytes, and less than 1% lymphocytes and neutrophils, and was used in these studies.

Virus preparation

Herpes simplex type 1 virus (strain VR/3) was obtained from the laboratory of Dr A. Nahmias (Emory University, Atlanta, GA) and passaged in VERO cells grown in RPMI medium containing 5% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Virus stocks (7.5×10^7 PFU/ml as titrated on VERO cells) were stored in 1 ml ampoules and frozen at −80°C until used.

Drugs

Recombinant human IFN-α (rhuIFN-α) was obtained from Hoffmann-La Roche and stored carrier free in PBS at 4°C at a concentration of 1×10^6 international units/ml. This interferon was diluted in RPMI just prior to use.

Polyinosinic:polycytidylic acid conjugated to lysine carboxymethylcellulose (poly I:C-LC) was obtained from Dr Hilton Levy (NIH, Washington, DC) at a concentration of 2 mg/ml and was diluted in RPMI.

Acyclovir was a generous gift from Burrough's Wellcome Laboratories, Research Triangle Park, NC, and diluted in complete RPMI prior to adding to infected cells. Ribavirin was obtained from the Antiviral Studies Group, USAMRIID, Fort Detrick, Frederick, MD.

In vitro culture of monocytes

Monocytes were allowed to differentiate in 'Sterilin' bacteriological Petri dishes in RPMI-1640 containing 10% human type AB serum, 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate (complete RPMI). Ten million cells suspended in 10 ml were cultured in each plate and detached at various times by washing with cold PBS (w/o calcium and magnesium). Cells were gently pelleted ($400 \times g$ for 7 min), counted, and added to flat bottom microtiter plates prior to infection with HSV-1. Virtually 100% of the cells handled in this manner were viable as determined by trypan blue exclusion. The number of cells added to each well was dependent on the age of the cells at the time of collection and is indicated in the figure legends. Those cells held in culture for at least 14 days were much larger than freshly isolated monocytes and were highly susceptible to lytic HSV-1 infection.

Virus infection and addition of drugs

Fourteen-day-old cultured cells (3×10^4) suspended in a volume of 0.2 ml of complete RPMI were added to each well (except for peripheral wells) of a 96-well (flat bottom) microtiter plate and incubated in the presence of optimal and sub-optimal concentrations of poly I:C-LC or recombinant human interferon-α for 18–24 h prior to infection. These cells were either mock infected (RPMI without serum) or infected with 0.025 ml of the VR/3 strain of HSV-1 at a multiplicity of 0.5, 1.0 and 2.0 PFU per cell. Virus was adsorbed at 37°C (rocking plates every 15 min) for 1 h after which 0.2 ml of complete RPMI medium was added. When testing nucleoside analogs such as acyclovir or ribavirin, which do not require a preincubation period, drug was added in complete RPMI to each well following the infection procedure described above.

Evaluation of HSV-1 cytopathic effects

Cell viability following virus infection was measured by removing the media from infected cells and adding 0.2 ml of a filtered (0.22 U), neutral red (0.01% final concentration in RPMI) solution containing 1% human serum and 25 millimolar HEPES. Plates were incubated for 1 h at 37°C and the neutral red solution removed. Sorenson's citrate ethanol buffer (pH 4.1) in a volume of 0.1 ml was added to each well and the plates put on a shaker for several minutes. Dye extracts were measured in a colorimeter at 540 nm and the cell viability index expressed as the ratio of dye uptake by infected cells in the presence or absence of test drug to dye uptake by uninfected cells in the presence or absence of test drug.

Immunocytochemical staining of infected cells

Viral antigens expressed by infected cells were detected using a standard indirect antibody procedure in which alkaline phosphatase was conjugated to the secondary antibody. Briefly, infected cells were washed with PBS and then fixed with 2% paraformaldehyde in PBS for 30 min at room temperature. Fixed cells were washed once in PBS and permeabilized with 0.5% Nonidet P-40 for 5 min at room temperature. Fixed/permeabilized cells were washed twice with 10 mM glycine and blocked with 10% normal goat serum in HEPES-buffered saline (pH 7.3). After removing the blocking buffer, cells were incubated with rabbit anti-HSV-1 (Dakopatts B-114 at 1/250 dilution) antibody for 30 min at 37°C. These cells were washed and $F(ab')_2$ goat anti-rabbit IgG $F(ab)_2$ conjugated to alkaline phosphatase added for 30 min at 37°C. After washing four times with saline and once with substrate buffer (0.1 M Tris/HCl, pH 8.8), visualization of HSV-1 antigen was performed using a solution consisting of 0.4 mg/ml of Fast Red (BioRad), 1 mg/ml naphthol-phosphate (BioRad) and 0.001 M levamisole (Sigma) in substrate buffer.

Results

Characteristics of cultured monocytes

Both freshly isolated and cultured monocytes were examined in a flow cytometer using forward angle light scatter to determine size and monoclonal antibodies to detect specific cell surface antigens. As illustrated in Fig. 1, monocytes kept in culture for two weeks increased in size and revealed altered Leu M3 and HLA-DR surface markers. Additional studies revealed that cultured cells were (i) esterase positive and peroxidase negative, (ii) highly phagocytic, and (iii) IL-1 positive when stimulated with LPS (data not shown). As shown below, cultured cells were susceptible to infection with HSV-1 while fresh monocytes were resistant.

Quantitation of HSV-1 cytopathic effects

As illustrated in Fig. 2 (panels A–D), when cultured cells were infected with HSV-1, viral antigen expression increased as the degree of CPE intensified over time. In contrast, no viral antigen or CPE was observed when fresh monocytes from the same donor were infected. Note that acyclovir (Fig. 2, E–H) was able to prevent the induction of recognizable CPE, but did not fully prevent the expression of some HSV-1 antigens.

Fig. 2 also illustrates the pattern of virus-induced cellular changes in cultured cells. Thus, virus-infected cells rounded up 8 h after infection and fused with neighboring cells 4–8 h later (panel B). Cell fusion progressed to syncytial formation and was temporally related to the input MOI and cell density. Cell lysis and death was complete 48–72 h after infection (panels C and D). As expected, the loss of pinocytic activity correlated with increasing CPE.

Fig. 3 illustrates the relationship between the intensity of cell lysis and multiplicity of infection in 14-day-old cultured monocytes. Note that cell lysis was complete at high MOI (5–10 ph/cell) 48–72 h after infection, while lower MOI (0.5–1 ph/cell) did not result in complete lysis. The later observation appeared to be donor dependent since some donor cells were either more or less susceptible to lysis at low multiplicities of infection (data not shown).

Human monocyte susceptibility to HSV-1 lysis: dependence on in vitro culture and differentiation

Freshly isolated monocytes were resistant to HSV-1 infection; however, cells held in culture for more than 10 days lost their resistance and became sensitive to infection. To illustrate this change in sensitivity, monocytes were cultured for various times, removed from culture dishes, added to 96-well plates and infected. As illustrated in Fig. 4, fresh monocytes were not susceptible to lytic infection with HSV-1, but became susceptible following an 11–18 day incubation period. Susceptibility to virus lysis correlated with the development of classical macrophage morphology and differentiated features.

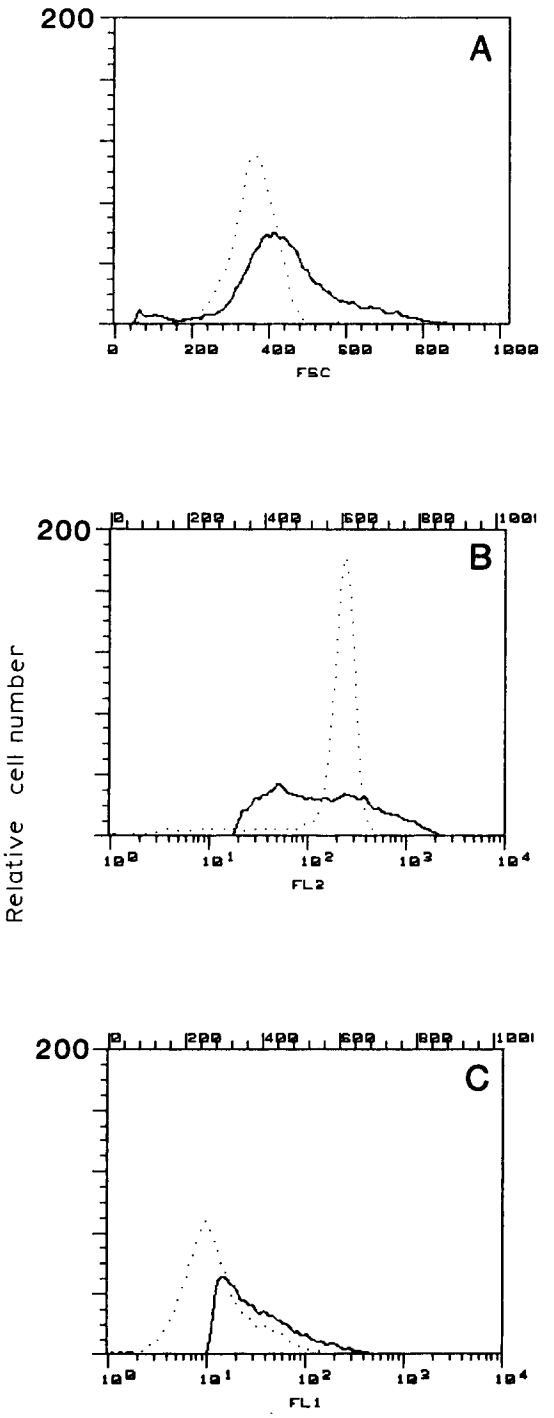

Fig. 1. Flow cytometric analysis of freshly isolated and cultured monocytes. Freshly isolated (·····) and 14-day-old cultured (—) monocytes were examined by forward angle light scatter for size (panel A), monoclonal antibody to Leu M_3 (panel B), and monoclonal antibody to HLA-DR (panel C).

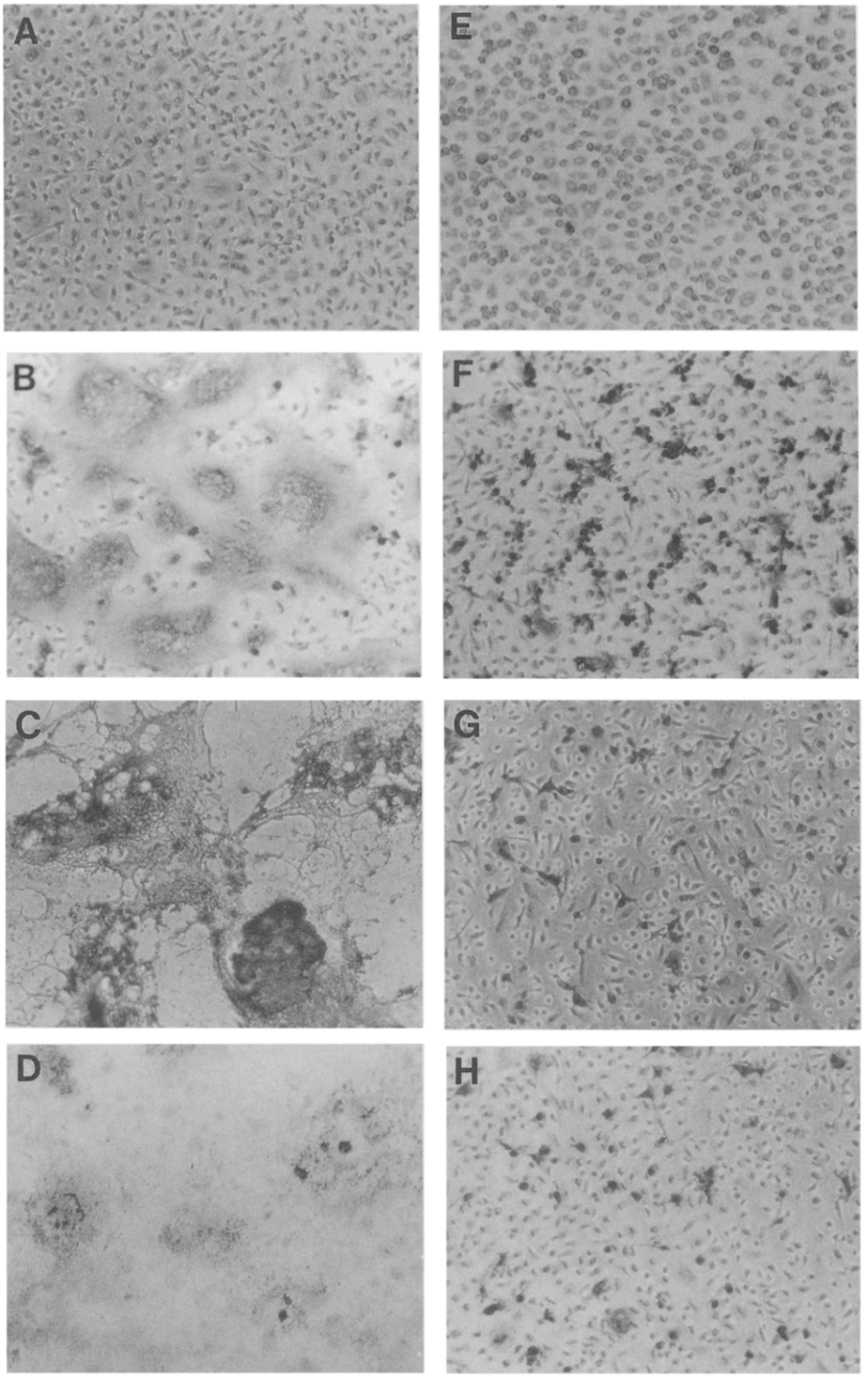

Fig. 2. Immunocytochemical staining of HSV-1 membrane antigen. Monocytes (14-day-old cultures) were infected with HSV-1 at a multiplicity of 3 and stained with an alkaline phosphatase conjugated antibody as described in Materials and Methods. Column A through D no drug treatment (A=0, B=24, C=48, D=72 h post infection). Column E through H acyclovir (100 ng/ml) treated (E=0, F=24, G=48, H=72 h post infection).

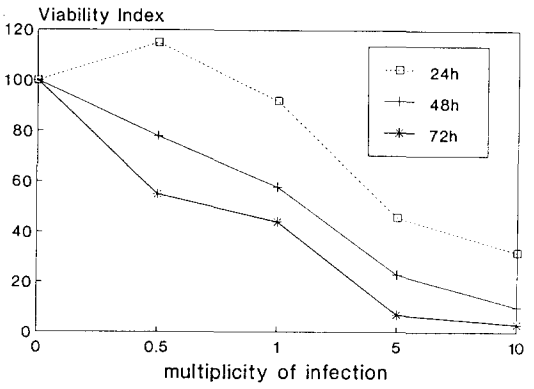

Fig. 3. Progression of the cytopathic effect of HSV-1 on in vitro differentiated monocytes: use of the neutral red dye uptake for quantification. Two-week-old macrophages were plated at 3×10^4 cells per well (0.2 ml) adhered overnight, infected as described with an MOI of 0.5, 1, 5 and 10. Neutral red dye uptake was evaluated 24, 48 and 72 h post infection. Viability index was established based on the following formula:

$$\frac{OD_{540} \text{ infected cells}}{OD_{540} \text{ uninfected cells}} \times 100$$

Correlation between observed cytopathic effects and release of infectious virus

To determine the relationship between neutral red dye uptake and the extent of infectious virus released by culture monocytes, plaque titrations (Gangemi et al., 1987) were performed on cell supernatants at selected times following infection. Ten day old cultured monocytes (2×10^5 cells) were added to 24-well tissue culture plates and infected with HSV-1 at a multiplicity of 3. As shown in Table 1,

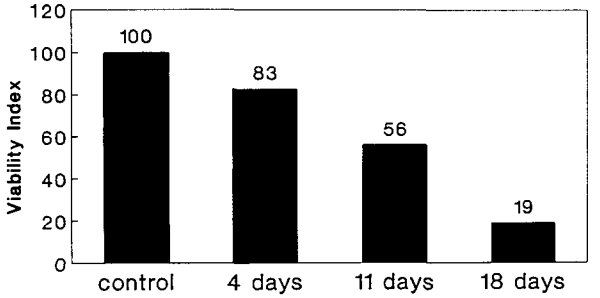

Fig. 4. Human monocyte susceptibility to HSV-1 induced cytopathic effects: dependence on in vitro differentiation. Cells from the same donor were cultured for 4, 11, 18 days detached and cryopreserved as described by Stevenson et al. (1983). (Instead of fetal calf serum, human serum was used.) These cells together with the cryopreserved monocytes were thawed, plated to establish confluent monolayer (3×10^5 cells/well for monocytes, 10^5 cells/well for 4-day-old cells and 3×10^4 cells/well for 11 and 18-day-old cells). After overnight adherence, the cells were infected with an MOI of 2. The viability index was determined 72 h after infection.

TABLE 1

HSV-1 replication in ten-day-old cultured human monocytes

Hours after infection	Virus in supernatant (PFU/ml)[a]	Cell morphology	Viability index[b]	Thermal inactivation (PFU/ml)[c]
24	4×10^6	Syncytia	50	7×10^5
48	3×10^6	Cell rupture	20	6×10^4
72	6×10^6	Total cell lysis	0	2×10^4
96	4×10^6	Total cell lysis	0	2×10^3

[a]Release of infectious virus as determined by plaque titrations on VERO cells.

[b]As determined by the uptake of neutral red. Viability index = $\frac{\text{OD test cells}}{\text{OD control cells}}$.

[c]Inactivation of virus inoculum 7×10^5 PFU/ml in complete RPMI at 37°C in absence of monocytes.

release of infectious virus was observed at 24 hours post infection and remained elevated up to the time of cell lysis (72 h). Note that large syncytia were observed 24 h post infection but they were still pinocytic. However, by 72 h post infection, none of the cells was able to pinocytose neutral red and complete cell lysis was evident.

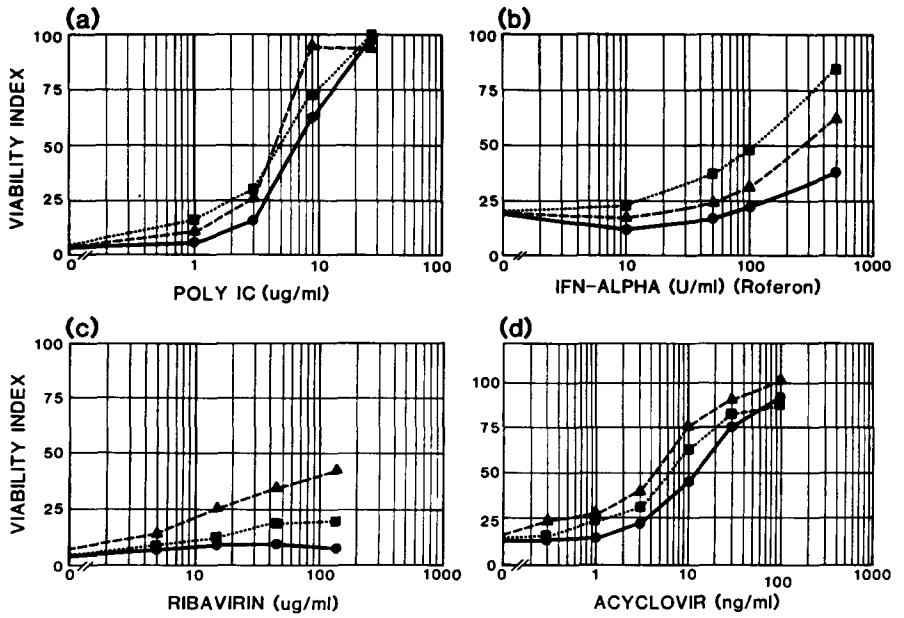

Fig. 5. Modulation of HSV-1 induced cytopathic effects by selected pharmacological agents. Monolayers of two-week-old macrophages were established as described. The cells were co-cultured overnight with poly I:C-LC (1, 3, 9 and 30 μg/ml) or IFN-α (10, 50, 100, 500 IU/ml) supernatants aspirated and infected at MOI of 0.5 (-▲-), 1 (-■-), 2 (-●-). When antivirals (acyclovir 0.3, 1, 3, 10, 30, and 100 ng/ml and ribavirin 5, 15, 45 and 150 μg/ml) were tested, the drugs were added immediately following infection. Seventy-two hours later, the viability index for each drug concentration was determined and ED_{50} established.

Modulation of HSV-1 induced cytopathic effects by selected pharmacological agents

The antiviral activity of immunomodulators and nucleoside analogues were examined in 14-day-old mononuclear phagocytes. Fig. 5 illustrates the effect of incubating these cells with poly I:C-LC (5a) or rhuIFN-α (5b) for 18 h prior to virus infection. The addition of poly I:C-LC resulted in a dose-dependent response in which inhibition of HSV-1 cytopathology was observed at concentrations ranging between 3 and 27 μg/ml with an ED_{50} of approximately 6 μg/ml. These values were independent of the MOI examined. Likewise, interferon-α exhibited a dose dependent response and inhibition of viral cytopathology was observed between 10 and 100 units/ml. However, unlike poly I:C-LC, the degree of protection afforded by interferon-α was dependent on the multiplicity of infection.

In contrast to immunomodulators, the presence of nucleoside analogs was required throughout the virus incubation period. Ribavirin (Fig. 5c) is a poor antiherpetic drug and was included in these studies as a negative control. Note that ribavirin was only marginally effective in human monocytes when examined at a very low multiplicity of infection and no activity was observed at a multiplicity of 1 or 2 PFU/cell. Concentrations of ribavirin greater than 300 μg/ml were toxic and could not, therefore, be examined. In contrast to ribavirin, the preferred antiherpetic agent, acyclovir (Fig. 5d), was quite effective at concentrations ranging from 1–100 ng/ml with an approximate ED_{50} of 6 ng/ml. This value was not dependent on the multiplicity of infection.

Discussion

The discovery of novel antiviral drugs should be based on systems that take into account: (a) the human origin of the cell target, (b) the relevance of the cell to viral pathology, and (c) the different mechanisms of action of antiviral agents (virustatic vs immunomodulatory). Ideally such a system could be provided by mononuclear phagocytes which are primary targets for a number of viral infections including HSV-1, HIV and other lentiviruses (Daniels et al., 1978; Koening et al., 1986; Gendelman et al., 1985).

While abortive infections have been observed in human blood monocytes infected with HSV-1 (Daniels et al., 1978), productive HSV-1 infections appear to require cell differentiation (Tenney and Morahan, 1987). Since abortive infections are difficult to quantitate, the development of murine systems or the use of differentiated human macrophage cell lines which are susceptible to productive infection could be used for the discovery of novel antiviral agents. However, these cell systems have limitations such as species specificity for interferons in the case of murine cells or need for chemical agents that are required to induce differentiation and which may alter the effects of the antiviral agents being tested (Tenney and Morahan, 1987).

In this report we have described a cellular system which is based upon the use of spontaneously differentiated human monocytes which are susceptible to pro-

ductive HSV-1 infection. We have shown that this productive infection can be abrogated by agents such as interferons, inducers of interferon and nucleoside analogs. Based upon this observation, an assay to evaluate antiviral agents was designed. This assay takes into account both the intensity of infection, as determined by the multiplicity of infection, and the dosage of drug. It should be emphasized that the intensity of infection and drug dosage are two of the most significant variables in the determination of potency and efficacy. Thus, acyclovir or poly I:C-LC show high potency as well as efficacy, while on the other hand, interferon-α and ribavirin show efficacy but not potency as indicated by the 50% inhibitory concentrations at the three multiplicities of infection examined.

The human mononuclear phagocytes described in this study appear to be more sensitive than other more commonly used indicator cells such as rabbit kidney and murine embryonic cells. Thus, we were able to show an ED_{50} of 6 ng/ml for acyclovir in our system while others have reported values ranging from 200 ng/ml in the case of primary rabbit kidney cells to 10 ng/ml in murine embryonic cells (De Clercq et al., 1986). Furthermore, when bromovinyl-deoxyuridine (BVDU) and S-hydroxy-2-phosphonyl-methoxypropyl adenine (s-HPMPA) were examined in this cellular system and compared to values reported in the literature (De Clercq et al., 1987), a 10–100-fold respective difference was observed. However, the order of potency remained the same (i.e. BVDU>acyclovir>(s)-HPMPA>ribavirin) (manuscript in preparation).

In conclusion, we believe that the human mononuclear phagocyte assay described in this study offers a suitable and relevant model for the evaluation and discovery of novel antiviral agents. This system could prove useful in the evaluation of drugs which may be effective in the treatment of other macrophage tropic viruses which result in lysis or inhibition of normal metabolic and pinocytic functions.

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