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Susceptibility of Clinical Isolates of Cytomegalovirus to Human Interferon

BOSKO POSTIC* AND JOHN N. DOWLING

Department of Medicine, University of Pittsburgh School of Medicine, and the Medical Service, the Oakland Division of the Veterans Administration Hospital, Pittsburgh, Pennsylvania 15240*

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Human cell culture-derived interferon was shown to inhibit human cytomegalovirus in vitro. A prototype strain, Davis, and six clinical isolates of cytomegalovirus were tested. All six isolates showed uniform susceptibility to interferon, exceeding that of the Davis strain by two- to fourfold. The latter virus was found to be 32 to 4 times less susceptible than the sensitive indicator, vesicular stomatitis virus. However, the laboratory finding of susceptibility to an antiviral material may not relate to its clinical effectiveness.

Human cytomegalovirus (CMV) causes a broad spectrum of clinical illness (21). The devastating effects of congenital disease have long been recognized. More recently, subtle sequelae, including decreased mentation and auditory impairment, have been described, even among children with subclinical presentation at birth (18). Although acquired CMV infection is generally benign, significant morbidity and mortality occurs, especially in organ transplant recipients and immunosuppressed patients (16, 20; S. Suwansirikul, N. Rao, J. N. Dowling, and M. Ho, Arch. Intern. Med., in press).

Therapeutic trials have been attempted with a number of agents. CMV is inhibited in cell culture by the nucleoside analogues, 5-iodo-2'-deoxyuridine, adenine arabinoside, and cytosine arabinoside (7). In practice, these compounds may transiently suppress urinary excretion of CMV, but generally fail to eradicate the virus or alter the natural course of the disease (4, 5, 13, 15, 19). Administration of massive interferon doses may also affect CMV excretion in the urine. Viruria was completely inhibited in three of nine patients with congenital or acquired infection treated by Emodi et al. (6), and in five patients, viruria was transiently suppressed. Viremia was not affected. Arvin et al. (3) obtained transient suppression of viruria in only one of six courses of therapy, but the doses of interferon employed were lower. No clinical response was appreciated in these trials, but this was difficult to evaluate.

These results are not surprising in view of the relative nonsusceptibility of CMV to interferon in cell culture. Early work by Glasgow et al. (8) demonstrated that CMV is more resistant to the antiviral action of interferon than other viruses, including Sindbis, vaccinia, and vesicular stomatitis virus (VSV), to which it was compared. However, the amount of interferon employed was relatively small. The antiviral effect of interferon against CMV needs to be reassessed in view of the massive doses now available for human administration. In this study, the in vitro inhibition of CMV by human interferon was characterized quantitatively. The antiviral effect on the Davis strain of CMV was compared with that observed with VSV. In addition, the susceptibility of six clinical isolates of CMV was determined.

MATERIALS AND METHODS

Viruses. The Indiana strain of VSV was used as indicator for the assay of interferon (1). CMV of the Davis strain was originally obtained from Thomas H. Weller (Harvard School of Public Health, Boston, Mass.) and had undergone numerous passages in human embryo fibroblast cells in this laboratory. Clinical isolates of CMV were obtained from the buffy coat or urine of patients, as described in preceding studies (2, 10).

Cell culture. MA-184 (Microbiological Associates, Bethesda, Md.) human foreskin cell monolayers were produced in tissue culture tubes. For the growth and maintenance of cultures, as well as viruses and interferon dilutions, medium 199 was supplemented with 2% newborn calf serum, 0.2% $NaHCO_3$, penicillin, streptomycin, and nystatin at 100 U, 100 μg, and 50 U per ml, respectively.

Human interferon. Human interferon was prepared by the hyperinduction procedure (9) in FS-4 cells kindly supplied by Jan Vilcek. Two batches of interferon were prepared. Their titers were 40,000 and 25,000 IU, respectively, in MA-184 cell cultures by the microassay of Armstrong (1), adapted for human interferon (11). The interferon units determined by this assay are contained in 0.1 ml. The microwells were each seeded with 20,000 MA-184 cells and used in assays 48 to 72 h later. The stan-

dard human interferon, G-023-901-527, distributed by the Research Resources Branch, National Institutes of Health, had a titer of 23,000 U in the microassay. A local standard human leukocytic interferon, with a geometric mean titer of 2,560 reference units, was used in each experiment to standardize the test titers obtained by the microassay (1, 11, 12, 17).

Inhibition of cytopathic effect (CPE). Human interferon was applied to MA-184 cultures in appropriate dilutions by adding 1 ml to each tube, which had been seeded with 200,000 cells 48 to 72 h previously. After the time intervals designated in Results, the medium with interferon was drained, and the cultures were infected with either VSV or CMV. The infectivity of the inoculum, contained in 0.1 ml, was determined by parallel titration in the same cell culture system and expressed as the mean tissue culture infective dose ($TCID_{50}$). The virus was absorbed for 15 h in cultures on a stationary rack at 37°C, and the tubes were fed with medium alone or medium containing interferon. During incubation at 37°C, medium was changed weekly. Tubes infected with CMV were examined for the characteristic CPE at least three times weekly for 6 weeks. Infection with VSV resulted in total destruction of control monolayers by 72 h; these cultures were examined daily. Monolayers were scored for CPE on a scale of 0 to 4+. Protection in interferon-treated cultures was judged as less than 1+ (25%) CPE in the cell sheet at the time when untreated controls first showed 3+ to 4+ (75 to 100%) CPE. Interferon titers in this system refer to 1 ml and represent reciprocals of highest dilutions that protected the cells. Cultures receiving the high interferon dose required for protection from CMV showed no cytotoxicity when examined in the microassay by the quantitative elution of the methylrosaniline dye (1).

CMV yield reduction. MA-184 cultures were inoculated with 0.1 ml, containing 100 $TCID_{50}$ of the Davis strain. After adsorption, the inoculum was aspirated, and each tube was washed with 10 ml of medium and fed either with medium alone (controls) or medium containing interferon. At intervals, four controls and four test tubes were removed from the incubator, frozen, and thawed, and their contents were pooled, as "controls" or "treated," and clarified by low-speed centrifugation. The infectivity of the supernatants was determined by titration in sets of four MA-184 culture tubes per 10-fold dilution and expressed as $TCID_{50}$ per 0.1 ml.

RESULTS

The effect of human interferon was examined first by inhibition of the CPE produced by the Davis strain in MA-184 cultures. The batch of interferon used in this experiment had a titer of 40,000 U by the microassay (1, 11). Since this method could not be applied to CMV, inhibition of CPE was attempted in duplicate sets of interferon-treated tubes. These were infected with VSV and CMV (Davis) for the purpose of comparison. To examine the relationship of timing of interferon treatment to infection, cultures were treated prior to, after, and before and after inoculation of virus, as detailed under Table 1. The infection due to VSV was completed (i.e., complete CPE was evident in untreated cultures) by 48 h, whereas the comparable CMV effect was evident by 28 days. The interferon titers determined in this experiment are shown in Table 1. As expected, VSV was more susceptible to the antiviral action of interferon than CMV. The relative potency of interferon in favor of VSV was greatest when treatment preceded infection. By comparison of groups 1 and 2, inhibition of VSV and CMV was 32 and 4 times greater, respectively, when interferon treatment preceded, rather than followed, infection. Since the interferon was removed from group 1 cultures at the time of challenge with CMV, it is clear that the antiviral effect persisted for several weeks. The combined treatment (group 3) was the most effective in protecting the cells from either virus. However, application of interferon after infection, which was the least sensitive method of treatment, was used in further experiments with CMV. This timing was considered to be more applicable to therapeutic attempts in established and chronic human infections.

The effect of the challenge dose of CMV on the antiviral effect of interferon was examined in two experiments. Cell cultures were inoculated with infectious virus in a volume of 0.1 ml. The inocula were determined by parallel titration. Infected cultures were incubated with twofold dilutions of interferon which, undiluted, contained 25,000 reference units per 0.1 ml. The protective dilutions are shown in Table 2. It is evident from the more complete experiment 2 that the increase in the challenge dose

TABLE 1. *Results of concurrent titrations of human interferon with VSV or CMV (Davis)*

Treatment[a]		Titer[b] measured with:		Relative[c] potency
Group	Interferon versus infection	VSV	CMV	
1	Before	4,096	128	32
2	After	128	32	4
3	Before and after	8,192	512	16

[a] Groups of human foreskin cell cultures (MA-184) were treated with interferon: 1, 18 h prior to; 2, continuously after; and 3, prior and after infection with 100 $TCID_{50}$ of VSV or CMV.

[b] Reciprocal of highest dilution of interferon inhibiting the viral CPE.

[c] Titer to VSV/CMV.

TABLE 2. *Effect of the CMV (Davis) challenge dose on the titer of human interferon*[a]

Log_{10} $TCID_{50}$	Interferon titer[b]	
	Expt 1	Expt 2
1.0	ND	256
2.0	128	128
3.0	128	64
4.0	<128	16
5.0	ND	8

[a] MA-184 cell cultures were infected with graded doses of virus. One hour later, interferon treatment was begun.

[b] Reciprocal of highest dilution protecting the cells from the viral cytopathic effect; ND, not done.

required a lower dilution (i.e., higher dose) of interferon to produce the antiviral effect. Also, the 100-$TCID_{50}$ inoculum yielded the same interferon titer of 1:128 in experiments 1 and 2, and 1:100 in a trial with a 2 log_{10} inoculum only, indicating fair reproducibility. This challenge was selected for subsequent experiments.

To confirm the antiviral effect of interferon by another method, the yields of CMV were measured in interferon-treated and untreated cultures. MA-184 cultures were infected with 100 $TCID_{50}$ of the Davis strain. Subsequently one set was fed with medium (control) and another was given 2,500 reference units of human interferon, as determined by the microassay (1, 11). This exogenous interferon had a titer of 1:16 by the CPE inhibition method with the Davis strain. Samples, consisting of four tubes from each set, were frozen, thawed, and pooled at various times during incubation. Infectious CMV content was determined by titration in MA-184 cell cultures. The results of this experiment are shown in Table 3. It is evident that the treated cultures had a reduced yield of infectious CMV, amounting to 2 log_{10} observed after 24 and 28 days of incubation. The observed antiviral effect was not due to the transfer of exogenous interferon into assay cultures, since its activity would have been effectively diluted with inocula beyond the dilution of 10^{-1}.

To examine the susceptibility of six clinical isolates of CMV to human interferon, stock virus suspensions were made by passing the original suspension of each isolate once in MA-184 cell cultures. When the cultures showed evidence of CPE, the cells were disrupted by a freeze-thaw cycle, and the supernatants were collected after low-speed centrifugation. The inoculum of each isolate was adjusted so that it contained 100 $TCID_{50}$. After infection of MA-184 monolayers, twofold dilutions of interferon were added to the culture tubes. The undiluted preparation contained 25,000 U/0.1 ml. The cultures were observed for 6 weeks for CPE. The results are shown in Table 4. It is evident that all CMV isolates were inhibited in a comparable way. The interferon susceptibility of these clinical isolates appears to exceed by two- to fourfold that of the Davis strain, since the latter yielded a titer of 128 in a parallel titration (experiment 1 in Table 2). Thus, CMV isolates originating from two different hospitals and six individual patients showed little variation in their susceptibility to the antiviral effect of interferon.

DISCUSSION

Human CMV enjoyed, until recently, the reputation of nonsusceptibility to the antiviral action of interferon. Glasgow and co-workers found that CPE due to CMV in human foreskin tissue was somewhat retarded by 120 U of interferon, in contrast to the complete protection achieved against VSV and Sindbis virus (8). In the same study, viruria in a chronic human carrier of CMV was not altered by the low circulating levels of interferon induced by live measles vaccine. Lang et al. demonstrated an interferon-mediated protection of human em-

TABLE 3. *Reduction of the yield of CMV (Davis) in interferon-treated human cell culture*

Interferon	Log_{10} $TCID_{50}$ of CMV on day[a]				
	4	16	20	24	28
0 (Control)	0[b]	0	1.0	3.0	2.0
2,500 U	0	0	0	1.0	0

[a] Yield per 0.1 ml of fluid from MA-184 cultures infected with 100 $TCID_{50}$ of virus on day 1. The inoculum was washed off before treatment with interferon.

[b] 0 relates to <1 $TCID_{50}$ per 0.1 ml.

TABLE 4. *Susceptibility of human CMV isolates to interferon*

Isolate		Patient		Interferon[b]
No.	Site	Group	Condition[a]	
1	Buffy coat	Adult	RT	512
2	Urine	Adult	RT	256
3	Urine	Adult	RT	512
4	Urine	Adult	RT	512
5	Urine	Child	OH	256
6	Urine	Child	OH	512

[a] RT, After renal transplant; OH, after open heart surgery.

[b] MA-184 cell cultures were incubated with interferon 1 h postinfection with 100 times the $TCID_{50}$ of the listed CMV suspensions. The reciprocal of the highest inhibitory dilution is shown.

bryonic fibroblasts against challenge with extracellular, but not intracellularly contained, CMV (14). These observations were extended by A. R. Holmes, L. Rasmussen, and T. C. Merigan (Fed. Proc., p. 624, 1976) who used potent human leukocytic interferon. An inoculum of CMV-infected cells required up to 300-fold increment in the interferon dose required for protection, compared with extracellular virus. With either inoculum, the increase in the infectious dose was associated with decreases in susceptibility to interferon. The most sensitive measurements of the antiviral action of interferon were decreases in the yield of infectious CMV and the intracellular inclusion formation. The production of CPE was somewhat less sensitive.

More recently, human leukocytic interferon was administered in massive and repeated doses of $\geq 10^5$ U daily to several patients with congenital or acquired CMV infections (3, 6). The responses varied. In some patients, viruria was completely inhibited, and in others, the effect was transient or not evident. The interferon susceptibilities of the particular clinical CMV isolates were not determined.

The present study addressed the relative susceptibility of clinical CMV isolates to interferon. The susceptibility of the Davis strain was first demonstrated by the inhibition of CPE in human foreskin cells and in the same assay system, to that observed with VSV, a sensitive indicator. Depending on the timings of the interferon treatment of cells and virus challenge, VSV was 4 to 32 times more susceptible (Table 1).

Data contained in Table 1 indicate also that the titration of interferon by CPE inhibition is less sensitive than the microassay. This comparison could be made with the (prophylactic) treatment group 1, challenged with VSV, which is also used in the microassay. The relationship between the microassay titer of 40,000 and that of 4,096 by the CPE assay in tubes suggests a tenfold lower sensitivity for the latter, assuming that the different volumes, 0.1 and 1.0 ml, as well as the obvious difference in the number of cells in the respective assays, allow a direct comparison. The CPE inhibition method for estimating interferon effect could be applied to both CMV and VSV, and it was therefore useful, despite the loss in sensitivity.

The method of CPE inhibition was found to depend on the dose of extracellular virus, which was the only type of challenge used in the current study. This finding is in keeping with those by Holmes and co-workers (above). Likewise, the inhibitory effect of interferon on the yield of infectious CMV was confirmed.

For the testing of clinical isolates, the interferon-mediated inhibition of CPE was selected because of relative ease and shorter time required for performance. Based on three separate experiments, in which the same interferon was titrated with 100 $TCID_{50}$ of CMV, the method was judged to be reproducible. Clinical CMV isolates were propagated only once after original isolation, so as to obviate the possibility of a change in interferon susceptibility. In parallel assays, all were found to be at least twice as susceptible to interferon as the Davis strain. By extrapolation from findings in Table 1, the clinical isolates were only 2 to 16 times less susceptible than VSV. It is far more difficult to predict whether these findings correlate with effectiveness in vivo. Arvin and associates found that doses producing a serum interferon level of 400 did not decrease the amount of CMV in the urine of a patient, whereas in others, lower levels did (3). In interpreting the finding relative to serum levels of interferon, one should bear in mind that the units refer to a sensitive assay with VSV. A decrease in interferon effectiveness can be expected when one considers CMV instead of the sensitive indicator (Table 1). Also, due to the intracellular character of virus infection, the serum level of a drug may not appropriately monitor its therapeutic efficacy.

In conclusion, six clinical isolates of human CMV were found to be susceptible to interferon when tested in the laboratory. This encourages further trials with interferon in patients with CMV infections, since the efficacy of treatment cannot be assured by laboratory results alone.

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