Response of Cloned Progeny of Clinical Isolates of Herpes Simplex Virus to Human Leukocyte Interferon

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First- and third-generation cloned progeny viruses were derived from clinical isolates of herpes simplex virus and examined for their sensitivities to human interferon by inhibition of plaque formation in Vero cells. The dose-response curves obtained with the first- and third-generation clones were similar to those obtained with the parental isolates, and both the parent and the clones showed similar sensitivities to interferon. These results suggest that clinical isolates of herpes simplex virus consist of a homogeneous population of virus particles with respect to interferon sensitivity. The dose-response curves obtained with herpes simplex virus demonstrated a shallower slope than those obtained with vesicular stomatitis virus. Vesicular stomatitis virus plaque formation was completely inhibited at high concentrations of interferon, whereas complete inhibition of plaque formation by herpes simplex virus did not occur at the highest concentration of interferon used. Cloned progeny were derived from plaques appearing in the presence of high concentrations of interferon. The dose-response curves and interferon sensitivities of these clones were similar to those of the parent and third-generation clone from which they were derived. There was no evidence for genetic heterogeneity with respect to interferon sensitivity.

The effectiveness of human interferon as an antiviral agent is currently being evaluated in several human infections, including infections with herpes simplex virus (HSV) (12). The herpesvirus group has been generally considered to be relatively resistant to the action of interferon, but it has been shown that HSV is in fact more sensitive than vaccinia virus to mouse interferon (8). Preliminary attempts in this laboratory to measure the sensitivity of clinical HSV isolates to human leukocyte interferon by a semi-micro, cytopathic effect reduction method (1) gave extremely variable results. In extreme cases, repeated assays of the same isolate gave results spanning three orders of magnitude. We therefore investigated other procedures, among them plaque reduction with the Vero line of African green monkey kidney cells. These cells have the advantage of being sensitive to human interferon but not producing interferon in response to viral infection (2). As will be shown, this assay was sufficiently reproducible to be useful but, in addition, presented an interesting dose-response problem which is the subject of this contribution. It is well known that the dose-response slopes observed when the plaque reduction system is used tend to be more shallow than the slope of the theoretical one-hit model, and Gif-

ford and Koch (3) presented several hypotheses to account for this. Ito and Montagnier (7) have presented evidence recently that, for uncloned vesicular stomatitis virus (VSV), a shallow dose-response slope was due to heterogeneity of the virion population with respect to interferon sensitivity. Cloned populations showed steeper slopes. In our hands, HSV showed an extremely shallow dose-response slope in plaque reduction assays on Vero cells with human leukocyte interferon. In all assays, there appeared to be a residual fraction of the HSV not inhibited by high concentrations of interferon. We further show that this pattern was phenotypic and not due to genetic heterogeneity of the virus populations.

MATERIALS AND METHODS

Viruses. Clinical isolates of HSV were obtained from throat washings of patients participating in a clinical trial (12) and were determined to be HSV type 1 by the method of differential plaque formation on primary chicken embryo cell cultures as previously described (10). Isolates were passaged twice in secondary rabbit kidney cell cultures (RK₂). The second-passage titers were between 10⁵ and 10⁶ plaque-forming units (PFU) per 0.1 ml on Vero cells. These second-passage progeny served as parents for clones.

Three generations of single-plaque clones were derived from each of four clinical isolates and kindly provided by Lucille O. Guevarra. Parental strains (no.

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544, 576, 732, and 751) were serially diluted and plaqued on RK₂ under an agar overlay medium containing 200 μ g of diethylaminoethyl dextran per ml. Four plaques (first-generation clones) were picked at random from the terminal dilutions and suspended in 1.0 ml of minimum essential medium (MEM). This procedure was repeated for each first- and second-generation clone. A total of 4 first-generation, 16 second-generation, and 64 third-generation progeny viruses were derived from each clinical isolate. Four third-generation clones, one directly descended from different first-generation clones, were selected for study. These, along with the first-generation clones from each parent, were passaged one time in RK₂, yielding titers of between 10^6 and 10^7 PFU/0.1 ml when assayed on Vero cells. First-generation clones were given identification numbers 1, 2, 3, and 4. The third-generation clones were identified by three-digit numbers (e.g., 111), the first two digits indicating the first- and second-generation clones from which it was derived.

Another clinical isolate (S₂517) was passaged twice in RK₂ and had a titer of 3×10^6 PFU/0.1 ml in Vero cells. VSV, Indiana strain, was propagated in chicken embryo cell cultures as previously described (1) and had a titer of 7×10^6 PFU/0.1 ml in Vero cells. HSV (S₂517) and VSV served as reference strains in the interferon assays.

Cell cultures. RK₂ cultures were prepared as previously described (1). Vero cells were propagated as monolayers in 32-ounce (ca. 0.946-liter) glass bottles in MEM supplemented with 10% heat-inactivated fetal calf serum and 0.08% NaHCO₃. Cells were detached from the surface of the bottle with trypsin and suspended at a concentration of 3×10^5 to 4×10^5 cells per ml in MEM containing 0.2% NaHCO₃. Two milliliters of the suspension was dispensed into each well of a six-well multidish plate (Linbro, Hamden, Conn.). Confluent monolayers were obtained after overnight incubation at 37°C in a CO₂ incubator and used for virus and interferon assays.

Interferon. Human leukocyte interferon (lot M76; specific activity, 0.5×10^6 U/mg of protein; obtained from Kari Cantell through the Antiviral Substances Program of the National Institute of Allergy and Infectious Diseases) was titrated against VSV in locally derived human embryonic fibroblast cells and contained 5×10^6 U/0.1 ml. The interferon was diluted 10-fold in MEM without NaHCO₃ and divided into 3-ml samples. The samples were frozen at -60°C.

Interferon assay. Interferon was thawed in a 37°C water bath and then diluted 10-fold in MEM supplemented with 10% fetal calf serum and containing 0.2% NaHCO₃ to obtain a 10^{-2} dilution of the original preparation. Serial fourfold dilutions were made to a final dilution of $10^{-6.5}$. The medium was removed from Vero cell culture monolayers, and 1 ml of each dilution of interferon was added to duplicate wells. One milliliter of MEM was added to duplicate wells to serve as a virus control. After incubation overnight at 37°C in a CO₂ incubator, interferon was removed and cells were inoculated with 0.1 ml of MEM containing approximately 100 PFU of the test virus. After adsorption for 1 h, cells were overlaid with MEM containing 2% fetal calf serum and 0.8% gum tragacanth (ICN Pharmaceu-

ticals, Inc., Cleveland, Ohio). Cultures were incubated at 37°C in a CO₂ incubator to allow for plaque development, 4 to 5 days for HSV and 1 day for VSV. The overlay was removed, and cultures were fixed and stained with gentian violet (1) to allow visualization of plaques.

Each parental isolate and its first- and third-generation clones were assayed together, and HSV (S₂517) and VSV were included as reference strains in each assay. Data were fed into a PL/PROPHET computer, and plot graphs were produced on a Tektronix plotter (Bolt Beranek and Newman Inc., Cambridge, Mass.). Interferon titers were interpolated from the graphs as the log₁₀ of the reciprocal of the dilution which reduced PFU by 50%.

RESULTS

Relative interferon sensitivities of HSV (S₂517) and VSV. Ten assays were performed, each of which included HSV (S₂517) and VSV as reference viruses. Table 1 lists the interferon titers in these assays. The interferon titers obtained with VSV as the challenge virus ranged between 5.6 and 6.3, with a mean of 5.87 (± 0.08 standard error). Considerably more variation appeared with HSV (S₂517) as the challenge virus. Interferon titers ranged from 4.1 to 6.5, with a mean of 5.33 (± 0.25 standard error).

There are variations in the responses of different viruses to the antiviral effects of interferon; VSV is generally considered to be of intermediate sensitivity, and HSV is considered to be among the least sensitive of viruses. However, our results indicated that the sensitivity of clinical isolates of HSV approaches that of VSV. In 6 of the 10 assays, the sensitivity of HSV (S₂517) to human leukocyte interferon was approximately equal to that of VSV, whereas the sensitivity was less in 4 of the 10 assays. Moreover, there is no statistical difference between the titers as determined by these two viruses ($P > 0.05$; t test for paired comparison).

Dose-response. Each HSV strain and the

TABLE 1. *Interferon titers with VSV and HSV (S₂517)*

Expt no.	Interferon titer (log ₁₀) ^a		Ratio of titers (log ₁₀)
	VSV	HSV (S ₂ 517)	
1	5.8	4.1	1.7
2	5.9	5.2	0.7
3	5.7	4.5	1.2
4	6.1	4.3	1.8
5	5.6	5.5	0.1
6	5.6	5.8	-0.2
7	6.2	5.9	0.3
8	5.8	5.8	0.0
9	5.7	5.7	0.0
10	6.3	6.5	-0.2

eight clones derived from it were assayed at least twice. The results of a typical assay are shown in Fig. 1 for the parental strain (no. 576) and its first-generation (Fig. 1A) and third-generation (Fig. 1B) clones. Included in the graph is a dose-response curve for the VSV strain used as a reference. Dose-response curves obtained with the HSV parental isolate and the first- and the third-generation clones were almost log linear at the low concentrations of interferon (i.e., at $\log_{10}$ interferon dilutions of ≥ 5.0). However, at the high concentrations of interferon, the curve flattened out. Complete inhibition of plaque formation by HSV did not occur at the highest concentration of interferon used in the assay (10^{-2} dilution). VSV plaque formation was completely inhibited at the highest concentration of interferon. The curve in Fig. 1 is not typical in that complete inhibition of VSV was usually seen at all interferon dilutions of less than $10^{-4.4}$. More typical is the curve shown in Fig. 2. The HSV dose-response curves demonstrated a shall-

lower slope than that obtained with VSV. The mean slope ± 1 standard deviation was $25 \pm 11\%$ per log dilution for five repetitions of the S₂517 strain of HSV. Slopes were measured in the steepest, almost-linear portion of the curve between 30 and 80% of the control plaque number. For VSV, the slopes were measured between 20 and 80%, which was possible because of the lack of a resistant fraction, and the mean of five slopes was $34 \pm 5\%$ per log dilution.

The first- and third-generation clones produced dose-response curves similar to that of the parental isolate (Fig. 1). Dose-response curves for the other parental isolates (nos. 544, 732, and 751) and their respective first- and third-generation clones were similar to those obtained with clinical isolate 576 (data not shown). Both the parental isolate 576 and its clones showed similar interferon sensitivities (Table 2). The interferon titers of the other

FIG. 1. Interferon dose-response curves for parental isolate 576, first- and third-generation clones, and VSV. (A) Symbols: +, parental isolate 576; *, 576:1; □, 576:2; ○, 576:3; △, 576:4; ◇, VSV. (B) Symbols: +, parental isolate 576; *, 576:142; □, 576:232; ○, 576:332; △, 576:432; ◇, VSV.

FIG. 2. Interferon dose-response curves for parental isolate 576, a third-generation clone, three persistent clones, and VSV. Symbols: +, parental isolate 576; *, third-generation clone 576:232; □, persistent clone 576:1421; ○, persistent clone 576:1422; △, persistent clone 576:1423; ◇, VSV.

TABLE 2. Interferon titers for parental isolate 576 and first- and third-generation clones

Virus designation	Interferon titer (log ₁₀)
Parent (no. 576)	6.3
First-generation clones	
1	6.3
2	6.2
3	6.1
4	6.3
Third-generation clones	
142	6.0
232	6.1
332	6.1
432	5.6

parental isolates and the range of titers of their first- and third-generation clones are shown in Table 3. These results, therefore, suggest that clinical isolates of HSV consist of a homogeneous population of virus particles with respect to interferon sensitivity.

Persistent fraction. As mentioned above, complete inhibition of HSV plaque formation was not obtained in any of the assays of parental isolates and their clones. An experiment was designed to determine whether the observed persistent fraction was due to genetically resistant virus. Vero cells were pretreated with interferon as described above. After removal of interferon, cells were inoculated with 0.1 ml of MEM containing approximately 100 PFU of virus from the third-generation clone (no. 142) derived from parental isolate 576. After adsorption for 1 h, cells were overlaid with a mixture containing equal volumes of twice-concentrated MEM and agar containing 200 μ g of diethylaminoethyl dextran per ml. Cells were incubated at 37°C in a CO₂ incubator for 5 days and then stained with neutral red. Three plaques were observed at the 10⁻² dilution of interferon. These plaques were picked and suspended in 0.5 ml of MEM. Plaques were passaged once in RK₂, yielding titers of between 4 × 10⁶ and 5 × 10⁶ PFU/0.1 ml when assayed on Vero cells.

The cloned progeny were assayed for interferon sensitivity along with the parental isolate, a third-generation clone, and VSV (Fig. 2). (Because the supply of the third-generation clone [no. 142] from which the persistent clones were derived was exhausted, for purposes of comparison we chose to assay another third-generation clone [no. 232], which had a dose-response curve [Fig. 1B] and an interferon sensitivity [Table 2] similar to those of 142.) The cloned progeny

derived from plaques resistant to interferon (i.e., plaques observed in the presence of the highest concentration of interferon) have dose-response curves and interferon sensitivities similar to those of the parent and third-generation clone. The plaques picked from the persistent fraction, therefore, do not appear to be genetically resistant to interferon.

DISCUSSION

Previous reports in the literature have ranked members of the herpesvirus group as relatively resistant to interferon (4, 6), although only a few studies have compared directly the sensitivity of HSV with that of VSV (8, 9, 13). In the present study, the mean interferon titer of HSV (S₂₅₁₇) was 0.54 log₁₀ lower and not significantly different from that obtained for VSV. HSV was as sensitive to interferon as was VSV when assayed by plaque reduction in Vero cell cultures pretreated with human leukocyte interferon. However, in comparison with VSV, HSV isolates showed, in the presence of interferon, a shallow dose-response curve. One explanation might be that the isolates may consist of a heterogeneous population of viruses differing in their sensitivities to interferon. Ito and Montagnier (7) described an uncloned isolate of VSV which consisted of viruses having different sensitivities to interferon. Each clone derived from the parental isolate yielded a dose-response curve with a steep slope and a characteristic interferon sensitivity. The uncloned isolate gave a shallow dose-response curve and an intermediate interferon sensitivity when compared with the clones. These results are entirely in accord with the theoretical model developed by Gifford and Koch (3). However, when four isolates of HSV were tested, we observed no such heterogeneity. Four first- and four third-generation clones derived from each isolate produced dose-response curves similar to the parent, all of which were shallow, and all clones had the same interferon sensitivities as did the parental strains.

Complete inhibition of HSV plaque formation did not occur. In all experiments, plaque formation occurred in the cells treated with the highest concentration of interferon. Although we detected no heterogeneity in the initial inoculum, the persistent fraction might be more resistant to interferon. The presence of interferon or interferon-induced antiviral protein may have selected for interferon-resistant variants. There is little support for this hypothesis in the literature. Takemoto and Baron (14) have reported the occurrence of an apparently interferon-resistant fraction (10 to 15% of virus control) with encephalomyocarditis virus while attempting to select

TABLE 3. Range of interferon titers for parental isolates and first- and third-generation clones

Parental isolate no.	Expt no.	Interferon titer (log ₁₀) of:		
		Parent	First generation	Third generation
544	1	ND ^a	4.8-5.7 ^b	5.3-6.0 ^b
	2	5.5	5.2-5.8	4.9-5.5
576	1	6.3	6.1-6.3	5.6-6.1
	2	ND	3.3-4.2	3.2-4.2
732	1	4.9	4.7-5.8	4.8-5.2
	2	5.3	4.5-5.5	5.1-5.4
751	1	5.0	4.2-5.0	3.9-4.7
	2	5.8	5.2-5.8	5.0-5.4

^a ND, Not done.

^b Range of titers of the four clones of designated generation.

interferon-resistant mutants. After four passages in the presence of interferon, the progeny virus had the same interferon sensitivity as did the parental stock. Metz and Douglas (11) failed to produce interferon-resistant mutants of encephalomyocarditis virus despite treatment of virus with mutagens and repeated passage in the presence of interferon. To prove that the persistent tail of the dose-response curve which we observed with HSV does not consist of interferon-resistant mutants, plaques which arose in the cells treated with the highest concentration of interferon were picked and their sensitivities were tested. Clones derived from the persistent plaques showed the same interferon sensitivity as did the parental isolate and third-generation clone from which they were derived, and the dose-response curves were also similar.

The appearance of the persistent fraction of virus may be due to differences in the cellular response to the action of interferon. Gifford and Koch (3) suggested that a cell type with marked resistance to interferon in a population or a cell type with a particularly large burst size would result in the observation of an apparent interferon-resistant fraction. In our case, it is unlikely that a fraction of the cell population is resistant to interferon because, in parallel assays, VSV was completely inhibited at the higher concentrations of interferon. Another possibility is that HSV virions are phenotypically variable in their susceptibilities to interferon. This hypothesis could not be directly tested in our system. It is more likely that the persistent fraction is due to a virus-cell interaction which allows plaque formation in treated cells. Gifford and Koch (3) have postulated that a neighborhood of n unprotected cells is needed for a plaque to form. A long period of time before the release of virus may allow such a neighborhood of n unprotected cells to develop, and plaque formation could occur. Rasmussen and Farley (13) have shown that approximately 90% of antiviral activity for HSV in human fibroblasts treated with fibroblast interferon is lost after 1 day, whereas almost none is lost for VSV. Hallum et al. (5) had previously shown that, in chicken embryo cells, interferon-induced resistance to Newcastle disease virus decayed more rapidly than did resistance to VSV. These observations could explain why plaques formed with HSV and not with VSV at the highest concentration of interferon. The above theories may explain how a virus-cell

interaction could allow HSV plaque formation in an interferon-treated cell culture without apparent genetic heterogeneity of either virus or cell. The shallow slope and persistent resistance of HSV may be important should interferon, even in large doses, fail to eradicate a persistent or latent infection in humans.

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