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# Rapid Determination of Human Cytomegalovirus Susceptibility to Ganciclovir Directly from Clinical Specimen Primocultures

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**We were able to rapidly determine the susceptibility of human cytomegalovirus to ganciclovir using a late antigen synthesis reduction assay directly on primocultures of clinical specimens. This test was compared with a conventional susceptibility assay which was performed with cell-free virus obtained after cytomegalovirus isolation and in vitro passages. Both tests produced similar results. The rapid test, unlike conventional assays, is able to provide a result within 5 days after receipt of the specimen and could thus play a direct role in the therapeutic decision.**

Cytomegalovirus (CMV) resistance to ganciclovir (GCV) has been described in the clinical setting (6), and about 8% of patients with AIDS who take this drug for more than 3 months excrete GCV-resistant virus (5). The existing CMV susceptibility determination assays—plaque reduction assay (2), DNA hybridization assay (4), late antigen synthesis reduction assay (10), yield reduction assay (13), or in situ enzyme-linked immunosorbent assay (16)—require the isolation of CMV from clinical specimens. The isolate must then be passaged several times in cell culture to increase the virus titer and/or to obtain cell-free virus before testing. For this reason, these work-intensive conventional methods may select CMV strains with modified susceptibilities to GCV (5), and their results are generally not available before 6 weeks. Since GCV-resistant CMV may be susceptible to foscarnet (9), it is necessary to develop rapid CMV susceptibility assays since their results could play a major role in the therapeutic decision.

Susceptibility testing performed directly on primocultures of clinical specimens after a single replication cycle of the virus might provide a rapid result and a susceptibility determination of the overall CMV population present in vivo, without the drawback of the possible in vitro selection by passages. The objective of this study was to evaluate the feasibility of this direct approach by using a late antigen reduction assay (10) and to compare the results of the assay with those of a conventional method performed on cell-free virus after CMV isolation and in vitro passages.

## MATERIALS AND METHODS

**Clinical specimens.** Ten clinical specimens (three urine, one amniotic fluid, and six bronchoalveolar lavage fluid specimens) were studied. Eight samples were obtained from eight GCV-naive patients, and two were collected from two previously GCV-naive patients after 6 days of effective GCV therapy. They were consecutively sent to our laboratory for CMV detection and fulfilled the following criteria: (i) they were CMV positive as determined by a rapid 24-well plate culture method (11) and (ii) they contained at least 1 immediate-early antigen (IEA) infectious unit (IU; as defined below) per μl.

**Control strains.** The laboratory strain AD169 was used as a susceptible control strain, and a resistant mutant of the AD169 strain, designated RCL1, was selected as described by Biron et al. (1) by serially passaging the virus in the presence of increasing concentrations of GCV. Strain RCL1 was cloned by limiting dilution in 80 μM GCV, and after multiplication of the strain in the absence of the drug, the supernatant containing cell-free virus was stored (−80°C) until use.

The susceptibilities of the GCV-susceptible AD169 strain (mean 50 percent inhibitory dose [$ID_{50}$] = 5.3 μM; mean 90 percent inhibitory dose [$ID_{90}$] = 14.7 μM) and the GCV-resistant RCL1 strain ($ID_{50}$ = 58.9 μM; $ID_{90}$ = 170.9 μM) were verified in a plaque reduction assay (14).

In the present study, RCL1 was used as a resistant control strain in three late antigen synthesis reduction assays.

**Rapid direct susceptibility testing.** Samples were filtered (pore size, 0.45 μm) and divided into two fractions. One fraction was immediately used in a rapid titration assay. The second one was stored at 4°C for 18 h and was used in the susceptibility assay performed with a standardized virus input.

**(i) Rapid 18-h titration assay.** Samples were diluted fourfold (from pure to $4^6$) in BME (Whittaker). Each virus dilution (300 μl per well) was centrifuged (2,000 × *g* for 45 min) onto a 24-well plate containing MRC-5 cells (Bio-Mérieux, Lyon, France) grown to confluency (the culture medium was BME containing 10% fetal calf serum). Immunoperoxidase staining was performed 18 h postinfection by a standard method (11) with the E-13 antibody (Clonatec, Paris, France). IEA-expressing cells, designated as IEA IU, in each well were counted with an inverted microscope.

**(ii) Rapid 92-h susceptibility testing.** A stock solution of GCV (Syntex, Paris, France) was prepared in distilled water at a concentration of 20 mM, validated by a high-performance liquid chromatography assay (15), and stored at −80°C until use.

The sample fraction stored for 18 h at 4°C was diluted to 1 IEA IU per μl in BME, according to the results of the titration assay. The sample dilution was distributed (300 μl per well; multiplicity of infection, 2 IEA IU per $10^3$ cells) into each well of a 24-well plate containing confluent MRC-5 cells. Since the same viral inoculum provides two- to fourfold less late antigen-expressing cells than IEA IU (10a), the viral input used in the susceptibility test corresponded to 75 to 150 late antigen-expressing cells per well.

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For each sample, a cell-free dilution of the AD169 strain (300 IEA IU per well) was used as a susceptible control in a parallel plate.

The plates were centrifuged (2,000 × *g* for 45 min). The wells were washed twice with BME; and 1 ml of each GCV dilution per well, which was prepared in the culture medium at final concentrations of 0, 1.2, 3.7, 11.1, 33.3, and 100 μM, was added. Each drug dilution was tested in quadruplicate. The plates were incubated for 92 h at 37°C in 5% $CO_2$. The immunoperoxidase staining was performed as described previously (11); but the NEA-9220 antibody (DuPont, Billerica, Mass.) (2 μl/ml in phosphate-buffered saline), a monoclonal antibody against a late antigen of CMV, was used as the anti-CMV antibody. Brown-stained late antigen-expressing cells were counted in each well. Late antigen-expressing cell reduction was calculated relative to that in the drug-free wells. The $ID_{50}$s and $ID_{90}$s were calculated by linear regression analysis.

For each clinical sample, these values were compared with the $ID_{50}$s and $ID_{90}$s of the AD169 strain determined simultaneously. Sensitivity indices ($SI_{50}$ or $SI_{90}$) were defined as the $ID_{50}$ (or the $ID_{90}$) for the isolate divided by the $ID_{50}$ (or the $ID_{90}$) for the AD169 strain, which was tested simultaneously.

**Conventional susceptibility testing on cell-free virus.** The conventional susceptibility test, which was adapted from the test described by Mazeron et al. (10), was performed with each clinical specimen for comparison with the rapid direct susceptibility assay. Specimens were processed by a conventional cell culture method for the isolation of CMV. The isolates were passaged five to eight times to reach about 90% cytopathic effect. The filtered (pore size, 0.45 μm) supernatants containing cell-free virus were used.

Titration and susceptibility assays were performed as described above for the rapid test. For each susceptibility test, the susceptibility of strain AD169 was determined concomitantly. The $ID_{50}$s, $ID_{90}$s, $SI_{50}$s, and $SI_{90}$s were calculated as described above.

Three assays were performed with the RCL1 strain by the same procedure described above, including a control AD169 assay.

## RESULTS AND DISCUSSION

**Control strains. (i) AD169 strain.** Sixteen susceptibility tests were performed with strain AD169 as the control for the rapid and conventional assays. The mean ± standard deviation $ID_{50}$ was 6.5 ± 2.2 μM (range, 3.9 to 11.2 μM). The mean $ID_{90}$ was 36.6 ± 10.5 μM (range, 23.6 to 59.8 μM). These values were in agreement with those obtained in previous studies (7, 8) in which a plaque reduction assay with MRC-5 cells and cell-free strain AD169 was used.

Significant interassay variations in the $ID_{50}$s or $ID_{90}$s were observed. This phenomenon has been described by other investigators. AD169 $ID_{50}$s ranged from 1.6 μM (7) to 7 ± 3 μM (8) in the same assay system. These variations might reflect the heterogeneity of GCV phosphorylation by CMV-infected MRC-5 cells according to the MRC-5 batch or the number of passages.

**(ii) RCL1 strain.** The results obtained with the RCL1 and AD169 strains tested simultaneously are presented in Table 1, and an example of a regression curve is shown in Fig. 1. The $ID_{50}$ for RCL1 was about fivefold higher than the $ID_{50}$ for AD169. The $ID_{90}$ for RCL1 (four- to sevenfold greater than the $ID_{90}$ for AD169) was less reproducible. In these tests, the $SI_{50}$ for RCL1 was nearly constant, suggesting that this $SI_{50}$ is less dependent on the cell or technical conditions than is the absolute $ID_{50}$.

TABLE 1. Susceptibility of human CMV strains AD169 and RCL1 to GCV determined by results of three late antigen synthesis reduction assays[a]

| AD169 | | RCL1 | | | |
|---|---|---|---|---|---|
| $ID_{50}$ (μM) | $ID_{90}$ (μM) | $ID_{50}$ (μM) | $SI_{50}$[b] | $ID_{90}$ (μM) | $SI_{90}$[b] |
| 5.7 | 34.6 | 25.8 | 4.5 | 251.8 | 7.3 |
| 8.6 | 41.2 | 37.7 | 4.4 | 230.0 | 5.6 |
| 5.6 | 37.4 | 31.3 | 5.6 | 162.2 | 4.3 |

[a] The strains were tested simultaneously.
[b] $SI_{50}$ (or $SI_{90}$) = $ID_{50}$ (or $ID_{90}$) for RCL1/$ID_{50}$ (or $ID_{90}$) for strain AD169 tested simultaneously.

**Clinical isolates.** Ten clinical specimens were studied. Susceptibility tests were performed directly on a primoculture of the specimen (rapid test) and after isolation and in vitro passages (conventional test). Each test included a control AD169 susceptibility assay.

The $ID_{50}$s, $ID_{90}$s, $SI_{50}$s, and $SI_{90}$s observed with both tests are presented in Table 2. The mean ± standard deviation $ID_{50}$ obtained by the rapid assay was 5.1 ± 3.1 μM (range, 1.9 to 10.8 μM), which was similar to that observed in the conventional test (mean, 6.3 ± 3.0 μM; range, 2.9 to 10.8 μM). These values were very close to the $ID_{50}$s for AD169 (mean, 6.5 ± 2.2 μM; range, 3.9 to 11.2 μM). The range was in agreement with the results of previous studies performed with wild-type susceptible strains, which demonstrated $ID_{50}$s ranging from 0.65 to 6.3 μM (3) and 0.4 to 11 μM (12), i.e., more than a 10-fold variation.

For the rapid assay, the mean $ID_{90}$ was 30.7 ± 15.0 μM (range, 17.7 to 58.2 μM), which was slightly lower than that obtained in the conventional assay (mean, 46.3 ± 13.5 μM; range, 28.6 to 69.8 μM), but the number of tested samples was inadequate to validate a statistically significant difference.

To compare the results of rapid versus conventional assays for each sample, the $SI_{50}$ was chosen as the parameter which is less susceptible to variation according to the cell or technical conditions. The $SI_{50}$s were similar in nine of nine samples in both tests; for six isolates the $SI_{50}$ was <1,

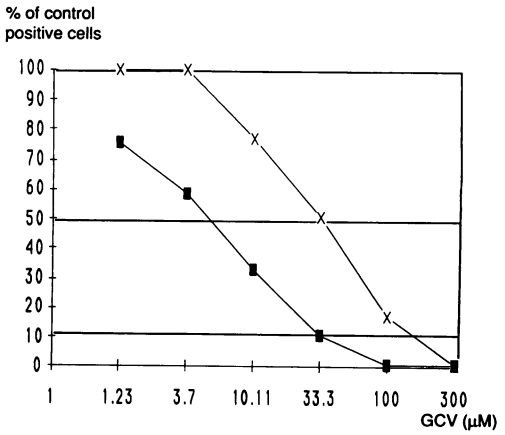


FIG. 1. Susceptibility of human laboratory CMV strain RCL1 (×) and susceptible control strain AD169 (■) to GCV. The strains were tested simultaneously and were assessed by late antigen immunoperoxidase staining.

TABLE 2. Susceptibility of human CMV clinical isolates to GCV[a]

| Sample no. | Sample[b] | Rapid test | | Conventional test | | Rapid test | | Conventional test | |
|---|---|---|---|---|---|---|---|---|---|
| | | $ID_{50}$ (μM) | $SI_{50}$[c] | $ID_{50}$ (μM) | $SI_{50}$ | $ID_{90}$ (μM) | $SI_{90}$ | $ID_{90}$ (μM) | $SI_{90}$ |
| 1 | BAL | 1.9 | 0.3 | 4.2 | [0.5] | 19.1 | [0.6] | 34.1 | [0.8] |
| 2 | BAL | 1.9 | | Contaminated | Contaminated | 16.4 | | Contaminated | Contaminated |
| 3 | U | 2.1 | 0.3 | 6.6 | [0.6] | 20.9 | [0.8] | 57.8 | [0.9] |
| 4 | BAL | 2.9 | 0.6 | 2.9 | [0.7] | 17.7 | [0.5] | 41.3 | [0.9] |
| 5 | A | 3.9 | 0.4 | 2.9 | [0.4] | 20.1 | [0.3] | 31.0 | [0.9] |
| 6 | BAL | 5.1 | 1.0 | 8.9 | [1.8] | 35.6 | [1.1] | 28.6 | [1.2] |
| 7 | BAL | 6.2 | 0.8 | 3.3 | [0.8] | 42.0 | [1.5] | 69.8 | [1.5] |
| 8 | BAL | 6.5 | 1.2 | 10.5 | [2.7] | 22.3 | [0.7] | 60.1 | [1.3] |
| 9 | BAL | 9.8 | 1.2 | 10.8 | [2.2] | 58.2 | [2.1] | 52.0 | [2.2] |
| 10 | U | 10.8 | 0.9 | 7.0 | [0.7] | 54.4 | [0.9] | 41.7 | [0.7] |

[a] Susceptibility was determined by comparison between the test performed on primoculture of the clinical sample (rapid test) and the test performed on cell-free virus after CMV isolation (conventional test).
[b] BAL, bronchoalveolar lavage fluid; U, urine; a, amniotic fluid.
[c] $SI_{50}$ (or $SI_{90}$) = $ID_{50}$ (or $ID_{90}$) for the isolate/$ID_{50}$ (or $ID_{90}$) for the AD169 strain tested simultaneously.

and for three isolates the $SI_{50}$ was ≥1 in both tests. Interestingly, even with a slight difference, for eight of nine samples the $SI_{50}$ in the conventional assay was greater than or equal to the $SI_{50}$ in the rapid test. The possible presence of the drug in the clinical specimens could not explain this difference, since only one of these samples (sample 3) was obtained from a patient treated with GCV. Furthermore, the residual drug possibly present in the sample was eliminated by washings after centrifugation. This difference could also indicate that the passages that were performed in the conventional test favored the in vitro selection of CMV variants against which GCV was slightly less active.

Another observation concerns two CMV isolates (isolates 8 and 9) for which the $ID_{50}$s were 10.5 and 10.8 μM, respectively, in the conventional assay. These isolates, for which the $ID_{50}$s were more than twofold greater than those for AD169 ($SI_{50}$s, 2.7 and 2.2, respectively) might be considered resistant to GCV in vitro. Since the isolates were recovered from two GCV-naive patients who were successfully treated with GCV after sample collection, they were, in fact, susceptible to GCV from a clinical standpoint. This raises the problem of the definition of in vitro resistance of CMV to GCV. Since the absolute $ID_{50}$ for an isolate may vary according to the cell or technical conditions, it is difficult, even for a consensus standardized test, to set an absolute threshold value for in vitro resistance defined from the mean $ID_{50}$ values for GCV-naive isolates. Since the $ID_{50}$ for the AD169 strain was similar to the $ID_{50}$s for the GCV-naive isolates and because the AD169 strain can be tested in each run, an isolate might be considered resistant to GCV in vitro if the $ID_{50}$ for the isolate is significantly greater than the $ID_{50}$ for strain AD169 tested simultaneously, i.e., if the $SI_{50}$ is significantly greater than 1. Results of the study described here suggest that the $SI_{50}$ must be ≥3 (isolate for which the $ID_{50}$ is greater than or equal to threefold greater than the $ID_{50}$ for the simultaneously tested strain AD169) to suspect CMV resistance to GCV in vitro.

In conclusion, the study demonstrated that a late antigen reduction assay allows rapid and direct determination of CMV susceptibility to GCV and clearly distinguishes between susceptible and resistant cell culture-adapted strains of CMV. The rapid susceptibility test compared favorably with a conventional assay performed with cell-free virus after CMV isolation and in vitro passages and was easier to perform. Furthermore, it does not have the drawback of possible in vitro selection by cell passages of strains with modified susceptibility to GCV. The feasibility of the rapid assay was satisfactory since we were able to obtain results from all samples tested within 5 days. For this assay, a sample containing cell-free virus is necessary. It is therefore not suitable for blood specimens, but it can be successfully applied to bronchoalveolar lavage urine, or amniotic fluids. Its limitation might concern the necessity of obtaining a sample containing a sufficient amount of cell-free CMV. For this reason, and since, unfortunately, during this study we did not receive any CMV-positive samples from patients on long-term GCV treatment, the practicability of the test in patients who possibly excrete GCV-resistant strains in their urine (5) must be studied. Since this test is the only rapid susceptibility test described to date, it could be attempted when GCV resistance is suspected (e.g., CMV-positive urine specimens in a patient treated with GCV for a long period of time), because the result could be obtained within 5 days and thus could play a direct role in the therapeutic decision.

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