Inhibition of Human Cytomegalovirus Replication in a Human Retinal Epithelial Cell Model by Antisense Oligonucleotides

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Purpose. The antiviral activity of first and second generation antisense oligonucleotides on human cytomegalovirus (CMV) replication was evaluated in two cell systems, the traditional system on human fibroblasts and on human retinal pigment epithelial (HRPE) cell culture system.

Methods. To evaluate CMV replication strategies within the retina, an HRPE cell system permissive to CMV replication was developed. In this study, the antiviral activity of the antisense oligonucleotides, ISIS 2922 (Vitraven) and ISIS 13312, was evaluated in the traditional fibroblast antiviral assay and in the HRPE cell system. Antiviral activity was measured by evaluating inhibition of virus induced cytopathic effect, virus plaque formation, and virus gene expression.

Results. Both oligonucleotides produced concentration-dependent inhibition of CMV cytopathic effect and CMV plaque formation in both human RPE cells and a human fibroblast cell line, MRC-5. The oligonucleotide, ISIS 2922, demonstrated a mean 50% inhibitory concentration (IC_{50}) of 0.04 and 0.24 μM in HRPE and MRC-5 cells, respectively. The second-generation oligonucleotide, ISIS 13312, yielded similar results with IC_{50} levels of 0.05 and 0.3 μM in HRPE and MRC-5 cells, respectively. Similar findings were obtained with a CMV clinical isolate. In addition, initiation of effective oligonucleotide treatment could be introduced 6 days after CMV infection in HRPE cells, whereas, in the fibroblast cell line, oligonucleotide treatment was only effective up to 3 days after infection. Semiquantitative RT-PCR analysis demonstrated significant inhibition of CMV intermediate early and late mRNAs by both oligonucleotides.

Conclusions. These studies demonstrate that HRPE cells were significantly more sensitive than fibroblasts to the antiviral actions of ISIS 2922 and ISIS 13312. Moreover, the data indicate that the anti-CMV potency of the two oligonucleotides was similar. The enhanced potency of these oligonucleotides in HRPE cells may be associated with a delay in viral gene transcription and slow viral replication and spread in these cells. (*Invest Ophthalmol Vis Sci.* 2001;42:163–169)

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Human cytomegalovirus (HCMV) is a ubiquitous herpes virus that usually causes mild or subclinical disease in immunocompetent adults. However, CMV may cause severe morbidity or mortality in neonates or immunocompromised individuals, such as bone marrow transplant recipients and patients with AIDS.[1–3] In fact, disseminated CMV infections are common in patients with AIDS and are often associated with gastroenteritis and sight-threatening chorioretinitis.

Management of these infections has relied on the administration of ganciclovir, foscarnet, and cidofovir. In patients with AIDS, the prolonged maintenance therapy has resulted in drug toxicity and the emergence of resistant virus strains that have limited the effectiveness of these compounds and demonstrate the need for new drugs and treatment strategies.[4,5] Antisense oligonucleotides represent a new approach to antiviral therapeutics. Importantly, this therapy may overcome some of the difficulties associated with conventional antiviral therapies. Inhibition of virus replication using antisense oligonucleotides has been reported for several viruses including human immunodeficiency virus, herpes simplex virus, influenza virus, Rous sarcoma virus, vesicular stomatitis virus, and papilloma virus.[6–9]

Antisense oligonucleotides specific for the treatment of CMV have also been developed, and ISIS 2922 is the first antisense oligonucleotide approved by the Food and Drug Administration. This antisense oligonucleotide targets viral mRNA complementary to the major immediate early transcription unit of CMV.[10–13] Although the exact molecular basis of the antiviral activity of ISIS 2922 has not been clearly defined, there is evidence of sequence-specific activity consistent with an antisense mechanism of action. Moreover, the recent identification of an HCMV mutant with sequence-dependent resistance to ISIS 2922 provides strong evidence supporting drug selectivity.[14]

ISIS 13312 is a chemically modified analog of ISIS 2922, which has the same nucleotide sequence and viral mRNA target. Changes in the chemical nature of ISIS 13312 include the substitution of 5-methyl cytosine (5-methylC) for cytosine in the sequence and the addition of 2′-*O*-alkyl substitutes (2′-methylethoxy) on nucleosides on the 3′ end of the oligonucleotide and nucleosides on the 5′ end. These modifications were made to improve ocular tolerability and increase residence times in ocular tissues relative to ISIS 2922.[15,16]

HCMV-induced retinitis is associated with infection of the retinal pigmented epithelium (HRPE).[17–21] Because the HRPE cells play a basic role in maintaining the structural and physiological integrity of the neural retina, alterations in its structural and functional actions can result in loss of photoreceptors and vision.[22–24] Therefore, we have studied the mechanisms of HCMV replication in this cell. Previous studies have shown that regulation of HCMV infection of HRPE cells differs from that of human fibroblasts at both the level of virus entry and transcription of the viral genome.[17,25–27] Because CMV infects these epithelial cells in vivo, the HRPE cell model system appears to

be a more appropriate and relevant system for evaluation of this virus infection.

The purpose of this study was first to evaluate the antiviral activity of ISIS 2922 in two cell systems, the traditional antiviral assay (human fibroblasts) and an HRPE cell culture system. This latter cell system is more relevant to the treatment of local infection in the eye. Moreover, this study also characterizes the antiviral activity of the second-generation oligonucleotide (ISIS 13312) using both human fibroblasts, MRC-5 cells, and HRPE cells.

Materials and Methods

Cell Cultures

Human RPE cells were obtained and propagated as described previously.[28] When subcultured cells reached confluency, they typically were hexagonal and formed monolayers with clear intercellular boundaries characteristic of epithelial cells. Two primary cell lines of human RPE (RPE-1 and RPE-3) between passages 5 and 10 were used in this study and in previous studies.[17,28–31] When these cell lines at different passage levels were tested for the presence of cytokeratin, 100% of the cells reacted positively with monoclonal antibodies (Mab) to cytokeratin. This reactivity supports the presence of epithelial cells. Immunoblotting analysis further confirmed the presence of cytokeratin and indicated that cytokeratin 18 (42 kDa) is the predominant form. At the time of first passage, HRPE cells reacted positively with Mab developed against HRPE.[32]

MRC-5 is a human fibroblast cell line obtained from American Type Culture Collection (CCL171; ATCC, Rockville, MD). All cells were maintained in minimum essential medium supplemented with antibiotic–antimycotic mixture (Gibco BRL, Grand Island, NY) and 2% to 5% fetal bovine serum.

Oligonucleotides

ISIS 2922 was synthesized and purified as previously described.[12,13] The sequence of ISIS 2922 is 5′-GCG TTT GCT CTT CTT CTT GCG-3′, which corresponds to nucleotide coordinates 170,120 to 170,140 on the HCMV AD169 genome. ISIS 2922 is complementary to sequences present on major immediate early region (IE) mRNA encoding 55- and 86-kDa polypeptides. ISIS 3383 is a control antisense oligonucleotide used in this study. Reversed-phase HPLC-purified material was used in this study.

ISIS 13312, a modified phosphorothioate oligonucleotide, was synthesized and purified by reversed-phase HPLC before removal of trityl protecting groups, as previously described.[12,13] The sequence of ISIS 13312 is 5′-GCG TTT GCT CTT CTT CTT GCG-3′, which is complementary to the immediate early region 2 (IE2) mRNA of human CMV. In addition to phosphorothioate linkages, this oligonucleotide contained 2′-methoxyethoxy substituents on the seven nucleotides on the 5′-end (residues 1–7) of the oligonucleotide and six nucleotides on the 3′-end (residues 15–20) of the oligonucleotide (underlined). ISIS 13312 also contains 5-methyl cytosine in place of cytosine throughout the sequence.

Viruses

Cytomegalovirus, AD169 strain, was used in this study. For some studies, a clinical isolate of HCMV was used. The virus was isolated from peripheral blood lymphocytes of a patient who underwent bone marrow transplantation at The George Washington University Medical Center. The virus was passaged twice in human fibroblasts. Virus stocks were prepared by propagation in MRC-5 cells. Infected cultures were harvested by freezing and thawing one time, followed by centrifugation for 20 minutes at 2000 rpm.

Virus Infectivity Assays

Virus infectivity was assayed by two methods, observations of cytopathic effect (cpe) and plaque formation. In the cpe assays, triplicates of cells propagated in 96-well microplates were incubated with a 0.1 ml volume of serial 10-fold dilutions of the virus. After a 2-hour adsorption period at 37°C, inocula were removed, and cells washed, refed with media containing 2% to 5% heat-inactivated FBS, and incubated at 37°C. Infectivity was recorded as the induction of cpe by serial 10-fold dilutions of the virus, which was identified as tissue culture 50% infectious dose ($TCID_{50}$). In the plaque assays, triplicate samples of cells propagated in 24-well plates were incubated with a 0.5 ml volume of serial 10-fold dilutions of the virus. After a 2-hour adsorption period at 37°C, inocula was removed, and cells were washed with media, refed with 1.0 ml of media containing 0.75% methylcellulose and 2% FBS, and incubated at 37°C. Cultures were refed every 2 to 3 days with media. When plaques were seen, cells were harvested by removing the methylcellulose media. Cells were washed, fixed in alcohol, and stained with Giemsa stain. Infectivity was recorded as plaque forming units (pfu).

Stock virus pools were titrated on both HRPE and MRC-5 cells. Infectious viral titers on MRC-5 cells were approximately 2 logs higher than the infectious virus titers observed in HRPE cells. Therefore, to generate equivalent amounts of infectious virus in MRC-5 and HRPE cells, 100 times more virus was required in the inocula for the HRPE cells.

Virus Inhibition Assays

The standard virus inhibition assays were performed in the following manner. In the virus cpe assay, 100 $TCID_{50}$ units of HCMV were used to infect cells propagated in 96-well plates. After a 2-hour adsorption period at 37°C, the inoculum was removed, and cells were washed and then refed with media or media containing various concentrations of oligonucleotides. Cpe was recorded on a daily basis. In a typical experiment, cpe was recorded on days 5 to 8 for MRC-5 cells and days 8 to 15 for HRPE cells. The IC_{50} concentration of the oligonucleotide was identified as that concentration that inhibited development of cpe by 50%.

In the plaque assay, approximately 100 pfu of HCMV were used to infect cells propagated in 24-well plates. The conditions were similar to those described above. In a typical experiment, pfu was recorded at day 8 for MRC-5 cells and day 16 for HRPE cells. The IC_{50} concentration of the oligonucleotide was identified as that concentration that inhibited 50% of the plaques.

RT-PCR Analysis of Viral RNA

RNA was isolated according to the RNA STAT-60 protocol (Tel Test, Friendswood, TX). The final preparation was resuspended in DEPC-treated water and quantitated spectrophotometrically. Reverse transcription was carried out using an RT-PCR kit (Roche Molecular Sys, Inc, Branchburg, NJ) according to manufacturer's instructions. The mixtures were incubated at room temperature for 10 minutes and then placed in a thermocycler 9600 at the following conditions: 1 cycle: 42°C for 15 minutes, 99°C for 5 minutes, and 5°C for 5 minutes.

Amplification of cDNA was performed in the following manner. After an initial incubation at 95°C for 105 seconds, the reaction mixture was subjected to PCR cycles as follows: 95°C for 15 seconds and 60°C for 30 seconds. For each gene product, the optimum number of cycles was determined experimentally and was defined as that number of cycles that will achieve a detectable concentration well below saturating conditions. Primers used for HCMV IE 55/86 (IE2) were as follows: sense, 5′-GCA-CAC-CCA-ACG-TGC-AGA-CTC-GGC-3′; antisense: 5′-TGG-CTG-CCT-CGA-TGG-CCA-GGC-TC-3′. Primers used for HCMV pp65 were as follows: sense, 5′-CAC-CTG-TCA-CCG-CTG-CTA-TAT-TTG-C-3′; antisense, 5′-CAC-CAC-GCA-GCG-GCC-CTT-GAT-GTT-T-3′.[33] PCR amplification resulted in fragments of 659 bp for HCMV IE86 and 400 bp for HCMV pp65. To verify that equal amounts of RNA were added in each RT-PCR reaction, primers for "the housekeeping gene," actin, were used. The resulting products were probed as described below.

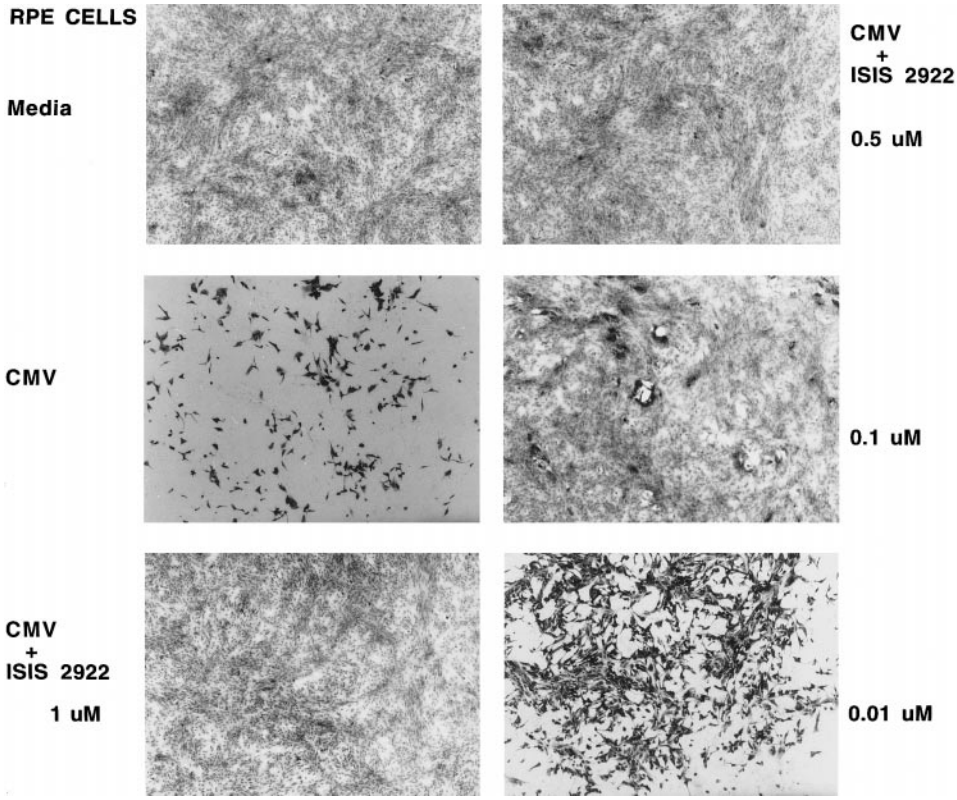

FIGURE 1. Antisense oligonucleotide, ISIS 2922, inhibition of CMV replication. Photomicrographs illustrate a concentration-dependent inhibition of CMV induced CPE in HRPE cells by the antisense oligonucleotide ISIS 2922. Confluent HRPE cell cultures grown in 96-well plates were inoculated with CMV (AD169 strain). After a 2-hour adsorption period, inocula were removed, and cells were refed with media alone or media containing varying concentrations of ISIS 2922. Culture media were replaced with media alone or with media containing ISIS 2922 every 2 days. After 16 days, cultures were fixed in alcohol, stained with Giemsa, and photographed.

Detection of Amplified Product by Southern Blot Analysis

Ten microliters of the PCR products were run on a 4% agarose gel at 80 V for 20 minutes. The gel was denatured by soaking two times for 15 minutes each in 1.5 M NaCl and 0.5 M NaOH, followed by neutralization in 1.5 M NaCl and 1 M Tris (two times for 15 minutes each). The DNA was transferred to a nylon membrane by capillary action overnight at room temperature. The DNA was UV cross-linked to the membrane using an UV Stratalinker 1800. Blots were prehybridized at 65°C for 2 hours in a hybridization solution consisting of 5× SSC, 1% w/v blocking agent, 0.1% sarcosyl, and 0.02% SDS. After 2 hours the hybridization solution was replaced with 10 ml of new hybridization solution plus probe (25 ng/ml) labeled with digoxigenin. The membranes were hybridized overnight at 65°C. After hybridization the membranes were washed twice (5 minutes each) in 2× SSC, 0.1% SDS at room temperature, and then twice (15 minutes each) in 0.5× SSC, 0.1% SDS at 65°C followed by detection using anti-digoxigenin antibodies, and CSPD (Boehringer Mannheim, Indianapolis, IN).

RESULTS

Cellular Uptake of Oligonucleotide

Preliminary studies were performed to determine whether ISIS 2922 was capable of entering both HRPE and MRC-5 cells. Cells were propagated on 8-well chamber slides and incubated with 5 μM concentrations of FITC-labeled ISIS 2922. After 2- and 24-hour incubation periods, cells were washed and evaluated under a fluorescence microscope. Approximately 80% and 100% of the cells evaluated at 2 and 24 hours, respectively, gave positive immunofluorescent staining. These studies indicated that ISIS 2922 was capable of entering both HRPE and MRC-5 cells (data not shown).

Inhibition of HCMV Replication by ISIS 2922

The ability of ISIS 2922 to inhibit HCMV (AD169) replication in HRPE and MRC-5 cells was evaluated by two quantitative virus assay systems: the inhibition of 50% of cpe induced by 100 $TCID_{50}$ units of HCMV and inhibition of 50% of the CMV plaques. HCMV was added to cells for a 2-hour adsorption period, inocula were removed, and cells were refed with media alone or media containing varying concentrations of the oligonucleotide. Cultures were then maintained for 7 to 21 days and evaluated for the development of cpe or pfu. An example of HCMV-induced cpe and the inhibition of this cpe by ISIS 2922 is shown in Figure 1. The concentrations of ISIS 2922 producing 50% reduction of virus relative to the control are shown in Table 1. The mean IC_{50} in MRC-5 cells was 0.3 μM in the virus cpe assay and 0.24 μM concentration in the virus plaque assay. However, the mean IC_{50} in HRPE cells was 0.07 μM in the inhibition of virus cpe assay and 0.04 μM in the virus plaque assay. These data demonstrate that both assay systems generated similar values and that the concentration of ISIS 2922 required to inhibit virus replication in HRPE cells was significantly less than that required to inhibit virus replication in MRC-5 cells ($P < 0.002$).

A typical dose response for the ISIS 2922–induced inhibition of HCMV in HRPE cells is shown in Figure 2A. Similar IC_{50} levels (0.025 and 0.03 μM) for ISIS 2922 were observed in

TABLE 1. Comparison of ID_{50} for Oligonucleotide ISIS 2922 on CMV Replication in Fibroblasts (MRC-5) and HRPE Cells

	ID_{50} (μM)	
Cell Type	**CPE Assay**	**Plaque Assay**
MRC-5 cells		
Range	0.25–0.5	
Mean	0.3*	0.24*
HRPE cells		
Range	0.05–0.1	0.01–0.06
Mean	0.07*	0.04*

*HRPE versus MRC-5: $P < 0.002$.

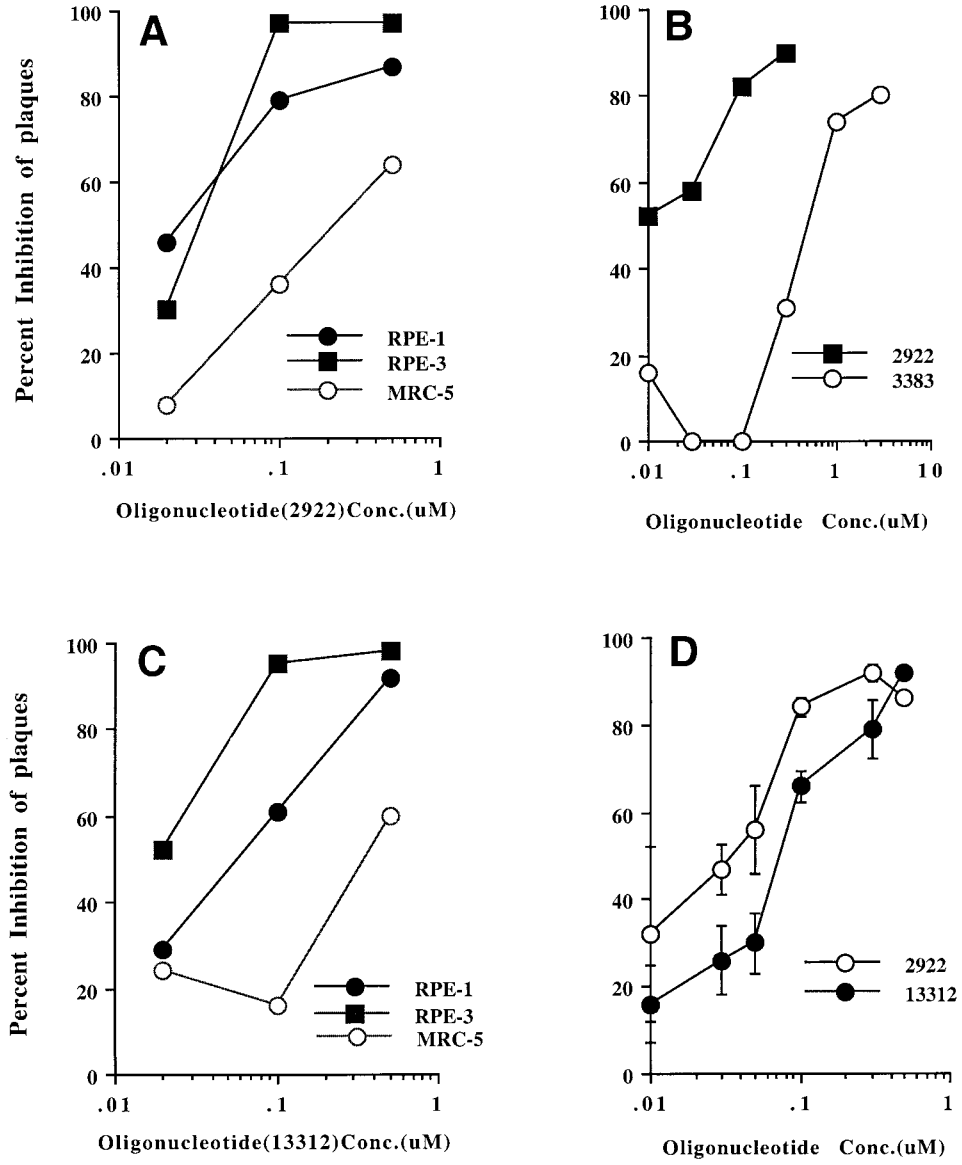

FIGURE 2. Inhibition of CMV plaque formation by antisense oligonucleotides. Confluent HRPE and MRC-5 cell cultures grown in 24-well plates were inoculated with approximately 100 pfu of CMV (AD169 strain). After a 2-hour adsorption period, inoculum were removed, and cells refed with methycellulose overlay media alone or overlay media with varying concentrations of oligonucleotide. (**A**) Inhibition of CMV plaque formation on MRC-5 cells and HRPE cells by ISIS 2922. RPE-1 and RPE-3 are two cell cultures derived from different donors. (**B**) Inhibition of CMV (AD169) plaque formation on HRPE cells by ISIS 2922 and ISIS 3383 (control oligonucleotide). (**C**) Inhibition of CMV (AD169) plaque formation on MRC-5 cells and HRPE cells by ISIS 13312. (**D**) Antiviral activity of ISIS 2922 and ISIS 13312 against CMV replication in HRPE cells. The data presented are the mean ± SEM for six separate experiments.

HRPE cell cultures derived from different donors (identified as RPE-1 and RPE-3). An evaluation of cell morphology and cpe or pfu formation revealed that the oligonucleotide limited the number and size of the HCMV-induced plaques (data not shown). Moreover, an evaluation of cpe formation revealed that cpe was limited to smaller focal areas, suggesting that the oligonucleotide may have decreased the virus spread from cell to cell.

A control oligonucleotide, designated ISIS 3383, was also evaluated in this system. The percent HCMV plaque inhibition in HRPE cells treated with varying concentrations of ISIS 2922 or ISIS 3383 is shown in Figure 2B. In this experiment the IC_{50} for ISIS 2922 was 0.01 μM. In contrast, the ISIS 3383 oligonucleotide did not alter CMV plaque formation at concentrations of 0.01, 0.025, or 0.1 μM. The IC_{50} concentration for ISIS 3383 was 0.55 μM. In two separate experiments, ISIS 3383 at a concentration of 0.1 μM had no effect on CMV replication, whereas ISIS 2922 at a 0.1 μM concentration induced an 87% inhibition of CMV plaque formation. These data support our previous reports demonstrating that unrelated oligonucleotides, when used at concentrations of 0.5 μM and above, can alter HCMV replication.[11–13] However, sequence-specific oligonucleotides are effective at significantly lower concentrations.

Efficacy of ISIS 2922 under Varying Conditions

Preliminary studies showed that the IC50 for ISIS 2922 in HCMV-infected HRPE and MRC-5 cells was similar when the oligonucleotide was added 24 hours before virus or 2 hours after virus adsorption. Because HRPE cells could also be maintained in serum-free media, we were able to evaluate the effect of serum on the efficacy of ISIS 2922. The IC_{50} for ISIS 2922 in HCMV-infected HRPE cells was similar when the cells were maintained in 2% serum or in serum-free conditions (data not shown).

Studies were also performed to determine the length of time after infection that the initiation of oligonucleotide treatment was still effective in blocking HCMV replication. After a 2-hour adsorption period with 100 $TCID_{50}$ units of HCMV, cells were washed and incubated with media. HCMV-infected cells were initially exposed to ISIS 2922 (0.5, 0.1, or 0.05 μM) at increasing intervals from 2 hours to 7 days after infection. The efficacy of ISIS 2922 under these conditions is presented in

TABLE 2. Potency of ISIS 2922 when Treatment Was Initiated at Varying Times after Infection

	IC_{50} Concentration of ISIS 2922 (μM)	
Time of Addition	**HRPE Cells**	**MRC-5 Cells**
2 hours	0.05	0.5
24 hours	0.1	0.5
2 days	0.1	0.5
3 days	0.1	0.5
4 days	0.1	None (>0.5)
5 days	0.1	None
6 days	0.1	
7 days	None (>0.5)	

Table 2. When ISIS 2922 was first added at 2 hours after infection of HRPE cells, the IC_{50} was 0.05 μM. When ISIS 2922 was first added at 1, 2, 3, 4, 5, or 6 days after infection, the IC_{50} was 0.1 μM. However, when ISIS 2922 was first added at day 7 after infection, inhibition of virus cpe was not observed at concentrations as high as 0.5 μM. In the MRC-5 cell system, the addition of ISIS 2922 at 2 hours, day 1, 2, or 3 resulted in virus inhibition with an IC_{50} of 0.5 μM. The addition of ISIS 2922 at day 4 or later did not result in the inhibition of virus-induced cpe. These data demonstrate that ISIS 2922 at a concentration of 0.1 μM was effective in inhibiting HCMV cytopathology up to 6 days after infection of HRPE cells. In contrast, ISIS 2922 at a concentration of 0.5 μM effectively inhibited virus replication when it was added to the MRC5 cells within 3 days of infection.

Second-Generation Oligonucleotide ISIS 13312

Although phosphorothioate oligonucleotides are effective therapeutic antisense molecules, it is desirable to develop second-generation molecules with improved properties such as increased stability and decreased toxicity. ISIS 13312 is a methoxyethoxy-modified version of ISIS 2922 designed to provide longer residence time in the eye and improved tolerability. The ability of ISIS 13312 to inhibit HCMV (AD169) replication in HRPE and MRC-5 cells was initially evaluated by the inhibition of HCMV plaque formation. As is shown in Figure 2C, ISIS 13312 significantly inhibited HCMV plaque formation in a dose-dependent manner in two different HRPE cell lines and in MRC-5 cells. The IC_{50} observed in both HRPE cell lines (0.02 and 0.06 μM) was markedly lower than that observed in the MRC-5 cells (0.5 μM).

A composite comparison of the two oligonucleotides (ISIS 2922 and ISIS 13312) on HRPE cells is shown in Figure 2D. The data presented are the mean of six separate experiments. Both oligonucleotides produced a concentration-dependent inhibition of CMV plaque formation. Mean IC_{50} values for ISIS 2922 and ISIS 13312 were 0.04 and 0.07 μM, respectively. These data demonstrate similar potency against CMV by both oligonucleotides.

CMV Gene Expression in Oligonucleotide-Treated HRPE Cells

To determine the effect of oligonucleotides on HCMV gene expression, the presence of mRNA encoding immediate early protein, IE-86, and mRNA encoding late protein, pp65, were analyzed by semiquantitative RT-PCR. Monolayers of HRPE were incubated with HCMV at an input multiplicity of 5. After a 2-hour adsorption period, cells were washed three times with media alone, refed with media alone or media containing 0.5 μM concentration of ISIS 2922 or ISIS 13312. Total RNA was extracted from HCMV-infected and uninfected cells at 6 days after infection. Southern blot analysis of RNA extracted from these cultures is shown in Figure 3. Density of the bands was analyzed with the NIH image analysis program, and the percentage of inhibition of CMV gene expression in treated samples was compared with CMV gene expression in untreated HRPE cells. HCMV IE-86 mRNA expression was inhibited by 37% with ISIS 2922 and by 64% with ISIS 13312. CMV pp65 mRNA expression was inhibited by 77% with ISIS 2922 and by 85% with ISIS 13312.

Effect of ISIS 2922 and 13312 on an HCMV Clinical Isolate

The ability of ISIS 2922 and ISIS 13312 to inhibit the replication of an HCMV clinical isolate was evaluated. The IC_{50} for oligonucleotide inhibition of a CMV clinical isolate in a single experiment is shown in Figure 4. The IC_{50} for ISIS 2922 was 0.35 μM in MRC-5 cells and 0.08 μM in HRPE cells, and the IC_{50} for ISIS 13312 was 0.32 μM in MRC-5 cells and 0.08 μM in HRPE cells. These levels are similar to those observed with the HCMV (AD169) strain, indicating similar potency.

DISCUSSION

We have shown that both antisense oligonucleotides, ISIS 2922 and ISIS 13312, produced a concentration-dependent inhibition of HCMV cytopathic effect and HCMV plaque formation in both human RPE cells and a human fibroblast cell line, MRC-5. Antisense oligonucleotides, ISIS 2922 and ISIS 13312, were developed to inhibit CMV replication by preventing the synthesis of proteins encoded by the major IE transcriptional units that are critical for virus replication.[11–13] Semiquantitative RT-PCR analysis demonstrated significant inhibition of HCMV IE and late (pp65) mRNA by both of the oligonucleotides. These studies demonstrated that the HRPE cell was significantly more

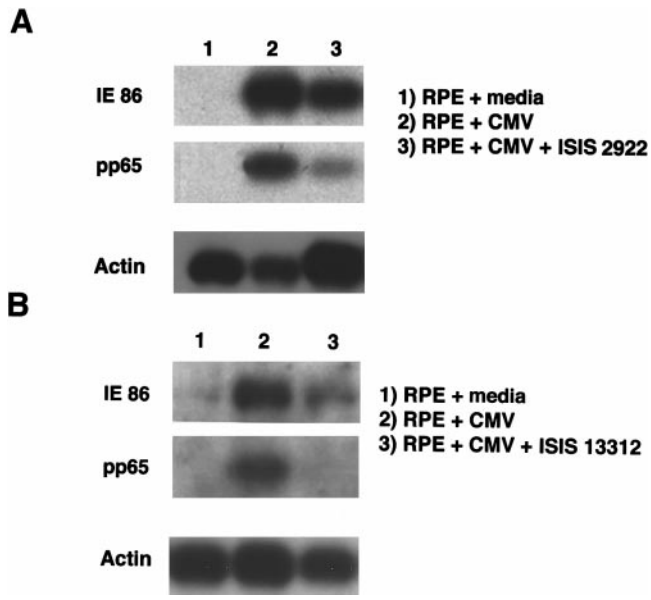

FIGURE 3. RT-PCR analysis of CMV IE86, CMV pp65, and actin mRNA expression in CMV-infected HRPE cells. Monolayers of HRPE cells were incubated with CMV at an input multiplicity of 5. One set of CMV-infected cultures were treated with either ISIS 2922 (0.5 μM) or with ISIS 13312 (0.5 μM). Uninfected and CMV-infected cells were harvested at day 6 after inoculation. After mRNA extraction and reverse transcription, IE86, pp65, and actin cDNA regions were amplified by PCR. Southern blot analysis was performed with digoxigenin-labeled probes.

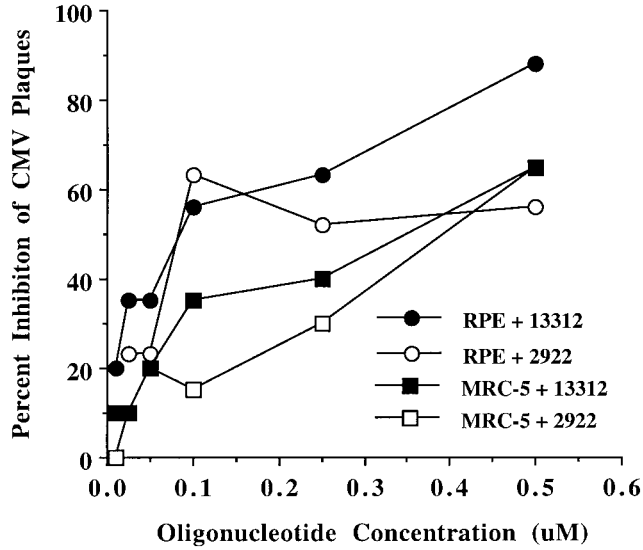

FIGURE 4. Inhibition of CMV (clinical isolate) replication in MRC-5 cells and HRPE cells by ISIS 2922 and ISIS 13312. Experimental conditions were the same as those presented for Figure 2.

sensitive than MRC-5 cells to the antiviral actions of these oligonucleotides. Moreover, initiation of effective oligonucleotide treatment could be introduced 6 days after CMV infection in HRPE cells. This was 3 days later than initiation of effective oligonucleotide treatment in the MRC-5 cell line.

Prior studies on the anti-HCMV activity of ISIS 2922 have used human fibroblast cell lines.[11–13] Human CMV has a tropism for the human retina.[3] However, until now, our ability to investigate HCMV replication within the human retina has been limited. Earlier studies have shown that HCMV was identified in the HRPE cell by electron microscopy, immunocytochemical staining, and virus isolation.[18–21] Our experimental approach to evaluate HCMV replication within the retina was based on the utilization of an in vitro model of HCMV replication in a clinically important ocular cell, the HRPE cells. HCMV IE gene expression and virus replication was delayed and slow in HRPE cells compared with gene expression and virus replication in fibroblasts.[17,25,27] This pattern of virus replication may reflect replication more accurately within specialized cells and may provide novel ways to evaluate factors that control HCMV gene expression and replication. The studies reported here indicate that ISIS 2922 and ISIS 13312 were both more effective in inhibiting HCMV replication in HRPE cells than in fibroblasts. The mean IC_{50} for these oligonucleotides were approximately 0.05 and 0.3 μM in HRPE and MRC-5 cells, respectively. The antiviral activity of both oligonucleotides was not attributable to nonspecific cytotoxicity because cell viability was not adversely affected under conditions used (concentrations up to 50 μM).

It is of interest to point out that there was an apparent nonspecific antiviral activity demonstrated by the control antisense oligonucleotide, ISIS 3383, but the potency was an order of magnitude less than that demonstrated for ISIS 2922. The sequence-independent antiviral activity of the control oligonucleotide is attributed to interference of virus adsorption at high concentrations in culture, rather than an effect on viral replication.[11–13]

The addition of 2′-methoxyethoxy modifications to phosphorothioate oligodeoxynucleotides increases affinity for the target mRNA. Moreover, the chemical modifications, including 5′methyl cytosine residues and 2′-methoxyethoxy modification of ribose in the backbone, also improve the ocular tolerability and increase ocular residence time.[15,16] ISIS 13312 was generated with these modifications and was shown here to have potent antiviral activity comparable to the activity of ISIS 2922 against HCMV (AD169) and a HCMV clinical isolate.

Enhanced antiviral activity of oligonucleotides in HCMV-infected HRPE cells compared with HCMV-infected fibroblasts may be associated with several factors, such as virus entry, viral gene transcription, and viral spread. First, the enhanced potency of oligonucleotides probably was not due to alterations in virus entry. Although less HCMV enters HRPE cells in comparison to fibroblasts, the culture conditions were initially established so that equivalent amount of virus (100 $TCID_{50}$ or 100 pfu) entered both cell types. Moreover, the oligonucleotide treatment was not initiated until after the 2-hour virus adsorption period. Second, prior studies showed that initiation of HCMV gene expression, IE, early, and late, were delayed in HRPE cells.[17] Expression of all three genes were seen in fibroblasts at 24 hours, whereas expression was not detected in HRPE cells until 3 to 5 days. The delay in viral gene transcription may allow the oligonucleotide to be present in high concentrations acting against a low number of HCMV IE gene-specific mRNAs. Finally, enhanced oligonucleotide potency may be associated with HCMV spread. Once cytopathology was observed, spread of the virus throughout the culture was very slow and progressive in HRPE cells in comparison to a more rapid viral spread in fibroblasts. Inhibition of viral replication may be more magnified under conditions where viral spread is slow and limited. Based on these observations, the delayed virus gene transcription and slow virus spread may contribute to enhanced potency of the anti-HCMV oligonucleotides observed in HRPE cells. Because this delayed, slowly progressive involvement of the retina is seen in HCMV retinitis, the HRPE cell model system may be more analogous to the retinal tissue destruction.

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