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Antiviral Research 33 (1997) 117–127

Antiviral Research

Inhibition of herpes simplex virus replication by retinoic acid

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Received 17 June 1996; revised 17 September 1996; accepted 24 September 1996

Abstract

The retinoic acid (RA) isomers all-*trans*-RA, 9-*cis*-RA and 13-*cis*-RA as well as other retinoids were tested for their ability to reduce the yield of herpes simplex virus-1 (HSV-1). RA isomers reduced HSV-1 replication whereas the other retinoids, retinol, retinal, β-carotene and amide derivatives of RA were not inhibitory. All-*trans*-RA reduced the yield of HSV-1 by 100-fold at 5 μg/ml but 9-*cis*-RA and 13-*cis*-RA reduced viral replication by 10-fold. At a concentration of 10 μg/ml all-*trans*-RA and 9-*cis*-RA reduced virus yield by 1000-fold while 13-*cis*-RA decreased HSV-1 production by 100-fold. RA isomers at a concentration of 10 μg/ml were not cytotoxic for the Vero cells used in these studies. Immunofluorescence studies showed that all-*trans*-RA treated cell cultures exhibited small foci of virus specific immunostaining while untreated cultures displayed intense HSV-1 immunoreactivity in virtually the entire cell population. RA-dependent inhibition of HSV-1 replication required the presence of RA with the virus. HSV-1 replication proceeded when RA was removed from infected cells. Treatment of cell cultures with RA did not induce gene expression for type-1 interferon (IFN) or for the type-1 IFN inducible genes studied suggesting that RA inhibition of HSV-1 replication is not mediated by IFN. These studies have established the ability of RA to reduce the replication of HSV-1 in vitro. Copyright © 1997 Elsevier Science B.V.

Keywords: Retinoic acid; Herpes simplex virus

1. Introduction

Herpes simplex virus infection is a common problem. Genital HSV infection is found in over 60 million people in the United States, most of whom are of childbearing age (Whitley, 1994;

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PII S0166-3542(96)01009-1

Corey, 1994). HSV shedding during birth results in approximately 85% of neonatal herpes infections (Whitley, 1994). There are many millions of herpes labialis cases each year and HSV infection is also the most common ocular infection with an estimated 500 000 cases reported annually in the United States (Nauss et al., 1985). Approximately 98% of non-neonatal ocular infection is the result of HSV-1 whereas 70–80% of ocular herpes in the first month of life is caused by HSV-2 (Kohl, 1994). HSV-1 causes most ocular, oral, labial, upper respiratory tract and central nervous system disease. Most genital and neonatal infections are due to HSV-2, though 12–50% of genital herpes is from HSV-1. Primary herpes infection is associated with the excretion of 10^6–10^8 plaque-forming units of HSV for as long as 14–21 days (Whitley, 1994), while recurrent HSV infection produces approximately 10^2 plaque-forming units of virus and lasts 3–5 days (Whitley, 1994). Not surprisingly HSV transmission during primary HSV infection is far greater than during recurrent episodes and the incidence of neonatal herpes infection is 10 times higher during primary genital infection than during subsequent infections (Whitley, 1994). Currently there is a need for effective treatments to reduce the spread of HSV.

Dietary intake of β-carotene and vitamin A have been shown to stimulate immune function and reduce the incidence and severity of a number of infections including measles, HIV and respiratory syncytial virus infection (Zhao and Ross, 1995; Hussey and Klein, 1990; Semba et al., 1994; Neuzil et al., 1994). Vitamin A supplementation can increase the number of activated macrophages, maintain epithelial barrier function and promote antibody dependent as well as non-specific immunity (Ross, 1991). Evidence is also accumulating which suggests that retinoids can directly modulate viral gene expression through retinoic acid receptors. This may involve viral enhancers as well as a direct protein-protein interaction between retinoic acid receptors and viral proteins (Ross, 1991; Ghazal et al., 1992; Hsu et al., 1993; Huan and Siddiqui, 1992; Sista et al., 1993). Studies with HIV indicate that all-trans-retinoic acid and 9-*cis*-retinoic acid can regulate HIV expression both increasing and decreasing virus production depending upon the host cell and the time of RA addition (Yamaguchi et al., 1994; Poli et al., 1992).

Since lowering the number of shed HSV particles should decrease viral transmission, the present study was undertaken to determine whether retinoids reduce HSV replication. The results suggest that the use of retinoids could lessen HSV production and consequently the risk of transmission between adults and from mother to infant.

2. Materials and methods

2.1. Cell cultures

Vero cells (African green monkey kidney cell line) and HeLa cells (cervical epithelial carcinoma cell line) were purchased from the American Type Culture Collection, Rockville, MD. Both cell lines were grown in RPMI 1640 containing glutamine (BioWhittaker, Walkersville, MD), 0.075% $NaHCO_3$, 0.2% gentamicin and 10% inactivated fetal bovine serum (BioWhittaker). The maintenance medium (MM) for Vero and HeLa cells was as described above but with 3% fetal bovine serum.

2.2. Virus

HSV-1 (strain F1) was obtained from Dr. R. Rubenstein (New York State Institute for Basic Research) and grown in Vero cells.

2.3. HSV-1 Titration

HSV-1 was titrated by inoculation of 10-fold dilutions into Vero cell cultures in 96-well microtiter tissue culture plates (Becton Dickinson, Lincoln Park, NJ). A virus dilution (0.1 ml) in MM was inoculated into each well with three wells per dilution. The plates were incubated for 5 days at 37°C and examined daily for cytopathic effect. Virus titers were calculated by the method of Reed and Muench (1938).

2.4. Retinoids

All-*trans*-RA, 13-*cis*-RA, retinol, retinal and β-carotene were purchased from Sigma Chemical, St. Louis, MO. 9-*cis*-RA was a gift from Hoffman LaRoche, Nutley, NJ. Fenretinide (4HPR) and *N*-(4-methoxyphenyl) retinamide (4MPR) were gifts from F. Formelli (Instituto Nazionale per lo Studio e la Cura dei Tumori, Milan, Italy). All three retinoic acid isomers and other retinoids were dissolved in dimethysulfoxide (DMSO) which was diluted 1:1000 in MM to obtain the desired concentration.

2.5. Assay of HSV-1 inhibition

Vero cells were seeded into 4 cm tissue culture dishes and grown to 1.5×10^5 cells/plate in growth medium at 37°C in a 5% CO_2–95% air atmosphere. Cultures were inoculated with HSV-1 at a multiplicity of infection (MOI) of 0.04 tissue culture infective doses ($TCID_{50}$) per cell in 1 ml of MM unless otherwise indicated. Cultures were incubated for 1 h at 37°C to permit virus adsorption. Virus inoculum was replaced with 2 ml of fresh MM containing RA at the indicated concentration. Plates were incubated at 37°C for 2 days following which the supernatants were titrated by the serial dilution endpoint method. Dilutions (10-fold) were made in MM. The 10^0–10^{-7} dilutions were inoculated onto monolayers of Vero cells, and the virus titers were determined as described above. The measure of viral inhibition was the difference between the titer ($\log_{10}$) of the control virus plates (no retinoid) and the titers of retinoid treated cultures.

2.6. Cytotoxicity assay

Cell counts were carried out using dividing cells following exposure to RA isomers for 24 h at a concentration of 12.5 μg/ml. Samples were done in triplicate and the value expressed are cell counts $\times 10^{-4}$. Values for control samples were 4.40 ± 0.87 and counts for all-*trans*-RA, 9-*cis*-RA and 13-*cis*-RA treated samples were 5.75 ± 0.78, 4.25 ± 0.21 and 4.15 ± 0.21, respectively. Studies were also done to measure the cytotoxicity of RA isomers using Vero cell monolayers exposed to RA over a 48 h period. Cell counts for control samples were 10.20 ± 3.15. All-*trans*-RA, 9-*cis*-RA and 13-*cis*-RA treated samples had cell counts of 9.23 ± 2.10, 9.80 ± 0.56 and 7.5 ± 0.32, respectively. As above these studies were done in triplicate and the values are cell counts $\times 10^{-4}$. Studies were also performed using trypan blue exclusion to determine if there were differences in the numbers of stained cells between control and RA isomer treated cultures. No differences were found. The effect of all-*trans*-RA on cell metabolism was determined using incorporation of [^{3}H]thymidine and [^{35}S]methionine. Cell monolayers were incubated over a 48 h period in the presence of radiolabelled precursor with and without all-*trans*-RA. Cells were washed in PBS and extracts prepared using 1% SDS. Incorporation of radiolabelled precursor was monitored following TCA precipitation. Each sample was assayed in triplicate. Control samples had $44\,720 \pm 6\,533$ dpm/10^5 cells and $55\,913 \pm 10\,290$ dpm/10^5 cells for labelled thymidine and methionine, respectively. All-*trans*-RA treated samples had $34\,654 \pm 1\,123$ dpm/10^5 cells for thymidine and $56\,968 \pm 15\,898$ dpm/10^5 cells for methionine. These values represent 78 and 102%, respectively of control values. Further evidence supporting the absence of cytotoxicity by all-*trans*-RA, 9-*cis*-RA and 13-*cis*-RA was achieved in studies using vaccinia virus. The 48 h treatment of Vero cells with all-*trans*-RA did not inhibit the yield ($TCID_{50}$) of this virus (data not shown).

2.7. Immunofluorescence

Cells were grown on cover slips in dishes as described above, infected with HSV-1 in the presence and absence of all-*trans*-RA (10 μg/ml) and after 48 h the MM was removed and the cells washed twice with RPMI 1640. Cells were then fixed with 2 ml of cold methanol and after 10 min washed with 2 ml of RPMI 1640. The cover slips were then air dried and stored at 4°C. When ready for staining cells were rehydrated with 2 ml of phosphate buffered saline (PBS) (pH 7.0) for 5 min at room temperature and blocked with 10% normal goat serum. The primary antibody against

HSV-1 (Herpes Virus CF Human) (Microbiological Associates, Bethesda, MD) was diluted 1:250 and incubated for 90 min at 37°C following which the cells were rinsed four times with PBS. The secondary antibody, goat anti-human immunoglobulin FITC conjugated, (BioSource, Camarillo, CA) was diluted 1:100 and incubated for 1 h at 37°C with the cells. Cells were then washed four times and fixed with 1% paraformaldehyde for 5 min at room temperature, washed twice with PBS and the cover slips were mounted to slides using an anti-quenching mounting medium (Giloh and Sedat, 1982).

2.8. Reverse transcription polymerase chain reaction (RT-PCR)

Vero or HeLa cells were grown in a monolayer to 2×10^6 cells per dish, washed with RPMI 1640 and then treated with MM containing either interferon-α (IFN-α) (5000 U/ml for 4 h), interferon-β (IFN-β) (1000 U/ml for 4 h), poly (rI)·poly (rC) (poly IC, a synthetic dsRNA) (50 μg/ml for 3 h) or all-*trans*-RA (12.5 μg/ml for 24 h). IFN-α, IFN-β and poly IC were purchased from Sigma, St. Louis, MO. RNA was isolated using a kit according to the manufacturer's protocol (Molecular Research Center, Cincinnati, OH). Briefly, the monolayer was lysed by adding TRI Reagent. The aqueous phase containing RNA was separated with chloroform and RNA was precipitated from the aqueous phase by mixing with isopropanol. RNA pellets were washed with 75% ethanol and solubilized in water.

RT-PCR was carried out with a StrataScript kit (Stratagene, LaJolla, CA). Total cellular RNA (10 μg) was mixed with 0.3 μg of *oligo* (dt) primer. The mixture was incubated at 65°C for 5 min, cooled at room temperature and treated with 50 U of Moloney mouse leukemia virus-reverse transcriptase, 40 U of RNase block ribonuclease inhibitor and 1 mM each of all four deoxynucleotide triphosphates (dNTPs). The reverse transcription reaction was carried out at 37°C for 1 h and stopped by heating at 95°C for 5 min. For PCR amplification, the cDNA products were mixed with 0.8 mM dNTPs, 2.5 U of *Taq* DNA polymerase and 1 μM of primers in a final reaction volume of 100 μl. Primers for IFN-α and IFN-β were purchased from Clontech, Palo Alto, CA and the β-actin primers from Stratagene, LaJolla, CA. The primers used for Mx (Baca et al., 1994) and P1/eIF-2 protein kinase (Thomis et al., 1992) and their amplified products were as described in the references and were synthesized by Biosynthesis, Lewisville, TX. The sequence of 2′,5′-*oligo*adenylate synthetase (Wathelet et al., 1986) was used to design PCR primers which were synthesized by Biosynthesis. The primer sequences are as follows: sense strand 5′-GACTATCTCTTGCCAGACAC-3′ (nucleotides 145–164); antisense strand 5′-ACTCCTC-GATGAGCTTGACA-3′ (nucleotides 622–641). An amplified product of 478 bp was produced. Amplifications were carried out on a Biometra PCR thermal cycler for 35 cycles (94°C for 1 min, 60°C or 55°C for 1 min, and 72°C for 2 min). The results were visualized on an ethidium bromide stained 0.8% agarose gel.

3. Results

All three retinoic acid isomers, all-*trans*-RA, 9-*cis*-RA and 13-*cis*-RA, inhibited HSV-1 replica-

Table 1
Specificity of Retinoids[a] for HSV-1 Inhibition

Retinoid	$TCID_{50}^{b}$
None	$10^{4.6} \pm 0.2$
Retinol	$10^{4.1} \pm 0.4$
Retinal	$10^{3.7}0 \pm 0$
Trans-RA[c]	$10^{0.6} \pm 0.2$
13-*cis*-RA[c]	$10^{2.5} \pm 0.2$
9-*cis*-RA[c]	$10^{0.4} \pm 0.5$
β-Carotene	$10^{5.5} \pm 0.5$
4MPR	$10^{4.4} \pm 0.2$
4HPR	Cytotoxic[d]

[a]Retinoids were used at a concentration of 10 μg/ml unless otherwise indicated.
[b]The $TCID_{50}$ is the mean ± S.D. of three separate experiments.
[c]All-*trans*-RA, 13-*cis*-RA and 9-*cis*-RA are three different isomers of retinoic acid.
[d]Cytotoxic above 1 μg/ml. No HSV-1 inhibition was found at concentrations of $\leqslant 1$ μg/ml.

tion at a concentration of 10 μg/ml (Table 1) while the corresponding alcohol, aldehyde and amide derivatives had no effect. When all-*trans*-RA was added with HSV-1, viral production was 4 $\log_{10}$ lower than in control cultures. This inhibition of HSV replication was shown not to be the consequence of cytotoxic activity by RA. Both cell survival (trypan blue exclusion) and cellular metabolism (DNA and protein synthesis) studies (see Materials and methods) indicated that cytotoxicity was not responsible for the reduction in viral yield. The addition of 9-*cis*-RA produced similar viral inhibition while 13-*cis*-RA was less effective and reduced the viral titer by 2 $\log_{10}$. Retinol and retinal did not significantly reduce viral production which was less than 1 $\log_{10}$ lower than control values. β-Carotene, which is a precursor of retinoids, also did not inhibit HSV-1 growth. 4HPR was cytotoxic at the same concentrations used for the other compounds and at lower concentrations had no effect on HSV-1 replication. 4 MPR, which is the major metabolic product of 4 HPR in vivo (Formelli et al., 1993), was not cytotoxic but did not inhibit viral replication even at 10 μg/ml.

The yield of HSV-1 was reduced in the presence of all three isomers of retinoic acid (Fig. 1). All-*trans*-RA was the most effective isomer at low concentrations. At 1 μg/ml all-*trans*-RA, HSV-1 production was reduced by 90% over a 48 h period (Fig. 1a). Below 5 μg/ml, 9-*cis*-RA did not appreciably reduce viral titers and 13-*cis*-RA was less effective than all-*trans*-RA (Fig. 1b and c). 9-*Cis*-RA dependent HSV-1 inhibition followed a similar pattern to all-*trans*-RA at concentrations of 7.5 μg/ml and above, reducing the yield of HSV-1 by 4 $\log_{10}$, while 13-*cis*-RA was less effective than the other two isomers at 7.5 μg/ml. Concentrations of 10 μg/ml or higher of all three RA isomers effectively reduced the HSV-1 yield. Differences in efficacy seen at RA concentrations below 10 μg/ml were obscured. Increasing the concentration of 9-*cis*-RA to as high as 20 μg/ml did not further reduce viral yield indicating that HSV-1 production could not be completely eliminated. Viral inhibition was less pronounced below 5 μg/ml of 9-*cis*-RA and at 5 μg/ml there was a 1 $\log_{10}$ drop in viral titer. These differences were obscured at concentrations above 10 μg/ml.

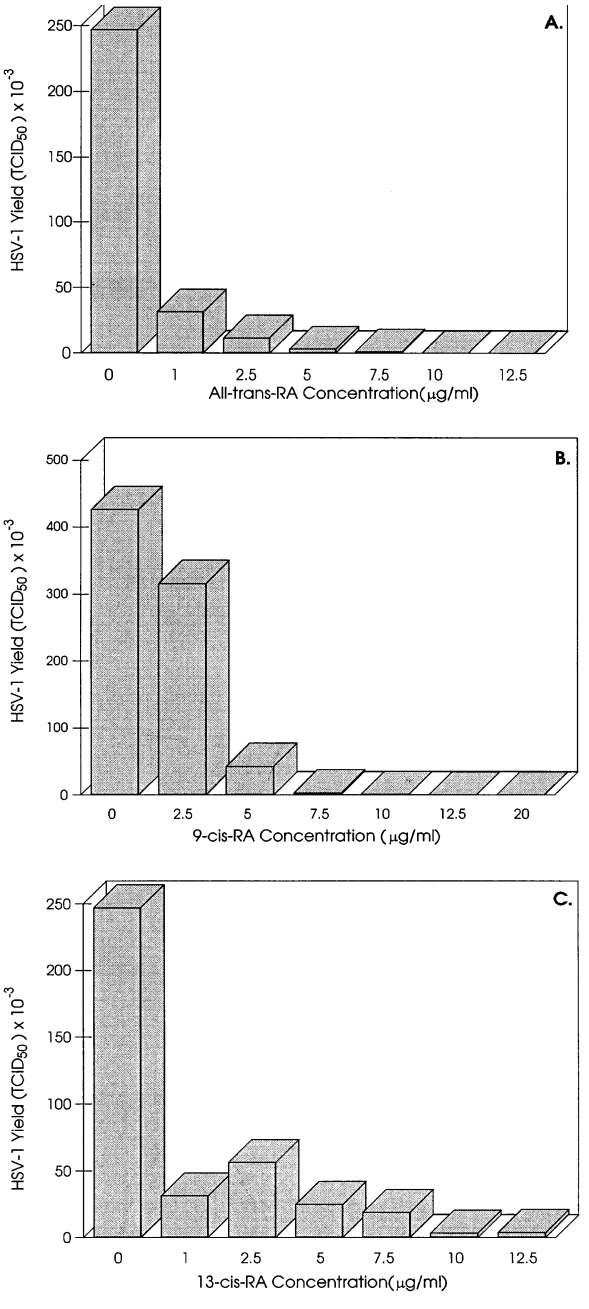

Fig. 1. Reduction of the yield of HSV-1 by varying concentrations of RA. (a) All-*trans*-RA. (b) 9-*cis*-RA. (c) 13-*cis*-RA. HSV-1 production was determined 48 h after viral infection of Vero cells.

The inhibition of virus replication was further investigated by immunofluorescence utilizing antibody to HSV-1 (Fig. 2). Cell monolayers which were not infected with virus did not show any immunofluorescence (Fig. 2a). When cultures were infected with HSV-1 and treated with all-*trans*-RA only small foci were observed in which the monolayer stained with bright green fluorescence indicating HSV-1 production (Fig. 2b). These foci may represent the residual HSV-1 infectivity found at a concentration of 10 μg/ml in Fig. 1a. The remainder of the monolayer which had normal cellular morphology was unstained and attached to the cover slip. The unstained cell monolayer in all-*trans*-RA treated samples was similar to cell monolayers which had not been infected with HSV-1 (Fig. 2a). In control cultures infected with HSV-1, but lacking all-*trans*-RA, all cells in the monolayer stained positive with a bright green fluorescence (Fig. 2c). These cells rounded and started to detach from the cover slip.

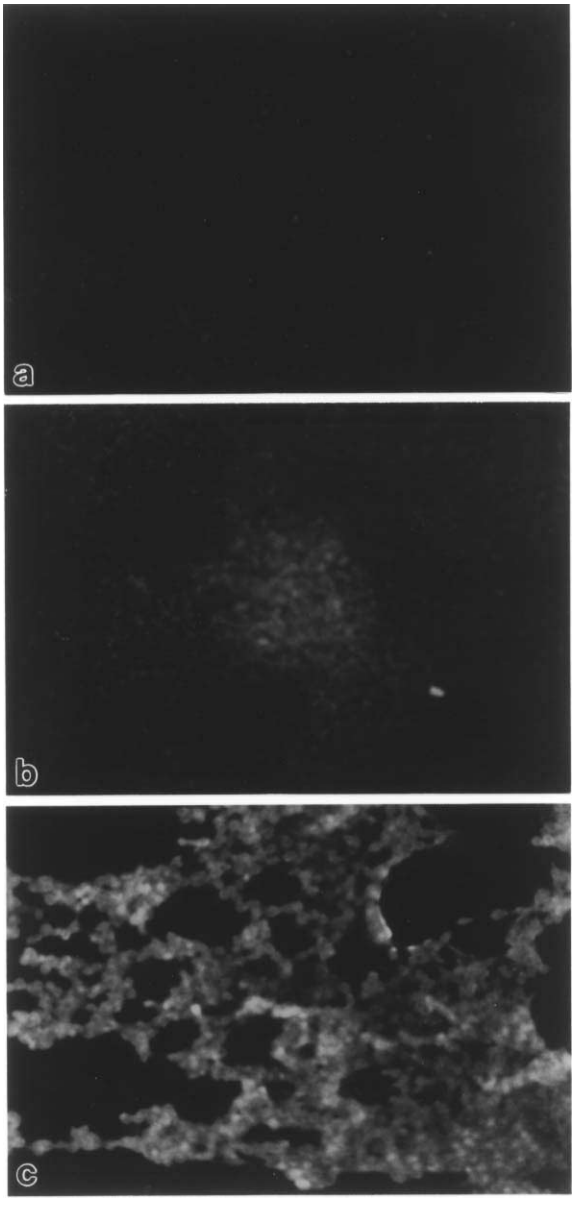

Fig. 2. Immunofluorescence staining of HSV-1 antigens produced in Vero cells. Detection of HSV-1 antigens resulted in bright green fluorescence. (a) Vero cell monolayer not infected with HSV-1. (b) Vero cells infected with HSV-1 and treated with all-trans-RA (10 μg/ml). (c) Vero cells infected with HSV-1, but not treated with RA. Viral growth was over a 48 h period. Final print magnifications are 100 ×.

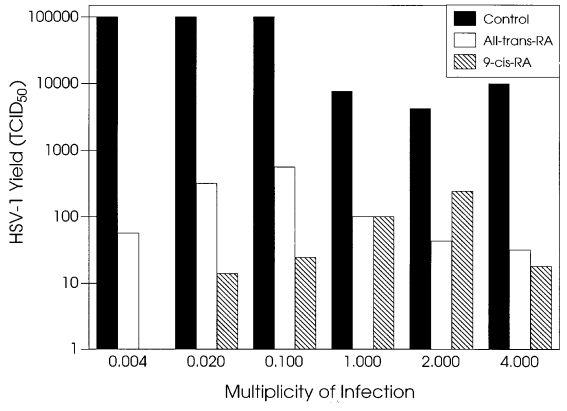

Fig. 3. Effect of the HSV-1 multiplicity of infection on all-*trans*-RA and 9-*cis*-RA inhibition of viral replication. RA (10 μg/ml) was added to Vero cells and HSV-1 production was measured after 48 h. The control virus titers produced at lower MOI (0.004–0.100) are greater than the control titers of higher MOI (1.000–4.000) as a result of being done as separate experiments. RA treated samples should be compared to their own controls.

Inhibition of HSV-1 replication was found at all multiplicities of infection tested (Fig. 3). At an MOI of 0.004, HSV-1 production was reduced by more than 3 $\log_{10}$ in the presence of all-*trans*-RA and no detectable virus was found with 9-*cis*-RA. Both all-*trans*-RA and 9-*cis*-RA inhibited HSV-1 production when the MOI was raised to as high as 4.0. Viral inhibition ranged from 2–3 $\log_{10}$ at each MOI tested above 0.004.

The presence of RA following viral adsorption to the cell monolayer was necessary to inhibit HSV-1 production. When all-*trans*-RA, 9-*cis*-RA or 13-*cis*-RA was added to cell cultures while they were dividing and forming the monolayer and then removed prior to viral infection, there was no reduction in the titer of HSV-1 produced (results not shown). These results are different from those found with interferon where prior exposure leads to an antiviral state which persists for several days following removal of interferon (Joklik, 1990). Experiments were then carried out to determine the effect on HSV-1 production of adding all-*trans*-RA with virus and removing it at varying times following viral inoculation. In addition, all-*trans*-RA was added at various times following virus adsorption (Table 2). When all-*trans*-RA was added up to 6 h after viral infection HSV-1 production was inhibited to the same extent as all-*trans*-RA addition immediately following virus inoculation. This resulted in a 4–5 $\log_{10}$ inhibition of viral production. Addition of all-*trans*-RA 24 h after infection resulted in 2.5 $\log_{10}$ virus production by 48 h and therefore a 2 $\log_{10}$ decrease in viral production as compared to control cultures. If all-*trans*-RA was added immediately following HSV-1 inoculation and then removed 1–3 h later no inhibition of viral production occurred after 48 h. Removal of all-*trans*-RA after 24 h resulted in a 2 $\log_{10}$ decrease in the titer of HSV-1 produced. Varying the time of addition and removal of all-*trans*-RA indicated that RA must be present simultaneously with the virus to inhibit its replication.

Table 2
Effect of the addition and removal of all-*trans*-RA[a] at different time points following viral adsorption on HSV-1 replication

	HSV-1 Infection (h)	$TCID_{50}$
Time of all-*trans*-RA addition	0	0
	3	0
	6	0
	24	$10^{2.5}$
Time of all-*trans*-RA removal	HSV-1 infection (h)	$TCID_{50}$
	0	$10^{4.8}$
	1	$10^{4.8}$
	3	$10^{4.6}$
	24	$10^{2.6}$
	Control[b]	$10^{4.8}$

[a]All-*trans*-RA was used at a concentration of 10 μg/ml following HSV-1 infection.
[b]All-*trans*-RA was not present in the controls. Virus production was determined after 48 h.

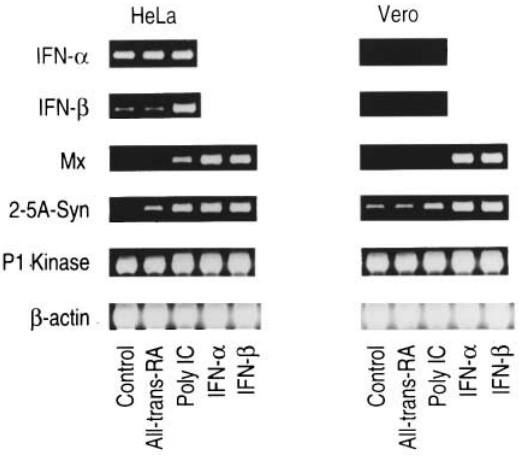

Fig. 4. All-*trans*-RA effect on expression of IFN and IFN-regulated genes. Cultures were either untreated or treated with all-*trans*-RA (12.5 μg/ml for 24 h), PolyIC (poly (rI)·poly (rC)) (50 μg/ml for 3 h), IFN-α (5000 U/ml for 4 h) or IFN-β (1000 U/ml for 4 h). Levels of IFN-α, IFN-β, Mx, 2′,5′-oligoadenylate synthetase (2–5A Syn) and P1/eIF-2 protein kinase (P1 kinase) were analysed in HeLa and Vero cells by RT-PCR. PolyIC was used as an IFN inducer. β-Actin served as an internal control. HeLa cells were used as a positive control for cells known to possess the genes for IFN-α and IFN-β.

In order to investigate the mechanism of inhibition of HSV-1 replication by all-*trans*-RA we have determined whether RA can increase the expression of type 1 IFN genes. IFN-α and IFN-β mRNA was not detected by coupled RT-PCR in Vero cells treated with all-*trans*-RA or the IFN inducer polyIC (Fig. 4). Interferon induction is therefore not the mechanism of all-*trans*-RA induced viral inhibition. However, to further explore the possible induction of interferon by all-trans-RA, similar studies were conducted in HeLa cells, in which interferon can be induced. IFN-α mRNA was found to be constitutively expressed in untreated HeLa cells and treatment

with RA or polyIC did not affect the level of IFN-α mRNA expression. HeLa cells showed constitutive expression of IFN-β message and treatment with RA did not alter the level of expression. PolyIC treatment of HeLa cells as expected resulted in increased IFN-β mRNA. All-*trans*-RA did not increase expression of three interferon regulated genes whose products cause inhibition of virus replication (Fig. 4). Treatment of Vero cells with all-trans-RA did not increase the expression of 2′,5′-*oligo*adenylate synthetase but it did increase expression in HeLa cells. PolyIC, IFN-α and IFN-β did increase expression of 2′,5′-*oligo*adenylate synthetase in Vero cells. All-*trans*-RA did not increase expression of the Mx gene in either Vero or HeLa cells, however, treatment of Vero cells with either type 1 IFN increased Mx gene expression. P1/eIF-2 protein kinase was constitutively expressed in Vero and HeLa cells and treatment with RA, poly IC or type 1 IFN did not alter kinase gene expression. These results suggest that RA inhibition of HSV-1 production is not by direct induction of IFN regulated gene products with an established antiviral function.

4. Discussion

The results presented in this study indicate that isomers of RA markedly reduce HSV-1 replication by a mechanism independent of the immune system. The specificity of inhibition of HSV-1 replication is such that retinol, retinal, 4 HPR, 4 MPR or β-carotene are ineffective. The presence of a carboxyl group appears to be needed for virus inhibition. In order for the other vitamin A derivatives and β-carotene to inhibit HSV-1 replication they would likely have to be converted to RA. At the concentrations employed in this study, RA was shown not to be cytotoxic nor interfere with cell metabolism. Inhibition appeared to be mediated by a specific effect of RA on the viral replication process. It is interesting that although retinol has been shown to bind to retinoic acid receptors (RARs) it did not inhibit HSV-1 replication (Repa et al., 1993). This may indicate that retinol binding to RARs does not activate the receptor or that RARs are not involved in inhibiting HSV-1 replication.

The immunofluorescence studies suggest that RA inhibits virus yield in most cells in the monolayer but that there are small foci which produce HSV-1 antigen. These foci may result from the initial successful infection of a cell by HSV-1 that overcomes RA inhibition. As virus is produced locally in high concentrations, the inhibition of virus replication in adjacent cells is overcome by the sheer local number of infectious virus particles. A similar situation is seen with interferon-dependent viral inhibition (Joklik, 1990). If the titer of virus is increased sufficiently, then virus replication overcomes inhibition by interferon. The remaining infectivity found after HSV-1 yield is reduced in the presence of RA is likely produced in the immunofluorescence positive foci. It cannot be determined from these results, however, whether virus yield per productively infected cell in RA treated cultures is comparable to per cell yield in untreated cultures.

Vero cells were chosen for these experiments because it had been reported that they lacked all type-I interferon genes (Wathelet et al., 1992; Diaz et al., 1988). This was confirmed in this study since we did not detect mRNA for α or β interferon by RT-PCR after induction with polyIC in Vero cells but α and βIFN mRNA was readily detected in HeLa cells. Our results showing that pretreatment of cells with all-trans-RA and its subsequent removal did not inhibit HSV-1 replication are consistent with a lack of IFN induction. It is thus possible that RA can regulate cellular antiviral pathways independently of IFN induction. Cytokines such as tumor necrosis factor-α have been shown to activate IFN regulated genes without using IFN as an intermediate (Wathelet et al., 1992). The level of expression of IFN regulated antiviral genes such as Mx, 2′,5′-*oligo*adenylate synthetase and P1/-eIF-2 protein kinase was not altered by RA in Vero cells. These three genes have been the best characterized as far as antiviral actions of IFNs involving a single cell response. It is possible however that RA induces other IFN dependent genes with antiviral activity that is not yet established.

RA has been found to control the transcription of cellular and viral genes through the RARs and retinoid X receptors (RXRs) which bind to consensus DNA sequences called retinoic acid response elements (RAREs). RAREs have been found in a number of viral genes which can be regulated by RA in transfection systems. RA activates the major immediate-early gene promoter of human cytomegalovirus (Ghazal et al., 1992), biphasically regulates hepatitis B surface antigen gene expression (Hsu et al., 1993), and activates the hepatitis B enhancer element (Huan and Siddiqui, 1992). RARs have been shown to bind to the long terminal repeat (LTR) region of HIV-1 (Orchard et al., 1993).

The ability of RARs and RXRs to bind to RAREs identified in viruses is not necessarily sufficient to modulate transcription of viral genes. Presence of an RAR and RXR binding site in the HIV-1 LTR region did not confer retinoic acid responsiveness in F9 cells (Orchard et al., 1993). The opposite is also true. In the absence of identifiable RAREs, RA can affect the rate of viral transcription. Both the simian immunodeficiency virus and HIV-1 LTRs show increased transcription after exposure to retinoic acid in U937 cells even when RAREs are not present (Maciaszek et al., 1994). RA has been shown to inhibit reactivation of Epstein-Barr virus as the result of protein interaction between RAR or RXR and the viruses' immediate early protein (Sista et al., 1993).

These results suggested that the ability of RA to affect transcription of viral genes and subsequent virus production is more complex than can be predicted by the presence or absence of a RARE. In fact, increased transcription of HIV mRNA has been shown after exposing HL-60 cells to retinoic acid but decreased mRNA translation blocked HIV replication (Semmel et al., 1994).

Whether RA will inhibit or enhance replication of a particular virus appears to depend on a number of factors. Different studies with HIV produce conflicting conclusions. In some studies RA treatment inhibits HIV replication (Nakashima et al., 1987), whereas in other studies RA increases the number of HIV particles produced in each infected cell (Turpin et al., 1992). Some studies show both increased and decreased viral replication in the same cell line depending on whether RA is added before or after viral infection (Poli et al., 1992). The ability of RA to decrease, increase or have no effect at all on HIV replication may be explained by differences in host cell type and the expression of RARs and RXRs (Yamaguchi et al., 1994). However, there is no simple explanation for the different results based on the time of RA addition relative to HIV infection of the cell cultures. It may be due to indirect effects on viral replication by activation or inhibition of other cellular genes.

The time of RA addition to cell cultures was critical for HSV-inhibition. When cells were grown in the presence of RA and the RA removed before HSV-1 infection, viral replication was unaffected. The presence of RA simultaneously with HSV-1 was necessary for inhibition of viral replication. If RA was added after viral infection, viral inhibition started at the time of RA addition. When RA was removed from infected cultures virus inhibition was also removed. Inhibition of HSV-1 replication requires the presence of RA. This is in distinction to studies with HIV in which exposure of cells to RA prior to virus infection increased HIV replication despite the absence of RA in the culture medium after viral infection (Turpin et al., 1992).

There have been reports in the literature that RA can reduce the severity of HSV-1 infections in the eye. The course of HSV-1 ocular infections in vitamin A deficient rats was more rapid and the disease more severe (Nauss et al., 1985). Studies using rabbits which were not RA deficient but infected in the eye with HSV-1 showed that vitamin A injections resulted in milder more rapidly healing epithelial lesions (Smolin et al., 1979). The ability of retinoic acid to decrease HSV-1 replication—as shown in the present study—would be important since HSV is the most common cause of infectious blindness in the developed world (Kohl, 1994). There have not been any reports in the literature suggesting that retinoic acid or other vitamin A derivatives stimulate HSV-1 replication in comparison to the conflicting results with HIV.

The ability of RA to reduce the rate of herpes simplex virus replication could be important not only for decreasing the incidence and severity of ocular infections, but also for reducing the vertical transmission of HSV from mother to infant. The risk of vertical HSV transmission is approximately ten times higher in women with primary or initial genital infection than in women with recurrent infection (Whitley, 1994). The use of RA or other retinoid derivatives, possibly in the form of a topical virucide, could potentially reduce the rate of HSV excretion during birth and have a major impact on HSV transmission since 85% of neonatal HSV infections are acquired during the intrapartum period. Additionally, if RA can reduce HSV replication, it could play a role in reducing the incidence of sexually transmitted herpes infection. Studies are needed to determine whether HSV excretion can be reduced in vivo using all-*trans*-RA or another RA isomer.

In summary, RA reduces HSV replication without inducing IFN or the IFN-regulated genes measured. Appropriate use of RA at mucosal surfaces could reduce the spread of herpes simplex virus and lessen the severity of disease by holding the virus in check as a supplement to other defense mechanisms.

Acknowledgements

This work was supported in part by a grant from the National Institute of Allergy and Infectious Disease (AI 39061). We thank Dr. Giuseppe LaFauci for designing some of the primers used in this study and for his helpful discussions, Premila S. Pullarkat for technical assistance, Richard Weed for photographic supervision and Anna Parese for preparing the manuscript.

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