Rose Bengal Inhibits Herpes Simplex Virus Replication in Vero and Human Corneal Epithelial Cells In Vitro

James Chodosh, M. Careene Banks, and William G. Stroop

Rose Bengal dye is thought to highlight corneal lesions induced by herpes simplex virus type 1 (HSV-1) by virtue of its binding to dead or dying HSV-1-infected epithelial cells. However, whether rose Bengal binds specifically to damaged versus normal corneal epithelial cells is unclear. To determine the binding properties of rose Bengal, the authors compared binding of the dye to HSV-1-infected and uninfected cells, determined the cellular binding sites of the dye, and investigated the effects of rose Bengal on HSV-1 replication in dye-treated cells in vitro. Spectrophotometric analysis revealed that uninfected and infected Vero cells bound equivalent amounts of dye at several times post inoculation, indicating that rose Bengal does not preferentially bind to HSV-1-infected cells. By light microscopy, rose Bengal was found to bind to the cell nuclei and perinuclear region of human corneal epithelial cells (HCEC) and Vero cells. Pretreatment of Vero and HCEC with different concentrations of rose Bengal and exposure to 148 \( \mu \text{W/cm}^2 \) of white light for 2 min reduced the ability of both cell types to support HSV-1 replication. Vero cells, in the absence of rose Bengal, supported HSV-1 replication, whereas pretreatment with 0.05% rose Bengal reduced the yield of HSV-1 by 99.99% \( (P < 0.000001) \) and 1% rose Bengal completely prevented HSV replication. HCEC supported HSV-1 replication in the absence of rose Bengal, but pretreatment with 1% or 0.05% rose Bengal completely prevented HSV-1 replication \( (P < 0.000001) \). In a dose-response study, treatment with greater than or equal to 0.06% rose Bengal was found to significantly reduce the yield of HSV-1 in Vero cells. In vitro, rose Bengal binds equally well to HSV-uninfected and infected cells, binds primarily to the nuclei of cells, and inhibits HSV-1 replication in cells at very low concentrations. Invest Ophthalmol Vis Sci 33:2520-2527, 1992

Herpes simplex virus type-1 (HSV-1), a double-stranded, enveloped DNA virus, is a leading infectious cause of corneal blindness in the United States. Rose Bengal dye is a fluorescein derivative used in clinical ophthalmologic practice to visualize many types of ocular surface disease, but is especially useful for the identification of herpes simplex epithelial keratitis.

Harnisch et al. have demonstrated by scanning electron microscopy that the dendritic pattern of herpetic keratitis apparently results from a combination of the spread of viral-induced cytopathic effect and epithelial cell movement. Clinically, rose Bengal highlights the edge of the dendritic ulcer and is believed to stain dead and devitalized corneal and conjunctival epithelial cells.

As a first step toward discernment of the differential adherence of rose Bengal to normal versus viral-infected corneal epithelial cells, we compared binding of the dye to infected and uninfected epithelial cells in vitro. We then examined epithelial cell dye-binding sites by light microscopy and investigated the effect of rose Bengal on HSV-1 replication.

Materials and Methods

Virus

The H129 strain of HSV-1 was used for all experiments. H129 was originally isolated in 1977 from a fatal case of herpes encephalitis by Klassen, Stroop, and Baringer. Dix, et al have shown that H129 is virulent in mice following peripheral infection, and using thymidine kinase assays, we have shown that H129 is
TK+ (data not shown). In a rabbit model of acute and immunosuppression-induced reactivated infections, H129 causes severe epithelial keratitis and focal temporal lobe necrotizing encephalitis.5–7

Cells

Vero cells, a continuous line of green monkey kidney cells, and human corneal epithelial cells (HCEC) were used in these studies and were maintained at 37°C in 5% CO2 and 95% humidity. HCEC were prepared from a pair of corneas from an 83-year-old woman, obtained from the Lion’s Eye Bank of Texas (Houston). The corneas were trimmed to remove the sclera, and three radial slits were made to allow the cornea to lie flat, endothelial side down. Corneal epithelial cells were scraped from the cornea using a no. 22 belly blade scalpel, vigorously resuspended, and explanted in MEM-10 OS that contained minimal essential media (MEM; Gibco, Gaithersburg, MD) and 10% heat-denatured fetal calf serum (FCS), and was supplemented with 100 μg/ml of gentamicin, 100 U of penicillin, 100 μg/ml of streptomycin, 10 ng/ml of mouse epidermal growth factor (EGF; Sigma Chemical Co., St. Louis, MO), and 5 μg/ml of insulin (Sigma). HCEC were maintained in MEM-10S for 1 wk, whereupon the media was changed to MEM-10 that contained 10% FCS plus all the supplements, except insulin and EGF. At confluence, HCEC were maintained in MEM-2 that contained 2% FCS plus all the supplements except insulin and EGF. HCEC cells were split at a ratio of 1:3 in MEM-10. HCEC used for the experiments reported here were used at the third subpassage.

Spectrophotometric Analysis of Rose Bengal Binding

Twenty-five cm² flask cultures of Vero cells were washed once with Hank’s salt solution (HBSS; Gibco) and infected at an approximate multiplicity of infection of 10 by addition of 7 × 10⁴ tissue culture infectious doses (TCID) of H129 suspended in 1 ml of HBSS. Control cell cultures were mock infected with 1 ml of HBSS without virus. After an adsorption period of 1 hr, cultures were washed twice with 5 ml of HBSS and incubated at 37°C in 5% CO2 and 95% humidity. At 2, 6, and 12 hr post infection, triplicate cultures of infected and uninfected cells were stained with 0.016% (150 μmol/l) rose bengal (N17478-261-10; Akorn, Inc., Abita Springs, LA), rinsed three times with 10 ml of normal saline, and either scraped or trypsinized from the flasks. The cells then were centrifuged at 500 × g for 5 min, lysed in 5 ml of 50% ethanol in normal saline for 1 min at room temperature, and centrifuged at 500 × g for 5 min. The absorbance at 550 nm was measured on 1 ml of the supernatant from the lysed cells.

Rose Bengal Application Prior to HSV-1 Inoculation

Twenty-five cm² flask cultures of Vero or HCEC were washed once with HBSS and stained for 1 min under ambient light conditions with 1% rose bengal (9.5 mmol/l) or with rose bengal diluted in HBSS to a final concentration of 0.05% (469 μmol/l). Control cell cultures were mock stained with HBSS. Cultures then were washed three times with 5 ml of HBSS per wash to remove unbound rose bengal and then infected at an approximate multiplicity of infection of 1.0. After an adsorption period of 1 hr, infected and control cultures were washed twice with 5 ml of HBSS, incubated at 37°C in 5% CO2 and 95% humidity, and later frozen at −70°C when maximum virus-induced cytopathic effect was observed. All cultures were exposed to incident room light (148 μW/cm²) for approximately 2 min while being stained and infected, and were washed prior to incubation and freezing. Frozen cultures of Vero or HCEC later were thawed to room temperature and the culture fluid was centrifuged at 500 × g to clarify them of cell debris. Cell-free supernatants were titered in triplicate in 96 well microtiter plates that contained confluent Vero cells. In an additional experiment to generate a dose-response curve, Vero cells were treated in the dark with serial dilutions of rose bengal; after a wash, viral adsorption, and another wash, but prior to incubation, the cells were exposed to 6.528 W/cm² of white light for exactly 2 min.

Results

Rose Bengal Binds Equally to Infected and Uninfected Cells

In clinical practice, rose bengal preferentially stains HSV-1-infected corneal epithelium as opposed to adjacent, uninvolved epithelium. To determine whether rose bengal bound selectively to HSV-1-infected cells, cells were infected at a multiplicity of 10. Infected and uninfected monolayer cultures of Vero cells then were stained at 2, 6, and 12 hr post inoculation (or mock inoculation), and the amount of bound stain at each time was measured spectrophotometrically. We chose 0.016% (150 μmol/l) rose bengal as the concentration of dye to stain cells because this was the lowest concentration that reproducibly stained uninfected Vero cells (data not shown). We found that rose bengal did not selectively stain infected versus uninfected epithelial cells (Fig. 1). The amount of rose bengal that bound to infected cells was overall more variable than that bound to uninfected cells (Fig. 1). This effect...
Fig. 1. Binding of rose bengal to vero cells at 2, 6, and 12 hr after infection with HSV-1. The amount of dye that bound to uninfected control (hatched bars) and infected cells (solid bars) was equivalent at all times after infection. Error bars indicate the standard error of the mean of cultures assayed in triplicate at each time point.

probably was a result of the lytic effect of HSV, which affected the total number of cells available for assay. This was especially evident at 12 hr after infection (Fig. 1), when most of the infected cell monolayer exhibited extensive cytopathic effect. However, there was no statistical difference between the groups regarding bound stain, regardless of the time after inoculation (Fig. 1). In a separate experiment, we found that 0.016% rose bengal coupled with exposure to ambient room light did not irreversibly harm the cells. Cells were stained, washed three times to remove unbound dye, and returned to the incubator. These cells were monitored microscopically for 3 d. The cells did not detach from the flask and continued to divide. We did not assess any long-term effects (beyond 3 d) on cell survival.

Rose Bengal Binds to Cell Nuclei

Because the spectrophotometric study demonstrated that rose bengal bound to uninfected cells, we wished to determine the subcellular site stained by the dye. Uninfected Vero cells and HCEC were stained with 0.016% (150 µmol/l) rose bengal, washed three times, and examined microscopically. We found that the majority of the dye bound to cellular nuclei of both cell types, but the perinuclear region of the cell also was stained (Fig. 2). This was more obvious in HCEC, because these cells had a greater proportion of cytoplasm, allowing easier visualization of the perinuclear region. The cytoplasm of both cell types was nearly devoid of stain.

Although we did not perform detailed timed studies, we noted that the adherence of rose bengal to epithelial cell nuclei was very rapid. The shortest length of time used to stain uninfected cells was 1 min, and...
this was sufficient to stain cell nuclei. In other experiments, we allowed the dye to flow over monolayers of cells while they were in place on the microscope stage. The dye stained the cells almost immediately.

**Rose Bengal Inhibits HSV-1 Replication in Epithelial Cells**

Because rose bengal is a photoreactive compound that appeared to preferentially bind to cell nuclei, and because HSV is a DNA virus that replicates in cell nuclei, we investigated whether rose bengal would directly interfere with HSV replication in Vero cells and HCEC. In particular, we wished to determine whether pre-treatment of cells with rose bengal and exposure to light would interfere with HSV-1 replication. This (pre-treatment) approach was taken because the replication of HSV is quite rapid (about 9–12 hr) and we wanted to maximize any effect of the dye. Triplicate cultures of cells were pre-treated with one of three different concentrations of rose bengal, exposed to ambient light (148 μW/cm²) for 2 min, washed to remove unbound dye, and then infected with HSV-1. Triplicate cultures of uninfected cells served as con-

![Fig. 3](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933394/) Cytopathic effect induced in vero cells by rose bengal and HSV-1 infection. Cells were unstained (A and D), or stained with 0.05% (B and E) or 1% (C and F) rose bengal, exposed to 148 μW/cm² of white light for 2 min, washed, inoculated with HSV-1 (D, E, and F) or left uninfected (A, B, and C), and photographed 3 d after infection. Typical HSV-1-induced cytopathology was seen in the absence of rose bengal (D), whereas the combination of rose bengal and HSV-1 infection caused vero cells to develop an unusual appearance (E and F). Virus production from these cells is shown in Figure 5. Bars = 75 μm.
trols. All cultures were monitored for the development of any cytopathic effect and were harvested for virus titration when maximum cytopathic effect was observed in the virus-infected cultures.

In Vero cells, viral-induced cytopathic effect was seen at 3 d post-infection only in those cells infected with virus but not pre-treated with rose bengal (Fig. 3D). HCEC developed virus-induced cytopathic effect by 5 d post-infection only in those cells infected with virus but not pre-treated with rose bengal (Fig. 4D). By the microtiter assay, Vero cells in the absence of rose bengal supported HSV-1 replication, whereas pre-treatment with 0.05% (469 µmol/l) rose bengal reduced the yield of HSV-1 by 99.99% (from 10^8 TCID/ml to 10^4 TCID/ml; P < 0.000001). One percent (9.5 mmol/l) rose bengal completely prevented HSV replication (Fig. 5). HCEC supported HSV-1 replication in the absence of rose bengal (10^4 TCID/ml), but pre-treatment with 1% (9.5 mmol/l) or 0.05% (469 µmol/l) rose bengal completely prevented HSV-1 replication (P < 0.000001; Fig. 5).

Of interest was that the appearance of the cytopathic effect seen in Vero cells and HCEC infected with HSV-1 and treated with rose bengal was not typi-

Fig. 4. Cytopathic effect induced in human corneal epithelial cells by rose bengal and HSV-1 infection. Cells were unstained (A and D), or stained with 0.05% (B and E) or 1% (C and F) rose bengal, exposed to 148 µW/cm² of white light for 2 min, washed, inoculated with HSV-1 (D, E, and F) or left uninfected (A, B, and C), and photographed 5 d after infection. Note that the cytopathology induced by HSV-1 infection in the absence of rose bengal (D) was different from the cytopathology induced by 0.05% rose bengal and HSV-1 (E). Cells treated with 1% rose bengal before infection (F) did not develop cytopathic effect. Virus production from these cells is shown in Figure 5. Bars = 75 µm.
yield of HSV-1 (99.9% reduction at 0.06% rose bengal; Fig. 6).

**Discussion**

In ophthalmology, rose bengal is used to aid in the diagnosis of various disorders of the ocular surface. How rose bengal stains epithelial cells remains uncertain, but uncontrolled observations with biomicroscopy of the human cornea as well as light microscopy of conjunctival epithelial scrapings have suggested that rose bengal stains epithelial cell nuclei to a greater degree than cytoplasm. Our in vitro studies with Vero and HCEC in monolayer culture confirm the finding that rose bengal binds predominantly to epithelial cell nuclei. Our studies do not support the clinical impression that rose bengal binds preferentially to dead or devitalized epithelial cells.

We hypothesize that the staining of abnormal corneal epithelium seen in vivo, such as in herpetic epithelial keratitis and keratoconjunctivitis sicca, is a result of an alteration in the relationship between corneal epithelium and tear film and may only indirectly reflect the vitality of the cells. In vitro, dynamic epithelial cell-tear film interactions are absent, and epithelial cells stain uniformly. This postulate must be viewed with some caution, because there are other...

**Fig. 6. Dose response curve of rose bengal.** Vero cells were treated with serial dilutions of rose bengal and exposure of 6.528 W/cm² of white light for 2 min (54.4 mW/cm²/sec). Pre-treatment with ≥0.06% (595 μmol/l) rose bengal significantly reduced the yield of HSV-1. Error bars indicate the standard errors of the means of cultures titered in triplicate at each concentration of dye.
factors in vivo not present in cell culture. For example, the multiple layers of corneal epithelial cells, the state of epithelial cell differentiation, the surface temperature, and the movement of the eyelids and globe also may affect the binding of rose bengal. However, propagation of corneal epithelial cells in vitro produced cells of variable age, and the cells were stained at room temperature, so these factors are relatively less likely to be involved. We believe it is more likely that the relationship between the surface layer of epithelial cells and the overlying tear film in vivo is central to preventing rose bengal from staining the non-diseased cornea. When this relationship is altered by a change in the quality or quantity in the tear film or by intrinsic ocular surface disease, the dye can bind to the affected site.

Rose bengal is tetrachlorotetraiodo-fluorescein. When combined with 550 nm wavelength light and oxygen, rose bengal generates singlet oxygen.\(^{10,11}\) Singlet oxygen can inactivate enzymes and damage single-stranded DNA\(^{14,15}\) and cell membranes,\(^{16,17}\) and it is believed to be the toxic product of light-activated rose bengal that kills a variety of microorganisms, including viruses,\(^{18}\) bacteria,\(^{19,20}\) and protozoa.\(^{21}\) Roat et al demonstrated the ability of rose bengal to reduce HSV-1 infectivity in an experiment in which HSV was mixed directly into serial dilutions of rose bengal.\(^{22}\) They also showed in a mouse herpetic keratitis model that the application of rose bengal prior to viral culture markedly reduced the incidence of positive culture, whereas fluorescein, a poor producer of singlet oxygen, did not significantly alter positive culture results. This suggests that the predictive value of a negative ocular surface culture for herpes simplex may be significantly lowered by the application of rose bengal prior to culture. Rose bengal application for demonstration of corneal epithelial morphology may be inappropriate in the laboratory animal or human research subject with HSV epithelial keratitis, unless applied at the endpoint of the study.

Our experiments show that, depending on the concentration of the dye employed, pre-treatment of Vero cells and HCEC in monolayer culture with rose bengal reduces or eliminates their capacity to support HSV-1 replication. Of interest was that Vero cells and HCEC treated with rose bengal before HSV-1 infection developed an unusual morphology that was distinct from that seen after virus infection alone. Because this unusual cytopathology was not seen in rose bengal-treated but uninfected cells, the effect was not a result of rose bengal and light exposure alone. At higher doses of rose bengal pre-treatment (1%), this effect was still apparent in Vero cells but hardly noticeable in HCEC infected with HSV-1. HCEC pre-treated with 1% rose bengal prior to virus infection appeared nearly identical to nonstained, uninfectcd HCEC. We speculate that the cytopathology induced in both cell types by rose bengal treatment prior to HSV-1 infection is a result of the combined effects of rose bengal phototoxicity on cell nuclear processes and virus-induced shutdown of host macromolecular synthesis.

We did not study the effect of rose bengal on established infection. Therapeutic trials of photoreactive dyes for herpetic keratitis\(^{23-27}\) were largely abandoned after O'Day et al in 1975 reported several cases of severe generalized epithelial erosions and iritis after the application of proflavine and light for treatment of herpetic epithelial keratitis.\(^{28}\) The subsequent development of safe and efficacious medications such as acyclovir and trifluridine have eliminated much interest in this direction. Phototherapy for herpes genitalis was similarly abandoned\(^{29}\) after a large, prospective, masked study of neutral red failed to show any benefit.\(^ {30}\) In addition, the application of photodynamic therapy to herpetic infection poses a theoretical risk of malignancy with those dyes known to intercalate into viral DNA.\(^{31-33}\) HSV, inactivated by intercalating dyes and light exposure, although no longer infectious, has been reported to cause tumors when injected into laboratory animals.\(^ {34}\) In addition, a few patients treated with neutral red and light for herpes progenitalis later developed Bowen's disease.\(^ {35}\) Rose bengal does not intercalate into DNA and it inactivates biomolecules via singlet oxygen. Therefore, it may pose less of a risk of oncogenesis than previously used photoreactive agents.

In summary, we found that rose bengal binds equally well to HSV-infected and uninfected Vero cells in monolayer culture. We then observed that rose bengal binds preferentially to the nuclei of Vero and HCEC in vitro. With the knowledge that HSV replicates within cell nuclei, we pre-treated these cells with rose bengal and then inoculated them with HSV. Depending on the concentration of dye employed, pre-treatment of Vero or HCEC with rose bengal significantly reduced their capacity to support HSV-1 replication. We conclude that nuclear-bound rose bengal alone can prevent herpes simplex viral replication in vitro and may have the same capacity in vivo.

**Key words:** herpes simplex virus, herpessvirus, HSV-1, phototherapy.

**Acknowledgments**

We wish to thank Alex Kogan and Gilma Miranda for photographic assistance, Drs. Kirk Wilhelmus and Alice Matoba for critical review of this manuscript, and Dave Beers and Jeng-Yang Ling for helpful suggestions.

**References**


3. Product insert: Rose Bengal 1% Sterile Ophthalmic Solution, Akorn, Inc., Abita Springs, LA.


