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Assessment of Antiviral Activity, Efficacy, and Toxicity of Prostaglandin A_2 in a Rabbit Model of Herpetic Keratitis

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Prostaglandin A_2 (PGA_2) inhibited the replication of herpes simplex virus type 1 in rabbit and human cornea stromal cells at concentrations of 1 to 5 μM while causing significant toxicity at 55 to 150 μM. Despite favorable therapeutic indices in cultured cells, PGA_2 was not effective as a therapeutic agent in the treatment of herpetic keratitis in a rabbit model. The sequelae of disease appeared more severe in animals receiving PGA_2 than in untreated or placebo-treated controls. The recovery of virus from tissues of latently infected rabbits was not affected by therapy. PGA_2 therapy alone induced breakdown of the blood-aqueous barrier, indicating that pharmacologically active concentrations of drug were achieved in the eye. Thus, PGA_2 had antiviral activity, but its proinflammatory effects appeared to be more detrimental than beneficial in the treatment of herpetic keratitis.

Prostaglandins are a group of molecules capable of producing highly diverse responses in a tissue-specific and/or cell-type-specific manner (10). These endogenously synthesized products of the arachidonic acid cascade are produced at sites in tissues in response to a variety of stimuli (11). The mechanism(s) by which prostaglandins function are far from clear and are likely to be as diverse as the responses they induce (7).

Cyclopentenone prostaglandins of the A series (PGAs) have been reported to have antiviral activity at concentrations which do not alter cell viability (17). Several features of this group of prostaglandins make them attractive as antiviral agents. The derivatives are physiologically stable in pharmacological formulations (4). The antiviral activity is, in many cases, broad spectrum in nature, including both RNA and DNA viruses (16); for example, the retrovirus human immunodeficiency virus and DNA viruses of the human herpesvirus family, such as herpes simplex virus (HSV), are inhibited from replicating in vitro by micromolar concentrations of PGAs (3, 23). The extent to which the various PGAs demonstrate antiviral activity appears to depend on subtle structural requirements associated with the molecule's cyclopentenone ring (23). The specific mechanisms responsible for the antiviral activities have not been specifically defined, but at least two general mechanisms must exist. In some cells, the key event appears to be associated with the production of heat shock protein 70 (HSP70) (16). In other cells, the activity is not actinomycin D-dependent and thus appears not to be related to the activities of inducible proteins (15). The antiviral activities of these compounds have rarely been evaluated in animals, and as far as we are aware, their anti-herpesvirus activity has not been evaluated with animal models.

PGAs are of particular interest for the treatment of ophthalmic viral disease because they can be used therapeutically to reduce intraocular pressure (22). The rabbit model of herpetic keratitis provides a useful system for evaluation of the therapeutic antiviral activity of PGAs because of the similarity of the disease to human disease and because of the sensitivity of rabbit cells to the antiviral effects of PGA_2 (12). In addition, rabbits are very sensitive to other physiologic effects of prostaglandin treatment. Effects such as flare, indicating increased protein content in the aqueous chamber, can be scored by an observer, thus providing a means of assessing the delivery and pharmacological activity of the drug. It is also of interest that functional sensory neurons of the trigeminal ganglia, a site of HSV latency, appear to be required for prostaglandins to exert many of their effects in eyes (19).

In the studies reported here, we document that PGA_2 has antiviral activity in both rabbit and human cornea stromal cells in culture at a concentration severalfold less than that required to produce measurable cytotoxicity. In the rabbit, however, PGA_2 did not induce a beneficial therapeutic response despite the fact that pharmacologically active concentrations were achieved within the eye, as evidenced by a dose-dependent flare response. In fact, the sequelae of disease were significantly worse in rabbits receiving PGA_2 than in those receiving the placebo.

MATERIALS AND METHODS

Virus. In all studies, the McKrae strain of HSV-1 was used. The original stock was a gift of M. Trousdale (Doheny Eye Institute, Los Angeles, Calif.). Stock cultures were prepared in cultures of the rabbit kidney cell line RK-13.

Cells. Cultures of rabbit and human cornea stromal cells were prepared from collagenase-digested corneal stroma (14). Briefly, the corneal epithelium and endothelium were removed from freshly excised rabbit and human donor corneas. Human tissue was obtained from the Wisconsin Lions Eye Bank. The rabbit corneas were obtained from eyes enucleated by a local rabbit market. The stromal layer was digested with 150 IU of collagenase (EC 3.4.2.4.3; Gibco-BRL, Gaithersburg, Md.) per ml for 16 to 20 h at 37°C. The stromal cells were recovered by centrifugation at 800 × *g* for 10 min and seeded into a tissue culture flask (Costar, Park Ridge, Ill.). Cells were allowed to grow to confluence and passaged at a 1:2 split ratio for four to six passages, at which time, the cells were used in either antiviral or cytotoxicity assays. Rabbit and human stromal cells were grown in Dulbecco's modified Eagle's medium (Gibco-BRL) containing 100 U of penicillin G per ml, 100 mg of streptomycin per ml, and 0.25 μg of amphotericin B per ml, and supplemented with 5% fetal bovine serum (Hyclone, Logan, Utah), 0.1% Mitoplus serum extender (Collaborative Research, Inc., Bedford, Mass.), 0.28 mg of glutamine (Sigma Chemical Co., St. Louis, Mo.) per ml, and 0.45 mg of glucose per liter. Medium for human cells also contained 50 μg of gentamicin (Gibco-BRL) per ml. Cultures were routinely screened for mycoplasma with Hoechst's stain (ICN Biomedical, Inc., Aurora, Ohio) after growth in medium without antibiotics.

Antiviral activity. The antiviral activity of PGA_2 (Cayman Chemical Co., Ann

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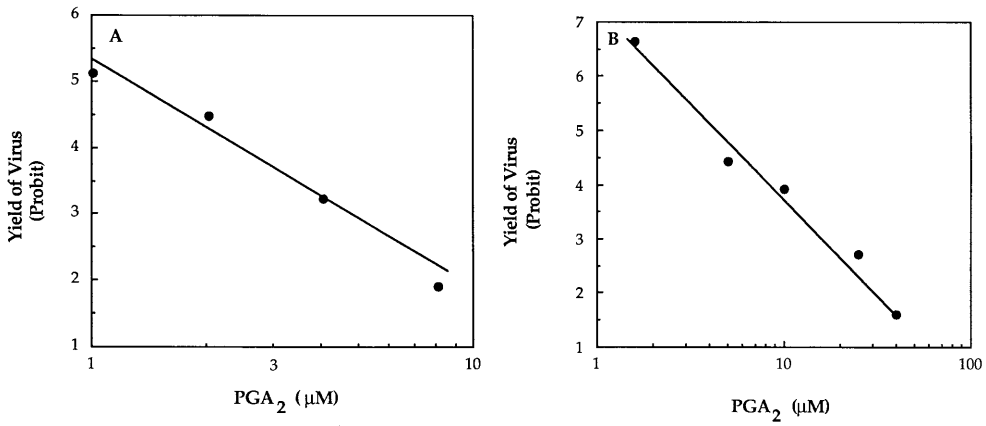

FIG. 1. Measurement of the antiviral activity of PGA_2 in rabbit and human cornea stromal cells. Rabbit (A) and human (B) corneal cells were infected with HSV at a multiplicity of infection of 5 PFU per cell and treated with various doses of PGA_2. At 24 h after infection, cultures were frozen and then thawed, and virus yields were measured by plaque formation on Vero cells. Virus yields in untreated human stromal cells were $(4.9 \pm 1.8) \times 10^7$ PFU/ml, and those in rabbit stromal cells were $(4.9 \pm 0.4) \times 10^6$ PFU/ml. The yield of virus as a percentage of that of the untreated control was plotted as a probit versus the log of the dose of PGA_2. Each datum point represents the average of three to six determinants.

Arbor, Mich.) was determined in vitro with yield reduction assays as previously described (20). Stock solutions of PGA_2 were prepared in ethanol. Aliquots of stock were dried under nitrogen and suspended in cell culture medium. Stromal cells were seeded into culture plates at 10^5 cells per cm^2. Twenty-four hours later, the confluent cell layers were infected with HSV-1 at a multiplicity of infection of 5 PFU per cell, and after a 1-h adsorption, the unadsorbed virus was removed and medium containing PGA_2 at various concentrations was added. After 24 h, cultures were frozen and thawed, and the lysate containing virus was assayed in triplicate for infectious virus by plating on Vero cells. The purity and stability of PGA_2 were evaluated spectrophotometrically as described below.

Cytotoxicity. Cytotoxicity in the cell culture was determined by treating triplicate confluent cultures of stromal cells with various amounts of PGA_2. Cultures were observed by phase-contrast microscopy several times during the 24 h after treatment. After 24 h of exposure to PGA_2, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT; Sigma Chemical Co.) in phosphate-buffered saline was added to cultures to achieve a final concentration of 0.25 mg/ml. Cultures were incubated for an additional 4 h at 37°C, the medium was decanted, and 3.0 ml of 0.04 N HCl in isopropanol was added to solubilize the reduced MTT-formazan. After extraction with the acidic isopropanol, the optical density at 570 nm was measured. The percentage of MTT-formazan in treated cultures relative to that in untreated cultures was determined (9), and the dose of PGA_2 required to cause a 50% reduction in the amount of reduced MTT was used as a measure of toxicity.

Animal model and evaluation of therapy. The animal model of acute disease was similar to that originally used by Wander et al. (21). New Zealand White rabbits (body weight, 2.3 to 2.7 kg), free of preexisting corneal defects, were sedated and anesthetized by intramuscular injection of 25 mg of ketamine-HCl (Fort Dodge Laboratories, Inc., Fort Dodge, Iowa) per kg, 5.0 mg of xylazine (Phoenix Pharmaceuticals Inc., St. Joseph, Mo.) per kg, and 1 drop of 0.5% proparacaine (Alcon, Humacao, Puerto Rico) per eye. Rabbits were infected bilaterally with 10^6 PFU of the McKrae strain of HSV-1 after scarification of the corneas with five overlapping circles with a 5.5-mm-diameter trephine set at a depth of 0.5 mm. Animals were assigned randomly to treatment groups, and therapy was initiated on the third day after infection and continued through the seventh day after infection.

The drug for therapy was prepared by dissolving PGA_2 in ethanol. Aliquots of the ethanol solutions were dried, and the prostaglandin was resuspended in an ophthalmic vehicle containing 0.5% hydroxypropyl methylcellulose 2910, 0.01% benzalkonium chloride, and sodium phosphate buffer (pH 7.0). A similar formulation has been used to provide stable PGA_2 for study of the physiologic effects of PGA_2 (4). The concentration and stability of PGA_2 in stock solutions were determined spectrophotometrically. PGA_2 had a maximum absorbance (λmax) at 220 nm with a molar extension coefficient of 9,600, while the principal breakdown product, PGA_2, had a λmax at 280 nm and a molar extinction coefficient of 30,000. In all cases, PGA_2 was stable and ≥98% pure.

Animals were given 20-μl eyedrops of the appropriate doses of PGA_2 or the vehicle as placebo every 2 h for five doses per day from day 3 through day 7 after infection. Eye disease was evaluated on alternate days in fluorescein-stained corneas. Corneal lesions were assigned scores between 0 and 4 on the basis of the fraction of the corneal surface involved (1 = one-fourth of the cornea, 2 = one-half of the cornea, etc.) (21). Corneal thickness was measured with an ultrasonic pachymeter (DGH Technology, Inc., Frazer, Pa.) (13). Flare and cell responses in the anterior chamber were evaluated with a slit lamp and scored from 0 to 3 on the basis of severity (4).

Recovery of latent virus from tissue. At various times after infection, rabbit eye disease was scored, and eyes were rinsed with 200 μl of phosphate-buffered saline. A 100-μl sample of the rinse was collected and tested for the presence of infectious virus on Vero cells. Animals were sacrificed, and the corneas and trigeminal ganglia were taken for further study. Trigeminal ganglia were cut into six pieces and digested in Hanks' balanced salt solution containing 150 IU of collagenase per ml. After 16 to 20 h of digestion at 37°C, the cells and debris were recovered by centrifugation at 500 × *g*, rinsed once in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, and seeded into 25-cm^2 flasks of confluent Vero cells. Cultures were observed for the presence of virus cytopathic effect (CPE). Virus isolates were regrown on Vero cells and confirmed as HSV-1 by neutralization with specific anti-HSV antibody (AXL 237; Accurate Chemical & Scientific Corp., Westbury, N.Y.). Cell free fractions of the collagenase digest were plated on Vero cells to determine if cell-free virus existed within the tissue.

Corneas were cut into six pieces and digested with collagenase in HBSS as described for ganglia. Cells were recovered by centrifugation and plated in 75-cm^2 flasks. Cultures were monitored microscopically for CPE and cell growth. If no CPE was observed within 30 days, the cells were harvested and stored for detection of viral RNA and DNA. If virus CPE was observed, the cultures were incubated until CPE was nearly complete and then frozen. Virus isolates were characterized by neutralization with HSV-specific antibody.

RESULTS

Antiviral activity and cytotoxicity in vitro. The antiviral activity of PGA_2 was evaluated in cultures of human and rabbit corneal stromal cells by yield reduction assays. Probit analysis was used to determine the dose required to reduce the single-cycle yield of the McKrae strain of HSV-1 by 50% (ED_{50}). In rabbit stromal cells, the ED_{50} was 1.2 ± 2.4 μM, while in human stromal cells, the ED_{50} was 4.5 ± 2.3 μM (Fig. 1).

The cytotoxicity of PGA_2 to confluent cultures of cornea stromal cells as determined by the dose required to inhibited the reduction of MTT by 50% (TD_{50}) was 148.5 ± 5.3 μM in cells of rabbit origin and 56.2 ± 3.9 μM in cells from human corneas (Fig. 2). Visual observation of the cultured cells treated with PGA_2 after 24 h indicated cell rounding and granularity at lower concentrations of PGA_2, while higher concentrations of PGA_2 produced cell detachment.

The chemotherapeutic index (CI), calculated as the ratio of TD_{50} to ED_{50}, was taken as a measure of the potential value of PGA_2 as a therapeutic agent. In rabbit cells, the CI was approximately 120, while in human cells, the CI was approximately 12. Thus, PGA_2 had chemotherapeutic potential as a treatment for eye disease.

Therapeutic response of HSV-1-induced keratitis to PGA_2 therapy. In the initial study, 15 rabbits were infected with the

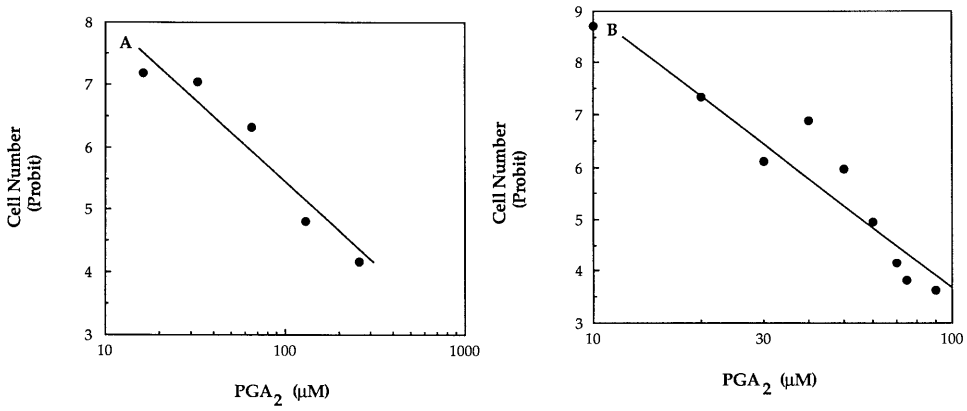

FIG. 2. Cytotoxicity of PGA_2 to rabbit and human cornea stromal cells. Rabbit (A) and human (B) corneal cells were treated with various doses of PGA_2 for 24 h, and cell viability was measured as A_{570} of reduced MTT. The amount of MTT reduced as a percentage of that of the control was plotted as a probit versus the log of the dose of PGA_2. Each datum point represents the average of three to nine determinants.

McKrae strain of HSV-1 bilaterally after epithelial scarification. Rabbits were assigned randomly to each of four treatment groups: placebo (vehicle), 0.5-mg/ml PGA_2, 1.0-mg/ml PGA_2, and 2.0-mg/ml PGA_2. On the third day after infection, therapy was initiated and was continued through day 7 after infection. Analysis of variance of lesion scores indicated that there were only 1 day and one dose at which a significant response to PGA_2 was observed (Table 1). Thus, the data strongly suggest that epithelial disease was not responsive to therapy in a dose-dependent manner. A second study in which 15 infected rabbits were assigned to three treatment groups confirmed the results of the initial study. Mean lesion scores on day 8 after infection were 1.2 (placebo), 1.7 (PGA_2 [1 mg/ml]), and 2.1 (PGA_2 [2 mg/ml]). A Kruskal-Wallis nonparametric analysis of variance indicated that the scores among the three groups were not significantly different ($P = 0.09$, $n = 10$ eyes for each group).

Corneal thickness, a second measure of corneal involvement in disease, was evaluated in both experiments. At 12 days after infection, the time of maximal stromal involvement, there was no significant difference among treatment groups ($P = 0.887$, one-way analysis of variance). Placebo-treated eyes had a mean corneal thickness of 570 ± 42 μm (mean ± standard error), while the corneal thicknesses of the PGA_2-treated groups were 473 ± 23, 582 ± 76, and 541 ± 42 μm for the 0.5-, 1.0-, and 2.0-mg/ml-treated groups ($n = 8$ for each), respectively. In the second experiment, there was also no significant difference in corneal thicknesses among treatment groups during the period of maximal stromal edema, 12 to 14 days after infection. The mean corneal thickness of the placebo-treated group was 496 ± 86 μm ($n = 6$), that with PGA_2 at 1 mg/ml was 479 ± 22 μm ($n = 10$), and that with PGA_2 at 2 mg/ml was 510 ± 41 μm ($n = 10$) ($P = 0.874$, one-way analysis of variance).

TABLE 1. Epithelial lesion scores of the eyes of HSV-1-infected rabbits treated with PGA_2

Treatment	Mean lesion score on the indicated day after infection[a]					
	3	5	7	10	12	14
Placebo[b]	1.1	2.0	2.7[c]	0.7	0.5	0.4
PGA_2 (mg/ml)						
0.5	0.5	2.6	2.0	0.5	0.5	0.2
1.0	0.6	1.8	1.6[c]	0.8	0.9	0.6
2.0	1.3	1.8	2.2	0.7	0.3	0.1

[a] Scores represent the mean lesion score in each group ($n = 6$ eyes for placebo and $n = 8$ eyes for all others).
[b] Vehicle alone.
[c] Significant difference ($P \leq 0.05$) as determined by the Kruskal-Wallis test and the Mann-Whitney U statistic.

The lack of therapeutic response was surprising because of the strong antiviral activity observed in vitro. One reason for the lack of response could be related to pharmacologic aspects of delivery. We selected the vehicle because others have delivered topical prostaglandins to eyes successfully with a similar mixture (4). As a measure of pharmacologic delivery, we scored the flare response in the anterior chamber in all eyes. Flare, in this case, was an estimate of protein in the anterior chamber based on the Tindell effect on a beam of light from the slit lamp. In both studies, pharmacologic levels of drug were being achieved because flare was significantly greater in the treatment groups receiving PGA_2 than in the placebo-treated group (5). For example, the mean flare scores were 0.06 for the placebo group ($n = 8$), 0.44 for the 0.5-mg/ml PGA_2-treated group ($n = 8$), 0.88 for the 1-mg/ml PGA_2-treated group ($n = 8$), and 1.03 for the 2-mg/ml PGA_2-treated group ($n = 6$) after 1 day of therapy. The flare scores of the PGA_2-treated corneas were significantly greater than the scores of the placebo-treated controls and displayed a dose-dependent response after 1 day of therapy ($P \leq 0.05$, Kruskal-Wallis one-way analysis of variance and Mann-Whitney U statistic). Analysis of the data on day 6 after infection (after 3 days of therapy) produced similar results (data not shown). Data collected from the second study produced similar results, suggesting that pharmacologically active doses of drug were reaching targets within the eyes.

Effects of PGA_2 therapy on the recovery of latent virus. The sequelae of infection among animals in the first experiment which were killed 67 days after infection were greater in groups receiving either 1 or 2 mg of PGA_2 per ml than in the placebo-treated group. For example, the mean corneal thicknesses (± standard error) on day 67 just prior to sacrifice were 480 ± 70 μm ($n = 4$) in the placebo-treated group, 569 ± 120 μm ($n = 4$) in a group receiving 1 mg of PGA_2 per ml, and 694 ± 124 μm ($n = 4$) in a group receiving 2 mg of PGA_2 per ml ($P = 0.029$, one-way analysis of variance and Bonferroni's t test). Similarly, in a second group of eyes taken 40 days after infection, corneas from PGA_2-treated eyes were significantly

TABLE 2. Isolation of infectious virus from trigeminal ganglia and corneas of HSV-1-infected rabbits

Treatment group	Infected	No. of samples positive for virus/no. tested				
		Cornea			Trigeminal ganglia	
		Eye wash	Cell free	Cultured	Cell free	Cocultured
None	No	0/6	0/6	0/6	0/6	0/6
Placebo[a]	Yes	0/4	0/4	1/4	0/4	4/4
Placebo[b]	Yes	0/4	0/4	0/4	0/4	4/4
PGA_2[a]	Yes	0/18	0/18	4/18	0/18	15/18
PGA_2[b]	Yes	0/12	0/12	5/12	0/12	10/12

[a] Tissue taken for analysis 40 days after infection.
[b] Tissue taken for analysis 67 days after infection.

thicker and had a greater incidence of necrotizing stromal disease and neovascularization than those from placebo-treated eyes. The corneal thickness of the placebo-treated groups was 490 ± 124 μm ($n = 4$) compared with that of PGA_2-treated groups, in which the thickness was 694 ± 93 μm ($n = 7$, $P = 0.025$, one-way analysis of variance and Bonferroni's t test). In studies in which eyes were taken for virologic assessment, 3 of 10 placebo-treated eyes had severe necrotizing stromal disease at the time of sacrifice, while 14 of 28 PGA_2-treated eyes had necrotizing stromal keratitis. Thus, even a short duration of PGA_2 therapy seemed to have long-term detrimental effects on the outcome of disease.

To determine whether virus was being shed prior to sacrifice, the eyes of all animals were sampled by eye rinses and the rinses were cultured on Vero cells. The corneas and trigeminal ganglia were then harvested, digested with collagenase, and separated by centrifugation into cellular and cell-free fractions. No infectious virus could be isolated from either the rinses of the ocular surface or the cell supernatants prepared from any of the infected animals (Table 2). Upon cocultivation of the cells recovered from the trigeminal ganglia, 25 of 30 yielded infectious virus after 6 to 20 days of cultivation. Cocultures of all six uninfected control animals were negative for virus. Cultures of corneal cells established from collagenase digests of corneas produced infectious virus from a total of 9 of 30 corneas. There were no statistical differences among the groups receiving PGA_2 in the recovery of virus from corneal cultures.

DISCUSSION

The antiviral activity of A-type prostaglandins was documented many years ago, but the mechanisms responsible for the activity remain to be definitively established (16). PGA_2, PGA_1, and several synthetic, stable, long-lasting analogs have demonstrated antiviral action in cell culture models not only on DNA viruses such as HSV-1 but also on retroviruses such as human immunodeficiency virus (3, 8, 23). Two keys to the activity of the prostanoids appear to be the maintenance of the cylopentenone ring and the ability to regulate protein synthesis in the target cell (8, 23). The induction of HSP70, a translational regulatory protein, appears to play a key role in some systems (16). In mouse cell lines which do not respond to prostaglandins by synthesizing HSP70, no antiviral activity on Sendai virus has been observed (1). It has been hypothesized that synthetic PGAs such as Δ^7-PGA_1 inhibit transcription and reduce the amount of mRNA of the immediate-early class of genes in HSV-infected cells (24). The accumulation of a PGA-induced protein appears to be required in some cases, but it remains to be definitively established whether or not that protein is HSP70. This feature of the antiviral mechanism appears not to be universally responsible for the broad-spectrum activity of PGAs; for example, although actinomycin D inhibits transcription, it does not inhibit the antiviral activity of PGAs for encephalomyocarditis virus or vesicular stomatitis virus (2, 15). The concentration at which PGA_2 inhibited HSV-1 replication in the studies reported here was in the low micromolar range, similar to that reported for other viruses. Likewise, the concentration which induced apparent cytotoxic effects was severalfold greater than the ED_{50}, implying selective antiviral activity. It is unclear whether prostaglandin receptors mediate the antiviral effects, but it is unlikely that the cytotoxic effects are receptor mediated, because concentrations in the range of 50 μM or greater are required to produce measurable effects.

The mechanism(s) responsible for the antiviral action of PGAs in vitro is complex, but it is likely that the in vivo responses to these prostanoids are considerably more complicated. Although the antiviral activity of PGAs has been demonstrated repeatedly in cell culture, studies with animal models have been limited. Dimethyl-PGA_2, injected intraperitoneally, has been shown to increase the survival of mice injected with a lethal dose of influenza virus A (18). Here we demonstrated that topical therapy with PGA_2 did not produce a highly significant therapeutic response in the herpetic keratitis model in rabbits. In fact, the ultimate sequelae of disease appeared significantly more severe in animals receiving therapy than in those treated with the placebo. Furthermore, it was clear that therapy did not alter the recovery of virus from trigeminal ganglia and thus did not appear to affect latency of the virus in neurons. Our data document that pharmacologically active drug in amounts necessary to induce biologic responses did reach sites within the eye. Flare, indicative of increased protein in the anterior chamber, increased in a dose-dependent manner in response to PGA_2 treatment. It is likely, therefore, that drug was delivered in adequate amounts to the ocular surfaces, the primary site of virus replication.

The observation that the outcome of disease was actually worse in the animals that received therapy than in those that received the placebo suggests that properties other than the antiviral activities of the prostaglandin system must be considered. PGAs have been demonstrated not only to reduce intraocular pressure in rabbits and other species but also to induce an inflammatory response in the anterior chamber of rabbits. This response, which we measured as flare, likely represents a breakdown of the blood-aqueous barrier (4). The infusion of components from the blood into the anterior chamber was obviously detrimental to the outcome of the disease. Specific mechanisms to inhibit inflammatory responses in the anterior chamber appear to have developed, but we did not study these responses (6). The cornea and anterior chamber are, under normal conditions, avascular, and as a result are not very susceptible to systemic immune or inflammatory responses. Thus, it is not unreasonable that the proinflammatory activities of PGA_2 in the eye were more detrimental than the antiviral activities were beneficial, a circumstance that may be unique to the eye.

Prostaglandins are endogenous mediators of a variety of biological reactions. Despite the fact that they may display potent antiviral activity in vitro, it is very difficult to predict their efficacy in vivo. Consideration must be given to the selection of an animal model selected for testing, because the response of animals to exogenously supplied prostaglandins varies greatly from one species to another. As more is learned about the structure-function relationships of these molecules, it may be possible to design prostaglandin analogs which retain

potent antiviral activity but which lack moieties that induce functions that may be detrimental to the therapeutic response.

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