Inhibition of Herpes Simplex Virus Replication in the Mouse Cornea by Drug Containing Immunoliposomes

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Monoclonal antibody to HSV glycoprotein D was derivatized with palmitic acid and incorporated into liposomes. These immunoliposomes bound specifically to intact mouse corneas infected with HSV-1 in vitro. Furthermore, in yield reduction assays, anti-gD immunoliposomes loaded with acyclovir proved far more effective at inhibiting viral replication in the cornea than free drug or drug delivered in untargeted liposomes. Site-specific sustained release immunoliposomes of this type are potentially an improved vehicle for drug delivery in the treatment of ocular HSV. Invest Ophthalmol Vis Sci 28:591–595, 1987

Since the pioneering studies of Kaufman et al1 in 1962, acute herpetic keratitis has been successfully treated with nucleoside analogues.2 Many drugs are clinically effective, but in all instances the treatment regimen requires inconveniently frequent topical administration. The application interval might be suitably delayed if drugs were slowly released from vehicles that remained anchored on or in the cornea. Drug-containing liposomes targeted with a lectin or antibody to antigens expressed on virus infected cells may provide a suitable vehicle.3 We have shown previously that immunoliposomes targeted with monoclonal antibody to a surface glycoprotein of herpes simplex virus (HSV) specifically attach to virus infected tissue culture cells and that immunoliposomes containing acyclovir (9-(2-hydroxyethoxymethyl)guanine) are more effective than free drug or nontargeted drug-containing liposomes at inhibiting virus replication.4 In the present study we evaluated the effectiveness of drug-containing immunoliposomes at inhibiting the replication of HSV in the isolated mouse cornea.

Materials and Methods

Virus Production and Titration

The HF strain of HSV-1 was propagated for 3 days in monolayers of SIRC (rabbit cornea) cells. After three cycles of freeze-thawing and removal of cell debris by centrifugation (1000 × g, 10 min), the virus was aliquoted and stored at −70°C. Samples to be titrated for virus were homogenized, freeze-thawed three times, centrifuged to remove cell debris, and titrated in quadruplicate using Vero cell monolayers grown in microtiter plates. After 4 days at 37°C, the plates were washed in phosphate buffered saline (PBS), stained with crystal violet, and examined for cytopathic effect.

Monoclonal Antibody

Hybridoma cells producing antibody specific for the glycoprotein D (gD) of HSV-1 (clone D4.2, provided by Dr Melvin Trousdale, Estelle-Doheny Eye Institute, Duarte, CA) were cultivated in Balb/c mice. Ascites fluids were harvested, and IgG purified using Protein A-Sepharose columns.5 Where necessary, antibody was radiolabeled with 125Iodine using Enzymobeads (Biorad, Richmond, CA). Antibody was derivatized with palmitic acid using the method of Shen et al.6 Antibody and N-hydroxysuccinimide ester of palmitic acid (NHSP) were mixed at a 1:10 molar ratio in 2% deoxycholate (DOC) and incubated for 10 hours at 37°C. Derivatized antibody was fractionated using a Sephadex G-75 column (Pharmacia, Piscataway, NJ) with PBS containing 0.15% DOC (PBS/DOC) as buffer. Peak fractions were pooled and concentrated to 2 mg/ml, dialysed against PBS/DOC and stored at −20°C.

Production of Immunoliposomes

Liposomes were produced using a modification of the dehydration-rehydration method of Kirby and Gregoriadis.7 Usually, 15 μmol of egg phosphatidylcholine (PC) and 1 μmol cholesterol in chloroform (found to yield maximum encapsulation efficiency) were dried under a stream of nitrogen, redissolved in ether, dried again to a thin film with nitrogen, and further dried in a vacuum dessicator. Lipids were vortexed in 1 ml of 1/20 PBS and, using a bath sonicator...
(Laboratory Supplies, Hicksville, NY), sonicated to opalescence to form a suspension of small unilamellar vesicles (SUV). Acyclovir (Burroughs Wellcome, Research Triangle, NC) to be encapsulated (1 mg in 1 ml 1/20 PBS) plus 500 μg derivatized antibody were added and the suspension frozen to a thin shell by swirling in an ethanol dry ice bath. After lyophilization overnight, the lipids were rehydrated with 100 μl dH₂O and left to stand for 30 min at room temperature. The resulting dehydration-rehydration vesicles (DRV's) were washed three times in PBS (30,000 × g, 10 min) to remove unincorporated drug and antibody. When necessary, liposomes were trace labeled with the radioactive lipid hexadecyl 3H-cholestanyl ether (3H-CE).

**Corneal Cultures**

Under sterile conditions, corneas were separated from the eyes of Balb/c mice immediately after sacrifice. Each cornea was placed in a separate well of a flat-bottomed tissue culture microtiter plate containing 100 μl RPMI supplemented with 10% fetal calf serum, L-glutamine, hepes, penicillin, and streptomycin. Corneas were used either immediately or after incubation overnight at 37°C. Animal studies conformed to the ARVO Resolution on the Use of Animals in Research.

**Detection of Viral Antigen**

Freshly prepared corneas in culture were infected with 10⁴ TCID₅₀ HF HSV-1 (provided by Dr Melvin Trousdale). After 1 hr adsorption, the corneas were washed three times and returned to 37°C. At different times postinfection (PI), infected and uninfected corneas were incubated with 125Iodine labeled anti-gD monoclonal antibody in 100 μl media for 1 hr at room temperature. Plates were rocked throughout the incubation to maximize cornea/antibody contact. Corneas were then washed five times, and associated radioactivity was measured, and the percentage of antibody bound calculated using the formula:

\[
\% \text{ Antibody bound} = \frac{\text{cpm bound}}{\text{cpm applied}} \times 100
\]

**Virus Replication and Inhibition With ACV**

Corneas in culture were infected with 10⁴ TCID₅₀ HF HSV-1, and at different times PI culture supernatant fluids and corneas were frozen at −70°C before appropriate processing and measurement of viral yield as described above. The antiviral efficacy of acyclovir (ACV) was measured by adding the drug at different dilutions immediately after infection and determining viral yield after 3 more days of incubation.

**Immunoliposome Binding**

Culture corneas infected 1 day previously with 10⁴ TCID₅₀ HF HSV-1 were incubated for 1 hr with 3H-CE labeled liposomes incorporating anti-gD antibody, anti-H2Kk antibody or no antibody. After careful washing, the corneas were solubilised in Protosol (New England Nuclear, Boston, MA) and associated 3H counts measured in a scintillation counter. The percentage of liposomes bound as calculated in the manner used for antibody. The effect of preincubation with polyclonal rabbit anti-HSV antibody was also investigated.

**Yield Reduction Assay**

Corneas in culture were infected with 10⁴ TCID₅₀ HF HSV-1. At 13 hr PI liposomes bearing anti-gD antibody and containing ACV were allowed to bind for 1 hr before careful washing. Corneas were incubated 20 more hours at 37°C before measurement of viral yield. Controls included liposomes without antibody and free ACV.

**Results**

**Appearance of Progeny Virus and Viral Antigen**

Figure 1 shows that progeny virus was first detected 15 hr after infection of the explanted cornea, with the viral yield approaching a plateau by 40 hr PI. From results using cell culture systems,⁴ viral glycoprotein D was known to appear at the cell surface approximately 2 hr prior to the development of infectious progeny virus. However, it proved difficult to pinpoint the exact time (in hours) for the appearance of viral gD on the infected mouse corneas. This was presumably because the number of cells infected (and therefore amount of antigen expressed) during the early stages of infection was too low to bind antibody in excess of the high background binding observed even with uninfected corneas. With a higher infecting dose, the background (measured 1 hr after infection) increased correspondingly, precluding the detection of specific binding (data not shown). However, significant levels of binding of radiolabeled monoclonal anti-gD antibody to infected corneas did not increase significantly over background throughout the 3-day infection period, demonstrating that specific binding was not mediated via Fc receptors expressed on HSV-infected cells (data not shown).

**Binding of Immunoliposomes**

To establish if immunoliposomes targeted with anti-gD monoclonal antibody would bind specifically to in-
Fig. 1. HSV-1 growth curve in explanted mouse corneas. BALB/c corneas maintained in tissue culture were infected with $10^4$ TCID$_{50}$ HSV-1, washed, incubated at 37°C, and at different times post infection, corneas and media were frozen at −70°C. Samples were thawed, homogenized and titrated for infectious virus as describe in Materials and Methods. Results represent the mean of 3 replicates ± SD.

Fig. 2. Binding of $^{125}$Iodine labeled anti-gD mAb to infected corneas. Corneas infected with $10^4$ TCID$_{50}$ HSV-1 (■) and uninfected corneas (□) were incubated at different times postinfection for 1 hr at room temperature with $^{125}$Iodine labeled anti-gD mAb. After five washes the associated radioactive counts were determined using a gamma counter. Results are expressed as percentage counts bound compared with input, and represent the mean of four replicates ± SD.

Fig. 3. Binding of Immunoliposomes. Radiolabeled $^{3}$H-CE control liposomes (■) and immunoliposomes bearing anti-gD mAb (□) were incubated with uninfected corneas and with corneas infected two days previously with HSV-1. After careful washing, the corneas and bound liposomes were solubilised and radioactive counts measured in a scintillation counter. Results are expressed as percentage counts bound compared with input, and represent the mean of four replicates ± SD. The effect of pre-incubation of the corneas with 1/100 dilution of polyclonal rabbit anti-HSV antiserum and with normal rabbit serum are shown. The binding of immunoliposomes prepared with monoclonal anti-H2K$^a$ antibody (□) is also shown.

a monoclonal antibody class homologous with anti-gD (but with no target antigen on the corneas) failed to bind. Furthermore, preincubation of infected corneas with rabbit anti-HSV serum, but not with normal rabbit serum, almost completely inhibited liposome binding. Such controls indicated that the binding by the anti-gD targeted liposomes was target antigen spe-
Fig. 4. Inhibition of HSV replication by ACV. Corneas were infected for one hour with 10⁴ TCID₅₀ HSV-1, before washing and incubation in the presence of different concentrations of ACV. After three days, the viral yields in the media (○) and homogenized cornea (●) were determined as described in Materials and Methods. Results represent the mean of 3 replicates ± SD.

Specific and not due to nonspecific binding via Fc receptors expressed by HSV infected cells. As with free antibody, it was not possible to demonstrate significant binding above background at an early stage of infection.

Inhibition of Virus Replication by Soluble ACV

The presence of soluble ACV at 10 µg/ml during the period of infection was sufficient to almost completely inhibit HSV replication in the explanted cornea (Fig. 4), with a dose of approximately 1 µg/ml mediating a 50% reduction. This dose is well within the delivery capabilities of the drug loaded immunoliposomes though higher than the dose required to inhibit HSV replication in tissue culture cells.

Inhibition of Viral Replication by Drug Loaded Immunoliposomes

The above results demonstrated the inhibitory effect of free ACV during corneal HSV infection and the binding capability of targeted immunoliposomes to those infected corneal cells. To test if targeted liposomes could deliver drug more effectively to the sites of infected cells, immunoliposomes containing ACV and control drug delivery systems were allowed to bind to corneas 13 hr after infection, a time before progeny virus was produced, but after viral gD was presumed to be expressed. Unbound liposomes and drug were then washed away and the infection allowed to proceed for a further 20 hr. It is apparent (Fig. 5) that at high drug concentrations, presentation by immunoliposomes was more effective than control liposomes but was not more effective than equivalent doses of free ACV.
drug. However, at lower drug levels, the targeted liposome delivery system was better at inhibiting viral replication than free drug or untargeted liposomes. Indeed, the dose of ACV required to exert a 50% maximal effect was approximately 0.001 μg/ml when delivered by immunoliposome compared with 1 μg/ml for free drug, a 1000-fold enhancement in efficacy.

**Discussion**

Our investigations demonstrate that liposomes targeted with palmitoyl derivatized monoclonal antibody to glycoprotein D of HSV and containing acyclovir provide an effective means of inhibiting virus replication in the infected cornea. The superiority of such immunoliposomes over free drug and untargeted drug-containing liposomes was most apparent at low doses of ACV. This was assumed to result from specific attachment of liposomes to virus infected cells, thus minimising their loss from the system. Attachment would be followed by leakage of drug at high local concentrations which could be preferentially absorbed by targeted cells and other nearby cells. It is also possible that infected cells could internalise the attached liposomes, but this topic requires further investigation.

From previous studies, we know that the type of immunoliposomes used release 40% of their drug load in 8 hr at 37°C, indicating that the delivery system will be useful for the prolonged administration of inhibitory concentrations of drug. Presumably, the reason both the free drug and untargeted drug-containing liposomes were less effective was because they were washed away following the initial 1 hr treatment period so their inhibitory effect would solely depend on cell uptake during this time. Others have used untargeted liposomes for enhanced topical delivery of drugs to the eye, but in our model, it is the unique affinity for the target cell which accounts for the large increase in efficacy.

Although an enhanced therapeutic effect was noted with our immunoliposome delivery system, it was not nearly as dramatic as that observed in cell culture monolayers. It is likely that HSV infection could spread to the stroma and this location is less accessible to drug. In contrast, all cells in an infected cell monolayer are accessible to drug. Our observation that replication of HSV in the explanted cornea system was apparently 10–100-fold less susceptible to inhibition by free drug than was replication of the same viral strain in cell monolayers supports the idea of impeded drug access. Alternatively corneal cells may take up ACV less effectively than corneal monolayer cells although this seems unlikely, both the extent of stromal cell infection in our model and the degree of stromal penetration by liposomes are topics currently under investigation in our laboratory.

Clearly the direction of our research must be to assess the use of immunoliposomes for drug delivery in vivo. In preliminary studies, immunoliposomes of the same construction have failed to bind to infected mouse eyes in vivo. One suspected reason for this is that the monoclonal antibody we have used binds with insufficient avidity to prevent mechanical removal. We are currently evaluating other ligands including different monoclonal antibodies as well as lectins in an attempt to find a binding system of greater efficiency. For in vivo use, it may be possible to optimize the physicochemical construction of liposomes so that penetration into the tissues of the stroma is facilitated.

**Key words:** liposome, acyclovir, HSV, targeting, cornea

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**References**