Optimized Viral Dose and Transient Immunosuppression Enable Herpes Simplex Virus ICP0-Null Mutants To Establish Wild-Type Levels of Latency In Vivo

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Received 7 February 2000/Accepted 6 April 2000

The reduced efficiency with which herpes simplex virus type 1 (HSV-1) mutants establish latent infections in vivo has been a fundamental obstacle in efforts to determine the roles of individual viral genes in HSV-1 reactivation. For example, in the absence of the “nonessential” viral immediate-early protein, ICP0, HSV-1 is severely impaired in its ability to (i) replicate at the site of inoculation and (ii) establish latency in neurons of the peripheral nervous system. The mouse ocular model of HSV latency was used in the present study to determine if the conditions of infection can be manipulated such that replication-impaired, ICP0-null mutants establish wild-type levels of latency, as measured by viral genome loads in latently infected trigeminal ganglia (TG). To this end, the effects of inoculum size and transient immunosuppression on the levels of acute replication in mouse eyes and of viral DNA in latently infected TG were examined. Following inoculation of mice with $2 \times 10^4$, $2 \times 10^4$, $2 \times 10^5$, or $2 \times 10^6$ PFU/eye, wild-type virus replicated in mouse eyes and established latency in TG with similar efficiencies at all four doses. In contrast, increasing the inoculum size of the ICP0-null mutants n212 and 7134 from $2 \times 10^4$ to $2 \times 10^6$ PFU/eye significantly decreased the levels of infectious virus detected in the tear films of mice from days 4 to 9 postinfection. In an attempt to establish the biological basis for this finding, the effect of viral dose on the induction of the host proinflammatory response was examined. Quantitative reverse transcription-PCR demonstrated that increasing the inoculum of 7134 from $2 \times 10^4$ to $2 \times 10^6$ PFU/eye significantly increased the expression of proinflammatory (interleukin 6), cell adhesion (intercellular adhesion molecule 1), and phagocyte-associated (CD11b) genes in mouse eyes 24 h postinfection. Furthermore, transient immunosuppression of mice with cyclophosphamide, but not cyclosporin A, significantly enhanced both the levels of acute n212 and 7134 replication in the eye and the levels of mutant viral genomes present in latently infected TG in a dose-dependent manner. Thus, the results of this study demonstrate that acute replication in the eye and the number of ICP0-null mutant genomes in latently infected TG can be increased to wild-type levels for both n212 and 7134 by (i) optimization of inoculum size and (ii) transient immunosuppression with cyclophosphamide.

Clinical interest in herpes simplex virus type 1 (HSV-1) and HSV-2 centers on their ability to reactivate from latency and cause recurrent herpetic diseases such as herpes labialis, stromal keratitis, genital herpes, and opportunistic infections of immunosuppressed individuals (37, 58). Despite long-standing interest in the problem (3, 13) and significant advances in our understanding of the molecular events in HSV-1 replication (38), the events that lead from latency to reactivation remain poorly understood. Two factors that have impeded our understanding of latency and reactivation are (i) the lack of definitive in vitro models of HSV latency and (ii) the fact that animal-based models of latency are not amenable to the analysis of HSV reactivation at the molecular level. Regarding the first point, although quiescent infections can be established in several different cell types (2, 9, 39, 47, 59), the relevance of existing “in vitro latency models” to HSV latency in vivo is unclear. Regarding the second point, although the establishment of latency in animal models closely parallels the natural history of HSV infection in humans (22, 41, 48), the effect of eliminating a viral gene product on reactivation is difficult to study because many HSV mutants replicate poorly in animals. Comparison of the reactivation efficiencies of null mutant viruses to that of wild-type virus is a potentially powerful approach to identifying viral genes involved in HSV reactivation. The effect of a mutation in a given gene on reactivation efficiency can be measured accurately, however, only when equal numbers of mutant and wild-type viral genomes are present in latently infected ganglia at the time of reactivation. Given that the efficient establishment of latency is dependent on viral replication at the site of inoculation (27, 42, 43), a fundamental obstacle to the use of viral mutants to study reactivation is that mutations in many “nonessential” viral genes impair the ability of HSV-1 to replicate in animals. For example, attempts to define the roles of ICP0, ICP22, and the virion host shutoff protein in HSV-1 reactivation have been inconclusive because null mutants in these genes replicate poorly in vivo and hence fail to establish latency in sensory ganglia as efficiently as wild-type virus (6, 36, 51). Consequently, the use of viral mutants to study reactivation has been most informative when mutations either (i) have little effect on viral replication in vivo (e.g., latency-associated transcript [LAT] mutants [22, 29, 33]) or (ii) eliminate a function that is absolutely essential for HSV-1 reactivation (e.g., thymidine kinase [8, 52]).

This paper describes efforts to develop new methods to facilitate the molecular genetic analysis of reactivation in a mouse model of HSV-1 latency. Because considerable in vitro and in vivo evidence suggests a role for the immediate-early protein, ICP0, in reactivation (4, 6, 7, 21, 28, 39), we chose to focus on mutants in the gene specifying ICP0. The hypothesis

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underlying the present study was that the efficiencies of acute replication and establishment of latency by ICP0-null mutants can be increased to wild-type levels in mice by altering the conditions of infection. The results of these studies demonstrate that by reducing the viral inoculum from 2 × 10^6 to 2 × 10^5 or 2 × 10^4 PFU/eye, the efficiency of acute replication of ICP0-null mutants in mouse eyes increases. Notably, the rapid inhibition of ICP0-null mutant replication observed at high viral doses correlated well with the enhanced expression of proinflammatory (interleukin 6 [IL-6], intercellular adhesion molecule 1 [ICAM-1]) and proinflammatory-associated molecules that are linked to the pathogenesis of cytomegalovirus (CMV) and human immunodeficiency virus (HIV). The viruses used in this study were wild-type HSV-1 strain KOS (p12 from original isolation [46]) and propagated as previously described (46). The deletion in 7134 that removes the LAT and ICP0 transcripts but contains three translational stop codons inserted at codon 212 (of 775) of the ICP0 open reading frame (6). A rescuant of 7134 has not yet been constructed. Therefore, it remains a possibility that the phenotypes of n212 may also be influenced by secondary mutations acquired in the construction of the virus.

Materials and Methods

Cells and Viruses. Vero and L7 cells, a Vero-derived, ICP0-complementing cell line (40), were propagated as described previously (26). The viruses used in this study were wild-type HSV-1 strain KOS (p12 from original isolation [46]) and the KOS-derived ICP0-null mutants, n212 (6) and 7134 (4). Viruses were propagated as previously described (4, 46). The deletion in 7134 that removes the ICP0 gene also removes −1 kb of the 3′ end of the LATs (10); consequently, 7134 is an ICP0−LAT− double mutant. In contrast, n212 produces full-length LAT and ICP0 transcripts but contains three translational stop codons inserted at codon 212 (of 775) of the ICP0 open reading frame (6). A rescuant of 7134 has been constructed (4) and has been found to behave like wild-type virus both in vitro and in vivo (6, 4), thus demonstrating that the mutation in the ICP0-LAT locus is solely responsible for the phenotypes of 7134. In contrast, although n212 has been found to be phenotypically identical to 7134 in all in vitro tests (6, 26, 61), a rescuant of n212 has not yet been constructed. Therefore, it remains a possibility that the phenotypes of n212 may also be influenced by secondary mutations acquired in the construction of the virus.

Measurement of viral titers in tear film and peripheral WBC counts. Titers of KOS were determined on Vero cell monolayers at various times after inoculation on Vero cell monolayers. Titers of n212 and 7134 were determined on monolayers of L7 cells which complement ICP0-null mutants. For transient immunosuppression, 0.1 ml of CSa (Sandoz Pharmaceutical Co., East Hanover, N.J.) was diluted to 15 or 30 mg/ml in castor oil and administered i.p. to achieve doses of 50 and 100 mg/kg, respectively. Vehicle-treated control mice received 0.1 ml of phosphate-buffered saline (PBS). Dexamethasone (DEX; Steris Laboratories Inc., Phoenix, Ariz.) was diluted to 1.2 mg/ml in PBS, and a volume of 0.1 ml was administered i.p. to achieve a dose of 4 mg/kg. CV (Pharmacia and Upjohn Co., Kalamazoo, Mich.) was administered i.p. in a volume of 0.25 ml of PBS (15 mg/ml to achieve a dose of 150 mg/kg).

Measurement of viral titers in tear film and peripheral WBC counts. Viral titers in tear film were measured as follows. Tear film samples were collected from both eyes with a cotton-tipped applicator, and the titer was transferred into 0.4 ml of complete cell culture medium. Titers of KOS were determined on Vero cell monolayers, and titers of the ICP0-null mutants were determined on L7 cells. Viral titers were determined by a microtiter plate plaque assay under medium containing 0.5% horse serum.

Levels of peripheral WBCs were determined on days 4 and 20 p.i. as follows: mice were bled from the retroorbital sinus with Natelson blood collecting tubes, blood was diluted 10 μl/200 μl in 5% glacial acetic acid, and WBC counts were determined on a hematocrit.

Competitive PCR measurement of viral DNA load. DNA was isolated from the pooled left and right trigeminal ganglia (TG) of each mouse by a standard phenol-chloroform DNA extraction procedure (54). Separate analyses of viral genome loads in the left and right TG of each mouse were determined by the method of Halford et al. (19, 20). Complementary DNA (cDNA) from each PCR sample was prepared by reverse transcription of mRNA by using uninfected TG DNA (33.3 ng/l) as the diluent. PCR assays were conducted as follows. i) A mixture of reagents that contained 1× Taq buffer (Promega Corp., Madison, Wis.), 50 μM (each) deoxyribonucleoside triphosphate, 0.25 μM (each) primer, and 160 ng of TA-RR-mimic per ml (−1,400 competitors per 50 μl of reaction mixture) was made. (ii) Forty-twow icrotiter aliquots of PCR reagents were placed in 0.65-ml tubes and overlaid with mineral oil. (iii) One hundred nanograms of TG DNA (3 μl) was added to each tube. (iv) Samples were brought to 90°C in a thermal cycler block (MJ Research, Watertown, Mass.). (v) Taq polymerase (Promega Corp.) was diluted in 1×Taq buffer (0.5 U/μl), and 5 μl was added per reaction. PCR samples were incubated for 35 thermal cycles of 94°C for 1 min 5 s, 57°C for 1 min 30 s, and 72°C for 40 s.

Measurement of viral genome load in mouse eyes increases. Notably, the rapid inhibition of ICP0-null mutant replication observed at high viral doses correlated well with the enhanced expression of proinflammatory (interleukin 6 [IL-6], intercellular adhesion molecule 1 [ICAM-1]) and phagocyte-associated (CD11b) molecules that are linked to the pathogenesis of cytomegalovirus (CMV) and human immunodeficiency virus (HIV). The viruses used in this study were wild-type HSV-1 strain KOS (p12 from original isolation [46]) and propagated as previously described (46). The deletion in 7134 that removes the LAT and ICP0 transcripts but contains three translational stop codons inserted at codon 212 (of 775) of the ICP0 open reading frame (6). A rescuant of 7134 has not yet been constructed. Therefore, it remains a possibility that the phenotypes of n212 may also be influenced by secondary mutations acquired in the construction of the virus.

Infection and transient immunosuppression of mice. Male ICR mice (6 to 8 weeks old, 20 to 25 g) were obtained from Harlan Sprague-Dawley (Indianapolis, Ind.) and were handled in accordance with The Guide for the Care and Use of Laboratory Animals (24). Mice were anesthetized by intraperitoneal (i.p.) administration of xylazine (6.6 mg/kg of body weight) and ketamine (100 mg/kg). (24). Mice were anesthetized by intraperitoneal (i.p.) administration of xylazine (6.6 mg/kg of body weight) and ketamine (100 mg/kg).
multiple groups at multiple time points (i.e., acute replication in eyes). Linear regression was used to evaluate the quantitative relationship of the standard curves for competitive PCR and RT-PCR.

RESULTS

Effect of optimized inoculum and transient immunosuppression on acute replication of ICP0 mutants in mouse eyes and viral genome loads in latently infected TG. HSV-1 ICP0-null mutants replicate poorly in mice and rabbits (14, 28) and establish latency much less efficiently in TG than wild-type virus (6), but the mechanism(s) that accounts for the impaired replication efficiency of n212 in vivo: (i) the size of the viral inoculum (antigenic mass) was reduced, and (ii) mice were transiently immunosuppressed.

The effects of simultaneously reducing the viral inoculum (Fig. 1A) and causing transient immunosuppression (Fig. 1B) were tested during acute replication of n212 in mouse eyes as follows. Groups of ICR mice were treated every other day from day −3 to +13 p.i. with either vehicle or immunosuppressive drugs. On day 0, control, vehicle-treated mice were inoculated with 2 × 10^5 PFU of KOS/eye and CsA-treated mice were inoculated with either 2 × 10^4 or 2 × 10^5 PFU/eye (n = 3 mice per group). Because titers of ICP0-null mutants replicate poorly in mice and rabbits (14, 28) and establish latency much less efficiently in TG than wild-type virus (6), but the mechanism(s) that accounts for the impaired replication efficiency of n212 in vivo: (i) the size of the viral inoculum (antigenic mass) was reduced, and (ii) mice were transiently immunosuppressed.

The enhanced replication of ICP0-null mutants in IFN-α/β receptor knockout mice (30) suggests a role for ICP0 in the resistance of HSV-1 to innate immunity. Likewise, the rapid rate with which titers of the ICP0-null mutant n212 decrease in the eyes of infected mice (data not shown) led us to hypothesize that n212 is sensitive to a rapidly induced component(s) of the innate immune response. Therefore, in preliminary tests, the following two approaches were taken to delay activation of the host immune response and thus improve the replication efficiency of n212 in vivo: (i) the size of the viral inoculum (antigenic mass) was reduced, and (ii) mice were transiently immunosuppressed.

To evaluate the effect of viral inoculum size and transient immunosuppression on the efficiency with which ICP0-null mutants establish latency as reflected by viral genome loads in latently infected TG, a competitive PCR assay was developed. The sensitivity of the assay was measured first. Coamplification of HSV-1 RR gene and competitor PCR products from standards containing known quantities of viral DNA showed that the logarithm of the ratio of the RR gene to competitor PCR products was linearly dependent on the logarithm of input viral genome copy number (input) in viral DNA standards. The ratio of the number of viral genomes/TG was calculated by multiplying the number of viral genomes/100 ng of TG DNA by 300, based on the fact that there is ~30 µg of total DNA in each TG.

**FIG. 1.** Effect of viral dose and transient immunosuppression on the efficiency of acute replication of n212 in mouse eyes. (A) Effect of viral dose and CsA treatment on levels of n212 shed in tear film. CsA-treated mice were inoculated with n212 at a dose of 2 × 10^4 or 2 × 10^5 PFU/eye (n = 3 mice per group), and eyes were swabbed at the indicated times. KOS-infected controls were treated with vehicle (no drug) and inoculated with 2 × 10^5 PFU/eye (n = 12 mice). (B) Effect of immunosuppression on levels of n212 shed in tear film. Mice treated with either vehicle, CsA, CsA plus DEX, or CsA plus CyP were inoculated with 2 × 10^5 PFU of n212/eye (n = 3 mice per group). CsA and DEX were administered every other day from day −3 to 13 p.i. at dosages of 50 and 4 mg/kg/day, respectively. CyP was given at a dosage of 100 mg/kg/day on days −3, −1, 1, and 3 p.i. and at a dosage of 20 mg/kg/day every other day from day 5 to 13 p.i. The significance of the differences in viral titers over time in mice receiving each treatment regimen was evaluated by two-way ANOVA.

**FIG. 2.** Effect of viral dose and transient immunosuppression on numbers of n212 genomes in latently infected TG. (A) Viral DNA standards for competitive PCR. HSV-1 RR and RR competitor PCR products were coamplified from standards containing (i) twofold dilutions of viral genomes, (ii) a constant amount of RR competitor template, and (iii) 100 ng of uninfected TG DNA. Duplicate blots of PCR products were hybridized to either an RR gene-specific probe (RR) or a competitor-specific probe (competitor). (B) Log-log plot of the ratio of RR to competitor product (output) as a function of viral genome copy number (input) in viral DNA standards. The ratio of the number of viral genomes/TG was calculated by multiplying the number of viral genomes/100 ng of TG DNA by 300, based on the fact that there is ~30 µg of total DNA in each TG. (C) KOS and n212 genome loads in TG on day 30 p.i. (n = 3 mice per group), compared to that for uninfected TG (−). The significance of the differences in numbers of viral genomes in TG was evaluated by one-way ANOVA. VEH, vehicle.
1 μg of DNA per mouse TG), the assay provided a linear measure of HSV-1 genome load over a range of \(3 \times 10^3\) to \(3 \times 10^6\) viral genomes/TG (Fig. 2B, \(r^2 = 0.99\)).

The effect of reduced viral inoculum (\(2 \times 10^4\) PFU/eye) and transient immunosuppression on the efficiency of the establishment of latency by n212 as measured by the number of viral genomes in latently infected TG was determined by competitive PCR (Fig. 2C). Consistent with the low levels of n212 detected in tear film (Fig. 1), the average numbers of n212 genomes in latently infected TG from vehicle- and CsA-treated mice were only 6 and 9% of the wild-type level, respectively (Fig. 2C). Although reducing the viral inoculum to \(2 \times 10^4\) PFU/eye enhanced n212 replication efficiency at the site of inoculation in CsA-treated mice (Fig. 1B), the average number of n212 genomes in TG of these mice was only 1% of the wild-type level (Fig. 2C). In contrast, transient immunosuppression with CsA plus DEX or CsA plus CyP significantly increased the number of n212 genomes present in latently infected TG (\(P \leq 0.05\)), such that n212 genome loads were 116 and 98% of the wild-type level, respectively (Fig. 2C). The unexpectedly low n212 genome loads in CsA-treated mice inoculated with \(2 \times 10^4\) PFU/eye underscored the need for independent analysis of the two variables: the size of the viral inoculum (i.e., viral dose) and transient immunosuppression.

Effect of viral dose on the efficiency of acute replication in eyes and genome load in latently infected TG. (i) Wild-type virus. The effect of viral dose on the efficiency of acute replication and establishment of latency in TG by wild-type virus was analyzed first. Five mice per viral dose were inoculated with \(2 \times 10^1, 2 \times 10^2, 2 \times 10^3, 2 \times 10^4, 2 \times 10^5,\) or \(2 \times 10^6\) PFU of KOS per eye, and tear film was collected at 4, 8, 12, and 24 h p.i. Following inoculation with all doses of KOS, little or no virus was detected in tear film from 4 to 12 h p.i. (Fig. 3A). By 24 h p.i., however, a linear relationship between the dose of virus in the inoculum and the amount of virus shed was observed (Fig. 3A, inset). At all subsequent time points, the amounts of KOS recovered from tear film did not vary significantly for mice infected with \(2 \times 10^3\) PFU/eye or greater. In contrast, KOS replication was only detected in the eyes of one of five mice inoculated with \(2 \times 10^1\) PFU/eye and in none of the mice inoculated with \(2 \times 10^2\) PFU/eye.

Competitive PCR demonstrated that the levels of KOS genomes in latently infected TG on day 30 p.i. were not significantly different in mice inoculated with \(2 \times 10^3\) PFU/eye or greater. In contrast, KOS replication was only detected in the eyes of one of five mice inoculated with \(2 \times 10^5\) PFU/eye and in none of the mice inoculated with \(2 \times 10^3\) PFU/eye.

FIG. 3. Effect of viral dose on the efficiency of acute replication of KOS in mouse eyes and numbers of KOS genomes in latently infected TG. (A) Effect of viral dose on the efficiency of acute KOS replication in eyes. Groups of five mice received the indicated inocula, eyes were swabbed, and virus titers were determined at the indicated times. (Inset) Effect of viral dose on viral titers in eyes at 24 h p.i. Differences in viral titers over time were evaluated by two-way ANOVA. (B and C) Effect of viral dose on KOS genome loads in TG on day 30 p.i. as measured by competitive PCR. (B) Primary data: blot of RR gene PCR products amplified from TG latently infected with KOS (five mice per dose, six viral doses). (C) Histogram of data shown in panel B. Numbers of KOS genomes in latently infected TG of mice receiving the indicated inocula are shown. The significance of differences in numbers of viral genomes in TG was evaluated by one-way ANOVA followed by Tukey’s post hoc t test.
~10^3 PFU/eye) and that, whenever productive infection is initiated, the level of KOS genomes detected in latently infected TG is high.

(ii) ICP0-null mutants. The n212 virus is phenotypically null for ICP0 function but expresses the N-terminal 211 amino acids that contain the ring finger domain of this 775-amino-acid protein (11). A rescuant of n212 has not yet been constructed, and thus it remains a possibility that the phenotypes of n212 may be influenced by secondary mutations acquired in the construction of the virus. Therefore, to control for (i) any functional effects of the 211-amino-acid ICP0 peptide made from n212 and (ii) any potential secondary mutations in n212, a viral deletion mutant lacking the entire ICP0 open reading frame, 7134, was also tested. Because the rescuant of 7134, 7134R, replicates like wild-type virus in mouse eyes and TG and reactivates with wild-type efficiency from latently infected TG, the in vivo phenotype of 7134 can be ascribed to the deletion in the ICP0 locus (6). Given that the LATs are encoded in part by the strand opposite that encoding ICP0, however, 7134 is also a LAT^ mutant that fails to express the major 2.0-kb LATs (6).

The effect of viral dose on the acute replication efficiency of n212 and 7134 was analyzed as follows. Five mice per dose were inoculated with 2 × 10^3, 2 × 10^4, 2 × 10^5, or 2 × 10^6 PFU of n212, 7134, or the positive control, KOS, per eye, and viral titers were determined on days 1 to 9 p.i. As expected, KOS replicated to similar levels in mice inoculated with 2 × 10^3, 2 × 10^4, 2 × 10^5, or 2 × 10^6 PFU/eye (Fig. 4). In contrast, the size of n212 and 7134 inocula had a significant effect on the course of acute replication in mouse eyes (P < 10^-3 for both mutants). Inoculation with the lowest dose (2 × 10^3 PFU/eye) produced detectable replication of n212 and 7134 in five of five mice per group. Although inoculation with 2 × 10^4 PFU/eye produced detectable replication of n212 and 7134 in five of five and four of five mice per group, respectively, viral titers in tear film at 24 h p.i. were highly variable. Inoculation with 2 × 10^4 or 2 × 10^5 PFU/eye, however, produced detectable levels of n212 and 7134 replication in 100% of mice, and viral titers in tear film at 24 h p.i. were highly consistent. Despite the consistency of establishing an acute infection, increasing the inoculum of n212 and 7134 from 2 × 10^4 to 2 × 10^5 PFU/eye significantly reduced the duration of viral shedding in both ICP0-null mutants (P < 10^-4). In mice inoculated with 2 × 10^5 PFU/eye, the mean duration of shedding of n212 and 7134 was 4 days p.i. In contrast, in mice inoculated with 2 × 10^4 PFU/eye, n212 and 7134 were still detectable in tear film on day 9 p.i. in three of five mice per group. Thus, for n212 and 7134, increasing the size of the viral inoculum to 2 × 10^5 PFU/eye actually reduced the levels of virus shed 4 to 9 days p.i. relative to levels observed in mice inoculated with the lower viral doses of 2 × 10^4 or 2 × 10^5 PFU/eye.

The effect of viral dose on the efficiency of acute replication of n212 and 7134 in eyes. Levels of KOS, n212, and 7134 shed in tear film following inoculation with 2 × 10^3, 2 × 10^4, 2 × 10^5, or 2 × 10^6 PFU/eye (n = 5 mice per dose per virus) are shown. The significance of differences in viral titers over time was evaluated by two-way ANOVA.

FIG. 4. Effect of viral dose on the efficiency of acute replication of n212 and 7134 in eyes. Levels of KOS, n212, and 7134 shed in tear film following inoculation with 2 × 10^3, 2 × 10^4, 2 × 10^5, or 2 × 10^6 PFU/eye (n = 5 mice per dose per virus) are shown. The significance of differences in viral titers over time was evaluated by two-way ANOVA.

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-FIG. 4. Effect of viral dose on the efficiency of acute replication of n212 and 7134 in eyes. Levels of KOS, n212, and 7134 shed in tear film following inoculation with 2 × 10^3, 2 × 10^4, 2 × 10^5, or 2 × 10^6 PFU/eye (n = 5 mice per dose per virus) are shown. The significance of differences in viral titers over time was evaluated by two-way ANOVA.
ANOVA followed by Tukey’s post hoc test.

Wild-type genome loads in TG (i.e., 100%) are defined as the average numbers 30 p.i. was measured by competitive PCR (latently infected TG. The effect of viral dose on viral genome loads in TG on day were analyzed: IFN- to test.

To test this hypothesis, two components of innate immunity immune response induced in a viral-dose-dependent manner.

Based on the findings of Leib et al. (30) and Mossman et al. (32), which demonstrate the sensitivity of ICP0-null mutants to infection (Fig. 6A). In contrast, ICP27-specific PCR primers detected high levels of viral mRNA in the eyes of 7134-infected mice but not in the eyes of mock-infected mice (Fig. 6A). Likewise, yields of IL-6, ICAM-1, CD11b, and IFN-γ RT-PCR products amplified from 7134-infected eyes were markedly higher than those from mock-infected controls (Fig. 6A), suggesting that cytokine expression and the recruitment of CD11b+ cells to the eye were induced in response to 7134 infection.

The effect of viral dose on transcription of IL-6, ICAM-1, CD11b, and IFN-γ mRNA in the eyes of 7134-infected mice at 24, 48, and 72 h p.i. was measured by quantitative RT-PCR. RT-PCR was performed on RNA samples isolated from the left eyes of mock-infected mice (n = 2 eyes per time point) and both left and right eyes of mice infected with 2 × 102 or 2 × 104 PFU of 7134/eye (n = 4 eyes per group per unit time). Serial dilutions of infected mouse eye RNA produced standard curves in each set of RT-PCRs (i.e., ICP27, IL-6, ICAM-1, CD11b, and IFN-γ) and established that a linear relationship between product yield and the logarithm of mRNA concentration existed (Fig. 6B; densitometric analysis not shown).

24 h p.i., levels of ICP27 mRNA were equivalent in eyes inoculated with either 2 × 102 or 2 × 104 PFU of 7134/eye (Fig. 6B and C). The 100-fold increase in 7134 inoculum (from 2 × 102 to 2 × 104 PFU/eye) induced 31-, 13-, 11-, and 5-fold-higher mean levels of IL-6, ICAM-1, CD11b, and IFN-γ mRNA in mouse eyes at 24 h p.i., respectively (Fig. 6B and C). Therefore, increasing the size of the 7134 inoculum resulted in significant increases in IL-6, ICAM-1, and CD11b mRNA levels in mouse eyes at 24 h p.i. (P < 0.05). At 48 and 72 h p.i., however, no significant differences in the levels of ICP27, IL-6, ICAM-1, CD11b, or IFN-γ mRNA between eyes inoculated with 2 × 104 versus 2 × 105 PFU of 7134 were detected (data not shown). Therefore, the results of quantitative RT-PCR analysis indicated that the rate of induction of proinflammatory (IL-6 and ICAM-1) gene expression in the eye was dependent on viral dose and suggested a role for inflammatory cells (e.g., CD11b+ phagocytes) in the viral-dose-dependent inhibition of acute n212 and 7134 replication in mouse eyes.

Optimization of a transient immunosuppressive regimen that enhances the efficiency with which ICP0-null mutants establish latency in TG. A series of experiments was conducted to develop a simple, effective immunosuppressive regimen to enhance the acute replication efficiency of ICP0-null mutants in mice. Treatment of mice with CsA alone (50 and 100 mg/kg/day) consistently failed to enhance levels of n212 and 7134 genomes in latently infected TG (e.g., Fig. 2C). Although CsA plus DEX given every other day from days −3 to 13 p.i. increased latent n212 genome loads in TG (Fig. 2C), this drug combination was not effective when given over a shorter period of time (days −3 to 3 p.i.; data not shown). Given that CsA alone was ineffective, the ability of the CsA-plus-CyP combination to increase n212 genome loads (Fig. 2) suggested that

FIG. 5. Effect of viral dose on numbers of KOS, n212 and 7134 genomes in latently infected TG. The effect of viral dose on viral genome loads in TG on day 30 p.i. was measured by competitive PCR (n = 5 mice per virus per dose). Wild-type genome loads in TG (i.e., 100%) are defined as the average numbers of genomes detected in all 20 KOS-infected mice. For each virus, the effect of viral dose on the numbers of viral genomes in TG was evaluated by a one-way ANOVA followed by Tukey’s post hoc test.

reduction in the numbers of 7134 genomes detected in latently infected TG (Fig. 5). Specifically, in four of the five mice in which virus shedding was undetectable by day 4 p.i., 7134 genome loads in latently infected TG were only 2 to 5% of the wild-type level. Therefore, the efficiency with which ICP0-null mutants establish latent infections in TG is highly dependent on the dose of virus in the inoculum, and n212 and 7134 differ in the efficiency with which they establish latency in TG following inoculation with 2 × 106 PFU/eye.

Effect of viral dose on the innate immune response. Following inoculation with 2 × 106 PFU of n212 or 7134/eye, levels of infectious virus were undetectable in 80% of infected mice by day 4 p.i. Based on the significantly reduced titers of ICP0-null mutants in eyes at early times p.i., we postulated that ICP0-null mutants may be inhibited by a component(s) of the innate immune response induced in a viral-dose-dependent manner. To test this hypothesis, two components of innate immunity were analyzed: IFN-α/β and proinflammatory gene expression.

Based on the findings of Leib et al. (30) and Mossman et al. (32), which demonstrate the sensitivity of ICP0-null mutants to IFN-α/β, we considered the possibility that systemic induction of IFN-α/β might occur in a viral-dose-dependent manner. It is also possible that induction of the nonspecific inflammatory response in the eye is enhanced in a viral-dose-dependent manner. Therefore, the following experiment was conducted to determine if (i) systemic induction of IFN-α/β or (ii) proinflammatory gene expression in the eye could account for the dose-dependent inhibition of 7134 replication in vivo. Groups of six mice were either mock infected or inoculated with 2 × 104 or 2 × 106 PFU of 7134/eye, and samples were collected at 24, 48, and 72 h p.i. IFN activity was evaluated in sera, and levels of several proinflammatory mRNAs were analyzed in mouse eyes by RT-PCR, as described below.

Mouse sera were tested for IFN-α/β activity based on the ability of innate IFNs to inhibit replication of EMC virus in Vero cells. Pretreatment of Vero cells with as little as 2 U of recombinant IFN-α per ml inhibited EMC replication in Vero cells. In contrast, none of the 30 serum samples collected from mice 24 to 72 h p.i. exhibited detectable levels of IFN activity (<2 U/ml; data not shown). Therefore, a gross increase in the levels of circulating IFN-α/β did not appear to account for the viral-dose-dependent inhibition of 7134 replication in mouse eyes.

RT-PCR was used to compare the induction of cytokine and proinflammatory gene expression in the eyes of uninfected mice relative to those for mock-infected mice or mice inoculated with 2 × 104 or 2 × 106 PFU of 7134/eye (Fig. 6). Before measuring cytokine mRNA levels, an initial screen was performed on RNA samples isolated from the left eyes of mice (n = 2 eyes per group per time point) to identify cellular genes induced by 7134 infection (Fig. 6A). As with the housekeeping gene for GAPDH, the initial screen indicated that levels of IL-1-α, TNF-α, and IFN-α mRNA in the eyes of mock- and 7134-infected mice at 24, 48, and 72 h p.i. were not obviously different (Fig. 6A). In contrast, ICP27-specific PCR primers detected high levels of viral mRNA in the eyes of 7134-infected mice but not in the eyes of mock-infected mice (Fig. 6A). Likewise, yields of IL-6, ICAM-1, CD11b, and IFN-γ RT-PCR products amplified from 7134-infected eyes were markedly higher than those from mock-infected controls (Fig. 6A), suggesting that cytokine expression and the recruitment of CD11b+ cells to the eye were induced in response to 7134 infection.

The effect of viral dose on transcription of IL-6, ICAM-1, CD11b, and IFN-γ mRNA in the eyes of 7134-infected mice at 24, 48, and 72 h p.i. was measured by quantitative RT-PCR. RT-PCR was performed on RNA samples isolated from the left eyes of mock-infected mice (n = 2 eyes per time point) to identify cellular genes induced by 7134 infection (Fig. 6A). As with the housekeeping gene for GAPDH, the initial screen indicated that levels of IL-1-α, TNF-α, and IFN-α mRNA in the eyes of mock- and 7134-infected mice were only 2 to 5% of the wild-type level. Therefore, the efficiency with which ICP0-null mutants establish latent infections in TG fol-
CyP alone might produce the desired result. Indeed, in preliminary tests, CyP given on days 2 and 1 p.i. enhanced the efficiency of acute replication in mouse eyes, as measured by levels of virus in tear film on day 7 p.i., in a dose-dependent manner (Fig. 7). To achieve a consistent immunosuppressive effect, an additional treatment day was added, such that the regimen adopted for study was administration of CyP on days 2, 1, and 3 p.i.

Experiments were then performed to identify an optimal dose of CyP that would maximize ICP0 mutant genome loads in latently infected TG. For this purpose, mice were inoculated with $2 \times 10^6$ PFU of 7134 and harvested at 24 h p.i. The significance of differences in RT-PCR product yield were evaluated by two-sided t tests.
ated with acute 7134 infection and that CyP caused a dose-dependent reduction in WBC levels (Fig. 8A). The transient nature of the immunosuppressive regimen was demonstrated by the fact that peripheral WBC counts had returned to normal in all treatment groups by day 20 p.i. (Fig. 8A). While mice treated with doses of CyP less than or equal to 150 mg/kg/day remained healthy, mice treated with 200 mg/kg/day experienced visible weight loss and a significantly lower rate of survival (Fig. 8A).

On day 30 p.i., competitive PCR analysis demonstrated that the mean levels of 7134 genomes in latently infected TG of vehicle- and CsA-treated mice were 33 and 23% of wild-type levels, respectively (Fig. 8B). In contrast, CyP treatment enhanced 7134 genome loads in latently infected TG in a dose-dependent manner. Specifically, 7134 genome loads in CyP-treated mice given doses of 50, 100, 125, 150, and 200 mg/kg/day were 33, 85, 99, 140, and 152% of the wild-type level, respectively (Fig. 8B).

Effects of optimal viral dose and CyP treatment on the efficiency of acute ICP0 mutant viral replication and the establishment of latency in TG. Having determined the optimal conditions for the efficient replication and establishment of latency by ICP0-null mutants as described above, a final experiment was performed. Briefly, following inoculation with $2 \times 10^5$ PFU/eye, levels of acute replication of KOS, n212, and 7134 shed in tear films of vehicle (VEH; $n = 10$ mice) and CyP ($n = 10$ mice; 150 mg/kg/day)-treated mice inoculated with $2 \times 10^5$ PFU/eye were compared (Fig. 9A). The significance of differences in viral titers over time was evaluated by two-way ANOVA. The number of KOS, n212, and 7134 genomes in latently infected TG of vehicle- and CyP-treated mice were determined by competitive PCR on day 30 p.i. ($n = 5$ mice per group). Viral genome loads for KOS-infected, CyP-treated mice could not be determined because none of the mice survived acute infection. The significance of differences in viral genome loads was compared by one-way ANOVA followed by Tukey’s post hoc t test.

FIG. 8. Effect of CyP on WBC counts and numbers of 7134 genomes in latently infected TG. (A) Peripheral WBC counts in mice 4 and 20 days after inoculation with 7134 ($n = 4$ mice per group). On days –1, 1, and 3 p.i., mice were treated with vehicle, CsA (100 mg/kg/day), or CyP (50, 100, 125, 150, or 200 mg/kg/day). The survival rates of vehicle- and drug-treated mice are based on the average observed in this experiment and 1 or 2 other independent experiments. (B) 7134 genome loads in TG of latently infected mice treated with either vehicle, CsA, or CyP (50, 100, 125, 150, or 200 mg/kg/day) during acute infection ($n = 8$ mice per group, except for CyP at 200 mg/kg/day in which there were three survivors). The significance of differences in numbers of viral genomes in TG was evaluated by one-way ANOVA followed by Tukey’s post hoc t test.

FIG. 9. Effect of CyP on the efficiency of acute replication of KOS, n212, and 7134 in eyes and numbers of viral genomes in latently infected TG. (A) Levels of KOS, n212, and 7134 shed in tear films of vehicle (VEH; $n = 10$ mice) and CyP ($n = 10$ mice; 150 mg/kg/day)-treated mice inoculated with $2 \times 10^5$ PFU/eye were compared (Fig. 8A). The significance of differences in viral titers over time was evaluated by two-way ANOVA. The number of KOS, n212, and 7134 genomes in latently infected TG of vehicle- and CyP-treated mice were determined by competitive PCR on day 30 p.i. ($n = 5$ mice per group). Viral genome loads for KOS-infected, CyP-treated mice could not be determined because none of the mice survived acute infection. The significance of differences in viral genome loads was compared by one-way ANOVA followed by Tukey’s post hoc t test.
30 p.i., infectious virus was not detected in TG taken from vehicle-treated, KOS-infected mice (0 of 10 TG), nor was virus detected in TG taken from Cyp-treated mice infected with n212 (0 of 10 TG) or 7134 (0 of 10 TG). Therefore, Cyp treatment of mice from days −1 to 3 p.i. did not prevent the establishment of latency in TG by day 30 p.i.

On day 30 p.i., competitive PCR analysis demonstrated that, in vehicle-treated mice, the average numbers of n212 and 7134 genomes in latently infected TG were significantly lower than the wild-type level (Fig. 9B; P = 0.001). In mice treated with Cyp at 150 mg/kg/day on days −1, 1, and 3 p.i., however, the average numbers of n212 and 7134 genomes in latently infected TG were 113 and 105% of the wild-type level, respectively (Fig. 9B). Based on the high rate of survival and the significant increase in n212 and 7134 genome loads in latently infected TG, the regimen adopted for use in subsequent studies of ICP0 mutants was 2 × 10^5 PFU/eye and administration of Cyp at 150 mg/kg/day on days −1, 1, and 3 p.i.

**DISCUSSION**

Effect of viral dose on the efficiency of acute replication and establishment of latency by wild-type virus. Prior to this study, the relationship between the amount of wild-type HSV-1 used to inoculate mice and the efficiency with which latency is established in sensory ganglia, based on viral genome loads, had not been rigorously analyzed by a sensitive PCR assay. The results of the present study indicate that for the KOS strain of HSV-1, ~10^5 PFU is the minimum inoculum needed to consistently establish a productive infection in the eyes of ICR mice. Increasing the inoculum of KOS from 2 × 10^4 to 2 × 10^5, 2 × 10^6, or 2 × 10^7 PFU/eye had no significant effect on the course of acute viral replication in eyes or on the number of KOS genomes in latently infected TG. Therefore, although it has been assumed that increasing the size of the viral inoculum will increase the numbers of viral genomes detected in latently infected TG (34), the empirical evidence does not support this hypothesis. While KOS inocula of 2 × 10^3 or 2 × 10^4 PFU/eye failed to establish productive infections in 9 of 10 mice tested, in other studies as little as 3 × 10^3 PFU of another wild-type HSV-1 strain (McKrae) was shown to establish productive infection efficiently in the eyes of ICR mice (18). Thus, the minimum dose of virus required to establish a productive infection in mouse eyes is a viral-strain-specific property.

Effect of viral dose on the efficiency of acute replication and establishment of latency by the ICP0-null mutants, n212 and 7134. Viral dose had a significant effect on the efficiency of acute replication of ICP0-null mutants in mouse eyes. A minimum inoculum of ~2 × 10^4 PFU/eye was required to establish productive infection with n212 and 7134. The consistency with which productive infection was established increased significantly when the inoculum of ICP0-null mutants was increased to ~2 × 10^5 PFU/eye. Unlike that of wild-type virus, however, when the inoculum of ICP0-null mutants was increased from 2 × 10^5 to 2 × 10^6 PFU/eye, titers of n212 and 7134 recovered from tear film decreased from high levels on day 1 p.i. to undetectable levels on day 4 p.i.

The ICP0-null mutants, n212 and 7134, differed in the efficiency with which they established latency in TG (Fig. 5). The n212 virus was generally more efficient than 7134 in its ability to establish latent infections in TG, and this was most evident when mice were inoculated with 2 × 10^6 PFU/eye. The latter result is remarkably similar to the observations of Cai et al. in which n212 and 7134 genome loads in TG of latently infected mice inoculated with 2 × 10^6 PFU/eye were compared (6). Although n212 and 7134 are both phenotypically null for ICP0's transactivating function, the two viruses are genotypically distinct. The deletion in 7134 that removes the ICP0 gene also removes ~1 kb from the 3' end of the LATs; consequently, 7134 is an ICP0^-LAT^- double mutant (4, 6, 10). In contrast, n212 produces full-length LAT and ICP0 transcripts, but translational stop codons are inserted at codon 212 (of 775) of the ICP0 open reading frame (4, 6). Possible differences between the two mutants that may account for the increased efficiency with which n212 establishes latency in TG relative to 7134 are (i) the LATs are expressed by n212 but not 7134, and available evidence argues strongly that the LATs play a central role in the establishment of latency (12, 35); (ii) secondary mutations may have been acquired in the construction of n212 (a rescuant has not yet been constructed); and (iii) n212 (but not 7134) may express some additional activity associated with the N-terminal 211 amino acids of ICP0. Regarding the last point, because the essential ring finger domain of ICP0 is intact in n212 (11), it is possible that the truncated ICP0 peptide encoded by this mutant may have as yet unrecognized biological effects.

The role of innate immunity in the replication-impaired phenotype of ICP0-null mutants in vivo. ICP0 is well known for its function as a potent and global transactivator of viral gene expression (5, 26). The present study demonstrates that the impaired replication of ICP0-null mutants in mice is not solely a consequence of the loss of an important viral function. Based on the consistently high levels of infectious virus detected in tear film at 24 h p.i., n212 and 7134 established productive infections efficiently in mouse eyes inoculated with 2 × 10^6 PFU/eye. Titers of infectious virus in tear film decreased to undetectable levels, however, by day 4 p.i. Theoretically, the rapid decrease in ICP0-null mutant titers in tear film could have been due to the inability of ICP0-null mutant viruses to sustain lytic replication in vivo or the susceptibility of ICP0-null mutant viruses to inhibition by components of the host immune response or both. Although available evidence has long supported a role for ICP0 in facilitating efficient HSV replication (4, 26), recent evidence demonstrates that the replication-impaired phenotype of ICP0-null mutants in vivo is also a consequence of active inhibition by the innate immune response. Thus, the levels of ICP0-null mutant virus shed in tear film are significantly enhanced in (i) IFN-α/β receptor knockout mice (30), (ii) mice inoculated with a reduced viral dose of 2 × 10^4 or 2 × 10^5 PFU/eye, and (iii) mice treated with the immunosuppressive drug Cyp. The relevance of each observation is discussed below.

(i) IFN-α/β. The innate IFNs, IFN-α and IFN-β, are among the most rapidly induced components of the innate immune response to viral infection (~4 h p.i.). Jamieson et al. (25) first demonstrated that IFN-α dramatically inhibits the replication of ICP0-null mutants in vitro. Leib et al. (30) have recently demonstrated that an ICP0-null mutant replicates much more efficiently in IFN-α/β receptor knockout mice than in normal mice. Therefore, the induction of an IFN-induced “antiviral state” appears to limit the replication and spread of ICP0-null mutants in normal mice. Mossman et al. (32) have recently shown that ICP0 mutants are hypersensitive to IFN-α in vitro and that this phenotype is not characteristic of mutants in other HSV-1 genes. Therefore, ICP0 appears to play a central role in the resistance of HSV-1 to the innate IFNs. In the present study, enhanced replication of ICP0-null mutants was achieved in normal mice by (i) reducing the size of the viral inoculum and (ii) depleting levels of circulating WBCs by >90% with Cyp. Because neither treatment should impair signaling through the IFN-α/β pathway, these findings suggest that other components of the innate immune response are...
required for maximal inhibition of ICP0-null mutant replication in vivo.

(ii) Effect of viral dose. The recruitment of phagocytes (e.g., CD11b+ cells) to the site of infection via the expression of proinflammatory cytokines (e.g., IL-1, IL-6, TNF-α) and cell adhesion molecules in blood vessels (e.g., ICAM-1) is an early line of defense in the innate immune response (4 to 96 h p.i.). In particular, large numbers of neutrophils (e.g., CD11b+ cells) are recruited to the mouse eye within 1 to 3 days after inoculation with wild-type HSV-1 (53). In the present study, quantitative RT-PCR demonstrated that increasing the inoculum of 7134 from 2 × 103 to 2 × 106 PFU/eye resulted in faster induction of proinflammatory (i.e., IL-6, ICAM-1) and CD11b gene expression in the eye. The correlation between the faster induction of the proinflammatory response in the eye and the rapid reduction in 7134 titers in tear films of mice inoculated with 2 × 106 PFU/eye suggests that ICP0-null mutant replication may be especially susceptible to inhibition by nonspecific inflammatory cells (e.g., CD11b+ cells) recruited to the site of inoculation. Therefore, it is possible that the reduced viral inoculum may enhance the replication of ICP0-null mutants by delaying the influx of inflammatory cells into the eye. Further investigation will be required to test this hypothesis.

(iii) Effect of CyP. CyP is an alkylating agent that is rapidly converted in vivo into metabolites that cause lethal DNA damage in rapidly dividing cells. These metabolites decrease to undetectable levels within 3 h after administration of 320 mg of CyP/kg of body weight to mice (1). The bone marrow progenitor cells responsible for maintaining normal levels of WBCs in the peripheral circulation are especially sensitive to the acute toxicity of CyP. The depletion of WBCs is transient, and all measures of immunocompetence return to normal within 10 days after terminating CyP treatment (31, 49, 55).

In the present study, doses of CyP that reduced peripheral WBC counts by greater than 90% significantly enhanced the acute replication of ICP0-null mutants in mice, and consequently higher levels of ICP0-null mutant genomes were detected in latently infected TG. In contrast, treatment with doses of CsA that should block lymphocyte activation did not enhance the acute replication of ICP0-null mutants in vivo. Furthermore, the decrease in ICP0-null mutant titers observed in mice inoculated with 2 × 106 PFU/eye was too rapid (i.e., less than 4 days) to be the result of an antigen-specific process. Because lymphocytes do not appear to be critical, the enhanced replication of ICP0-null mutants in CyP-treated mice may well be a consequence of the depletion of nonspecific WBC effectors (e.g., macrophages, neutrophils, and natural killer cells).

Implications for the genetic analysis of HSV-1 reactivation.

It is widely recognized that a fundamental obstacle to the genetic and functional analysis of the roles of individual viral genes in HSV-1 reactivation is that mutations in many nonsential viral genes impair the capacity of HSV-1 to replicate in animals. Thus, viral mutants are often unable to establish latency efficiently in ganglia. Understanding the mechanism(s) that underlies the replication-impaired in vivo phenotype of viral mutants allows for the rational development of approaches to enhance the efficiency with which they establish latency. For ICP0-null mutants, manipulations that impair the innate immune response constitute a simple approach to increasing the efficiency of ICP0-null mutant replication in vivo. Although this study focused solely on ICP0 mutants, CyP treatment is also known to enhance the efficiency of acute replication of HSV-2 US3 mutants in mice (60). Likewise, the absence of functional lymphocytes in the periphery allows VP16- and thymidine kinase-null mutants to establish persistent infections in scid mice (56, 57). The primary weakness of any immunomodulatory approach is that it is not possible to directly address concerns that a given manipulation (e.g., CyP treatment) does not have secondary effects on the establishment and reactivation of HSV latency. Given the diversity of tools available to manipulate host immunity (e.g., immunosuppressive drugs, monoclonal antibodies to deplete WBC subsets, genetically immunodeficient mice), however, such concerns should be readily addressable by testing a given hypothesis using multiple independent approaches.

In conclusion, this study demonstrates that under conditions in which the host immune response is delayed or impaired, HSV-1 ICP0-null mutants can achieve viral genome loads in TG of latently infected mice equivalent to that of wild-type virus. Thus, a definitive analysis of the role of ICP0 and its functional domains in HSV-1 reactivation from latency is now feasible. Further investigations will be required to determine if immunomodulation can be used to enhance the efficiency of the establishment of latency by other viral mutants such that genome loads of mutants approach that of wild-type virus. If these principles apply to mutants defective in other nonessential viral genes, a more comprehensive approach to determining the roles of individual viral gene products in reactivation of HSV-1 from latency can be taken.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service Program Project grant P01 NS 35138 from the National Institute of Neurological Disorders and Stroke. W.P.H. is the recipient of individual National Research Service Award AI 10147 from the National Institute of Allergy and Infectious Diseases.

We thank Hamid Bassiri for generously donating scid mice for a preliminary experiment that served as the impetus for this study, Bryan Gehhardt and Daniel Carr for providing many of the oligonucleotide primers for the RT-PCR, and John Balliet, David Davido, Jennifer Isler, Rob Jordan, and Luis Schang for critical input into the development of this work.

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