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Viral Replication Is Required for Induction of Ocular Immunopathology by Herpes Simplex Virus

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Corneal infection of BALB/c mice with herpes simplex virus type 1 results in a chronic inflammatory response in the stroma termed herpetic stromal keratitis (HSK). This disease is considered to be immunopathological and mediated primarily by $CD4^+$ T cells of the type 1 cytokine profile. However, the nature of the antigens, virus or host derived, which drive the inflammatory response remains in doubt. In this study, the relevance of infection with replicating virus for the subsequent development of HSK was evaluated with immunocompetent mice as well as with SCID mice reconstituted with herpes simplex virus-immune $CD4^+$ T cells. In the corneas of immunocompetent mice, infectious virus, viral antigen, and mRNA expression were detectable for only a brief period of time (≤7 days postinfection), and all were undetectable by the time clinical lesions were evident (10 to 15 days). Viral replication, however, was necessary for the development of HSK in both models, since infection with UV-inactivated virus or with mutant viruses which were incapable of multiple rounds of replication in vivo failed to induce HSK. The inactivated and mutant viral preparations did, however, stimulate T-cell immune responses in immunocompetent mice. The results are discussed in terms of possible involvement of host antigens exposed in response to transient progeny virion replication in the immune-privileged cornea.

Herpetic stromal keratitis (HSK) is a chronic inflammatory response that occurs in the human eye as a sequel to infection with herpes simplex virus (HSV). This common cause of human blindness usually results from frequent reactivation of latent virus from the trigeminal ganglion (12). Studies of animal models, particularly the mouse, have indicated that HSK represents an immunopathological disease in which $CD4^+$ T cells act as essential participants (1, 22, 26, 28). The disease is not manifested in athymic or SCID mice unless the mice are reconstituted with T cells of the $CD4^+$ phenotype (1, 22, 23). On the basis of cytokine profiles of cells recovered from the cornea or by suppression of the disease with anticytokine antibody, $CD4^+$ T cells that produce type 1 cytokines appear to be principally involved in mediating immunopathology (5, 16, 29, 30). Although the idea that HSK represents an immunopathological response is widely accepted, details of the disease pathogenesis are largely lacking. Indeed, as a virus-induced immunopathological reaction, HSK has some unusual features. Thus, most virus-induced immunopathological diseases involve viruses that are mildly cytolytic or noncytodestructive, and the replicating agent persists during the inflammatory reaction (2, 7, 9). However, HSK is caused by a highly cytolytic virus which is usually absent from the tissue during clinical HSK (19, 24). This observation is confirmed in the present communication and is extended by showing that viral mRNA is also absent during clinical HSK in immunocompetent mice. In addition, HSV-infected SCID mice may develop lesions when reconstituted with T cells in the absence of a detectable virus-specific immune response (22). Taken together, such observations may imply that infection with HSV causes the expression of nonviral components in the cornea which then act as target structures for the immunopathological reaction. Indeed, HSK might represent another example of determinant spreading, whereby virus infections induce immune reactions that subsequently involve self components (34, 37). In the present study, we have evaluated the role of virus replication in initiating HSK. Our results demonstrate that although virus and demonstrable antigens are absent during clinical HSK, only replication-competent virus is able to elicit the disease. Our results are discussed in terms of the likely consequences of a replicating cytolytic virus at a site that is usually sequestered from immune surveillance.

MATERIALS AND METHODS

Mice. Four- to 5-week-old euthymic BALB/c (Harlan Sprague Dawley, Inc. Indianapolis, Ind.) and CB-17 ($H2^d$)$^{scid/scid}$ (Taconic, German Town, N.Y.) mice were used for experiments. The animals were acclimated for a week before use in a specific-pathogen-free animal colony accredited by the American Association for Accreditation of Laboratory Animal Care. The SCID mice were housed in sterile microisolator cages. All food, water, bedding, and instruments were autoclaved or disinfected, and all manipulations were done in a laminar flow hood. All experimental procedures were conducted according to the Association for Research in Vision and Ophthalmology resolution on the use and care of laboratory animals.

Viruses. Wild-type HSV type 1 (HSV-1) RE and HSV-1 KOS1.1 were propagated and assayed on Vero cells by measurement of PFU or 50% tissue culture infective doses by standard protocols. The mutant viruses (*d*301 and *n*504) were engineered from the KOS1.1 strain. The *d*301 virus (14) contains a deletion in the ICP8 gene. This β gene product is a major viral DNA-binding protein and is required for viral DNA replication and late gene (γ2) expression. The *n*504 virus contains a nonsense mutation in the ICP27 gene (32). This immediate-early (α) protein is required for viral DNA synthesis and γ2 gene expression. Both mutants express the viral α, β, and γ1 protein products. In the absence of the complementing gene products, both viruses do not express the late structural γ2 genes and fail to produce infectious progeny viruses. The *d*301 and *n*504 mutants were propagated and titrated on S-2 cells expressing ICP8 and on V27 cells expressing ICP27, respectively. In the initial inoculum, equal numbers of PFU of the replication-defective mutant viruses have been demonstrated to deliver numbers of

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virion particles approximately similar to that delivered by the wild type as measured by viral DNA content (14, 32). UV inactivation of the wild-type virus was carried out for 2 min.

Corneal infection and clinical observation. The corneal surfaces of the deeply anesthetized (methoxyflurane [Metofane]; Pittman-Moore, Mondelein, Ill.) mice were scarified with a 27-gauge needle, and 1×10^6 PFU of the wild-type HSV-1 KOS and RE viruses, 1×10^6 PFU (prior to UV inactivation) of the UV-inactivated wild-type viruses, or 2×10^6 PFU of the mutant viruses was applied in 4-μl volumes and gently massaged with the eyelids. To increase the sample size and to assess the function of the mutant viruses, wild-type-HSV-immune, $CD4^+$ T-cell-reconstituted SCID mice were infected with wild-type HSV in one cornea and with one of the mutant viruses in the other cornea. In one experiment, HSV-1 RE (noninactivated and UV inactivated) was used in the euthymic mice at 5×10^6 50% tissue culture infective doses. Animals were examined at different days postinfection (p.i.) by slit lamp biomicroscopy (Keeler Instruments, Broomall, Pa.), and the severity of the stromal keratitis in each cornea was recorded as described elsewhere (26). At the termination of the experiments, eyes were collected for histological evaluation.

Limiting-dilution analysis. Single-cell suspensions were made from the splenocytes of euthymic mice infected with HSV-1 KOS1.1, *d*301, or *n*504 at the termination of the experiments (28 days p.i.), erythrocytes were lysed with an ammonium chloride-Tris solution (pH 7.4), and macrophages and dendritic cells were removed by adherence onto plastic. The T cells in the nonadherent cell population were enriched by depleting the B cells by two rounds of panning on rabbit anti-mouse immunoglobulin M-coated plates. The resulting T cells were used for limiting-dilution analysis for a virus-specific response. Thirty replicates of various dilutions of the responder T-cell population were cultured for 7 days with mock-infected syngeneic stimulator cells or with syngeneic stimulator cells infected with HSV-1 KOS1.1 that had been UV inactivated for 2 min (multiplicity of infection of 5 prior to UV inactivation). At the last 18 h of the culture period, 0.75 μCi of [^{3}H]thymidine was added to each well, and lymphoproliferation was measured by [^{3}H]thymidine incorporation in a Trace-96 β-dry counter (Inotech, Lansing, Mich.). Positive wells incorporated an amount of radioactivity greater than the mean counts per minute of control wells plus two standard deviations. Estimates of responding-cell frequency were obtained by the maximum-likelihood method (17).

Donor cell preparation and adoptive transfer. Immune donor cells were isolated from euthymic BALB/C mice that were immunized intraperitoneally 7 days earlier with HSV-1 KOS1.1 (5×10^6 PFU). Single-cell suspensions were made from the splenocytes, and T cells were prepared as described above. The resulting T cells were enriched for $CD4^+$ T cells by using the CD4 subset column (R & D System, Minneapolis, Minn.) for adoptive transfer. A minimum of 6×10^6 $CD4^+$ T cells per mouse, which is sufficient to induce HSK lesions (6), were transferred intravenously to SCID mice that had been infected 1 day earlier on the cornea with wild-type, replication-defective, or UV-inactivated viruses. In one experiment, control SCID mice were infected with wild-type HSV-1 KOS1.1 but were not reconstituted with T cells. The enriched $CD4^+$ T cells generally contained >80% $CD4^+$ T cells (6).

Virus isolation and titration. At various times after infection with HSV-1 RE (10^6 PFU), groups of three mice were sacrificed and corneas were dissected free of iris and scleral tissues. Pooled corneal samples were placed in 100 μl of assay medium (phosphate-buffered saline [PBS] containing 0.1% glucose and 1% fetal calf serum) and frozen at −70°C until used. The samples were thawed on the day of assay and minced into fine pieces on ice. Individual eye tissue homogenates were prepared from groups of four mice infected with *d*301 and HSV-1 KOS1.1. Intracellular virus was released from tissue homogenates by three freeze-thaw cycles. The tear films were collected on cotton swabs from groups of four individual mice infected with HSV-1 KOS1.1 and were placed in 1 ml of assay medium. All viruses were applied on the scarified cornea. The recovery of infectious virus from the pooled cornea or individual eye tissue homogenates or from tear film samples was determined by standard PFU assay on Vero cell monolayers for wild-type HSV and on S-2 cells for *d*301.

PCR analysis of viral gene expression and viral DNA in corneal samples. To measure viral DNA and gene expression, three corneas were collected at various times after infection. The total cellular RNA and DNA of the minced corneal tissue were prepared by using TRI reagent (Gibco BRL) as suggested by the manufacturer. All materials used for RNA and DNA isolation were RNase and DNase free, respectively. For analysis of viral mRNA transcripts, isolated total cellular RNA was further treated with DNase to digest any possible contaminating viral DNA. The DNase was inactivated by heating the reaction mixture at 65°C for 10 min. RNA was purified by phenol-chloroform extraction and ethanol precipitation. Total cellular RNA was reverse transcribed in a 40-μl reaction mixture (5 mM $MgCl_2$, 25 mM KCl, 0.1% Triton X-100, 40 U of RNase inhibitor, 2 mM deoxynucleoside triphosphates, and 10 mM dithiothreitol) containing 0.5 μg of random primers (Promega, Madison, Wis.) and oligo dT_{18} with 15 U of avian myeloblastosis virus reverse transcriptase at 42°C for 1 h. PCR was carried out in a 25-μl volume with specific primers, either with the cDNA sample or with the DNA, for 35 cycles. The PCR mixture consisted of 2 mM $MgCl_2$; 0.01% Triton X-100; 125 μM each dATP, dCTP, dGTP, and dTTP; 50 mM Tris HCl (pH 8.3); 1 U of *Taq* DNA polymerase; and 2 to 4% dimethyl sulfoxide. The first cycle of PCR was at 95°C for 5 min, 60°C for 2 min, and 72°C for 3 min; this was followed by 34 cycles at 94°C for 1 min 30 s, 55°C for 1 min, and 72°C for 2 min.

TABLE 1. Recovery of infectious virus from corneal swabs and tissue homogenates following topical corneal infection of BALB/c mice with HSV-1[a]

Day p.i.	Viral titer (log_{10} PFU) after infection with:	
	HSV-1 KOS[b]	HSV-1 RE[c]
1	4.9 ± 0.9	5.8
2	4.1 ± 0.1	ND[d]
3	2.8 ± 0.5	3
4	3.0 ± 0	ND
5	ND	4
7	ND	UD[e]
9	ND	UD

[a] HSV-1 KOS or HSV-1 RE (10^6 PFU) was inoculated on the scarified cornea, and at the indicated times the presence of infectious virus in corneal swabs or tissue homogenates was determined by standard plaque assay.

[b] Results are expressed as mean titer (± standard deviation) in individual eye swabs ($n = 4$).

[c] Results are expressed as mean titer in pooled corneal tissue homogenates ($n = 3$).

[d] ND, not done.

[e] UD, undetectable, i.e., below the sensitivity of the assay (<3 PFU).

Expression of β-actin and hypoxanthine phosphoribosyltransferase mRNAs in the corneal tissue sample was used as a control for RNA isolation, and amplified products were resolved on a 2% agarose gel. The following PCR primers (5′→3′) were used: gD, GC CCG AGA CCC CCA ACG CCA (sense) and CAG GCG GAA CAG GTG CGC GT (antisense); ICP27, ATG CTA ATT GAC CTC GGC CTG (sense) and GCC GTG CAC GTA CGG GGG GGC (antisense); DNA polymerase, CAT CAC CGA CCC GGA GAG GGA (sense) and GGG CCA GGC GCT TGT TGG TGT A (antisense); ribonucleotide reductase, GAC AGC CAT ATC CTG AGC (sense) and ACT CAC AGA TCG TTG ACG ACC G (antisense); β-actin, GTA GGG CGC CCC AGG CAC CA (sense) and GGC TTT GTA TTT GGC TTT TCC AGT (antisense); and hypoxanthine phosphoribosyltransferase, HPRT, ATG CCG ACC CGC AGT CCC AGC G (sense) and GGC TTT GTA TTT GGC TTT TCC AGT (antisense).

The quantification of ICP27 mRNA expression in tissue samples was done by a competitor PCR approach described in detail elsewhere (4). Briefly, a constant amount of cDNA sample was coamplified in exponential phase with various concentrations of a synthetic competitor standard containing ICP27 primer sequences. The construction and design of the internal standard were essentially similar to those of the one described previously (4). The ratios of the amplified ICP27 and competitor product intensities were quantified in a imaging densitometer (Bio-Rad model GS-670), and the number of mRNA copies was determined when the ratio of the amplified products was 1.

Immunohistochemistry for viral antigens in corneal specimens. Eyes infected with HSV-1 KOS1.1 or RE (10^6 PFU) were collected at different time p.i., embedded in O.C.T. compound (Miles, Elkhart, Conn.), and snap frozen in liquid nitrogen. Frozen sections (8 μm) were made, fixed in cold acetone for 10 min, and treated with 2% bovine serum albumin for 30 min. The sections were then treated with rabbit anti-HSV serum (1:500 dilution; Dako, Carpenteria, Calif.) for 30 min and then with biotinylated anti-rabbit immunoglobulin (1:20 dilution; Biogenex, San Ramon, Calif.) for another 30 min. The sections were washed with PBS, treated with avidin-biotin-peroxidase (Vector, Burlingame, Calif.), washed, and subsequently treated with aminoethyl carbozole substrate for 5 min. After being washed, the sections were counterstained with hematoxylin. Sections treated with non-HSV-specific rabbit immunoglobulin in the primary staining reactions were included as negative controls.

RESULTS

Viral replication and expression following infection. Following infection of the scarified corneas of groups of immunocompetent mice, corneal tissues were collected at various times and assayed for virus and viral antigen as well as for viral mRNA and viral DNA. The RE strain of virus was used routinely, but viral replication and antigen expression were also measured with HSV-1 KOS1.1, the wild-type parent virus for the replication-defective mutants discussed below. Infectious virus could be detected for up to 5 days but was absent in corneal samples tested at 7 days and later (Table 1). Up to and including day 7, multiple species of viral mRNA were detectable by

TABLE 2. Detection of viral mRNA and DNA and quantitation of HSV-1 immediate-early gene (ICP27) mRNA in the cornea at different times p.i.[a]

Day p.i.	No. of ICP27 mRNA copies/cornea	Viral mRNA in cornea			Viral DNA in cornea	
		DNA polymerase	Ribonucleotide reductase	Glycoprotein D	DNA polymerase	Glycoprotein D
1	4.92×10^8	+[b]	+	+	+	+
3	9.75×10^7	+	+	+	+	+
5	8.9×10^7	+	+	+	+	+
7	7.9×10^6	+	+	+	+	+
9	Neg[c]	Neg	Neg	Neg	+	+
12	Neg	Neg	Neg	Neg	+	+
15	Neg	Neg	Neg	Neg	+	+
17	Neg	Neg	Neg	Neg	+	+
22	Neg	Neg	Neg	Neg	+	+

[a] A quantitative estimation of HSV immediate-early (ICP27) mRNA expression and a profile of qualitative PCR analyses of early (ribonucleotide reductase and DNA polymerase) and late (glycoprotein D) mRNA expression and of the presence of viral DNA in the cornea at different times following infection with HSV-1 RE (10^6 PFU) are shown. Total cellular RNA was isolated from the corneal samples as described in Materials and Methods, and levels of expression of ICP27 mRNA were quantified by a competitive PCR approach as described in detail elsewhere (4) with a synthetic internal standard. For qualitative reverse transcriptase PCR analysis of early and late genes, an equal volume of cDNA sample was amplified with viral mRNA-specific primers. The DNA was isolated from the cornea by using TRI reagent according to the manufacturer's instructions. Viral DNA polymerase and gD primers were used for viral DNA amplification.

[b] +, amplified products were detected.

[c] Neg, amplified products were undetectable after 35 cycles of amplification.

reverse transcriptase PCR, but at 9 days and later, neither immediate-early, early, nor late gene viral mRNA was found (Table 2). In contrast, samples analyzed by PCR for viral DNA were positive on all occasions until at least 22 days p.i. (Table 2). In the cases of both viral replication and mRNA, when positive samples were quantified, marked concentration differences were not noted.

Samples from animals infected with either HSV-1 RE or KOS1.1 and tested for viral antigen by immunohistochemistry revealed readily detectable antigen in the epithelium at 24 h p.i. (Fig. 1A). With time, viral antigen detection was confined to patches in the epithelium (Fig. 1B), and little if any signal was present in the stroma. Traces of antigen were detected in patches in the epithelium until day 5 but at no time thereafter (Fig. 1C).

The time frames for virus replication and antigen expression are summarized in Fig. 2. It is apparent that with the exception of viral DNA, measurable evidence of viral activity, such as the presence of live virus, viral antigen, or viral mRNA in the cornea, was undetectable by the time when clinical lesions appear. Clinical lesions may become evident at 10 to 12 days p.i. but are not at their peak histologically until 14 to 21 days p.i.

Failure to develop HSK in immunocompetent mice infected with replication-defective or inactivated virus. Whereas mice infected with replication-competent virus readily developed clinical lesions, virus inactivated by UV irradiation prior to application induced neither clinical nor histopathological lesions (Fig. 3). The dose of virus used was sufficient to induce an immune response, because mice given UV-inactivated virus topically to the scarified cornea did develop an HSV-specific delayed-type hypersensitivity reaction and a virus-specific lymphoproliferation response (data not shown).

In other experiments, mice were topically infected with replication-defective viruses *d*301 (deficient in ICP8 [14]) and *n*504 (deficient in ICP27 [32]), and the clinical outcome was evaluated. These replication-defective viruses were shown previously to induce effective immune responses, which in fact closely resemble those induced by wild-type virus (25, 28). As shown in Table 3, mice infected with the mutant viruses failed to shed progeny virus which could be recovered by using permissive cell culture for mutant virus detection. However, topical corneal infection with at least the *d*301 virus was considered to result in infection, since mutant virus was recoverable from tissue homogenates at 4 h p.i. (eclipse phase) in approximately the same amount as was found at the eclipse phase in tissue homogenates from animals infected with wild-type HSV-1 KOS1.1. All animals exposed to a mutant virus failed to express HSK, even though the infecting virus concentration in some experiments was four times the dose of wild-type virus used (Fig. 4). In support of previous observations with subcutaneous immunization (25), topical corneal infection with mutant viruses did elicit virus-specific immune responses, although as measured by limiting-dilution analysis for antigen-specific proliferating T cells, the responses were 10- to 14-fold lower than those in mice infected with wild-type HSV-1 KOS1.1 (Fig. 5).

Replication-defective mutants fail to induce HSK in SCID mice reconstituted with virus-specific $CD4^+$ T cells. As shown previously, SCID mice readily develop HSK if, following infection with HSV, they are reconstituted with virus-immune T cells (22). The disease appears to be essentially identical to that in immunocompetent mice except that it occurs and progresses more rapidly. The SCID mouse model made it possible to ensure that the adoptively transferred cells were reactive with HSV and to eliminate the chance that the failure of mutant viruses to cause HSK was associated with the minimal immune response they might induce. In these experiments SCID mice were infected in the cornea either with replication-competent virus or with UV-inactivated or replication-defective virus. The mice subsequently received HSV-immune $CD4^+$ T cells and were monitored for the development of clinical signs of HSK (Table 4). Only eyes infected with replication-competent virus developed lesions. In some experiments, some animals received wild-type virus in one eye and mutant virus in the other. In all instances, only the eye receiving wild-type virus developed lesions (Table 4). In addition, in some instances mice infected with mutant viruses were reconstituted with three times the number of $CD4^+$ T cells that is usually adequate to permit HSK development if mice are infected with wild-type virus. Such mice still had no recognizable clinical response in the cornea.

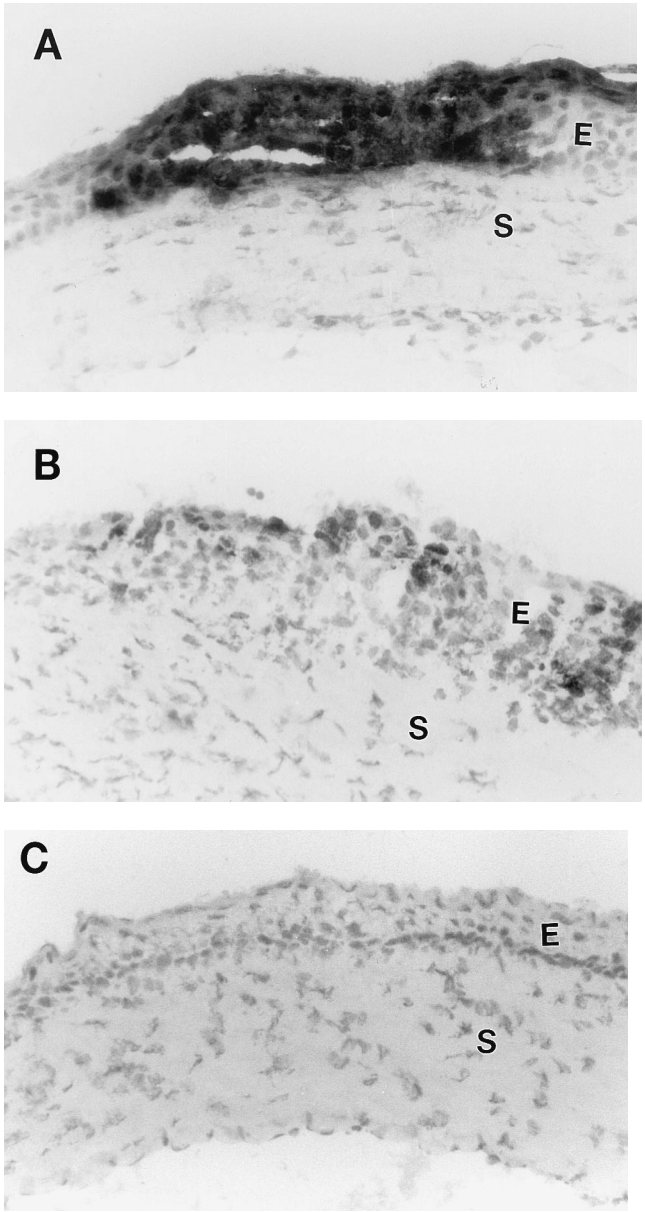

FIG. 1. Detection of viral antigens in BALB/c mouse corneal samples by immunohistochemistry. Frozen sections were prepared from corneal samples infected with HSV-1 KOS1.1 (10^6 PFU) and stained for viral antigens by using rabbit-anti HSV immunoglobulins as described in Materials and Methods. Sections stained with rabbit immunoglobulins were used as a negative control. Localization of viral antigen expressed in the cornea at days 1 (A), 4 (B), and 7 (C) p.i. is shown. E, epithelium; S, stroma. Original magnification, ×250. (Data are shown for KOS1.1 only.)

DISCUSSION

Topical corneal infection with HSV results in a chronic inflammatory response of the corneal stroma (HSK) mediated by $CD4^+$ Th1 cells (5, 16, 30). However, the molecular details of the disease process and the nature of the antigens recognized by T cells that serve to drive the chronic inflammatory response remain to be identified. In the present report, we confirm that replicating virus as well as viral mRNA is absent from the cornea at the time when lesions are clinically apparent, but we demonstrate that an initial period of viral replication is necessary in order to induce HSK. Accordingly, immunocompetent animals infected with a high dose of replication-defective or UV-inactivated virus failed to develop HSK. The infections did, however, result in demonstrable virus-specific immune responses. The replication-defective viruses also failed to elicit HSK in an adoptive transfer model in which a rapid onset of HSK lesions occurs in SCID mice infected with wild-type HSV and reconstituted with virus-immune $CD4^+$ T lymphocytes.

In humans as well as in the experimental disease in mice, HSK is considered to be an immunopathological lesion (12). The evidence favoring this conclusion includes the facts that HSK does not occur in T-cell-deficient mice (22, 23), that T-cell suppression may prevent the disease (27), and that reconstitution of virus-infected SCID or athymic mice with $CD4^+$ T lymphocytes restores the occurrence of HSK (1, 22).

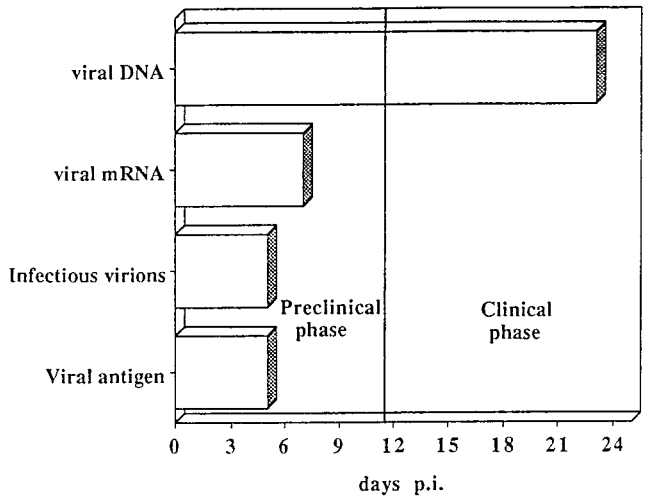

FIG. 2. Cumulative viral antigen recovery, antigen detection, and viral mRNA and DNA detection in corneal samples during the progression of HSK.

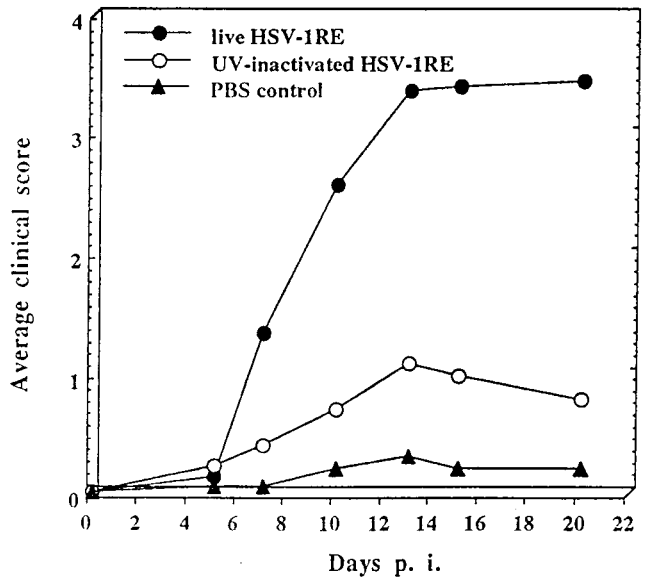

FIG. 3. Groups of seven euthymic BALB/c mice were infected on the cornea with 5×10^6 50% tissue culture infective doses of live or UV-inactivated HSV-1 RE on the cornea, and clinical signs of HSK were monitored with slit lamp microscopy and scored as described previously (26). Control animals received PBS on the scarified cornea.

TABLE 3. Viral titers in corneal swabs and eye tissue homogenates following topical corneal infection with replication-defective mutant and wild-type HSV-1[a]

Time p.i.	Viral titer ($\log_{10}$ PFU)[b] after infection with:		
	*d*301		HSV-1 KOS1.1 (eye tissue)
	Eye swab	Eye tissue	
4 h	UD[c]	1.24 ± 0	1.6 ± 0.5
1 day	UD	UD	5.6 ± 0.2
2 days	UD	UD	4.6 ± 0.6
3 days	UD	UD	3.7 ± 0.5
4 days	UD	UD	5.5 ± 2.8
5 days	ND[d]	UD	3.4 ± 0.5

[a] HSV-1 *d*301 or HSV-1 KOS1.1 was inoculated on the scarified cornea, and at the indicated times the presence of infectious virus in individual corneal swabs and eye tissue homogenates was determined. *d*301 and KOS1.1 were titrated on S-2 and Vero cells, respectively, by standard plaque assay.
[b] Expressed as mean ± standard deviation ($n = 4$).
[c] UD, undetectable, i.e., below the sensitivity of the assay (<3 PFU).
[d] ND, not done.

Exactly how HSV infection of the cornea gives rise to the immunopathology remains in doubt. Most virus-induced immunopathological reactions occur in response to active persistent virus infections in which the virus itself is often noncytolytic and the inflammation is sustained by T-cell recognition of viral antigens. Well-studied murine examples of viral immunopathology include lymphocytic choriomeningitis virus and Theiler's murine encephalitis virus infection, as well as hepatitis B virus infection in transgenic mice (2, 7, 9, 15). The situation with HSV infection appears to be quite dissimilar. In this instance the virus is usually cytolytic to cells that it infects, and virus is usually eliminated rapidly from peripheral sites of infection in the immunocompetent host (33).

Clearance of virus from the peripheral sites is thought to be mainly mediated by NK cells (20, 39) and $CD4^+$ T cells (26).

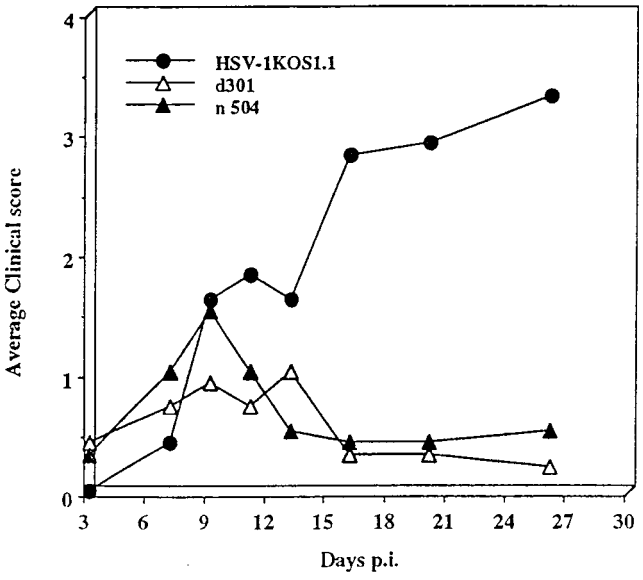

FIG. 4. Clinical scores of (26) euthymic animals ($n = 5$) infected with HSV-1 KOS or the *d*301 or *n*504 mutant. HSV-1 KOS (5×10^5 PFU) and mutant viruses (2×10^6 PFU) were inoculated on the scarified cornea, and the manifestation of HSK was observed at different times p.i. by slit lamp microscopy. At the termination of experiments, all samples were examined histologically.

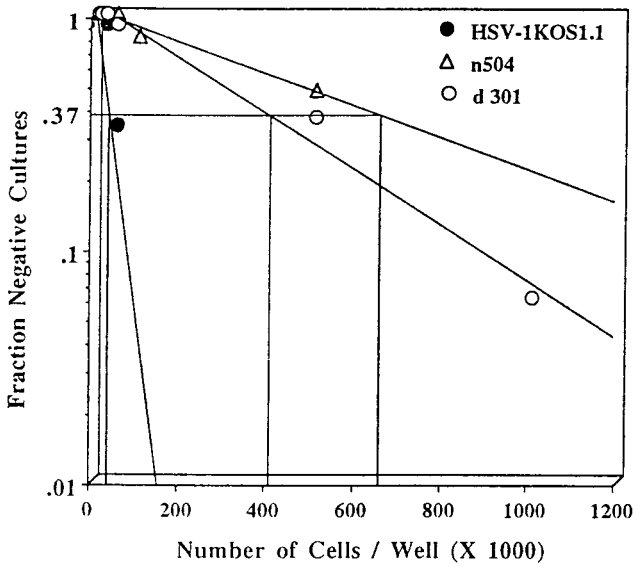

FIG. 5. Limiting-dilution analysis of virus-specific responding T cells among splenic cells of BALB/c mice infected on the cornea with wild-type HSV-1 KOS1.1 or with replication-defective mutant virus at the termination of the experiment (28 days p.i.). Thirty replicates of various responder cell concentrations were stimulated with mock-infected or HSV-1 KOS1.1-infected stimulator cells for 7 days, and the responding-cell frequency was determined as described in Materials and Methods.

Virus always remains in a nonreplicating state (latency) in the nervous system and is not considered to persist in any form at peripheral sites (13, 36). This issue, however, is controversial (8, 10, 24). For instance, by using an in situ PCR approach, HSV DNA was detected for weeks in the corneal epithelium (24). In the same study, however, no evidence of infectious virus or viral antigen was detectable after the initial phase of infection. As shown in this report, viral antigen is readily demonstrable in the epithelium but not in the stroma, which is the site of the chronic inflammatory response. Moreover, demonstrable viral antigen was absent after 5 days, and viral mRNA was undetectable beyond 7 days. Consequently, evidence of viral expression was absent well before histological evidence of HSK was at its peak (days 14 to 21 [5]).

Taken together, the observations described above indicate that the chronic inflammatory response that typifies HSK occurs at a location where and time when viral antigens cannot be demonstrated. Such observations could indicate that the T cells present in the cornea may be activated to orchestrate the inflammatory event not by reacting with viral peptides but perhaps in response to host-derived determinants unmasked as a result of the virus infection. Indeed, the old notion that some determinants exposed as a result of virus infection are involved as agonists in the chronic autoimmune inflammatory response is gathering increasing credence (21, 34, 37). It is also known that coimmunization with viral and self antigens may lead to a breakdown in tolerance (11).

With respect to the pathogenesis of HSK, neither viral nor host antigens involved in driving inflammation have been defined. What the present investigation indicates, however, is that the induction of viral or self antigens involved in HSK requires a productive virus infection in the cornea. Accordingly, infection either with viral mutants incapable of generating replication-competent progeny or with UV-inactivated virus failed to induce HSK. The failure of the mutant and inactivated viruses to induce HSK was not because of an in-

TABLE 4. Summary of HSK manifestation in SCID mice reconstituted with HSV-immune CD4$^+$ T cells and infected with replication-defective, UV-inactivated, and wild-type HSV[a]

No. of donor CD4$^+$ cells/mouse	Virus	Clinical severity[b]	Histological appearance
6×10^6	HSV-1 KOS1.1	4, 4, 3, 2	Marked PMN[c] infiltration with tissue damage
	HSV-1 KOS1.1 (UV inactivated)	0, 0, 0, 1	Clear cornea[d]
	*d*301	1, 0, 0, 0	Clear cornea
	*n*504	1, 1, 0, 0	Clear cornea
14×10^6	HSV-1 KOS1.1	5, 4, 4	Marked PMN infiltration with tissue damage
	*d*301	2, 2, 0, 0, 0, 0	Few PMNs and T cells in peripheral cornea in samples with mild clinical lesions
	*n*504	2, 1, 0, 0, 0, 0	Same as for *d*301
None (control)	HSV-1 KOS1.1	0, 0, 0, 0	Clear cornea[e]

[a] HSV-1 KOS1.1-immune CD4$^+$ T cells (6×10^6 or 14×10^6) from the donor BALB/c mice were transferred intravenously to SCID mice that had been infected with the various viral preparations 1 day prior to transfer. Three or four SCID mice per group were used. The mice were infected with HSV-1 KOS1.1 (5×10^5 PFU) in one cornea and with *d*301 (2×10^6 PFU) in the other. Separate groups of mice were infected with UV-inactivated HSV-1 KOS1.1 in one cornea and with *n*504 (2×10^6 PFU) in the other. Two nonreconstituted SCID mice infected with HSV-1 KOS1.1 were included as controls, and the animals were scarified at day 9 p.i., when clinical signs of encephalitis were noticed.

[b] Response (26) of individual corneas at the termination of the experiment (16 to 18 days after transfer).

[c] PMN, polymorphonuclear leukocyte.

[d] Lack of inflammatory cellular infiltration with edema in corneas with mild clinical lesions.

[e] Histological examination of the control animals at the termination of the experiment showed that the corneas were free of HSK lesions, as reported elsewhere (22).

ability to induce immune responses necessary for the immunopathology. Thus, immune responses were observed and, more importantly, HSK failed to occur in SCID mice infected with mutant virus and given large numbers of HSV-immune CD4$^+$ T cells. The replication-defective mutants used in the present study had mutations in the ICP8 and ICP27 genes and so may not express some key late antigens possibly involved in immunopathology. However, preliminary studies with other mutants, including the AN-1 virus, which can express all viral proteins but cannot package the virus effectively (35), also failed to generate HSK. Currently, we have no mechanistic explanation for the need for viral replication to induce HSK. One effect may be associated with extensive cell damage resulting from virus replication, which may serve to elaborate levels of chemokines adequate to signal the invasion of T cells and other inflammatory cells. Recently, HSV infection of the cornea, as well as infection of corneal cells in vitro, was shown to result in the generation of multiple chemokine mRNAs (18, 31). Another possibility worth exploring is that the generation of sufficient virus may result in its uptake by inflammatory cells, in particular by the abundant neutrophils found early in infection (38), and that when such cells die by apoptosis, altered host determinants could be generated. This type of effect was recently described for Sindbis virus, wherein apoptotic blebs were observed to contain clusters of autoantigen and viral glycoprotein which could possibly initiate an autoreactive response (3).

We are currently attempting to further understand the roles of viral and host components in HSK by defining the reactivity pattern of inflammatory T cells recovered from the cornea, as well as by ascertaining whether adoptive immunization with inflammatory corneal cells results in virus-specific as well as self-reactive T-cell responses.

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