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Corneal Herpes Simplex Virus Type 1 Superinfection in Patients with Recrudescent Herpetic Keratitis

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Purpose. Herpetic keratitis is a common sequel of a corneal infection with herpes simplex virus (HSV)-1. Recrudescent herpetic keratitis (RHK) may result in irreversible damage to the cornea. Recurrences may be caused by reactivation of endogenous HSV-1 or reinfection with exogenous HSV-1. The objective of this study was to determine the incidence and risk factors involved of HSV-1 superinfection in patients with RHK.

Methods. From 30 patients with RHK, sequential corneal HSV-1 isolates were genotyped by PCR amplification of the hypervariable regions located within the HSV-1 genes *US1*, *US10/11*, and *US12*. The clinical data from the patients obtained retrospectively were: ophthalmologic history, clinical picture during recurrences, number and time points of penetrating keratoplasty (PKP), and steroid or acyclovir treatment.

Results. Whereas the sequential corneal HSV-1 isolates of 19 (63%) of 30 patients had the same genotype (designated as group 1), the sequential isolates of 11 patients (37%) were genetically different (designated as group 2). Among the clinical data analyzed, only the time point of PKP was significantly different between the patient groups. Although no patients in group 1 had undergone transplantation between samplings, 4 of 11 patients in group 2 underwent PKP during the interrecurrence period in the same eye from which the corneal HSV-1 isolates were obtained.

Conclusions. The data demonstrate that RHK is frequently associated with corneal reinfection with a different HSV-1 strain and suggest that PKP is a risk factor for corneal HSV-1 superinfection. (*Invest Ophthalmol Vis Sci.* 2002;43:358–363)

Herpes simplex virus (HSV) infections may elicit a variety of serious diseases in humans, including chronic herpetic keratitis.[1,2] A hallmark of HSV and other neurotropic herpes viruses is their ability to establish latency in sensory nerve ganglia of the host.[1] Despite the induction of an acquired state of immunity after primary HSV infection, recrudescent herpetic lesions are often observed.[1] Patients who have had corneal HSV-1 infection risk recurrent corneal disease throughout life. Particularly prolonged or recurrent episodes of herpetic keratitis can result in decreased vision or blindness due to the development of herpetic stromal keratitis (HSK).[2,3]

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Recrudescent HSV infections are thought to result from reactivation of the HSV strain acquired during primary infection.[4–6] However, reinfection with a new HSV strain (i.e., superinfection) at the site of primary infection has also been documented.[6,7] The route or mode of HSV superinfection and its clinical consequences remain enigmatic. Genetically different HSV strains have been shown to induce different types of ocular lesions.[8] Furthermore, newly acquired herpetic keratitis may develop after penetrating keratoplasty (PKP) in patients who undergo transplantation for reasons unrelated to HSV infection, suggesting the possibility of HSV-1 transmission through cornea transplantation.[9] These issues underline the clinical importance of knowing whether recurrent corneal HSV-1 infections are caused by reactivation of latent virus or superinfection with a different virus strain. Molecular analyses of corneal HSV-1 isolates may allow distinction between both options.

The genome of HSV-1 consists of a unique long (U_L) and a unique short (U_S) component, each of which is flanked by a pair of oppositely oriented repeat elements. Several hypervariable regions have been identified in the HSV-1 genome. These regions encompass unique tandemly repeated sequences, reiterations (Re) that vary in copy number and nucleotide sequences (Fig. 1).[1,10,11] Generally, two types of restriction fragment length polymorphism (RFLP) analyses are used to differentiate HSV-1 isolates. One type is the variation due mostly to a gain or loss of a restriction enzyme cleavage site. The other appears as variation in length of cleaved fragments derived from Re-containing genomic HSV-1 regions.[11] Among the eight Re regions described for HSV-1, ReIV and -VIII (both located within the introns of genes *US1* and *US12*) and ReVII (located within the protein coding region of genes *US10* and *US11*) have been shown to remain stable during in vitro culture and have been used as sensitive and reliable markers to differentiate HSV-1 strains.[12–15]

We have recently developed a PCR method, based on the stability and strain-to-strain differences of ReIV, -VII, and -VIII that has facilitated the differentiation of up to 92% of unrelated HSV-1 strains.[12,15] The purpose of the present study was to determine the incidence and risk factors involved in corneal HSV-1 superinfection in patients with recrudescent herpetic keratitis (RHK).

Materials and Methods

Patients and Clinical Samples

Corneal swab specimens were obtained for diagnostic reasons from suspected herpetic corneal lesions and were used to inoculate human embryonic lung fibroblasts. Virus was harvested when approximately 75% of the monolayer showed cytopathic effect and was subsequently typed for HSV-1 or -2 by immunocytology and PCR.[15] Serial samples from 30 immunocompetent patients with recurrent corneal HSV infections were found in a databank of 408 frozen corneal HSV-1 cultures collected since 1980 at the Rotterdam Eye Hospital (Rotterdam, The Netherlands). The clinical items scored retrospectively were anatomic location (i.e., left or right eye), previous history of ocular disease, clinical picture at presentation of each recurrence, therapy regimen

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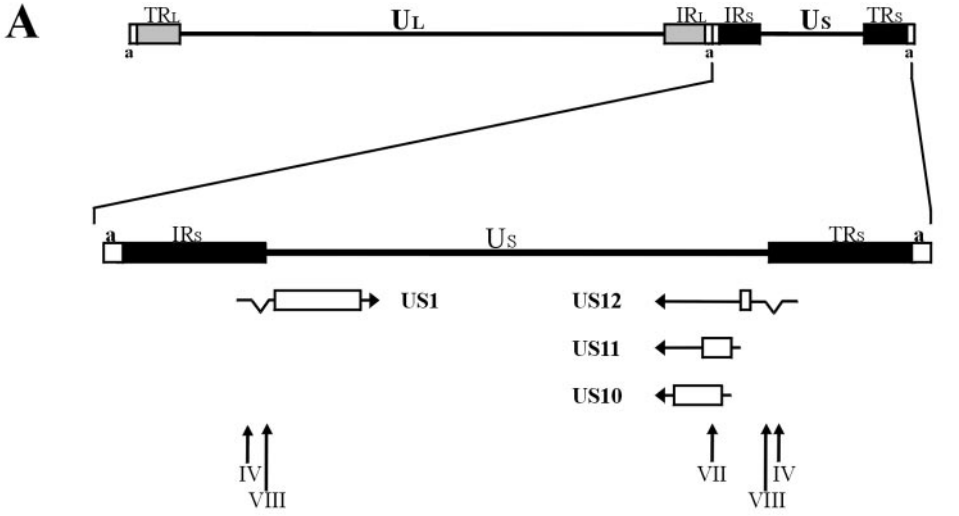

B

Reiteration	Sequence (5'→3')	Length (bp)
IV	CCCCCTCCTCCGCCCCCGCGTC	22
	CCCCCTCCTCCACCCCCGCGTC	22
VII	CCCCGGTCTCCCCGGGAG	18
VIII	GACGAGCAGGAAGCGGTCCACGCAACGGTCGCCGCCGGTCGCCTC	45

FIGURE 1. Map of the HSV-1 genome including the location and sequences of the Re regions tested. (**A**) The prototypic HSV-1 genome encompasses the covalently linked components L and S. Each component consists of unique sequences (U_L and U_S) bracketed by inverted repeat sequences (TR_L, IR_L, IR_S, and TR_S). A short sequence, a, is repeated directly at the termini of the genome and located at the L–S junction. The enlargement of the S component shows the 5′→3′ orientations of mRNA species as *horizontal arrows* with introns shown as *V-shaped indents*. Protein-coding regions are shown as *open boxes*. *Vertical arrows* indicate locations of Re regions, and Roman numerals indicate their locations as defined previously.[10,12] (**B**) Re-specific sequences have been described previously.[10,12] ReIV exists as two forms that differ from each other in a single residue.[10]

preceding the culture dates, total number of PKPs, and PKPs between virus culture dates. The classification of herpetic keratitis was defined on clinical criteria.[2] The present study was performed according to the Declaration of Helsinki, and informed consent was obtained.

Genotypic Analyses of Corneal HSV-1 Isolates

Genotypic analyses of the viral strains were performed by amplification of the hypervariable regions within the HSV-1 genes *US1*, *US10/11*, and *US12*. This method is based on strain-to-strain differences in the number of Re and point mutations within these hypervariable genomic regions.[10,12,13,15] DNA was extracted from the primary corneal HSV-1 cultures, lysed in a guanidine isothiocyanate buffer using a silica solution (Celite; Jansen Chemika, Beers, Belgium), as described previously.[15] The PCR primers and conditions for amplifying and detecting by Southern blot analysis of the hypervariable regions of the HSV-1 genes *US1*, *US10/11*, and *US12* have been described.[15] In case of small differences in length between amplicons (i.e., PCR products) from individual samples, the PCR products were run on denaturating (8 M urea) 6% acrylamide gels. The lengths of the amplicons were estimated by comparison to a 100- and 25-bp DNA ladder (Gibco BRL, Grand Island, NY). To confirm similarities or differences in amplicon length, all samples were finally electrophoresed in order of increasing length.

Statistical Analyses

The statistical evaluation of the results was performed using the Fisher exact test. Results were considered statistically significant at $P < 0.05$.

RESULTS

Patients' Characteristics and Genotypic Analyses of Sequential Corneal HSV-1 Isolates

The group of 30 patients with RHK included in this study consisted of 13 women and 17 men (mean age, 58.1 years; range, 17–78). From each patient, two ($n = 25$) or three ($n = 4$) sequential corneal HSV-1 isolates were obtained (mean time interval, 29.8 months; range, 0–170). Patient 22 had bilateral herpetic keratitis (Table 1).

To differentiate whether RHK is due to reactivation of latent HSV-1 or superinfection with another HSV-1 strain, the sequential corneal HSV-1 isolates of the patients with RHK were genotyped using a recently developed PCR-based DNA fingerprint assay.[15] The results of the PCR analyses, on the hypervariable regions of the genes *US1*, *US10/11*, and *US12*, performed on the corneal HSV-1 isolates are summarized in Table 1. As an example, the size fractionation and Southern blot analyses of the *US1*- and *US12*-specific amplicons obtained from the sequential samples of patients 1 through 5 and 20 through 24 are shown in Figure 2. The sequential corneal HSV-1 isolates of 19 (63%) of the 30 patients and 11 (37%) of the 30 patients showed either identical (patients 1–19; designated patient group 1) or different genotypes (patients 20–30; designated patient group 2), respectively (Table 1). The data suggest that more than one third of the corneas of the patients with RHK were superinfected with a different HSV-1 strain. In the case of patient 30, the newly acquired HSV-1 strain was cultured pending two post-PKP recurrences. This suggests that the newly acquired HSV-1 strain had colonized the recipient. Combining the results of the three amplified genomic regions showed that the majority of the distinguishable HSV-1 isolates displayed unique combinations of amplicons (Table 1).

In the case of patient 22, the data indicated that the bilateral herpetic keratitis was due to infections with different HSV-1 strains in either cornea. Patient 30 had two different HSV-1 strains identified. In the third episode sampled, the strain identified during the second recurrence was isolated (Table 1).

Comparison of Clinical Characteristics of Patients with RHK in Patient Groups 1 and 2

Compared with previous reports on patients with RHK,[2,9] our cohort consisted mainly of patients with severe entities of

TABLE 1. Patients' Characteristics and Genetic Characterization of Sequential Corneal HSV-1 Isolates from Patients with RHK

			Estimated Amplicon Length (bp)*									Months between HSV-1 Isolates†		
			US10/11 Region			*US1* Region			*US12* Region					
Patient	Sex	Age (y)	a	b	c	a	b	c	a	b	c	a–b	b–c	Eye Difference‡
Group 1														
1	F	17	215	215	—	295	295	—	240	240	—	11	—	+
2	F	34	220	220	—	320	320	—	305	305	—	15	—	+
3	M	64	215	215	—	280	280	—	220	220	—	8	—	−
4	M	54	225	225	—	280	280	—	260	260	—	27	—	+
5	M	44	225	225	225	420	420	420	270	270	270	56	3	+
6	F	59	215	215	215	270	270	270	370	370	370	8	11	−
7	M	39	215	215	—	280	280	—	270	270	—	170	—	−
8	F	72	220	220	—	410	410	—	220	220	—	78	—	−
9	M	74	225	225	—	320	320	—	310	310	—	22	—	−
10	M	69	225	225	—	220	220	—	200	200	—	12	—	−
11	F	73	—	220	—	230	230	—	240	240	—	28	—	+
12	M	29	215	215	—	305	305	—	300	300	—	22	—	−
13	M	60	220	220	—	280	280	—	260	260	—	21	—	−
14	M	67	225	225	—	220	220	—	220	220	—	11	—	−
15	F	69	225	225	—	295	295	—	290	290	—	36	—	−
16	F	74	225	225	—	360	360	—	310	310	—	6	—	−
17	M	52	230	230	—	260	260	—	260	260	—	77	—	−
18	F	55	210	210	—	520	520	—	240	240	—	40	—	−
19	F	78	230	230	230	300	300	300	260	260	260	3	4	−
Group 2														
20	M	63	225	225	—	*260*	*310*	—	*250*	*300*	—	7	—	−
21	M	46	*225*	*220*	—	*470*	*425*	—	*210*	*370*	—	22	—	−
22	M	69	220	220	—	*290*	*360*	—	*270*	*290*	—	0	—	+
23	F	32	225	225	—	*270*	*300*	—	270	270	—	2	—	−
24	F	75	225	225	—	*350*	*430*	—	*370*	*320*	—	3	—	−
25	F	38	215	215	—	*320*	*410*	—	*380*	*320*	—	85	—	−
26	M	72	225	225	—	*370*	*300*	—	*370*	*290*	—	38	—	−
27	M	65	220	220	—	*380*	*420*	—	*300*	*390*	—	2	—	−
28	M	78	225	225	—	*340*	*490*	—	220	220	—	2	—	−
29	F	77	225	225	—	*350*	*490*	—	210	210	—	28	—	−
30	M	46	225	225	225	290	290	290	*260*	*280*	*280*	125	43	−

* a, b and c represent the time points at which corneal HSV-1 isolates were obtained. Data showing differences in amplicon length between sequential samples of individual patients are in italics. Based on the genotypic analyses, patients 1 through 19 were designated as group 1 (genotype sequential isolates identical) and patients 20 through 30 as group 2 (genotype sequential isolates different).

† Patient 22 had bilateral herpetic keratitis. In patient 5, the first and second isolates were obtained from the left eye, and the third isolate was obtained from the right eye.

‡ − and + indicate that corneal HSV-1 isolates obtained were from the same or contralateral cornea, respectively.

HSV-induced keratitis, such as herpetic stromal and necrotizing keratitis. This is also reflected in the high number of PKPs in the patient cohort (Table 2; mean PKPs, 1.4 per patient; range, 0–6).

The clinical characteristics of the patients in groups 1 and 2 were compared, to identify the factors predisposing for corneal HSV-1 superinfection. Overall, the immune status and ophthalmic condition did not differ significantly between both groups (data not shown). Additionally, gender, inter-recurrence period, anatomic location of the lesions (left or right eye), ocular history, and clinical picture at time of recurrences were not statistically different between both groups (Tables 1, 2).

Comparison of Therapeutic Regimen for RHK in Patient Groups 1 and 2

The clinical outcome of corticosteroid treatment before or during the convalescence period was not statistically different between both groups. The potential effects of long-term (val)acyclovir treatment were not numerous enough to be interpreted (data not shown).

Although the mean number of PKP per patient did not significantly differ between both groups, indicating that both groups were comparable in disease severity, a correlation between corneal HSV-1 superinfection and time point of PKP was observed. Whereas no patient in group 1 received a corneal transplant between the sampled recurrences, 4 of the 11 patients in group 2 underwent a PKP during the inter-recurrence period in the same eye from which the sequential corneal HSV-1 isolates were obtained (Table 2; $P = 0.012$). Patient 30 received a corneal allograft between the first and second sampled recurrence.

DISCUSSION

HSVs have the ability to reside in latent form within neurons of the sensory ganglia that innervate the initial site of infection. It is therefore assumed that recurrent herpetic lesions are due to reactivation of the HSV strain acquired during the primary infection.[1,4–6] In contrast, HSV superinfection in patients with recrudescent herpetic lesions has been documented.[6,7] Patients with recurrent herpetic keratitis risk the development of HSK, a leading cause of corneal blindness worldwide.[2,3] The objective of the present study was to examine the two types of

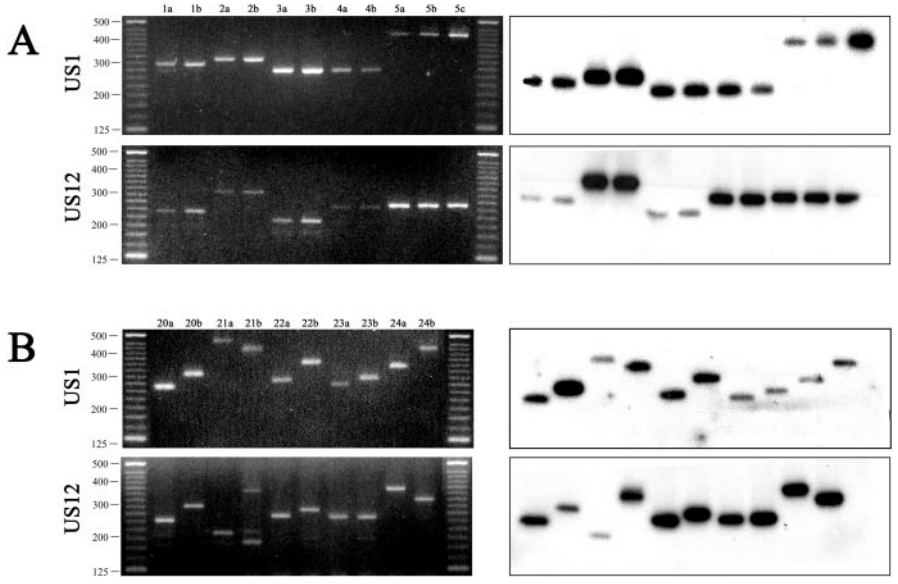

FIGURE 2. Amplicons of the hypervariable regions *US1* and *US12* amplified from sequential corneal HSV-1 isolates from patients with RHK. *Left*: Amplicons were electrophoresed on 2.5% agarose gels and were visualized by ethidium bromide staining. Representative sequential samples (a, b, and c) of 10 patients are shown: patients 1 through 5; (**A**) group 1, and patients 20 through 24; (**B**) group 2. A 25-bp molecular size marker was run in parallel. Numbers on the *left* are in base pairs. *Right*: autoradiograph of DNA in gel after Southern blot hybridization with appropriate reiteration-specific probe.

origins and risk factors involved in corneal HSV-1 superinfection in 30 patients with HSV-1–induced RHK.

Genotypic analyses of sequential corneal HSV-1 isolates from 30 patients with RHK demonstrated that 63% of the patients (patients 1–19; designated as group 1) had evidence of reactivation of the same HSV-1 strain. From five patients in group 1, the isolates were obtained from separate eyes. HSV-1 infection of the contralateral cornea most likely occurred through the external route (cross-infection). It was interesting that sequential isolates of 37% of the patients (patients 20–30; designated as group 2) had a different genotype, suggesting corneal HSV-1 superinfection in the inter-recurrence period.

Alternatively, the instability of the analyzed hypervariable regions may account for these differences. HSVs, similar to other DNA viruses, have less genomic variability than RNA viruses and are genetically more stable after in vitro passages.[11,15] In addition to standard RFLP, several hypervariable regions within the HSV-1 genome have been used to differentiate HSV-1 isolates genetically.[11] Intratypic variation of the regions results from differences in the number of Re and point mutations.[10,12,13] The stability of the eight HSV-1–specific Re regions described varies extensively.[11] Genotypic analyses of HSV-1 single-plaque clones compared with their parental strain have shown that the hypervariable regions located within the HSV-1 genes *US1*, *US12,* and *US10/11* remain stable during in vitro culture.[13,15] Moreover, the mean inter-recurrence period of patient group 1 (30.4 months) and the proofreading activity of *Pfu* DNA polymerase, implies that the intraindividual HSV-1 genotype differences are most likely not due to a genetic alteration of the initial strain or errors in amplifying these highly GC-rich DNA sequences, respectively.

Analogous to our study, reinfection with new HSV-2 strains has been described in two of three patients with recurrent HSV-2 genital herpes.[7] The latter study and our data indicate that HSV superinfection is not as rare as previously suggested.[4–6] To differentiate HSV strains, most investigators have used RFLP analyses with 6-bp recognizing restriction enzymes (REs).[4–6] The lower efficacy of 6-bp RE, compared with the 4-bp RE, to differentiate HSV-1 strains may account for the different frequencies of HSV superinfection described.[11]

Generally, corneal HSV-1 infection results in the development of herpetic epithelial keratitis in approximately two thirds of patients.[2] In the present study, however, the patient cohort consisted predominantly of patients with severe entities of herpetic keratitis (Table 2). Selection of individuals with a higher susceptibility for corneal HSV-1 infection may have occurred. Alternatively, the patients in group 2 may have been superinfected with a more virulent HSV-1 strain.

Among the clinical data analyzed, only the time point of PKP was significantly different between the patient groups. Although no patients in group 1 had undergone transplantation between sampling, 4 of 11 patients in group 2 underwent PKP during the inter-recurrence period in the same eye from which the corneal HSV-1 isolates were obtained. The data suggest that PKP is a risk factor for corneal HSV-1 superinfection. Primary graft failure and endothelial abnormalities of cultured eye bank corneas have been associated with the presence of HSV-1 DNA in affected corneal allografts.[16] The high prevalence of HSV-1 DNA in eye bank corneas (~ 10%)[16] has led to the hypothesis of HSV-1 latency in corneas. Although expression of HSV-1 latency-associated transcript, a marker of latency, has been detected in latently infected rabbit corneas and human HSK corneas, corneal HSV-1 latency remains controversial.[16,17] Recently, Zheng et al.[18] have demonstrated HSV-1 transmission through PKP in an experimental rabbit model. HSV-1 DNA was detected in recipient corneal rims and the innervating trigeminal ganglion (TG) of naive rabbits that received corneal allografts from latently infected rabbits. Moreover, infectious HSV-1 was recovered from the tear film of the rabbits that had undergone transplantation.[18] Besides true ocular viral latency, putative HSV-1 transmission through PKP may be due to coincidental shedding of small amounts of infectious virus from the allograft or a low level of viral replication in corneal resident cells in the allograft at time of PKP.[18,19]

Alternatively, the TG may harbor a mixture of HSV-1 strains with which the patients were previously latently infected, before PKP. In animal model studies, corneal trauma (similar to PKP) has been shown to induce reactivation of HSV-1 causing corneal HSV-1 infection.[20,21] Assuming that the human TG can be latently infected with multiple HSV-1 strains, PKP may serve as a powerful reactivation stimulus to certain portions of the TG, allowing multiple strains to reactivate.[22]

In conclusion, this study is the first to demonstrate a high frequency of corneal HSV-1 superinfection in patients with RHK. Although we could not determine the source or mode of corneal HSV-1 superinfection in patient group 2, the data suggest that PKP may be a risk factor for transmission of HSV-1 with subsequent reactivation of the donor-derived HSV-1 strain in the corneal allograft. Recently, we have genetically characterized HSV-1 DNA isolated from a donor cornea before and

TABLE 2. Clinical Characteristics and Cornea Transplantations Performed on Patients with RHK

Patient*	History of HSV-Mediated Eye Disease†	Diagnosis at Time Point of Sampling†			PKPs (*n*)	PKPs between Samples§
		Time Point a	Time Point b	Time Point c		
Group 1						
1	None	Blepharitis	Blepharitis		0	No
2	None	IEK	IEK		0	No
3	RecISK	IEK	IEK		0	No
4	RecISK	IEK	IEK		0	No
5	BilISK	IEK	IEK	IEK	0	No
6	recISK and uveitis	IEK	NSK	NSK	3	No
7	recISK	IEK and ISK	IEK and ISK		0	No
8	NSK in PKP	IEK and ISK	IEK and ISK		3	No
9	recISK	IEK and ISK	IEK		0	No
10	Bil/recISK	IEK in PKP	IEK in PKP		1	No
11	Bil/recISK and uveitis	IEK in PKP	IEK in PKP		2	No
12	NewHSV in PKP	IEK in PKP	IEK in PKP		1	No
13	NSK in PKP	IEK in PKP	NSK in PKP		2	No
14	recISK	NSK in PKP	IEK in PKP		1	No
15	newHSV in PKP	NewHSV in PKP	IEK in PKP		2	No
16	NSK	IEK and uveitis	IEK		0	No
17	recISK in PKP	IEK in PKP	IEK in PKP with GR		2	No
18	None	IEK in ISK	IEK and HKU		0	No
19	NSK	IEK in PKP	IEK in PKP	IEK in PKP	1	No
Group 2						
20	ISK and uveitis	blepharitis	IEK		0	No
21	Endothelitis	IEK	IEK		0	No
22	recIEK and ISK in PKP	IEK	IEK		2	No
23	newHSV in PKP	IEK	IEK		1	No
24	IEK	IEK	NSK		0	No
25	NSK in PKP	IEK in PKP	IEK in PKP		2	*Yes*
26	NSK	IEK in PKP	IEK in PKP		1	No
27	NSK in PKP	IEK and ISK	IEK and ISK		3	*Yes*
28	NSK in PKP and uveitis	IEK and uveitis	IEK and uveitis		1	No
29	NSK in PKP	ISK in PKP	IEK in PKP		3	*Yes*
30	newHSV in PKP	NSK in PKP	ISK in PKP	NSK in PKP	6	*Yes*

* The sequential corneal HSV-1 isolates of patients 1 through 19 (designated as group 1) and 20 through 30 (designated as group 2) had identical or different genotypes, respectively.

† HSV-mediated corneal diseases diagnosed were: immune stromal keratitis (ISK), necrotizing stromal keratitis (NSK), infectious epithelial keratitis (IEK) and herpetic keratouveitis (HKU). PKP, penetrating keratoplasty; LKP, laminar keratoplasty, and GR, graft rejection; rec, recurrent; bil, bilateral; new, newly acquired.

‡ Total number of preceding PKPs performed on each patient.

§ PKP between sample dates in the patient cohort with identical versus different HSV-1 genotypes of the sequential corneal HSV-1 isolates were statistically significant (Fisher exact test; $P = 0.012$). In patient 30 PKP was performed between time points a and b.

after PKP in a patient with newly acquired herpetic keratitis. The DNA sequences were identical in both strains, providing conclusive evidence for graft-to-host transmission of HSV-1 through corneal allograft.[23]

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