PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 179, 55–67 (1985)

Herpes Simplex Virus Latency in the Rabbit Trigeminal Ganglia: Ganglionic Superinfection (42064)

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Abstract. It has been confirmed and further documented that infection of the rabbit cornea with the E-43 strain of HSV-1 precludes superinfection of the corresponding trigeminal ganglia by another HSV strain, i.e., the challenging virus does not establish latency and can not be recovered from the ganglia. It was shown that after primary infection, a state of resistance is established in the neuronal cells of the ganglia, and although the challenging strain reaches the ganglia, it does not cause discernible acute infection, and does not displace the resident virus in the ganglia. This protection was present 6 months after primary infection, was independent of immune factors such as circulating or secretory antibodies, and was localized to the point of entry of the primary infecting strain and the sensory neurons that innervate that site. The smallest inoculum that provided protection from ganglionic superinfection was that which produced overt disease in the eye, although different degrees of disease resulted from varying inocula above this minimum. Asymptomatic primary infections produced by subminimal inocula of the E-43 strain or by the HSV recombinant strain, F(MP)F, which is avirulent for the rabbit eye, protected against severe disease and death, but the degree of protection against ganglionic superinfection was variable and depended on the time of challenge. These findings suggest that susceptible neurons in the trigeminal ganglion, when "occupied" by an infecting strain, cannot be superinfected by a second strain.

A striking characteristic of herpes simplex virus is its ability to persist in a latent form in the host between episodes of disease. Many adults have been exposed to this virus, as evidenced by HSV antibody levels in individuals, but only a fraction of this population has had acute episodes of herpetic disease. Presumably, latency is the more common status of the virus, and as such, deserves intense investigation to improve our understanding of its salient characteristics. Work in our laboratory has focused on several factors in latency such as virus shedding, superinfection of the ganglia, and colonization of the ganglia.

We have used the New Zealand white rabbit as a model for herpetic ocular disease. In this model, infection with herpes simplex virus type 1 (HSV-1) causes dendritic ulcers in the epithelial layer of the cornea. After the clinical disease runs its course, the virus remains in the trigeminal ganglia in a latent form and can be easily recovered by cocultivation of ganglionic explants onto susceptible cell monolayers in tissue culture (1). The neurons in the trigeminal ganglia serve as the source of virus for recurrent episodes of the disease (2–5). Reactivation of the latent virus does occur, as evidenced by spontaneous asymptomatic shedding of virus, as well as recurrent herpetic disease.

We have demonstrated that in this model, initial infection and colonization of the trigeminal ganglia by one strain of HSV prevents superinfection of the same ganglia by another HSV strain and protects the animals from death by encephalitis when the superinfecting strain is a more virulent one (6). When the previously infected animals are challenged by infection with the McKrae strain, the resulting disease is less severe, although virus remains in the ocular tissue for several days and is shed into the tear film. Also the virus recovered from the trigeminal ganglia is not the superinfecting McKrae strain, but the initial E-43 strain. Not all the factors involved in this protection are completely understood, but this kind of protection may be the basis for the mechanism that prevents overt herpetic disease in many individuals who have been exposed to this virus.

0037-9727/85 $1.50

The purpose of this study was to further characterize our previous findings and to determine the roles of several factors involved in providing this protection. Specifically, we sought to determine (a) the role of humoral and secretory antibodies, (b) whether the protection is dose dependent and related to the amount of latent virus in the ganglia, (c) whether there is a relationship between the number of infected neurons and amount of viral growth at the point of entry, and (d) whether the time of challenge is a determinant in the identity of the virus strain isolated from the ganglia.

Materials and Methods. *Cells and viruses.* RK-13 cells (Microbiological Associates) were grown in basal minimal medium supplemented with 10% fetal calf serum, 1% glutamine, sodium bicarbonate, and antibiotics. Vero cells grown in MEM minimal essential media (Eagle's), supplemented with 10% calf serum, were used for the DNA work.

Two of the virus strains used were the E-43 and the McKrae strain; both were grown in RK-13 cells. The E-43 strain is a clinical isolate that has been passed in RK-13 cells only twice. The McKrae strain is a well-established laboratory strain, which has been used extensively in the New Zealand white rabbit as a model for recurrent herpetic disease. The E-43 strain was used for primary infection and the McKrae strain was used as the challenge strain.

The MP strain and the recombinant F(MP)F strain were also used (7, 8). The MP strain, a mutant that is deficient in the production of gC, produces stromal disease and 100% mortality in rabbits (9). The F(MP)F strain was chosen because of its lack of pathogenicity in the rabbit eye (10). The recombinant F(MP)F was used for primary infection and the MP strain was used as the challenge strain.

Antibodies. Four rabbits were immunized intramuscularly with the E-43 strain of HSV-1 grown in RK-13 cells. For the detection of secretory antibodies, the rabbits were immobilized in a straight jacket, and a Schirmer test paper was placed in the conjunctiva for 10 min. The amount of tears collected on the test strip was equivalent to 10 lambda per eye. The test strips were placed in sterile vials and stored at −70°C. After thawing, the papers were pooled by day of collection and eluted in excess PBS overnight in the cold; the samples were concentrated by means of a Millipore concentration apparatus. The level of antibodies to HSV in the tears and serum was determined in a microneutralization assay, as previously described (6).

Animal model. New Zealand white rabbits (1 to 3 kg body wt) were used as the ocular disease model. To infect each eye, the cornea was lightly scarified, 50 μl of a virus suspension was dropped onto the surface of the cornea, and the lid was rubbed over the cornea twice. Three days after infection, all eyes were examined by means of the slit lamp to confirm the presence of HSV ocular disease.

Three major types of experiments were designed for use with this model:

(a) Ganglionic colonization. Twenty animals were infected in both eyes with 10^5 PFU of the E-43 strain of HSV-1. Six months later, the animals were challenged with 10^5 PFU of the McKrae strain. One day before challenge, the animals were bled and their tears collected to ascertain their immune status. For virus isolation, the tear film was cultured from all eyes for 35 days after challenge. Six weeks after challenge, the animals were sacrificed and the left and right ganglia were processed for virus recovery.

Five rabbits were infected with the same dose of the E-43 strain, but only in the right eye. Six weeks later, the animals were challenged with the McKrae strain in both eyes. Five weeks after challenge, the ganglia were removed and processed for virus recovery.

(b) Inoculum size. To investigate whether inoculum size is related to ganglionic colonization and subsequent protection, several groups of three animals each were infected with various log dilutions of the E-43 strain. Five weeks after infection, the animals were challenged with 10^5 PFU of the McKrae strain. Three weeks after challenge, the animals were sacrificed and the left and right ganglia were removed and processed for virus recovery and virus yield.

(c) Virulence. The F(MP)F strain was used to determine whether HSV strains that are avirulent and cause no disease in the rabbit eye can protect from ganglionic colonization. In these experiments, five rabbits were in-

fected with 10^7 PFU of the F(MP)F strain and challenged with the MP strain. Three weeks after challenge, the animals were sacrificed, and the ganglia removed and processed for virus recovery.

In vivo titer. To determine how well the E-43 strain grows in the corneal tissue, an *in vivo* growth curve was made. Twelve rabbits were infected in both eyes with 10^5 PFU of the E-43 strain. Two animals were sacrificed on Days 3, 5, 7, 10, and 12; the eyes were removed and the epithelium scraped and cultured in RK-13 cell cultures for 48 hr. The virus yield of each cornea was then determined.

Trigeminal ganglia. Virus recovery. The trigeminal ganglia were excised and washed with phosphate-buffered saline (PBS) containing 100 μg gentamicin per milliliter PBS. The tissue was minced aseptically and subjected to collagenase and trypsin digestion as described by Nesburn (11). The entire sample of digested tissue was seeded onto RK-13 monolayer cultures and incubated at 37°C for a minimum of 2 weeks. All positive cultures of the same ganglia were pooled, dispensed into small vials, and stored at −70°C for later use.

Immunofluorescence. The trigeminal ganglia were removed, mounted in O.C.T. compound (Miles Laboratories), and quick-frozen at −30°C. Serial sections, 6 μm in thickness, were cut, fixed in cold acetone for 10 min, overlaid with rabbit anti-HSV-1 antiserum, and incubated at 37°C in a moist chamber for 30 min. The slides were then washed twice for 10 min with PBS, overlaid with fluorescein-conjugated goat anti-rabbit IgG serum, and incubated for an additional 20 min followed by three washes with PBS. Coverslips were mounted with buffered glycerol (Aquamount, Lerner Laboratories); slides were examined by means of a Zeiss fluorescence microscope.

To document the specificity of this assay, several controls were included. Some sections were overlaid with normal rabbit serum instead of rabbit anti-HSV serum and other sections were overlaid with fluorescein-conjugated anti-rabbit IgG to test for nonspecific staining. For electron and light microscopy, the tissue samples were fixed with glutaraldehyde and osmium tetroxide according to standard procedures. For light microscopy, sections 0.5 to 1 μm in thickness were cut and stained with toluidine blue.

Viral DNA purification. Confluent monolayers of Vero cells were infected with the viral isolates at a multiplicity of approximately 1 PFU/cell and incubated at 37°C in phosphate-free medium containing 100 μCi [^{32}P]orthophosphate. Twenty-four to 36 hr after infection, the medium was decanted and 0.5 ml of 1% Triton and 1 m*M* EDTA in 10 m*M* Tris, pH 7.8, were added to each culture. The suspension was kept for 10 min at room temperature, then aspirated 10 times with a Pasteur pipet, and transferred to 1.5-ml Eppendorf centrifugation tubes. Sodium chloride was added to a final concentration 0.5 *M*, and the cell lysates were stored at 4°C for 3 hr. The nuclear debris was pelleted at 10,000 rpm for 5 min and discarded. Sodium dodecyl sulfate (SDS) was added to the supernatant to a final concentration of 0.5%. The cytoplasmic RNA was degraded by treatment with 100 μg of RNase A for 20 min at 37°C, 250 μg of Proteinase K (Boehringer-Mannheim Biochemical) was added to each culture, and the suspension was incubated for 1 hr at 56°C. The resulting lysate was extracted twice with phenol, followed by three ether extractions to remove the residual phenol. Twenty-five micrograms of sonicated salmon sperm DNA (Sigma) was added as carrier, and the total DNA was removed by ethanol precipitation overnight at −20°C.

Endonuclease digestion and electrophoresis. Viral DNA was digested with either *Bam*HI or *Kpn*I endonuclease (New England Biolabs) in a 50-μl reaction mixture as recommended by the supplier. As an internal control, λ phage DNA (New England BioLabs) was cleaved as described above and subsequently used as a sizing marker during electrophoresis. Restriction enzyme digestions were subjected to electrophoresis on 0.8% or 1% agarose horizontal slab gels at 2 V/cm. The stock loading solution contained 0.05% bromphenol blue, 0.05% xylene cyanole and 50% sucrose in 0.01 *M* Tris, pH 7.8. The running buffer, containing 5 m*M* sodium acetate and 1 m*M* EDTA in 40 m*M* Tris, pH 7.8, was circulated continuously. Following electrophoresis, the gels were stained in a 1 μg/ml ethidium bromide bath, visualized under uv

light, and dried. Autoradiography was performed with Dupont Chronex Lightning-Plus intensifying screens and Kodak XAR-5 X-ray film at −70°C for 1–4 days.

Results. *Definition of animal model.* To evaluate the mechanisms involved in protection against ganglionic superinfection, we first found it necessary to define the parameters of our animal model. To this end, we followed the course of ocular herpetic disease and the establishment of latency in rabbits infected with a standard 50 μl inoculum containing 10^5 PFU of E-43 strain HSV-1.

Ocular disease was evident at Day 3 after infection in all animals. From Days 3 to 12 after infection, excised corneas were processed as described under Materials and Methods and the HSV titer per day was determined. The amount of virus recovered from the corneas reached a peak on Day 7, and subsided thereafter. By Day 12, the eyes were clear, but virus could still be recovered from the cornea (Table I).

Virus inoculation resulted in an acute infection in the ganglionic tissue within 3–5 days, as evidenced by necrosis of the neuronal tissue and the production of virus particles (Fig. 1). The infection subsided within 2 weeks. Six weeks later, the virus was in the latent state; no infectious virus could be recovered from homogenates of the neuronal tissue, nor were infectious virus particles seen by electron microscopy.

With this degree of infection at the point of origin (i.e., the cornea), 100% protection against ganglionic superinfection is achieved.

TABLE I. *In Vivo* Titer of the E-43 Strain in Corneas of New Zealand White Rabbits

Time postinfection	Virus yield per cornea (PFU)
3	8.0×10^4
5	5.8×10^6
7	4.9×10^8
10	6.0×10^4
12	9.0×10^3

Note. Rabbits were infected in both eyes with 10^5 PFU of the E-43 strain. On each of the indicated days, two animals were sacrificed, corneas removed, and the virus yield per cornea was determined. The figures represented are the average plaque count of four eyes.

This is the model we used subsequently to study the mechanisms involved in this protective phenomenon.

Effect of time of challenge. The results of this experiment extend and support our previous findings that initial ocular infection with the E-43 strain precludes superinfection of the trigeminal ganglia when the animals are challenged with the McKrae strain 6 weeks after primary infection. In this experiment, 20 rabbits (Group A; Table II) were infected in both eyes with the E-43 strain and challenged 6 months later with the McKrae strain to determine the effect of a longer interval between primary infection and challenge (6 months vs 6 weeks). It seemed possible that the ganglia might be susceptible to superinfection with a second strain at a later time, when the animals had a reduced immunological defense and the amount of virus in the ganglia was considerably less.

Our results showed that under these conditions, ganglionic superinfection did not occur (Table II). The challenging virus produced ocular herpetic disease in all animals, as documented both by slit lamp examination and positive virus cultures at 3 and 5 days after challenge. Recurrent disease was observed in both control and doubly infected animals. In the control group, which was infected with only the primary E-43 strain, 12 recurrent episodes were seen in 19 animals. In the doubly infected animals (primary E-43/challenge McKrae), 20 recurrences were observed in 18 animals. Asymptomatic shedding of virus in both groups followed the same pattern. Antibodies to HSV were present in the tears and blood samples obtained 1 day before challenge, but at a lower level than those measured 6 weeks after primary infection. The titer of circulating antibodies in group A was 1:64 and the antibody titer in tears was 1:8.

The virus isolated from all doubly infected rabbits was identified as the initial E-43 strain by restriction endonuclease analysis (Fig. 2 and 3).

In the second part of the experiment (Group B, Table II), the animals were infected initially with the E-43 strain only in the right eye. Challenge with the McKrae strain 45 days after initial infection showed no super-

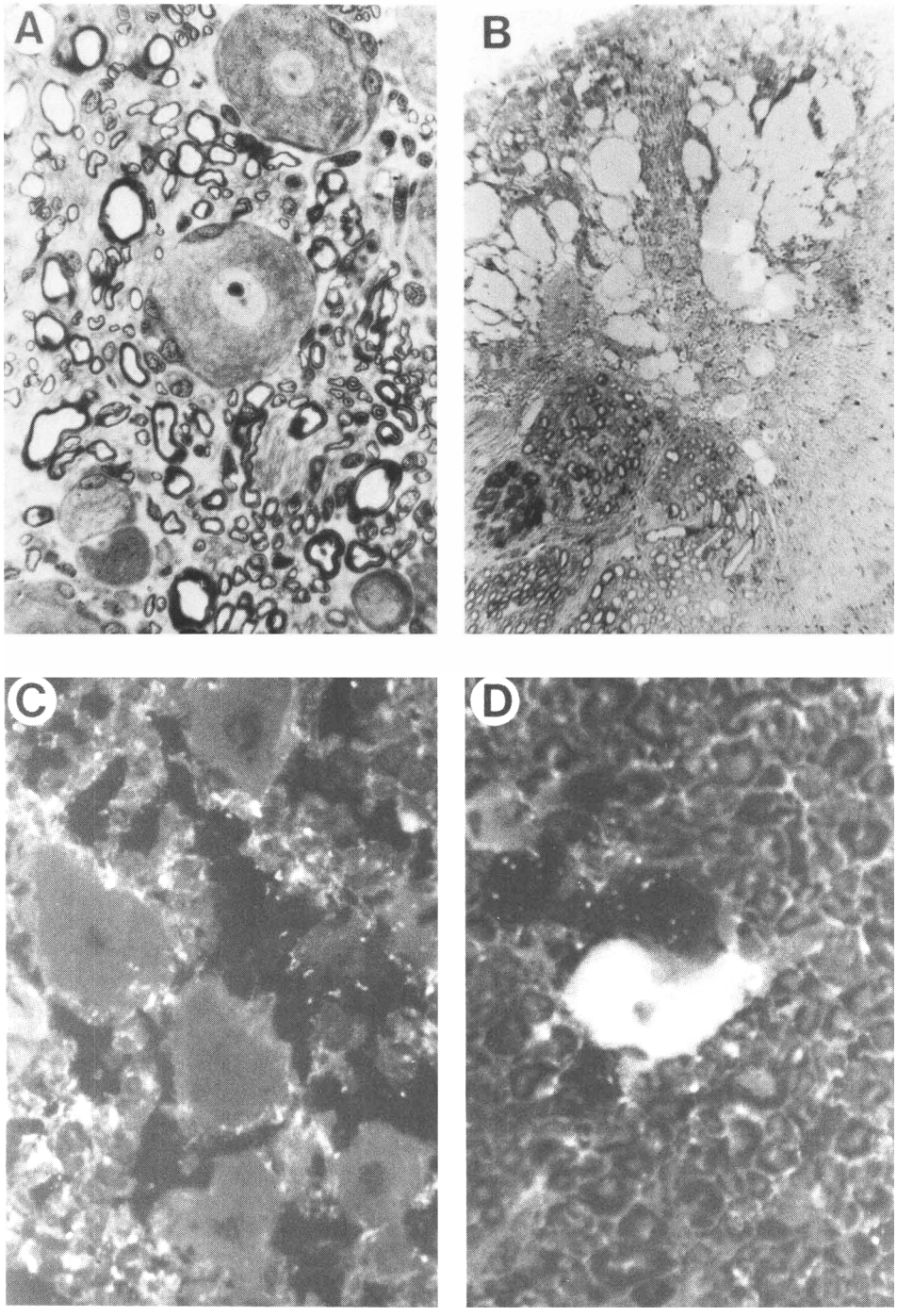

FIG. 1. Photomicrographs of rabbit trigeminal ganglion cells during the acute stage of infection with E-43 strain HSV-1. (A) Typical normal neurons from an uninfected animal. (B) Tissue necrosis seen in the acute phase of infection (5 days postinfection). Toluidine blue, 400×. (C) Negative immunofluorescence staining of neuronal cells from an uninfected animal. (D) Positive immunofluorescence staining of the cytoplasm of neurons from the trigeminal ganglion of an acutely infected animal (5 days postinfection). Both sections were stained with rabbit anti-HSV-1 antiserum followed by fluorescein-conjugated goat anti-rabbit IgG antiserum. 400×.

TABLE II. GANGLIONIC SUPERINFECTION: EFFECT OF TIME OF CHALLENGE

Treatment	No. of rabbits	Antibody titers at time of challenge		Ocular disease after challenge	Ganglionic superinfection by McKrae strain	Identity of virus in ganglia
		Tears	Serum			
Left and right eye infected with E-43 strain; challenged with McKrae strain 6 months later (Group A)	20	1:8	1:64	Right eye: yes Left eye: yes	0/16[a]	E-43
Right eye infected with E-43 strain; challenged with McKrae strain 45 days later (Group B)	5	1:16	1:256	Right eye: yes Left eye: yes	Right: 0/5 Left:5/5	Right: E-43 Left: McKrae

Note. NZW rabbits were infected in both eyes with the E-43 strain and left untouched for 6 months (Group A). Five rabbits (Group B) were infected in a similar manner but only in the right eye and left untouched for 45 days. Both groups of animals were challenged in both eyes with the McKrae strain (10^5 PFU). Neither colonization of the ganglia nor establishment of latency was seen in the previously infected animals. In the second group (B), protection from ganglionic superinfection was seen only in the previously infected eye.

[a] Sixteen ganglia (right and left) were sampled; none showed McKrae strain virus.

infection in the right trigeminal ganglia, but the left trigeminal ganglia were readily colonized by the challenging McKrae strain. One day before challenge, Group B had a serum titer of 1:256 and a titer of 1:16 in tears. Two conclusions can be drawn from these observations: (a) protection from ganglionic superinfection is restricted to the point of entry of the primary infecting virus and the corresponding trigeminal ganglia; and (b) although the animals were immune as a result of primary infection in the right eye, this had no apparent effect on the colonization of the left trigeminal ganglia by the challenging strain.

The fact that superinfection of the ganglia did not occur in animals with varying levels of immunity, either at 45 days or 6 months postinfection, implied that immunity alone is not the main factor involved in this apparent protection. To further characterize this relationship, we proceeded to evaluate the effect of immunization on ganglionic superinfection.

Effect of immunization. Animals that had undergone both local and systemic immunization were infected in both eyes with McKrae strain HSV-1. All animals developed clinically identifiable ocular disease, with typical dendritic ulcers. However, the disease was less severe than that seen in nonimmune animals.

Thirty days after infection, the immune animals were sacrificed and the ganglia removed and processed for viral recovery. McKrae strain virus was recovered from all the trigeminal ganglia. The titer of antibodies to HSV in the blood was 1:256 and the titer of the tear antibodies was 1:32, determined from samples obtained 1 week before infection.

These findings indicated to us that although antibodies, either humoral or secretory, do provide protection from herpetic ocular disease, they do not prevent ganglionic colonization (Table III). We next sought to determine if the protective mechanisms involved in the prevention of ganglionic superinfection are related to the physiology of the neuronal cells and/or the nature of the trigeminal ganglia as a privileged site.

Effect of inoculum size on protection from superinfection. To explore the question of how much virus is required to be present in the cornea or latent in the ganglia to preclude superinfection, rabbits were given inocula of various sizes, and challenged with a second strain in the latent period (Table IV). Inocula containing 10^5, 10^4, or 10^3 PFU of the E-43 strain produced evident ocular disease, and

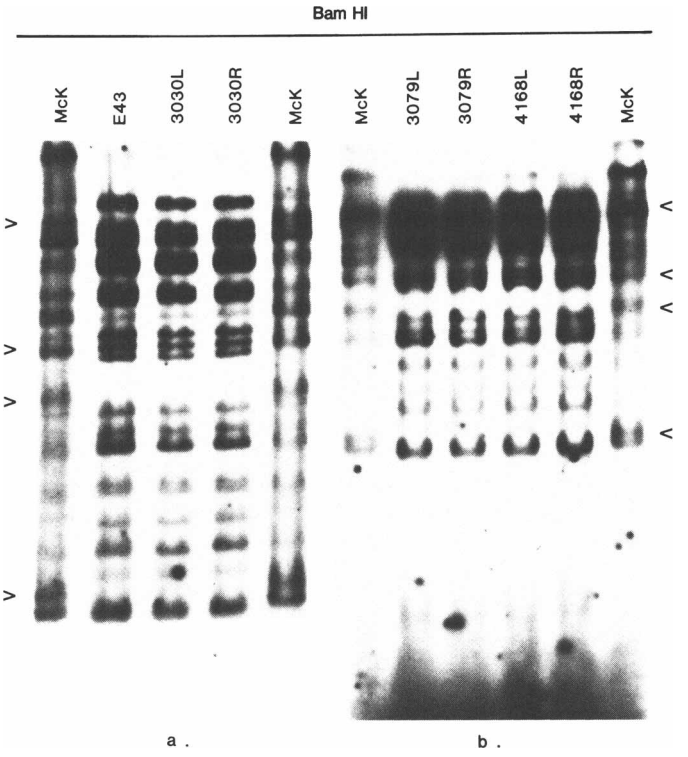

FIG. 2. *Bam*HI endonuclease analysis of viral DNA isolated from the trigeminal ganglia of doubly infected rabbits. Panels a and b are examples of the findings from a representative sample of this population. The digestion profiles of the DNA of all the viral isolates are similar to that of strain E-43 and distinctly different from that of the McKrae strain. Rabbits 3030 and 4168 are examples of animals in which virus shedding was observed. No virus shedding was observed in animal 3079. Within the figure, arrows mark distinct cleavage differences between the E-43 and McKrae strains.

protected the animals from ocular disease and death when challenged with the McKrae strain. In contrast, rabbits that received lower inocula and showed no discernible disease after primary infection were not protected from subsequent ocular disease and/or death.

The question of which strain is found in the ganglia of the doubly-infected animals that received various amounts of the primary virus was investigated next. The rabbits were sacrificed 6 weeks after challenge, and the viral DNA recovered and analyzed by restriction endonuclease assay. The ganglia from the rabbits that received the three larger inocula (10^5, 10^4, and 10^3 PFU) and that displayed definite ocular herpetic disease yielded the initial infecting E-43 strain (Fig. 4). It is apparent that, in this model, the E-43 strain provided protection against subsequent challenge with the McKrae strain only when the primary inoculum produced overt ocular disease and achieved ganglionic colonization.

No data could be obtained from animals infected with low inocula of the E-43 strain (10^1 and 10^2 PFU), as they died before the end of the 6-week postchallenge period. A study of asymptomatic infections is described below.

Our findings thus far suggest two questions: (a) In the animals that received the smaller inocula (10^2, 10^1 PFU), which resulted in asymptomatic infections and no protection, did the primary infecting virus ever reach the ganglia? (b) In the animals that received the larger inocula (10^5, 10^4, 10^3 PFU), which resulted in symptomatic infection and protection from superinfection, did the challenging strain reach the ganglia and establish an infection?

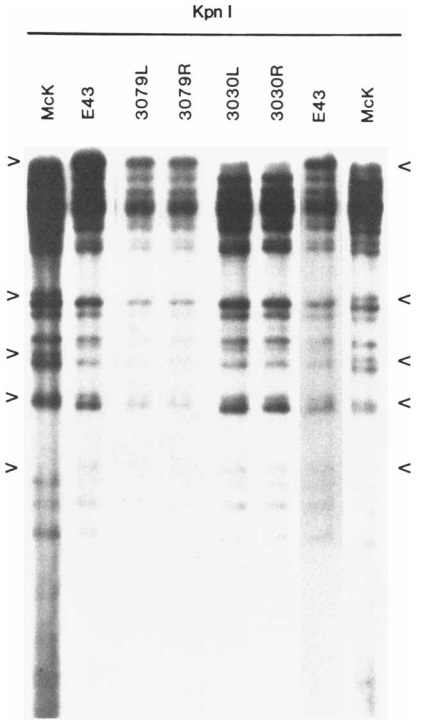

FIG. 3. The DNA of viral isolates from the right and left trigeminal ganglia of doubly infected rabbits was further analyzed with *Kpn*I endonuclease. The isolates were identified as the initial E-43 strain; however, both isolates from rabbit 3030 showed an altered migration of a single fragment distinct from that of the parental E-43 profile. Within the figure, arrows denote specific cleavage differences between the E-43 and McKrae strains. Successive exposure times have been spliced together for ease of comparison of the *Kpn*I digests.

Asymptomatic infections. Infections produced with smaller inocula, in which no discernible disease was seen, were studied first. Since the amount of virus in the ganglia may be small, virus recovery and the differentiation of the primary virus from the challenging virus could be difficult; therefore viruses that differ in their cytopathogenic effects, e.g., rounded cells versus polykaryocyte formation, were chosen to facilitate identification where mixed infections were possible. In one set of experiments, F(MP)F, a recombinant strain that causes no apparent disease in the rabbit eye, was used as the primary infecting virus. The MP strain, which is fatal in rabbits, was used as the challenging virus. The trigeminal ganglia of those animals infected with F(MP)F strain alone were examined for latent virus. The results were always negative; no infectious virus could be recovered from the explanted tissue, perhaps because of the low amounts of virus or the genetics of the infecting strain.

Two groups of animals infected with the F(MP)F strain were challenged with the MP strain 7 or 30 days after primary infection. Although both groups were protected from death caused by the MP strain, the identity of the virus recovered from the ganglia varied with the time of challenge. The ganglia from animals challenged 7 days after infection yielded the challenging MP strain, while the ganglia from animals challenged 30 days after infection yielded either the primary F(MP)F strain or the challenging MP strain, roughly in a ratio of 1:1 (Fig. 5). These results show that although the F(MP)F strain is avirulent in the rabbit eye, it does protect the animals from the usual 100% mortality associated with the MP strain. It also appears that the recombinant strain does reach the ganglia and can be recovered from that tissue upon challenge with another virus strain. It is not clear why the F(MP)F strain cannot be recovered after primary infection. A plausible explanation is that the F(MP)F strain does not reactivate by itself.

From what is known of the events in the ganglia, we suggest the following hypothesis: Infectious virus placed on the scarified cornea reaches the trigeminal ganglia, where it produces acute infection. In the 30 to 45 days after primary infection, the virus sets up a state of resistance and protection. Although a second virus strain may achieve entry into the ganglia, such a superinfecting strain cannot successfully take up residence in the ganglia when latency has been established by a primary infecting strain, i.e., when all the susceptible neurons are already colonized by the latent strain.

Symptomatic infections. This hypothesis was further supported by direct observation of the state of the neurons in animals infected with 10^4 PFU of the E-43 strain which we have demonstrated is sufficient to protect from severe ocular disease and ganglionic superinfection.

The animals were challenged with the MP strain (10^5 PFU) 6 weeks after infection and sacrificed 5 days after challenge. The ganglia

TABLE III. GANGLIONIC COLONIZATION: EFFECT OF IMMUNIZATION

Treatment	No. of rabbits	Antibody titer	Ganglionic colonization	Latency established
Systemic immunization	5	1:256 (serum)	5/5	Yes
Ocular immunization	2	1:32 (tears)	2/2	Yes

Note. NZW rabbits were immunized in each hind leg with live virus (McKrae strain). One ml of a 1:1 mixture of immunogen:Freund's adjuvant was injected weekly for 3 weeks, and boosted 10 days later. The titer of the antiserum was 1:256. Another group of animals received topical vaccination with uv-inactivated virus (McKrae) for 3 weeks. The titer of tear antibodies was 1:32. These immunized animals were then infected in both eyes with 10^4 PFU of McKrae strain HSV-1. All animals showed manifestations of ocular disease. The virus replicated in the cornea and established latency in the trigeminal ganglia, indicating that antibodies, either secretory or humoral, did not prevent ganglionic colonization.

were removed and subjected to electron microscopy, histology, and explant culture to determine whether and to what extent the challenging virus achieved an acute infection in the ganglia.

We found that the challenging MP strain did reach the ganglia, but did not establish a clearly defined, acute infection with evident necrosis, as had been observed when this strain was employed to establish primary

TABLE IV. OCULAR DISEASE AND GANGLIONIC SUPERINFECTION: EFFECT OF INOCULUM SIZE

Inoculum size (PFU)	Rabbit number	Ocular disease: 7 Days after infection, E-43 OD	7 Days after infection, E-43 OS	5 Days after challenge, McKrae OD	5 Days after challenge, McKrae OS	Recovered from ganglia
10^5	3896	1	2S[c]	0	0	E-43
	3897	1	2S	0	0	ND
	3898	$1\frac{1}{2}$	3	0		ND
10^4	3899	3	2S	0	0	E-43
	3900	$\frac{1}{2}$	2S	0	0	E-43
	1557	$\frac{1}{2}$	1S	0	0	E-43
10^3	1558	1	1	0	0	E-43
	1559	$\frac{1}{2}$	$\frac{1}{2}$	0	0	E-43
	1560	0	0	0	0	ND
10^2	1555	0	0	Died before challenge		
	1551	0	0	3	3	*
	1552	0	0	$\frac{1}{2}$	2	*
10^1	1556	0	0	Died before challenge		
	1553	0	0	$\frac{1}{2}$	1	*
	1554	0	0	$\frac{1}{2}$	2	*

Note. Rabbits were infected in both eyes with the designated amounts of E-43 strain. The severity of the epithelial disease was graded (by slit lamp examination) from 1 to 4, with 1 = mild; 4 = severe. At 5 weeks postinfection, the animals were challenged in both eyes with 10^5 PFU of the McKrae strain. Disease was graded at 5 days postchallenge. The animals that survived were sacrificed at 6 weeks after challenge, and the virus recovered from the ganglia was identified by restriction endonuclease analysis. S indicates stromal disease; *rabbit died before 6 weeks postchallenge.

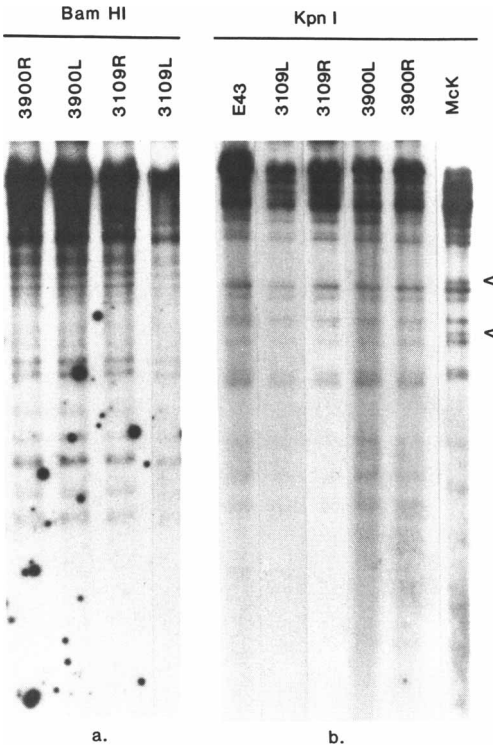

FIG. 4. Restriction endonuclease analysis of the virus DNA isolated from animals infected with different size inocula of the E-43 strain and challenged with 10^5 PFU of the McKrae strain. Panels a and b show the *Bam*HI and *Kpn*I digestion profiles, respectively, of two different rabbits. Rabbit 3109 is representative of rabbits receiving 10^5 PFU of E-43 strain. Rabbit 3900 is representative of those receiving 10^3 PFU of E-43 strain. Within the figure, arrows denote specific cleavage differences between the E-43 and McKrae strains. The virus isolated from these animals was the initial E-43 strain.

infection. The only signs of infection were a few condensed and compacted neurons. Virus particles were seen in a few Schwann cells, but not in the neurons (unpublished observation).

The ganglionic explants were cultured for 3 weeks. Spontaneous shedding of virus was seen at 12 days, and supernatants of these cultures were harvested and saved for virus identification. An aliquot of the supernatant was plated out in monolayer cultures of Vero cells with a methylcellulose overlay to aid in plaque identification. The characteristic syncytial plaque morphology of the MP strain was seen, averaging only 1 or 2 per 200 plaques counted per plate. These results indicate that the challenging strain achieved only very minimal, if any, acute infection in the ganglia.

Next we investigated whether this challenging strain can establish latency. The experiment was repeated, with the ganglia removed 30 days after challenge. The virus was isolated in culture and plated on Vero cell monolayers. All of the recovered virus showed the plaque morphology of the E-43 strain; no syncytial plaques characteristic of the MP strain were observed, indicating that the second challenging strain (MP) was not present and had not established latency.

These findings explain and confirm our previous results, that animals colonized by the primary infecting E-43 strain can be

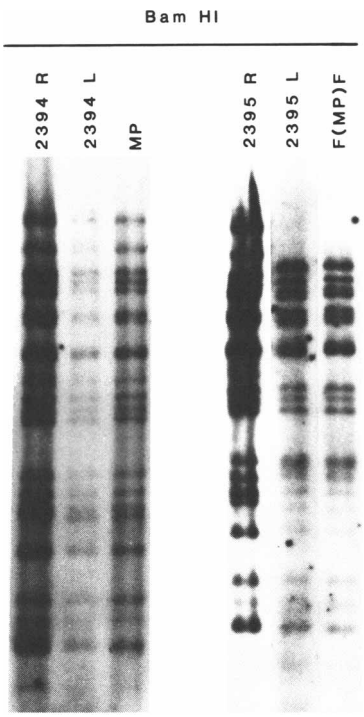

FIG. 5. Agarose gel electrophoresis of viral DNA digested with restriction endonuclease, *Bam*HI. Rabbits were infected in both eyes with the F(MP)F strain. Rabbit 2394 was challenged with the MP strain 7 days later and rabbit 2395 30 days later. The ganglia were removed 30 days after challenge. The MP strain was recovered from the left and right ganglia of rabbit 2394. In contrast, the ganglia from the rabbit challenged 30 days after primary infection gave either the F(MP)F strain or the MP strain.

challenged and infected with the McKrae strain, but yield only the E-43 strain when the latent virus is recovered from the ganglia.

It appears that there is no simple relationship between the relative size of the inoculum and increasing or decreasing protection. For symptomatic infections in which all the animals exhibited clinical disease, the presence of overt disease seemed to be correlated with the emergence of a state of resistance that is indicative of a specific amount of latent virus in the ganglia. For asymptomatic infections without disease, the protection from ganglionic superinfection was variable, depending on time of challenge. In this case, the presence of more than one virus in the trigeminal ganglia was possible.

In this study, it seemed that overt disease at the port of entry was directly related to subsequent protection against further ganglionic superinfection and colonization.

Discussion. This work has further documented that infection of the rabbit cornea with 10^5 PFU of the E-43 strain of HSV-1 produces colonization of the trigeminal ganglia and precludes subsequent colonization of the same ganglia by a second, challenging strain. We discuss below some conditions that elicit this mechanism of protection.

Three of our observations indicate that immune factors are not the main reason for this apparent protection: (a) Colonization of the trigeminal ganglia was readily achieved in animals that were immunized both topically and systemically. (b) Animals infected with the E-43 strain resisted superinfection even 6 months later, when their antibody titers were considerably reduced. (c) Animals that were infected in the right eye only and that developed evident disease and subsequent immunity by virtue of this infection were challenged in both eyes; the left trigeminal ganglia were readily colonized by the challenging virus but the right trigeminal ganglia resisted superinfection.

It is recognized that in mice, immunological responses (both antibodies and cell-mediated immunity) have an effect on the severity of disease and the outcome of infection, and these factors may also have an effect on the number of neuronal cells that harbor the virus. Oakes and co-workers (12, 13) showed that intraperitoneally injected HSV neutralizing antibodies prevented the spread of virus and the death of the animals. In addition, monoclonal antibodies specific for the major groups of HSV glycoproteins have been shown to promote recovery from both subcutaneous and ocular herpetic infection. However, the mechanisms by which this protection is achieved are not yet clearly defined, particularly as it is known that HSV is disseminated preferentially by intra-axonal rather than hematogenous transport (14). Antibody has been seen to produce an effect on the acute infection in the ganglia (15), on the establishment of latency (16), and on the number of neuronal cells that harbor the latent virus, but antibody does not prevent ganglionic colonization (17). Our findings are in agreement with these reports. In this investigation, previous exposure to the virus, with the production of antibodies to HSV, reduced the severity of subsequent infection and provided protection from death by encephalitis, but did not prevent the establishment of latency (Table III).

We found that different sizes of inocula produced variation in the severity of the initial disease, but so long as the animals did exhibit overt disease, ganglionic superinfection by the challenging strain was not achieved (Table IV). These animals apparently develop a state of resistance to ganglionic superinfection, and we postulate that this resistance is present locally at the level of the trigeminal ganglia and may be related to the state of the specific neuronal cells at that site, as infections with different strains at nearby sites have been reported (18).

This idea is supported by the protective effects seen in the animals initially infected with sufficient amounts of E-43 strain to produce disease and establish latency. Although the challenging strain reached the ganglia, it did not establish a discernible acute infection in these ganglia. Also, the character of the virus yield from these animals supports this observation. More than 99% of the observed plaques were caused by the initial E-43 strain; only a very few had the characteristic plaque morphology of the challenging MP strain. When the ganglia were removed during the latent stage (more than 30 days postchallenge), no MP-type plaques were seen. These results indicate that a resis-

tance state existed in the ganglia and because of that, a new infection could not easily be established. We speculate that when these neurons are "occupied," they cannot be infected by a second strain.

We believe that the results of these animal experiments are relevant to the natural course of HSV infections in humans. HSV has been found in the ganglia of persons with no history of herpetic disease, and Lonsdale *et al.* (19) showed that all cervical ganglia from such individuals harbored the same virus strain. More recently, Lewis *et al.* (20) assayed multiple cervical ganglia from 20 cadavers for the presence of latent virus, and determined the identity of the virus strains by restriction endonuclease analysis. Isolates from all the ganglia of 18 of these individuals were identical; different strains were seen in one case within the same ganglia and in another case in a closely related neuroanatomical site. The authors concluded that such variation could have been a result of exogenous superinfection.

Our results are in agreement with their findings. As we have shown, a challenging strain can reach the ganglia, but it does not displace the resident latent virus. Furthermore, the potential of the challenging virus to establish an acute infection depends on the state of resistance that exists in the ganglia. Our experiments involving large and small inocula lend additional support to the idea that the state of resistance may be related to the amount of virus in residence in the ganglia. Ganglia infected with a large inoculum of the wild-type strain, E-43, always yielded the primary infecting virus strain, but the recombinant strain, F(MP)F, which did not cause disease, gave variable results. In some instances, the initial virus was recovered from the ganglia and in other cases, the challenging strain was recovered. Also, the F(MP)F strain gave some protection from ganglionic superinfection when the challenge was performed 30 days after primary infection, but not before. In these experiments, at least 50% of the ganglia retained the initial recombinant strain and 50% yielded the challenging strain. We interpret this to be related to the state of resistance present at the time of challenge.

Similar experiments using HSV recombinants have been recently reported in mice (21). Animals were inoculated in the right eye with the intertypic recombinant CD7 and challenged in both eyes with the HSV-1(F) strain. The challenging strain colonized all the left ganglia and approximately half of the right ganglia. The authors concluded that the state of resistance induced by the recombinant strain used for primary infection was not adequate to prevent ganglionic superinfection. These findings may be related to the fact that the CD7 strain alone establishes latency with less efficiency that the HSV-1(F) strain and also to the fact that the amount of virus recovered from the ganglia of the CD7-infected animals was very low.

Furthermore, Gerdes and Smith (22) have recently reported results in rabbits that confirm our work. They showed that although latently infected animals could be acutely infected, the establishment of latency by the second, challenging strain was inhibited. The McKrae strain, a wild type, high recurrence phenotype which is the same strain used in our laboratory, was used in these studies. Their studies demonstrated that the genotype of the infecting strain determines some of the events in the disease process, e.g., the ability to cross over to the opposite eye during acute infection. Also in animals infected bilaterally with a different strain in each eye, the high or low frequency of recurrence phenotype of each strain was maintained.

In summary, we have drawn the following conclusions from the results of the work discussed here: (a) In overt primary ocular infections, the predominant strain found in the ganglia after challenge is the initial strain. (b) Protection from superinfection is restricted to the point of entry and the sensory neurons that innervate that site. (c) The size of the infecting dose of virus is a determining factor in the development of overt disease, the establishment of latency, and the potential for subsequent superinfection by a second strain. (d) The relationship between inoculum size and latency shows a threshold effect. Above the minimum necessary inoculum size, latency is established in all the susceptible neurons in that ganglion, and the establishment of latency by another strain is inhibited; below the minimum inoculum size, superinfection is possible. (e) Latency provides protection against superinfection when all

susceptible neurons are "occupied" by the infecting virus. These findings seem to support the hypothesis that initial infection with herpes simplex virus probably lasts a lifetime, and may also have implications in our understanding of the mechanism by which the majority of the human population escapes overt herpetic ocular disease.

This work was supported in part by Public Health Service Grants EY02389, EY02672, EY07073, and EY02377 from the National Eye Institute, National Institutes of Health, Bethesda, Maryland, as well as a Foreign Scholars Fellowship (Dr. Tian) from Research to Prevent Blindness, Inc., New York City.

1. Nesburn AB, Cook ML, Stevens JG. Latent herpes simplex virus. Isolation from rabbit trigeminal ganglia between episodes of recurrent ocular infection. Arch Ophthalmol **88**:412–417, 1972.
2. Baringer JR. Herpes simplex virus infection of nervous tissue in animals and man. Prog Med Virol **20**:1–26, 1975.
3. Baringer JR, Swoveland P. Recovery of herpes simplex virus from human trigeminal ganglions. New Engl J Med **288**:648–650, 1973.
4. Cook ML, Bastone VB, Stevens JG. Evidence that neurons harbor latent herpes simplex virus. Infect Immun **9**:946–951, 1974.
5. Hill TJ. Mechanisms involved in recurrent herpes simplex. In: Nahmias AJ, Dowdle WR, Schinazi RF, eds. The Human Herpesviruses, an Interdisciplinary Perspective. New York, Elsevier North-Holland, pp241–244, 1981.
6. Centifanto-Fitzgerald YM, Varnell ED, Kaufman HE. Initial herpes simplex virus type 1 infection prevents ganglionic superinfection by other strains. Infect Immun **35**:1125–1132, 1982.
7. Hoggan MD, Roizman B. The isolation and properties of a variant of herpes simplex producing multinucleated giant cells in monolayer cultures in the presence of antibody. Amer J Hyg **70**:208, 1959.
8. Ruyechan WT, Morse LS, Knipe DM, Roizman B. Molecular genetics of herpes simplex virus II. Mapping of the major viral glycoproteins and of the genetic loci specifying the social behaviour of infected cells. J Virol **29**:677, 1979.
9. Taktikos A, Aurelian L. Experimental study of the disease of the corneal stroma caused by herpes simplex virus. Amer J Ophthalmol **62**:1136, 1966.
10. Centifanto-Fitzgerald YM, Yamaguchi T, Kaufman HE, Tognon M, Roizman B. Ocular disease pattern induced by herpes simplex virus is genetically determined by a specific region of viral DNA. J Exp Med **155**:475–489, 1982.
11. Nesburn AB, Dunkel EC, Trousdale MD, Enhanced HSV recovery from neuronal tissue of latently infected rabbits. Proc Soc Exp Biol Med **163**:398–401, 1980.
12. Oakes JE, Hornbeak HR. Antibody-mediated recovery from subcutaneous herpes simplex virus type 2 infection. Infect Immun **21**:489–495, 1978.
13. Oakes JE, Lausch RN. Role of Fc fragments in antibody-mediated recovery from ocular and subcutaneous herpes simplex virus infections. Infect Immun **33**:109–114, 1981.
14. Rector JT, Lausch RN, Oakes JE. Use of monoclonal antibodies for analysis of antibody-dependent immunity to ocular herpes simplex virus type 1 infection. Infect Immun **38**:168–174, 1982.
15. Price R, Schmitz J. Route of infection systemic host resistance and integrity of ganglionic axons influence acute and latent herpes simplex virus infection of the superior clinical ganglion. Infect Immun **23**: 373–383, 1979.
16. Openshaw H, Sekizawa T, Wohlenberg C, Notkins AL. The role of immunity in latency and reactivation of herpes simplex virus. In: Nahmias AJ, Dowdle WR, Schinazi AJ, eds. The Human Herpesviruses, an Interdisciplinary Perspective. New York, Elsevier North-Holland, pp287–296, 1981.
17. Walz MA, Yamamoto H, Notkins AL. Immunological response restricts the number of cells sensory ganglia infected with herpes simplex virus. Nature (London) **264**:554–556, 1976.
18. Buchman TG, Roizman B, Nahmias AJ. Demonstration of exogenous genital reinfection with herpes simplex virus type 2 by restriction endonuclease fingerprinting of viral DNA. J Infect Dis **140**:295–304, 1979.
19. Lonsdale DM, Brown SM, Subak-Sharpe JH, Warren KG, Koprowski H. 1979. The polypeptide and the DNA restriction enzyme profiles of spontaneous isolates of herpes simplex virus type 1 from explants of human trigeminal, superior cervical and vagus ganglia. J Gen Virol **43**:151–171, 1979.
20. Lewis ME, Leung WC, Jeffrey VM, Warren KG. Detection of multiple strains of latent HSV-1 within individual human hosts. Eighth International Herpesvirus Workshop, Oxford, England, p171, 1983 (Abstract).
21. Meignier B, Norrild B, Roizman B. Colonization of murine ganglia by a superinfecting strain of herpes simplex virus. Infect Immun **41**:702–708, 1983.
22. Gerdes JC, Smith DS. Recurrence phenotypes and establishment of latency following rabbit keratitis produced by multiple herpes simplex virus strains. J Gen Virol **64**:2441–2454, 1983.

Received October 1, 1984. P.S.E.B.M. 1985, Vol. 179.
Accepted January 18, 1985.