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Studies of herpes virus latency in the sensory spinal ganglia of rabbits

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SUMMARY

Experimental latent herpes infection of rabbit dorsal root ganglia (DRG) is reported. The simian herpes virus used was derived from fatal natural infection in owl monkeys and has limited neurotropism in the rabbit. Following intradermal injection of the flank it causes a local lesion followed only by dorsal root ganglionitis; segmental paraesthesia and/or sensory loss going on to clinical recovery. Methods were developed for mapping sensory losses.

Virus could be immediately re-isolated from skin or DRG homogenates in the acute (first week) stage but from 8–550 days by DRG organ culture only. Spontaneous recurrence does not occur but reactivation can be provoked.

The system provides an improved analogue model for the study of the pathogenesis and symptomatic treatment of herpes zoster.

INTRODUCTION

The latency of herpes simplex virus in human tissues and the related phenomena of recurrent labial and genital herpes and of herpes zoster, pose questions about the pathogenesis of these diseases which are still largely unanswered. Their solution and if possible the development of methods for preventing latency, curing the established latent infection or at least limiting its clinical recurrence would be of direct value. Of less general use, but important to laboratory workers, would be the resolution of the factors which determine whether rhesus monkeys which have recovered from oral or genital lesions of B virus and become sero-positive will manifest reactivation of their latent infection and become a hazard to their human contacts. (Boulter, 1975; Vizoso, 1975*a*; 1975*b*).

Although most studies of herpes latency have been made in experimental animals, the starting point in these developments was probably Professor Rose's early successful surgical extirpation of the trigeminal ganglion (for the relief of *tic douloureux*) in Kings College Hospital (Rose, 1892). This led others and particularly Harvey Cushing (1904*a*; 1904*b*; 1905) to undertake a successful series of such

operations. In one of his cases Cushing observed that the extensive cervico-facial crop of herpes blisters provoked by the surgery spared totally the denervated trigeminal skin area while affecting the surrounding dermatomes. About the same time Howard (1905) reported the post-mortem finding of ganglionic lesions associated with herpes febrilis, *ante mortem*. These studies paved the way for systematic experimental investigations.

Not until 1952, however, was a systematic study made to confirm the clinical observation that in man trigeminal sensory root section proximal to the ganlion, as opposed to extirpation of the trigeminal ganglion, was regularly followed by facial herpes simplex recurrence in the corresponding skin area (Carton & Kilbourne, 1952; Carton, 1953). Carton (1953) further showed that section of the sensory root of the trigeminal ganglion precipitated an outcrop of herpes lesions only on the skin area supplied by the two intact divisions of the nerve, no lesions occurred in skin within that area which had been denervated by previous neurotomy of the third division distal to the ganglion.

Attempts to isolate virus from trigeminal ganglia excised from known herpetics using conventional extraction methods and tissue culture were unsuccessful (Burnet & Lush, 1939; Richter, 1944; Carton & Kilbourne, 1952; Ellison, Carton & Rose, 1959). Later, the presence of herpes simplex virus in ganglia of patients who had died with or without evidence of recent herpetic disease was demonstrated by the use of organ fragment culture techniques, in trigeminal ganglia removed post-mortem (Bastian *et al.* 1972; Baringer & Swoveland, 1973) and in sacral ganglia (Baringer, 1974). The presence of herpes virus varicellae in the trigeminal nerve and ganglion of a patient with ophthalmic zoster who had died was demonstrated by immunofluorescence and electron microscopy (Esiri & Tomlinson, 1972).

The earliest attempts to study herpes virus latency in experimental animals used herpes simplex virus infection of the rabbit brain. Perdrau (1938) found that brain removed from rabbits which had recovered from encephalitis although not infectious at the time of killing, yielded infectious virus after storage in glycerol. These results are still difficult to evaluate and seemingly have never been repeated.

Rabbits which had recovered from herpes simplex encephalitis were used in the first successful attempts to demonstrate reactivation of clinical disease and the reappearance of infectious virus. Good (1947) and Good & Campbell (1948) used anaphylactic shock while Schmidt & Rasmussen (1960) injected adrenaline. However, this experimental model is subject to several limitations. First, it is not possible to exclude the presence of infectious virus in the brain prior to the reactivation stimulus. Secondly, lack of knowledge of where in the brain reactivation will take place, coupled with the difficulties involved in random sampling such a large organ by fragment culture, make it very difficult to study systematically either the sites of latency in the brain or the stages in the reactivation process. Thirdly, rabbit encephalitis does not exemplify the common forms of recurrent herpes in man; herpes labialis or genitalis or zoster would be best studied in a model system involving the recurrence of herpetic lesions in animal skin.

A partial answer to some of these problems was furnished by Anderson, Margruder & Kilbourne (1961). Although they failed in their attempts to induce cutaneous reactivation in mice, guinea pigs and rabbits using as stimuli, epidermal

sensitization, artificial fever, local and systemic hydrocortisone injections, ultraviolet irradiation and intravenous administration of influenza virus, they were nevertheless able to demonstrate that healed herpetic keratitis in the rabbit could be reactivated by a local corneal Arthus reaction using horse serum. Subsequently, Laibson & Kibrick (1966) showed that the same condition could be reactivated by adrenaline injection. This experimental system allowed sequential studies to be made of the presence of infectious virus in the cornea, so that the time sequence of viral reactivation following specific stimuli could apparently be followed. However, Nesburn, Elliot & Leibowitz (1967) showed that following healed primary infection, spontaneous recurrence of herpetic keratitis in rabbits was not unusual. This complicates the interpretation of reactivation experiments using this model. Nevertheless, the reappearance of virus in this site following specific stimuli is at least consistent with the suggestions of Cushing (1904*b*) and many subsequent authors that the trigeminal ganglion is the site of persistence of the infective agent. An important development was the use of organ cultures to demonstrate that herpes simplex virus persists in a latent form in the trigeminal ganglia of rabbits following corneal infection (Stevens, Nesburn & Cook, 1972). Experiments to exclude the cornea itself as an additional site of virus latency by division of the ophthalmic nerve prior to a specific reactivating stimulus do not seem to have been done in rabbits. Another of the complications of the use of corneal infection in rabbits can be the spread of virus from the ganglion to the brain with resultant encephalitis. Knotts, Cook and Stevens, 1973, showed that virus persisted as a latent infection in the brain stem although the exact anatomical site was not determined.

Attempts have been made to develop other model systems. Stevens & Cook (1971) followed the progress of acute mouse infection after footpad inoculation and found that herpes simplex virus persisted after recovery as a latent infection in sacral dorsal root ganglia (DRGs). Infectious virus was recovered only after maintenance of explanted ganglia as organ cultures for 7–14 days. Virus could also be recovered from explanted fragments of spinal cord (Knotts, Cook & Stevens, 1973). However, despite vigorous stimulation, no good evidence of reactivation of the disease was observed in these experiments and free infectious virus could not be detected in any tissues (Stevens & Cook, 1973).

Although Underwood & Weed (1974) reported recurrent herpes infections in hairless mice the first reports of reactivation of herpes simplex virus infection in immune competent mice were the observation of spontaneous virus shedding from the ear skin (Hill, Field & Blyth, 1975), the reactivation in sacral ganglia but not in skin following induced pneumococcal pneumonia by Stevens, Cook & Jordan (1975) and the discovery that cyclophosphamide and epilation under ether anaesthesia could produce in latently infected mice a zosteriform skin eruption from which virus could be recovered (Hough & Robinson, 1975). However, the reliablity and extent of reactivation was limited and the relative contribution of cyclophosphamide and of epilation, to the reactivation is not clear.

That recurrent infections by herpes group viruses occur naturally in domestic animals was shown by Shope's classical studies (1935). Pigs which had recovered from Aujeszky's disease were shown to act as sources of pseudorabies virus from which future cattle outbreaks could stem. In infectious bovine rhinotracheitis

spontaneous recurrences are also known to occur (Snowdon, 1965). In addition, Sheffy & Davies (1972) have demonstrated that specific reactivation of IBR infection can be induced by treatment with adrenal corticosteroids or with ACTH, infectious virus being recovered from nasal discharges. Virus was also isolated, following steroid treatment, from a wide range of tissues, using organ fragment culture techniques. Unfortunately, the need for steroid treatment was not proven in their experiments since the organ fragment culture technique was not used without prior steroid treatment. A little later Davies & Carmichael (1973) showed that, as in man, cutting the proximal connexions of the trigeminal ganglion but leaving the ganglion and the three divisions of the Vth nerve intact resulted in reactivation of the IBR infection which had become latent in the ganglion. For this effect no steroids were necessary, virus and lesions both appearing in the nasal mucosa a few days after surgery.

It is now accepted that all five human herpes viruses induce latency and McCarthy & Tosolini (1975*a*) further suggested that all the 37 primate herpes viruses they listed might have this capability. Recently Ritchie & Timbury (1980) have drawn attention to the structural similarities between herpes genomes and transposable genetic elements perhaps giving all herpes viruses an inherent capacity for latency. However, no animal infection, natural or experimental, accurately models the clinical and pathological presentation of human herpes zoster: the prolonged latency; the pain and paraesthesia; the segmental distribution; the rarity of generalized spread; the occasional motor paralyses and the provocation by immunosuppression.

The serendipitous isolation in 1970 of an apparently new simian herpes virus from recently imported owl monkeys (*Aotus trivirgatus*) suffering from a lethal disease and presenting clinical evidence of dorsal ganglionitis has provided a new model for the study of virus latency. Preliminary studies (Tosolini & McCarthy, 1975; McCarthy & Tosolini, 1975*a*) with this virus designated KM 322 (McCarthy & Tosolini, 1975*b*) have shown that infection of rabbits with this agent seems to meet many of the criteria of a good model for studying both latency and reactivation.

Although the infection occurring naturally in the monkeys showed both clinical (itching and scratching) and histological evidence of dorsal root ganglionitis in thoracic and lumbar regions it was essentially a generalized exanthematous disease with a fatal outcome and as such was useless for long term study. However, in rabbits the virus produced a local skin lesion following intradermal injection; there was no dissemination by the bloodstream but the associated sensory ganglia did become infected. The rabbits remained otherwise healthy and suitable for long term studies of latency and reactivation. The placid nature of rabbits allowed cutaneous sensory mapping to be made over the injected flank area of the animals without significant discomfort for the rabbit or infection risk to the operator from bites and scratches.

MATERIALS AND METHODS

Virus. The virus strain used (KM/322) was one of four new closely related viruses of varying virulence isolated from owl monkeys (McCarthy & Clarkson,

unpublished). It was selected on the basis of its comparatively low virulence for the rabbit. Virus stocks were grown in RK-13 cell monolayers with 199 medium and 2% fetal calf serum (FCS) and were stored at −70 °C. The virus was titrated in VERO cell monolayers with 199 medium and 2% FCS, and the cytopathic effect (CPE) was observed in 16 mm diameter stoppered tubes by low-power dark-ground microscopy and by phase contrast microscopy (McCarthy, 1960; McCarthy & Taylor-Robinson, 1967). Localized plaques were produced with marked syncytial formation. Primary plaques appeared at about 20 h post-infection and became fully developed by 40 h. Secondary plaques were not visible at this time, so that quantitative assays could be made without an overlay.

Rabbits. Adult New Zealand white male rabbits of 4–5 kg were close-clipped on both flanks or where appropriate. They were examined daily.

Safety. The virus used is serologically related to Herpesvirus tamarinus (unpublished results). It has limited neurotropism in the rabbit but is lethal in the owl monkey. Since its possible infectivity or virulence for man was not known it was treated throughout by us in both laboratory and animal house as though it might be a human pathogen. For 3 weeks after infection or when reactivations were to be attempted animals were kept in air-lock protected and filtered animal rooms. Operators wore boots, gloves, glasses, gowns and masks and for the safety of the operators making the injections the animals were tranquilized using ketamine hydrochloride (Parke–Davis) intravenously. Xylocaine was used locally in the ear to make the i.v. injection painless.

A clear plastic box was positioned as a safety shield covering syringe and needle while injections were being made and when killing the animals with barbiturate i.v. or when taking skin samples.

Laboratory work was conducted on the open bench so as to be able to concentrate the maximum care on avoiding cuts and pricks. The use of 150 × 16 mm rubber-stoppered culture tubes avoided risk of spillages or aerosols (see below).

When post-mortem dissections were made to remove ganglia from the carcass appropriate stages were undertaken under a shield suitable for trapping droplets and flying bone fragments.

Intradermal infection of rabbits. Three intradermal (i.d.) injections of 0·05 ml. each containing 500 p.f.u. of virus were made into the lateral aspect of the left flank, within the three dermatomes T12 to L2, on a paravertebral line joining the costal margin to the iliac crest and 10–12 cm from the midline. Hair tufts were avoided where possible, to facilitate later examination of lesions.

Animals were examined daily for skin lesions and when appropriate were tested for changes in cutaneous sensation in the relevant skin areas. When lesions first appear they usually cause the animal to itch and scratch and even bite itself. The initiation of this activity loop and the consequent skin damage can be totally avoided by fitting a flexible 30 cm diameter 'Eton collar' of soft vinyl floor-covering round the animal's neck for a few days. This protection allowed rapid resolution of the three primary lesions and evidently caused no discomfort to the animals.

Sensory mapping. This was attempted initially using several modes of stimulation, i.e. light touch, heat, electrical Faradic stimulation and light pinprick. The only successful technique evolved used pinpricks, which produced just perceptible

muscular twitching which was easily detected by a gloved hand held firmly on the spinal muscles or by slight visible twitches of other trunk muscles. The animal's responsiveness sometimes faded after a few pricks and no further immediate response could be obtained until it was discovered that a single firm prick on the rump away from the test area fully restored the animal's responses for a further period. This facilitation procedure could be repeated if necessary to allow mapping to be completed. Animals were reclipped when necessary before sensory testing. Each mode of stimulus was first tried out on the authors and found to involve minimal discomfort.

Sampling of tissues. At appropriate times animals were killed and exsanguinated. Samples of tissues for virus examination were taken and processed at once. Dorsal root ganglia and the spinal cord were exposed by laminectomy using bone forceps and rongeurs. Dorsal root ganglia from T9 to L6 on both sides were routinely removed.

Isolation of virus from rabbit tissues. Tissues were either ground in a mortar and the extract inoculated onto VERO cell monolayers with 199 medium and 2% FCS, or diced with a scalpel and 0·5 mm cuboid fragments inoculated directly onto VERO cell monolayers in tubes with Eagle's medium and 20% FCS. The fragments of one complete dorsal root ganglion were inoculated into two tubes of VERO cells and the tubes were incubated stationary at 36 °C. The medium on these pairs of organ fragment cultures was changed weekly with separate single pipettes following microscopic examination. The medium was not changed in tubes in which virus lesions had developed in order to minimize the risk of cross-infection between tubes. Where appropriate, serological and animal tests of the identity of the virus isolates were made.

Neutralizing antibody titrations. Reaction mixtures containing dilutions of inactivated sera and 100 p.f.u. of virus in medium 199+2% inactivated FCS, were incubated in a 37 °C waterbath for 60 min. 0·2 ml of each reaction mixture was inoculated into each of five tubes containing VERO cell monolayers, adsorbed stationary at room temperature for 1 h and incubated stationary at 36 °C after addition of 1 ml of 199 medium plus 2% FCS. Plaques were counted by dark ground microscopy after 40 h incubation.

RESULTS

Clinical disease. Two days after i.d. inoculation with 500 p.f.u. of virus at each of three sites on the left flank, an erythematous papule 3–5 mm in diameter developed at almost every inoculation site. By day 4, the papule reached its maximum size, with a diameter of 10–12 mm and the centre of the papule often became necrotic. Although this virus caused a generalized fatal infection in the owl monkey (McCarthy & Clarkson, unpublished) generalized spread did not occur in the rabbit and encephalitis did not develop. The skin lesion started to regress by day 7 (Plate 1) and was healed by day 14.

On day 5–6 after infection and without any visible change in the appearance of the lesion, severe local irritation seemed to occur in the skin in the region of the inoculation site, as evidenced by continuous biting and scratching at the site which resulted in severe excoriation. This was subsequently anticipated and circumvented by fitting a collar on the fourth day as mentioned above.

Table 1. ***Free virus isolated in cell culture from 28 rabbits* killed in acute stage of infection (up to 12 days)***

Sites of isolation	Rabbits positive (No. tested)
Left DRGs (ipsilateral)	23† (28)
Right DRGs (contralteral)	4 (28)
Spinal cord	4 (28)
Skin at inoculation site	27‡ (28)
Spleen	0 (18)

* Animals inoculated on left flank only.

† Of the five negative rabbits, three were killed on the 3rd day post-infection and two on the 4th day.

‡ The one negative rabbit was killed 12 days after infection.

Between day 6 and 8 after infection loss of sensation developed in the skin around the lesion. In most cases, the sensory loss extended to involve the whole of the dermatome in which the lesion was situated from the dorsal midline to the ventral midline. A slow recovery of sensation began after 4–6 weeks, but recovery was sometimes incomplete (Plate 2).

Histology. Six days after infection, haematoxylin and eosin-stained sections of the skin at the inoculation site showed marked oedema and congestion, with polymorphonuclear and mononuclear cell infiltration, and some small areas of necrosis. The corresponding DRG showed oedema and some neuronal destruction, with polymorphonuclear and mononuclear cell infiltration.

Isolation of virus from 28 rabbits in the acute stage of infection. Up to 12 days after infection virus could be isolated from the skin of the inoculation sites. Up to the third day this was the only positive site. Out of 28 rabbits, 27 were positive for skin virus (Table 1).

In the DRGs of the infected side, virus was first detected in rabbits killed on the 4th day after infection but only in one out of three rabbits tested. In all 23 rabbits, killed in the period from 5 to 12 days after infection, virus was isolated from the DRGs serving the infected dermatomes by inoculating homogenized extracts of the tissues onto VERO cell monolayers in which cytopathic effect (CPE) was produced in 1–2 days (Table 1). This can be taken to indicate the presence, at the time of killing, of freely multiplying virus.

Virus could also be recovered from acutely infected ganglia using the fragment explant technique, but of course at this stage isolation by this method did not distinguish between free and latent virus. In 4 of 28 rabbits, virus was isolated from the adjacent part of the spinal cord and from the corresponding DRGs on the opposite side of the body. A few rabbits showed evidence of lower motor neurone paralysis limited to the abdominal musculature of the dermatomes bearing the primary lesions. Virus was not isolated from the spleen (Table 1), nor from blood, brain, liver or kidney on random sampling. In rabbits killed later than 12 days post-infection, virus could no longer be isolated from DRG homogenates; that is, free virus was no longer present in the DRG after this time, marking the end of the acute stage of infection.

In some rabbits DRGs were divided and tested for virus by both homogenization

Table 2. *Time distribution of* first *virus isolation in tissue culture from* 460 *DRGs from* 23 *rabbits killed in acute stage of infection* (*up to* 12 *days*)

	Time to appearance of CPE	
	1 Week	2 Week
No. of rabbits yielding a positive DRG culture	18*	23†

* Homogenate and/or fragment culture.
† Additional five by fragment culture only.

Table 3. *Time distribution of* all *virus isolations from* 460 *DRGs from* 23 *rabbits killed in acute stage of infection* (*up to* 12 *days*)

	Time to appearance of CPE		
	1 Week	2 Weeks	3 Weeks
No. of virus isolations	42*	61†	64†

* Homogenate and/or fragment culture.
† Additional isolations all resulted from fragment cultures.

and fragment culture techniques. The *in vitro* times for the first appearance of CPE from homogenates of DRGs from each acutely infected rabbit were always less than 1 week (Table 2). In contrast when the fragment culture technique was used in the acute stage, although CPE usually appeared within 1 week, in five rabbits virus isolation was delayed until the second week indicating either slow release of free virus or that latency had already supervened. In many instances, virus was isolated from more than one DRG from an individual rabbit (Table 3). Again, homogenates, if they were to yield virus, did so within 1 week of *in vitro* culture. All later isolations were achieved only by fragment culture techniques. The intradermal inoculations were positioned within the presumptive area of dermatomes T12, L1 and L2 and of the double chain of ten sampled (T9 to L6) these were the DRGs most often positive (Table 4). No deaths occurred following i.d. inoculation of this strain of virus.

Infection of rabbits by other routes. In addition to the detailed study of events following i.d. inoculation, other clipped rabbits were inoculated intravenously (i.v.), subcutaneously (s.c.), intramuscularly (i.m.) and intracerebrally (i.c.). Death occurred 6–10 days after i.c. inoculation in five of six rabbits and virus was recovered from the brain of one of two animals examined. Following i.v. inoculation five of six animals survived and in these no evidence of generalized skin lesions was seen. One animal developed a skin lesion at the inoculation site, from which virus was recovered. One rabbit died 8 days after infection, and virus was isolated from blood and kidney. Following i.m. inoculation in the leg of two rabbits, infection of the corresponding DRGs occurred even though no visible skin lesions developed. Paralysis was not seen. In 14 rabbits inoculated s.c. in the flank, no acute deaths occurred. One rabbit developed weakness in both hind limbs at 12 days, and virus was isolated from left and right DRGs and from the cord. Virus was also recovered from the DRGs of two other rabbits killed after s.c. inoculation.

Table 4. *Segmental distribution of free virus in individual DRGs (T9–L6) of 23 rabbits killed in acute stage of infection (up to 12 days)*

No. of rabbits positive	Dermatome									
	T9	T10	T11	T12	L1	L2	L3	L4	L5	L6
Left DRGs (ipsilateral)	0	2	2	5	5	6	3	3	1	1
Right DRGs (contralateral)	0	1	1	1	1	1	1	1	1	1

Table 5. *Latent virus isolated by fragment cultures* from all 54 rabbits killed in post-acute stage of infection (up to 550 days)*

Sites of isolation	Rabbits positive (No. tested)
Left DRGs (ipsilateral)	52† (54)
Right DRGs (contralateral)	21 (51)
Spinal cord	0 (51)
Skin at inoculation site	0 (19)
Spleen	0 (19)

* Free virus never isolated from tissue homogenates.
† Two rabbits positive on right (contralateral) side only.

Isolation of latent virus. To determine whether the virus remained in a latent form in the DRGs of post-acute (recovered) rabbits, virus recovery was attempted by explanting the DRGs as organ fragment cultures on VERO cell monolayers. DRGs were removed from rabbits killed at least 30 days after i.d. inoculation of virus into the left flank. Virus was isolated from one or more ganglia of all of 54 rabbits, some of which had been infected as long as 550 days previously (Table 5). Virus could never be recovered from homogenates of latently-infected DRGs. In 52 of the 54 rabbits, virus was isolated from DRGs on the same side as the i.d. inoculation (Table 5). In 21 of the 51 rabbits tested virus was isolated from DRGs on the opposite side of the body and this included the two rabbits from which virus was not isolated from DRGs on the same side as the inoculation. Viral multiplication was detected by the development of CPE in the VERO cell monolayer, but in contrast with homogenates of acutely infected DRGs, CPE did not occur for at least 8 days after explantation of the ganglion fragments (Table 6). The first appearance of virus from DRG fragment cultures of recovered rabbits normally occurred in the second week *in vitro* (Table 6), but in one case was delayed as long as 15 weeks. Commonly, more than one isolation occurred from individual rabbits, and these were most frequent in the second and third weeks *in vitro* (Table 7).

After the acute stage of infection, virus has never been recovered from explants of skin taken from the inoculation site or elsewhere and maintained *in vitro* for at least 6 weeks, nor from explants of spinal cord or spleen (Table 5).

The segmental distribution of latent virus is shown in Table 8. The DRGs most frequently found positive correspond to the dermatomes bearing the primary skin lesions and to the distributions of free virus shown in Table 4. This concentration

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Table 6. *Time distribution of* first *virus isolation in fragment culture from* 1050 *DRGs from* 54 *rabbits killed in post-acute stage of infection* (*up to* 550 *days*) (*cumulative totals*)

	Time in weeks to appearance of CPE															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16–25
No. of rabbits yielding a positive DRG culture	0	45	51	51	53	53	53	53	53	53	53	53	53	53	54	54

Table 7. *Time distribution of* all *virus isolations in fragment culture from* 1050 *DRGs from* 54 *rabbits killed in post-acute stage of infection* (*up to* 550 *days*) (*cumulative totals*)

	Time in weeks to appearance of CPE															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16–25
Number of virus isolations	0	92	129	135	141	145	146	146	147	147	147	147	147	147	148	148

Table 8. *Segmental distribution of latent virus in DRGs (T9–L6) of 54 rabbits killed in post-acute stage of infection (up to 550 days)*

No. of rabbits positive	Dermatome									
	T9	T10	T11	T12	L1	L2	L3	L4	L5	L6
Left DRGs (ipsilateral)	1	1	10	15	19	18	16	12	6	1
Right DRGs (contralateral)	0	0	3	5	7	4	3	1	2	1

of virus in comparatively few DRGs, together with the infrequent recovery of virus from the cord in the acute stage of the infection (Table 1) and the rarity of spread to the contralateral chain of ganglia (Tables 4 and 8) are in accord with the restricted neurovirulence of this particular strain of owl monkey herpes virus in the rabbit. Furthermore, virus isolated from latently infected ganglia behaved like the original i.d. inoculated virus. New uninfected rabbits injected i.d. with this recovered virus developed a local skin lesion and a latent infection in the corresponding DRG, from which the virus could again be isolated by fragment culture. There was no sign of increased neurovirulence.

Infection of rabbits with Herpesvirus tamarinus. Although the owl monkey herpes virus is not immunologically identical to Herpesvirus tamarinus, it is closely related (unpublished observations) and attempts were therefore made to ascertain whether our findings with the owl monkey herpes virus could be reproduced with Herpesvirus tamarinus. Preliminary findings showed that Herpesvirus tamarinus could be recovered from DRG fragment cultures of all of five rabbits killed between 7 months and 3½ years after i.d. inoculation of virus. Appleton (1975) has noted a possible spontaneous reactivation of Herpesvirus tamarinus affecting six cotton-topped marmosets which had been resident in a colony for 17 months.

DISCUSSION

The current concepts of the pathogenesis of primary infections in man with Herpesvirus hominus and Herpesvirus varicellae, and the natural history and pathogenesis of recurrences, have been synthesized from the results of a large number of clinical observations and comparatively few experimental studies, some of which have been mentioned in the Introduction. The infection pattern in each of these two human diseases is unique and there has been no naturally occurring disease in animals nor experimental animal model which fully mimics either of them.

In this paper, we describe an experimental model in rabbits in which a herpes virus inoculated intradermally produces a local lesion, spreads to the associated dorsal root ganglion causing a transient ganglionitis with disturbance of sensory function, and persists in the ganglion in latent form for periods of at least 550 days (or 3½ years for Herpes tamarinus). No spontaneous recurrence has been seen, but in a preliminary paper we showed that the virus could be experimentally reactivated from the latent form; it then passes to the skin and causes typical herpetic epidermal lesions (McCarthy & Tosolini, 1975*b*). This provoked reactivation can be repeated at will. A full account is in preparation. The

disadvantages and advantages of using a new simian herpes virus in rabbits merit consideration. Because little was known of the human pathogenicity of the agent, we adopted extra precautions. We used isolation animal rooms with filtered exhaust air and wore protective clothing and gloves for all acute animal experiments. We also disinfected rooms after use with formaldehyde. We always anaesthetized rabbits before injecting them with virus and we used a clear plastic box to enclose almost completely the syringe needle and injection site to protect against accidental spray.

In considering the advantages of the system it is necessary to bear in mind the alternatives which have been used successfully by others. Herpes simplex infection of the sensory ganglia of mice has been extensively used as a model for human herpes simplex infection. Like the human condition spontaneous reactivation occurs in mice but it is also possible to provoke more extensive recurrence by external stimuli such as epithelial stripping with cellophane (Hill, Harbour & Blyth, 1980). The disadvantage of that system is that spontaneous and induced reactivation cannot easily be distinguished. In the system we report here infection produces a circumscribed local lesion at a chosen site in a particular dermatome and gives rise to ganglionic infection in a controlled manner. There is no haematogenous dissemination and no widespread invasion of the CNS. Furthermore the rabbits survive for years without spontaneous reactivation enabling long-term studies to be made. Unlike monkeys or mice, conscious, unrestrained, infected rabbits could be easily and safely examined and tested for sensory and motor function. Likewise neurotomy or other surgery could be simply and safely performed.

Infection in the rabbit gives rise to sensory disturbances including segmental anaesthesia and evident irritation leading to self-biting unless restrained with a protective collar. These sensory manifestations together with the prolonged latency provide an experimental system with many features in common with herpes zoster. The fact that there was also no virus latent in the skin and that, as we show elsewhere, reactivation could be regularly and repeatedly provoked with the production of virus-yielding cutaneous lesions in the affected dermatome, adds to its suitability as a model for zoster.

Rabbits are generally free from endogenous herpes virus infections, largely removing the possibility of uncertainty as to the identity of latent virus isolates.

The results show that there is only limited spread of the virus from the ganglia supplying the dermatomes bearing the three primary lesions (T12, L1, L2), either up and down the cord or to the DRGs of the contralateral side. There is no obvious neural pathway to account for the latter. The negative spleen cultures suggest that no generalized infection occurred.

The results of experiments to determine the anatomical distribution of the virus in rabbits killed from days 4–12 post-infection have been combined. We aimed to place the three primary skin inoculations within dermatomes T12–L2, but no dermatome maps of rabbits are available and the extent of anatomical variation in skin innervation is unknown to us. The degree of scatter of DRGs showing the presence of virus may reflect variations in the accuracy of placement of the inoculations, but more probably indicates spread of virus up and down the ganglion chain.

In acutely infected rabbits, the delay in the appearance of virus in some DRG fragment cultures until the second and third weeks *in vitro* may be due to virus having already become latent in the DRGs. An alternative explanation is that a small amount of virus present in a single DRG may have taken more than one week to diffuse or grow out of a DRG fragment and produce CPE in the VERO cell feeder layer. No delay in virus isolation was seen when homogenates of acutely infected DRGs were inoculated onto VERO cell monolayers.

In the post-acute stage of infection, DRG homogenates were invariably negative. At this stage fragment cultures of DRGs were positive. The first isolation of virus from these was, however, always delayed until the second week *in vitro* and sometimes later. This contrasts with the earlier (first week usually) isolation in the acute stage and is evidence that latency had supervened.

At least one virus isolation was made from DRG fragment cultures of every one of 54 rabbits killed in the post-acute stage of infection. Although the site of inoculation was on the left flank, virus was isolated from the DRGs of the right side in 21 of 51 rabbits examined. Surprisingly, in two of the rabbits, virus was isolated from only the right side DRGs. On the right side, virus was isolated more frequently in the post-acute stage than in the acute stage of infection. Latent virus was never detected in the spinal cord after the acute stage. An important point in relation to our success, reported elsewhere, in producing reactivation of the skin lesions is that despite the presence of virus in the skin of the primary inoculation site during the acute stage, virus was not found to persist in the inoculation site in any of the 19 rabbits tested in the post-acute stage by fragment culture techniques.

When rabbits were killed in the post-acute state of infection and their DRGs explanted for co-cultivation, there was as mentioned above a delay of at least a week *in vitro* before any virus was isolated; in one animal the delay was 15 weeks. Often, isolations of virus were made from several DRGs in the same rabbit, with *in vitro* delays up to 9 weeks. After the acute stage was over, the time taken for the reappearance of virus *in vitro* was unrelated to the interval between primary infection and killing of the rabbit.

It can be seen from the results that the segmental distribution and frequency of isolation of latent virus parallels fairly closely the distribution and frequency in the acute stage of infection. The quantity of virus injected into the skin at each primary inoculation site was close to the minimal infective dose and this may be reflected in the relatively low frequency of isolation from the DRGs in both acute and latent stages of the infection.

In order to establish to our satisfaction the validity of latent virus isolations, the entire work of inoculation, reading and changing the medium of fragment culture tubes was undertaken personally. Since 20 DRGs as well as other organ fragments were taken from each rabbit, using in general two tubes per specimen, and since 82 rabbits were killed in the present experiments and cultures had to be maintained for many weeks with weekly changes of medium, there were in some weeks more than 2000 tubes changed. The possibility of cross-contamination with the virus had to be excluded. By means of a closed suction system, medium was gently removed using a separate 'drinking' straw for each pair of tubes. In early experiments, tubes were examined microscopically each day, but subsequently

only once a week, immediately prior to a medium change. Any tube showing the characteristic viral CPE was not opened, and was returned to the incubator without a medium change. Similarly, other procedures with the virus which might conceivably have produced aerosols, were avoided in the laboratory at the time of changing medium.

It was often found that changing the medium on DRG fragment cultures precipitated the appearance of viral CPE within 48 h; the appearance of CPE in the latter part of the week was uncommon. Although cross-contamination might theoretically have produced such a timing of events, we believe that this is not so for the following reasons. The first signs of CPE in the VERO cell feeder layer frequently occurred immediately adjacent to a DRG fragment and progressed slowly enough for the site of origin to be identified. Sometimes the spread of CPE resulted in detachment of the fragment. A second reason for discounting cross-infection as the source of CPE in DRG fragment tubes is that from any one rabbit were set up, as well as 40 DRG fragment tubes, up to 30 other tubes containing skin, spinal cord and spleen fragments or control tubes, and in none of these other tubes was virus ever isolated.

A finding which may indicate that only a small amount of virus is latent in any one DRG, is that frequently only one of a pair of DRG fragment-culture tubes become positive. As mentioned above, however, the dose of virus injected into the skin was close to the lower limit for the production of a skin lesion and the resulting minimal lesion rarely exceeded 12 mm in diameter. Little seems to have been published about the innervation of the rabbit skin, in regard to the position of the dermatomes, the density of the different modalities of sensory receptors within them and the extent of overlap of the innervation of adjacent dermatomes. Nevertheless, it is likely that only a small proportion of neurones in a DRG would be infected from a 12 mm skin lesion.

In the early stages of the experiments, when it became apparent that infected rabbits suffered first a sensory disturbance and then anaesthesia of the skin, a number of preliminary studies were made in clipped normal rabbits to determine the best method of sensory testing. The choice lay between clinical observation of reaction to stimuli such as pinpricks, and electrophysiological detection and measurement of impulses in exposed nerves in anaesthetized animals as a measure of sensory function. The latter was of course unsuitable for mapping changes in sensation on a daily basis and the complexity of the apparatus was incompatible with use in an infectious system. Using muscle twitching as evidence of response to a sensory stimulus, we could detect no reaction to light touch, cold probe or electrically-heated wire loop in the latter case even when the temperature and time of application were well beyond those which produced an instant response in human subjects. Deep pinching of the skin also failed to produce a reliable response. Electrical stimulation of the skin of conscious rabbits with a peripheral nerve stimulator was too imprecise because of direct stimulatory effects on motor nerves. The simple method of response to pinpricks, as described in Materials and Methods, proved to be the most suitable. It was sensitive and reproducible, provided recourse was made to the facilitation technique of occasional pinpricks in the very sensitive rump area.

Using the pinpricks method, the extension and subsequent reduction of the area

of anaesthesia was easily followed over periods of many weeks. In the acute stage of the infection, the large area of anaesthesia indicated extensive involvement of the DRGs, which at this stage were congested and oedematous. It is not known whether the subsequent recovery of sensation was simply due to resolution of the inflammatory changes in the DRGs or in some measure to recovery of function of virus-infected neurones or to ingrowth from neighbouring areas. Further work is necessary to determine the exact site of virus latency within the DRG.

The experimental model described in this paper has considerable potential for further development and for improving our understanding of the pathogenesis of other recurrent herpes virus infections. We are also using it to investigate possible therapeutic measures. As a second and unexpected bonus from this work, the loss of sensitivity to pinpricks coupled with the evident onset of cutaneous irritation in the animals may provide an animal model for testing the efficacy of analgesics for this modality of pain in man.

The production of long-term latency in the DRGs of rabbits is not a unique property of the owl monkey herpes virus used in these studies, since, as mentioned above, a strain of herpes tamarinus has been recovered from the DRGs of rabbits which had remained healthy for a period of three and a half years following primary skin infection.

REFERENCES

ANDERSON, W. A., MARGRUDER, B. & KILBOURNE, E. D. (1961). Induced reactivation of Herpes simplex virus in healed rabbit corneal lesions. *Proceedings of the Society for Experimental Biology and Medicine* **107**, 628–632.

APPLETON, H. (1975). Hepatitis transmission in marmosets. *Journal of Infectious Diseases* **132**, 500–505.

BARINGER, J. R. (1974). Recovery of Herpes simplex virus from human sacral ganglia. *New England Journal of Medicine* **291**, 828–830.

BARINGER, J. R. & SWOVELAND, P. (1973). Recovery of Herpes simplex virus from human trigeminal ganglions. *New England Journal of Medicine* **288**, 648–649.

BASTIAN, F. O., RABSON, A. S., YEE, C. L. & TRALKA, T. S. (1972). Herpesvirus hominis: Isolation from human trigeminal ganglion. *Science* **178**, 306–307.

BOULTER, E. A. (1975). The isolation of monkey B virus (Herpesvirus simiae) from the trigeminal ganglion of a healthy seropositive rhesus monkey. *Journal of Biological Standardization* **3**, 279–280.

BURNET, F. M. & LUSH, D. (1939). Herpes simplex studies on the antibody content of human sera. *Lancet* i, 629–631.

CARTON, C. A. (1953). Effect of previous sensory loss on the appearance of Herpes simplex following trigeminal sensory root section. *Journal of Neurosurgery* **10**, 463–468.

CARTON, C. A. & KILBOURNE, E. D. (1952). Activation of latent Herpes simplex by trigeminal sensory-root section. *New England Journal of Medicine* **246**, 172–176.

CUSHING, H. (1904*a*). Perineal Zoster. *Johns Hopkins Hospital Bulletin* **15**, 172.

CUSHING, H. (1904*b*). Perineal Zoster with notes upon cutaneous segmentation postaxial to the lower limb. *American Journal of Medical Sciences* **127**, 375–391.

CUSHING, H. (1905). The surgical aspects of major neuralgia of the trigeminal nerve. *Journal of the American Medical Association* **44**, 773–779, 860–865, 920–929, 1002–1008, 1088–1093.

DAVIES, D. H. & CARMICHAEL, L. E. (1973). Role of cell-mediated immunity in the recovery of cattle from primary and recurrent infections with infectious bovine rhinotracheitis virus. *Infection and Immunity* **8**, 510–518.

ELLISON, S. A., CARTON, C. A. & ROSE, H. M. (1959). Studies of recurrent Herpes simplex infections following section of the trigeminal nerve. *Journal of Infectious Diseases* **105**, 161–167.

ESIRI, M. E. & TOMLINSON, A. H. (1972). Herpes Zoster: Demonstration of virus in trigeminal

nerve and ganglion by immunofluorescence and electron microscopy. *Journal of Neurological Sciences* **15**, 35–48.

GOOD, R. A. (1947). Recovery of Herpes simplex virus from rabbit brain nine months after inoculation. *Proceedings of the Society for Experimental Biology and Medicine* **64**, 360–362.

GOOD, R. A. & CAMPBELL, B. (1948). The precipitation of latent Herpes simplex encephalitis by anaphylactic shock. *Proceedings of the Society for Experimental Biology and Medicine* **68**, 82–87.

HILL, T. J., BLYTH, W. A. & HARBOUR, D. A. (1978). Trauma to skin causes recurrence of Herpes simplex in the mouse. *Journal of General Virology* **39**, 21–28.

HILL, T. J., FIELD, H. J. & BLYTH, W. A. (1975). Acute and recurrent infection with Herpes simplex virus in the mouse: a model for studying latency and recurrent disease. *Journal of General Virology* **28**, 341–353.

HILL, T. J., HARBOUR, D. A. & BLYTH, W. A. (1980). Isolation of Herpes simplex virus from the skin of clinically normal mice during latent infection. *Journal of General Virology* **47**, 205–207.

HOUGH, V. & ROBINSON, T. W. E. (1975). Exacerbation and reactivation of Herpesvirus hominis infection in mice by cyclophosphamide. *Archives of Virology* **48**, 75–83.

HOWARD, W. T. (1905). Further observations on the relation of lesions of the Gasserian and posterior root ganglia to herpes occurring in pneumonia and cerebrospinal meningitis. *American Journal of Medical Sciences* **130**, 1012–1019.

KNOTTS, F. B., COOK, M. L. & STEVENS, J. G. (1973). Latent Herpes simplex virus in the central nervous system of rabbits and mice. *Journal of Experimental Medicine* **138**, 740–744.

LAIBSON, P. R. & KIBRICK, S. (1966). Reactivation of herpetic keratitis by epinephrine in rabbit. *Archives of Ophthalmology* **75**, 254–260.

MCCARTHY, K. (1960). An adaptor for examining tissue cultures in test tubes by phase contrast microscopy. *Virology* **12**, 313–316.

MCCARTHY, K. & TAYLOR-ROBINSON, C. H. (1967). Rubella. *British Medical Bulletin* **23**, 185–191.

MCCARTHY, K. & TOSOLINI, F. A. (1975*a*). A review of primate herpes viruses. *Proceedings of the Royal Society of Medicine* **68**, 145–150.

MCCARTHY, K. & TOSOLINI, F. A. (1975*b*). Hazards from simian herpes viruses: reactivation of skin lesions with virus shedding. *Lancet* i, 649–650.

NESBURN, A. B., ELLIOT, J. H. & LEIBOWITZ, H. M. (1967). Spontaneous reactivation of experimental Herpes simplex keratitis in rabbits. *Archives of Ophthalmology* **78**, 523–529.

PERDRAU, J. R. (1938). Persistence of the virus of Herpes simplex in rabbits immunised with living virus. *Journal of Pathology and Bacteriology* **47**, 447–455.

RICHTER, R. B. (1944). Observations bearing on the presence of latent Herpes simplex virus in the human Gasserian ganglion. *Journal of Nervous and Mental Disease* **99**, 356–358.

RITCHIE, D. A. & TIMBURY, M. C. (1980). Herpes viruses and latency: possible relevance to the structure of the viral genome. *FEMS Microbiology Letters* **9**, 67–72.

ROSE, W. (1892). The Lettsonian Lectures on the surgical treatment of trigeminal neuralgia *Lancet* i, 71–73, 182–184, 295–302.

SCHMIDT, J. R. & RASMUSSEN, A. F. JR (1960). Activation of latent Herpes simplex encephalitis by chemical means. *Journal of Infectious Diseases* **106**, 154–158.

SHEFFY, B. E. & DAVIES, D. H. (1972). Reactivation of a bovine Herpesvirus after corticosteroid treatment. *Proceedings of the Society for Experimental Biology and Medicine* **140**, 974–976.

SHOPE, R. E. (1935). Experiments on epidemiology of pseudorabies: mode of transmission of disease in swine and their possible role in its spread to cattle. *Journal of Experimental Medicine* **62**, 85–99.

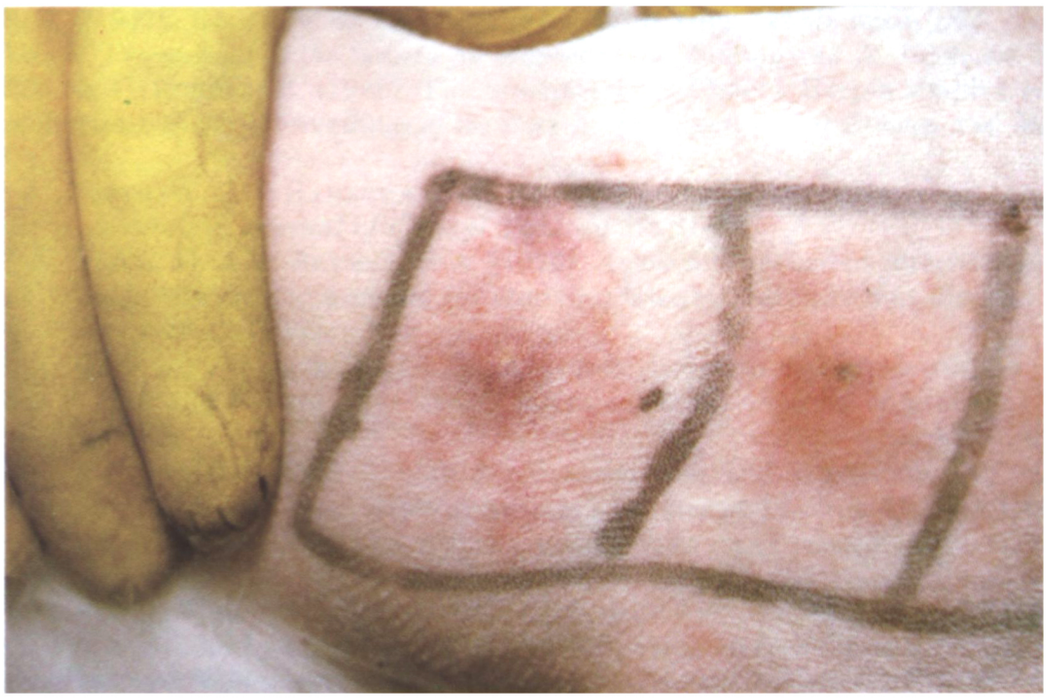
SNOWDON, W. A. (1965). The IBR–IPV virus: reaction to infection and intermittent recovery of virus from experimentally infected cattle. *Australian Veterinary Journal* **41**, 251–254.

STEVENS, J. G. & COOK, M. L. (1971). Latent Herpes simplex virus in spinal ganglia of mice. *Science* **173**, 843–845.

STEVENS, J. G. & COOK, M. L. (1973). Latent infections induced by Herpes simplex viruses. *Cancer Research* **33**, 1399–1401.

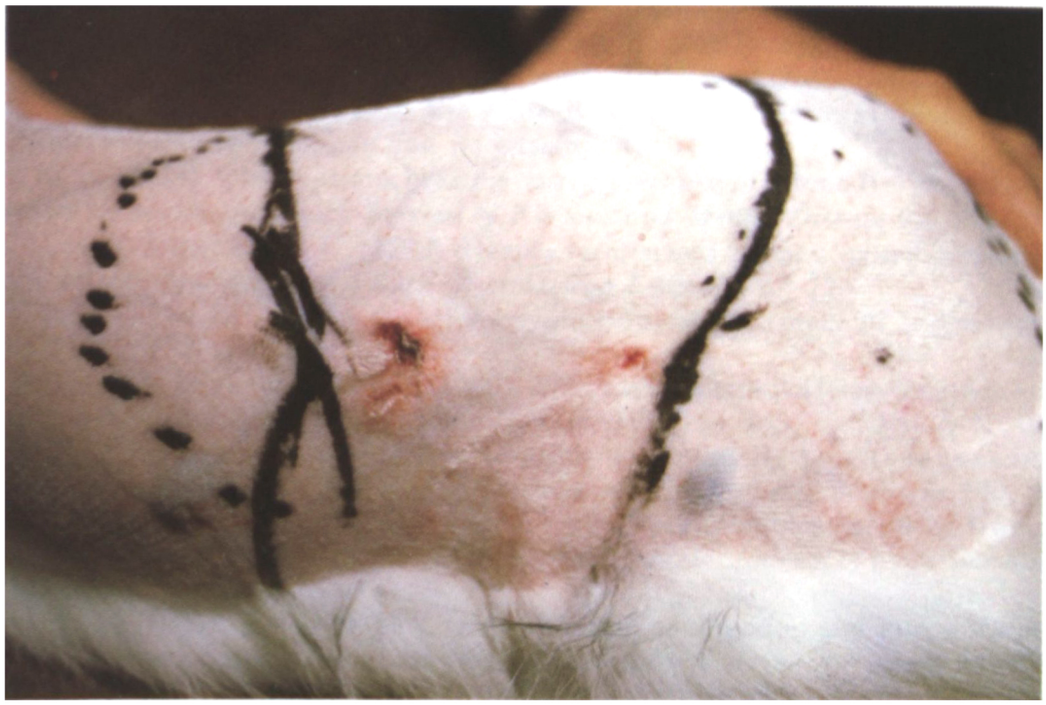
STEVENS, J. G., COOK, M. L. & JORDAN, M. C. (1975). Reactivation of latent herpes simplex virus after pneumococcal pneumonia in mice. *Infection and Immunity* **11**, 635–639.

STEVENS, J. G., NESBURN, A. B. & COOK, M. L. (1972). Latent Herpes simplex virus from trigeminal ganglia of rabbits with recurrent eye infection. *Nature New Biology* **235**, 216–217.



Skin lesions 7 days after infection showing slight central necrosis. Regression has commenced.

Plate 2



Segmental anaesthesia; T12, L1, L2: complete (——) and partial (· · · ·), 95 days after infection. There are two new traumatic lesions resulting from a resumption of biting which necessitated the brief re-fitting of a protective collar.

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TOSOLINI, F. A. & MCCARTHY, K. (1975). Herpes virus latency in the nervous system. *Proceedings of the Royal Society of Medicine* **68**, 150.

UNDERWOOD, G. E. & WEED, S. D. (1974). Recurrent cutaneous Herpes simplex in hairless mice. *Infection and Immunity* **10**, 471–474.

VIZOSO, A. D. (1975*a*). Recovery of Herpesvirus simiae (B virus) from both primary and latent infections in rhesus monkeys. *British Journal of Experimental Pathology* **56**, 485–488.

VIZOSO, A. D. (1975*b*). Latency of Herpesvirus simiae (B virus) in rabbits. *British Journal of Experimental Pathology* **56**, 489–494.

EXPLANATION OF PLATES

PLATE 1

Skin lesions 7 days after infection showing slight central necrosis. Regression has commenced.

PLATE 2

Segmental anaesthesia, T12, L1, L2: complete (——) and partial (. . . . .). 95 days after infection. There are two new traumatic lesions resulting from a resumption of biting which necessitated the brief re-fitting of a protective collar.