In vitro and *in vivo* ocular studies using herpes simplex virus types 1 and 2

by

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in

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SUMMARY

The biological properties of three HSV strains were characterized with reference to ocular disease in the rabbit. Two HSV-1 strains, 17 and McKrae, and the HSV-2 strain HG52 were studied and the following parameters were assessed; clinical disease; virulence; spontaneous shedding of HSV; induced shedding of HSV; neural latency; and corneal latency. Intratypic and intertypic differences were apparent. The HSV-1 strain 17 was pathogenic to rabbit eyes and neuropathogenic with increasing titres of inoculum. It had a low frequency of spontaneous shedding and an intermediate frequency of induced shedding. The HSV-1 strain 17 was able to establish latent infections within trigeminal ganglia. The HSV-1 strain McKrae was pathogenic to rabbit eyes and particularly neuropathogenic. It had a high frequency of both spontaneous and induced viral shedding. The McKrae strain was able to establish latent infections within trigeminal ganglia but differed in maintaining a latent infection within the cornea. The HSV-2 strain HG52 was non-pathogenic to rabbit eyes and non-neuropathogenic. It had a very low frequency of spontaneous and induced shedding. The HSV-2 strain HG52 was able to maintain latent infections within the trigeminal ganglion.

Twelve corneas from patients suffering from herpes simplex keratitis were collected and analysed by light microscopy, electron microscopy and organ culture. Two of the twelve corneas released HSV after at least seven days in organ culture. The released virus was identified as HSV-1 by restriction endonuclease analysis.

Primary cultures of rabbit corneal epithelial cells,

keratocytes and endothelial cells were established. The identity of the cells was confirmed by electron microscopy and indirect immunofluorescence techniques. The one step growth kinetics of HSV-1 in the three distinct cell types were established. Latent infections were established in the distinct cell lines *in vitro* using supra optimal temperatures. Cellular stress proteins were demonstrated at supraoptimal temperatures. The antiviral agent acycloguanosine was unable to eliminate latent HSV infections at the supraoptimal temperature (42°C), and the reactivation of HSV from acycloguanosine treated cell cultures was no different from the control group when cells were restored to 37°C.

Latently present genomes were detectable in epithelial cells following superinfection after up to 14 days at 37°C. Wild type genomes and recombinant genomes were recovered following superinfection.

The results presented in this thesis confirm that HSV can be recovered from human corneas after organ culture and extend this observation to HSV infected rabbits. Latent HSV infections can be induced in rabbit corneal cells under conditions of heat shock, and latently present HSV genomes can be detected in corneal epithelial cells after long term (14 day) latent infections at 37°C. These findings suggest that the cells of the cornea are able to maintain latent HSV infections both *in vivo* and *in vitro*, and are thus an additional site to neurones for HSV latency.

Abbreviations

ACG	acycloguanosine
ara-C	cytosine arabinoside
BHK21C13	baby hamster kidney cells
B.S.A.	bovine serum albumin
CAV	cell associated virus
Ci	Curies
CMV	cytomegalovirus
CNS	central nervous system
cpe	cytopathic effect
CRV	cell released virus
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EBV	Epstein Barr virus
EDTA	sodium ethylene diamine tetra acetic acid
EM	electron micrograph
g	gram
HSV	herpes simplex virus
HIV	human immunodeficiency virus
hr	hour
IE	immediate early
IFN	interferon
K	kilo
kg	kilogram
L	late
M	molar
ml	milli litre
mm	milli metre
mM	milli molar
m.o.i.	multiplicity of infection
mRNA	messenger RNA
mol wt	molecular weight
nm	nano metre
PBS	phosphate buffered saline
pfu	plaque forming unit
PAGE	polyacrylamide gel electrophoresis
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SEM	scanning electron micrograph

CHAPTER 1

INTRODUCTION

Classification

The current classification of herpes viruses is based upon biological properties. Three subfamilies of herpesvirus are recognised on the basis of host range, duration of reproductive cycle, cytopathology and the characeristics of latent infection. Briefly alpha herpes viruses including human herpesviruses 1, 2 and 3 [herpes simplex virus 1 and 2, (HSV-1 and -2) and varicella zoster virus, (VZV)] are restricted to man *in vivo*, have restricted host range *in vitro*, a short reproductive cycle of less than 24 hours causing widespread cell destruction, and an ability to maintain latent infections in neurones (Gilden *et al.*, 1983; Hyman *et al.*, 1983). The beta human herpesvirus 5, [cytomegalovirus, (CMV)] has a narrow host range and a reproductive cycle of greater than 24 hours, which slowly causes lytic foci. Latent infections may establish within secretory glands, lymphoreticular cells, the kidneys and other tissues. The gamma human herpesvirus 4, [Epstein-Barr virus, (EBV)] has a restricted host range and its latent infections occur frequently in lymphoid tissue, but may occur in other tissues (Roizman, 1985). A sixth human herpesvirus has been isolated recently from patients with the human immunodeficiency virus-2 (HIV-2) associated acquired immune deficiency syndrome and patients with other haematological disorders (Salahuddin *et al.*, 1986).

syn$^+$	non syncytial
TEM	transmission electron micrograph
TEMED	N,N,N,N tetramethylethylenediamine
tk	thymidine kinase
ts	temperature sensitive
ts$^+$	wild type for temperature sensitivity
uCi	micro Curies
ug	micro gram
ul	micro litre
um	micro metre
v/v	volume per volume
V_{mw}	molecular weight of virus induced polypeptide
VZV	Varicella zoster virus
V	volt
wt	wild type
w/v	weight per volume

Epidemiology of ocular herpes disease

Herpes simplex virus infections are endemic throughout the world. Different studies have found that around 90% of the tested populations have antibodies against HSV (Buddingh et al. (1953), Leopold and Sery, 1963). The primary infection is most often asymptomatic but may be manifest as a pharyngitis. Infections are characteristically acquired in childhood and adolescence. However the pattern of disease may be changing, as a recent review (Anonymous, 1981) showed that the prevalence of adults with antibodies against HSV was declining. Smith et al. (1967) showed that between 36-48% of medical students and student nurses had antibodies to HSV-1. Glezon et al. (1975) reported finding the antibody in only 30% of students in North Carolina. This suggests that infection in childhood may be less common.

Man is the natural host species for the human herpesviruses and no animal or insect reservoir is known. Disease is spread by personal contact, often unwittingly by an asymptomatic virus shedder. In general only one strain of HSV, identifiable by restriction endonuclease analysis, can be cultured after explantation from different sites of the peripheral nervous system within an individual (Lonsdale et al., 1979). However the isolation of more than one virus strain from an individual has been reported (Buchman et al., 1979). This variation will be reviewed later.

The most important biological properties of HSV-1 and -2 affecting pathogenesis of disease are the functions of latency and recurrence/recrudescence. These will be discussed in depth later.

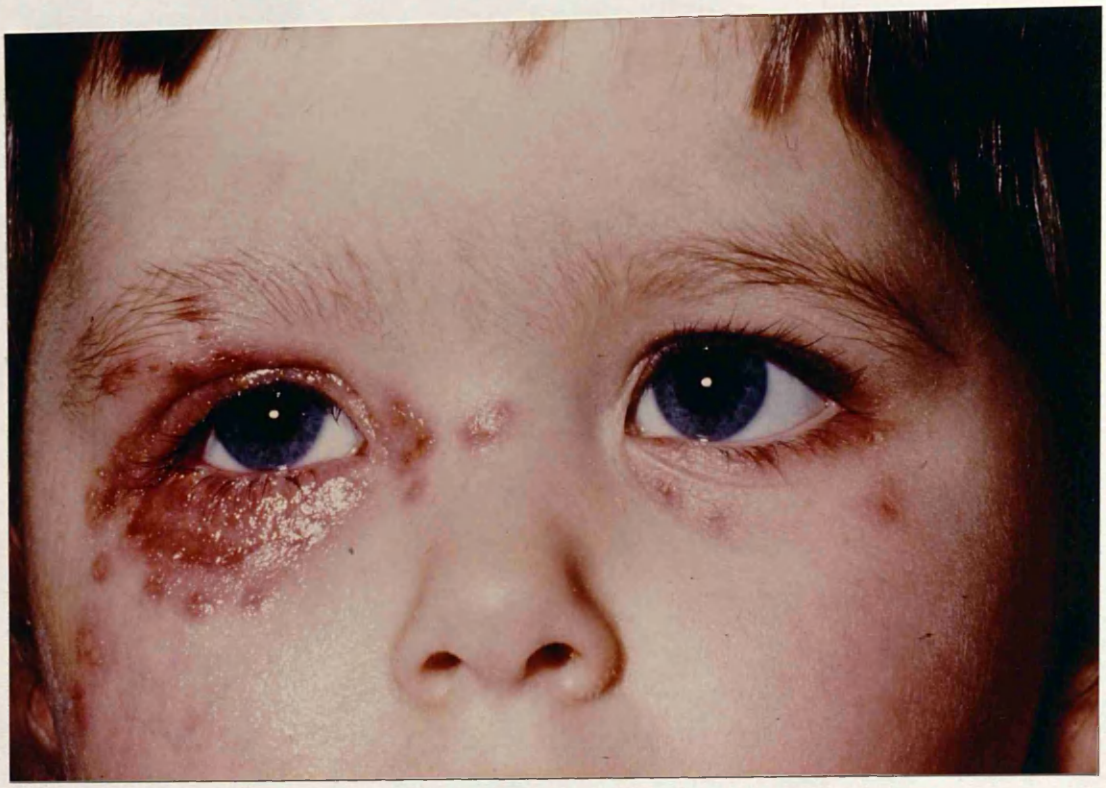
The six human herpesviruses have all been associated

with ocular disease; herpes simplex virus types 1 and 2 cause primary and recrudescent disease affecting the anterior segment (Hogan et al., 1964), the uveal tissue (Patterson et al., 1968) and the neural cells of the retina (Pepose, et al., 1985). VZV recrudescent disease causes significant morbidity and can result in blindness following an associated optic neuritis (Glaser, 1986). EBV is associated rarely with orbital lymphomas (Henle and Henle, 1974), and similarly infectious mononucleosis is occasionally associated with a conjunctivitis (McCollum, 1970). CMV may be acquired in utero and be manifest as a retinochoroiditis often associated with periventricular calcification (Lonn, 1972). Most adults with active CMV ocular disease are immunosuppressed and have a retinochoroiditis (Smith, 1964). Recently CMV retinitis has been noted as a late feature of the human immunodeficiency virus (HIV) induced acquired immune deficiency syndrome. The CMV retinitis has poor prognostic implications for the affected patient (Humphry et al., 1986). The novel human herpesvirus 6 has been detected in the retina of patients suffering from acquired immune deficiency syndrome retinitis. Immunohistochemical techniques and the polymerase chain reaction were used to detect antigen and DNA respectively (Qavi et al., 1988).

It can be seen that the clinical spectrum of ocular herpetic disease is wide. This thesis is confined to the human herpesviruses HSV-1 and -2 and their ocular effects.

Pathogenesis of ocular herpetic disease

a (i) Primary disease. A primary herpetic infection is characterised by a rising titre of antibodies against



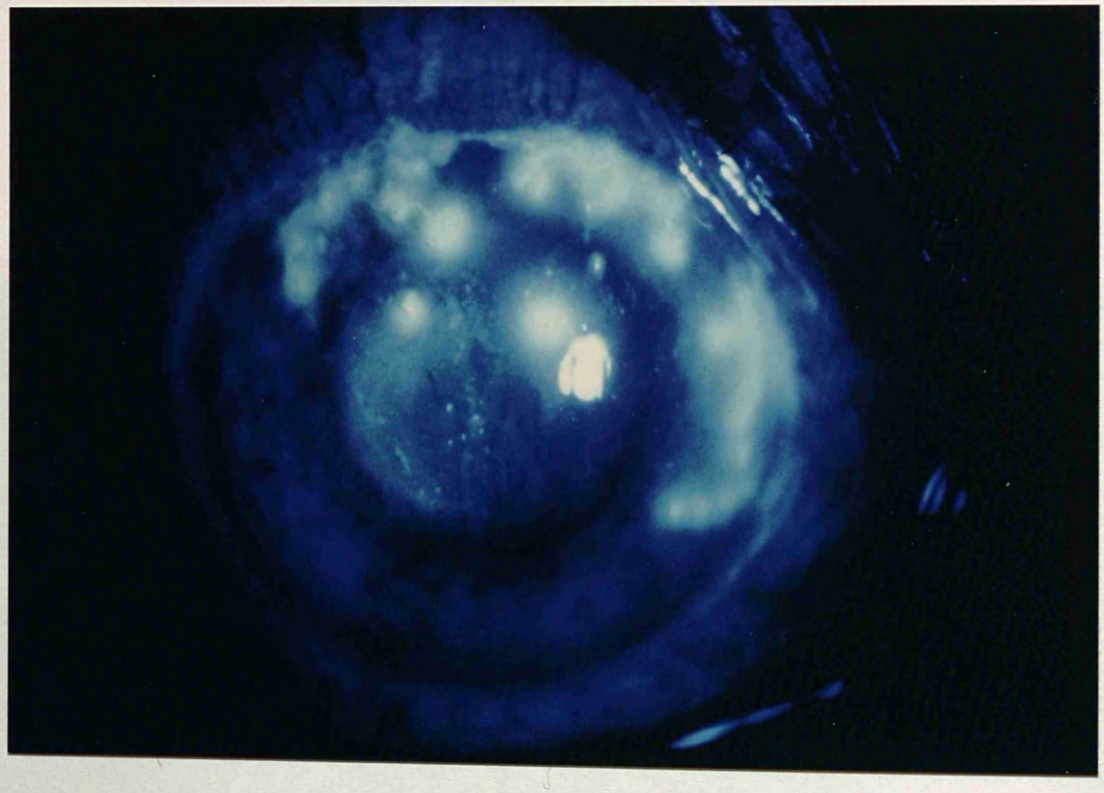


FIGURE 1

A child with a primary periocular HSV infection. Herpetic vesicles are present around the eyelids. Primary HSV infections are diagnosed on the basis of a rising titre of antibodies to HSV.

FIGURE 2

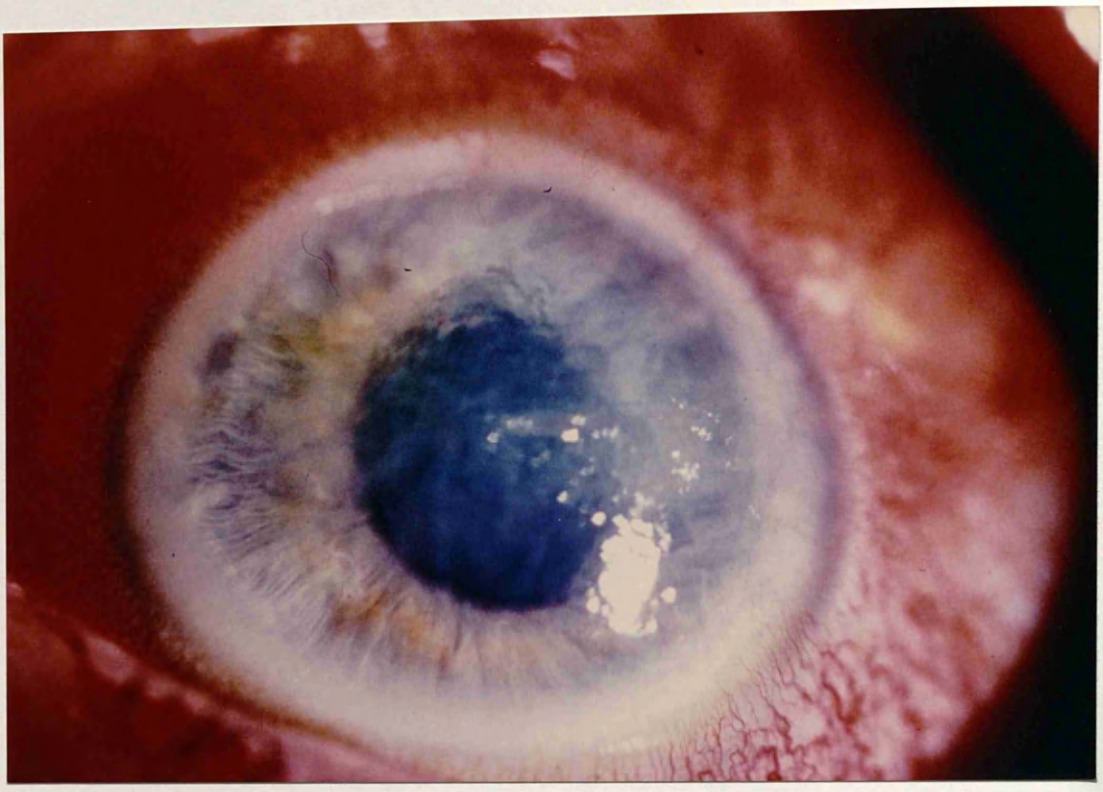
A dendritic ulcer caused by viral replication within the epithelium. The ulcer, caused by loss of epithelial cells, stains green with fluoroscein under a cobalt blue light.

HSV. Clinically it is impossible to make an absolute distinction between primary and recrudescent disease, but the signs of primary disease may be more widespread due to the absence of protection afforded by antibodies against HSV. When the anterior segment of the eye is involved, a follicular conjunctivitis occurs, often associated with pre-auricular lymphadenopathy. Herpetic vesicles may be present around the eyelids (fig. 1) and multiple dendritic ulcers representing sites of viral replication may be present within the conjunctival and corneal epithelium. Healing usually occurs within 7-10 days. HSV-1 and -2 can cause a primary infection. An epidemiological study in man has shown that both HSV types have been separately isolated from individual ocular infections. HSV-2 was present in 2% of patients where virus was isolated, and two of the three HSV-2 cases reported, had severe clinical disease (Neumann-Haefelin et al., 1978). Animal studies using different strains of HSV-1 and -2 show wide inter and intratypic strain variation in the severity of induced primary disease (Stevens and Cook 1971, Oh and Stevens 1973 and Wander et al., 1980).

(ii) Pathology of primary disease. During a primary pseudorabies virus infection, virus replication occurs at the inoculation sites before entry into the nerve endings (Field and Hill, 1975). HSV may replicate in corneal epithelium, stroma or endothelium depending upon the severity of disease. However since most patients have no history of a primary ocular HSV infection, and a subclinical pharyngitis is generally regarded as the site of primary infection (Buddingh et al., 1953), an

alternative route for spread to and from the eye is required. Tullo et al. (1982a and b) proposed that spread of HSV to neurones not supplying the site of primary infection occurs via the "back door" route, within the time span of the primary infection. Following HSV inoculation to either mouse lip or cornea, the spread of HSV was traced from the inoculation site to the mandibular or ophthalmic divisions respectively of the trigeminal ganglion. HSV then spread to the brain stem (the CNS), and from there back to all divisions of the trigeminal ganglion. In other words HSV infection can occur in non ophthalmic neurones following ocular herpes infections and HSV infections can occur in ophthalmic neurones following non ocular infections. Goodpasture and Teague (1923) observed that rabbits injected in mid flank with HSV developed a band-like ipsi lateral lesion akin to the lesion of VZV in humans. Simmons and Nash (1984) suggested that this zosteriform spread of HSV during a primary infection might be used as a model of recrudescence, because clinically normal skin became infected with HSV via nerve endings.

Animal studies, which are to an extent artificial in view of the high inoculum, showed that following intrastromal inoculation, HSV particles were seen within the nuclei of epithelial cells and keratocytes after 2 hours. Polymorphonuclear cells and lymphocytes were seen at the limbus within 7 hours post infection. By day 7 there were areas of neovascularization with polymorphonuclear cells, plasma cells, macrophages and lymphocytes in the surrounding stroma. By day 35 inflammatory cells were no longer present in the stroma,



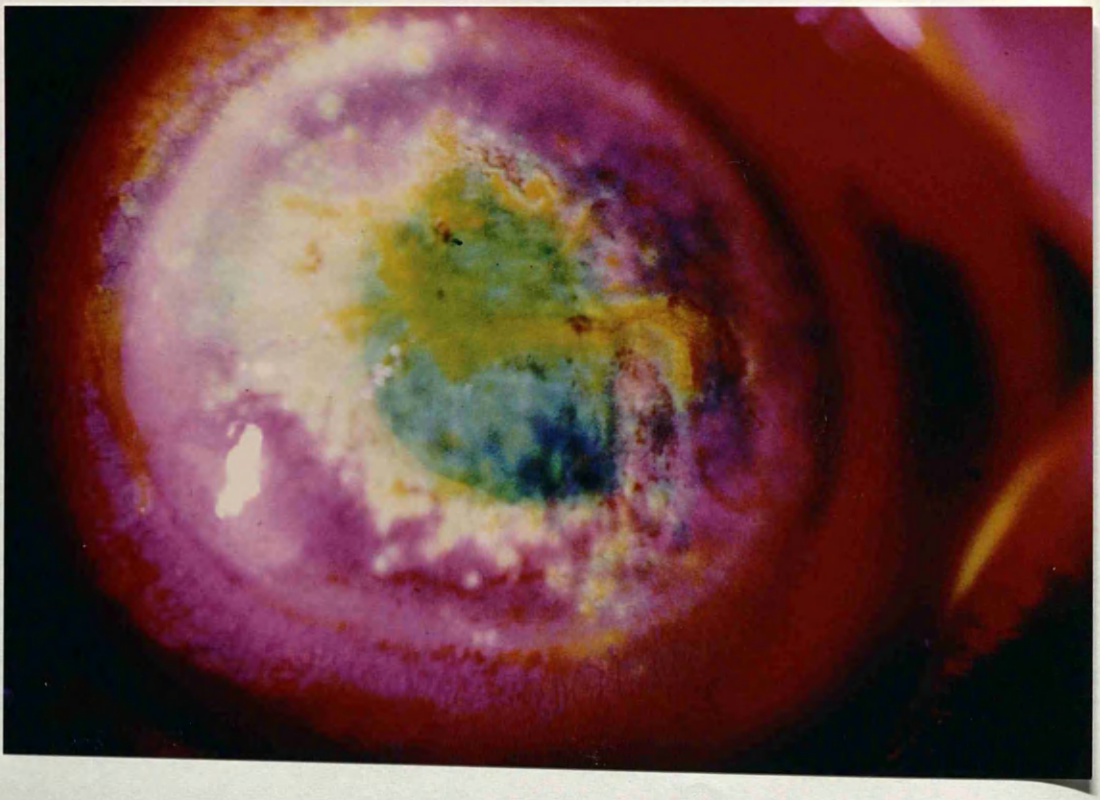


FIGURE 3

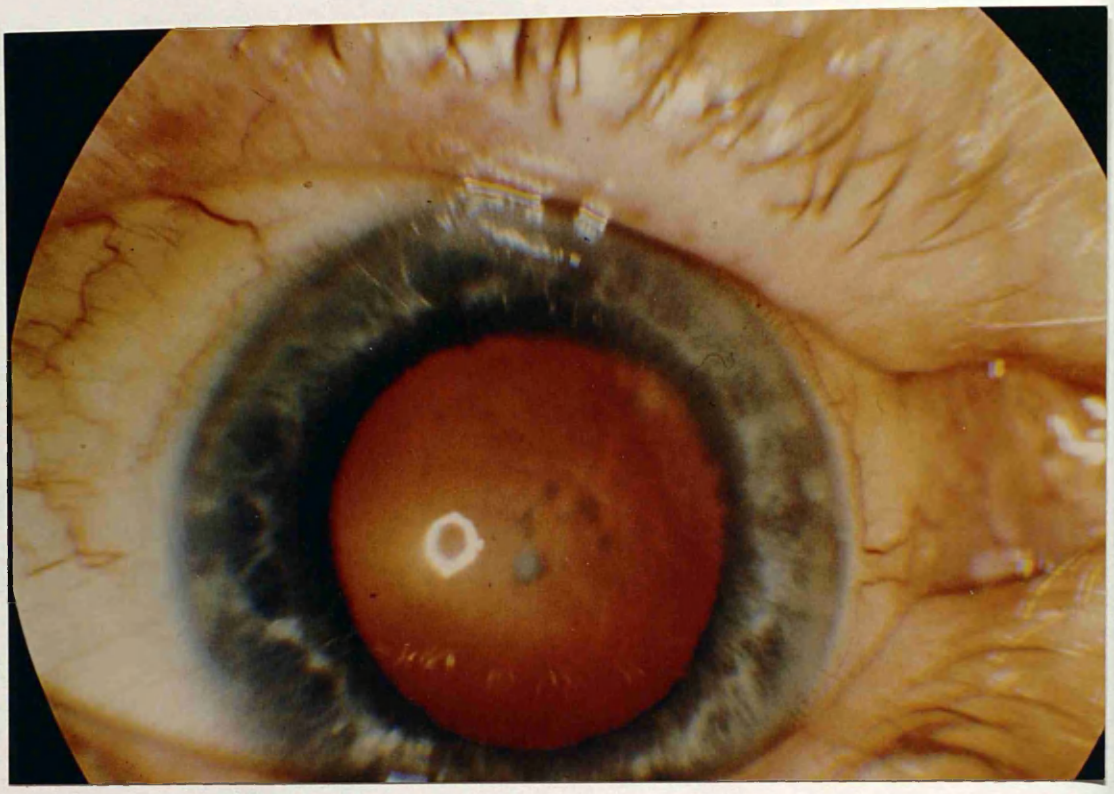
(a) Epithelial disease with deeper stromal involvement. Scarring of the cornea is a likely consequence of this recrudescence.

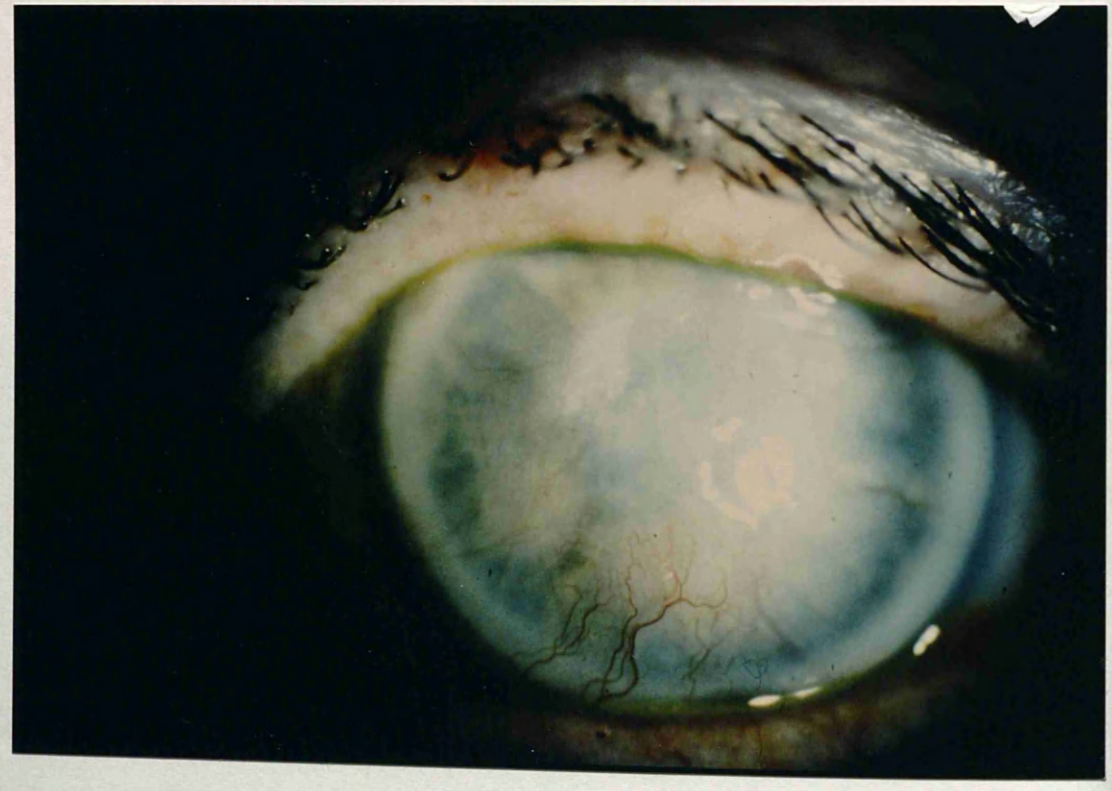
(b) The same cornea stained with fluoroscein (green) and rose bengel (pink). The right edge of the ulcer stains with rose bengel. This sign is said to identify virus within cells.

FIGURE 4

(a) Disciform keratitis. The central cornea is hazy - HSV replication within the endothelial cells affects cell function and leads to oedema in the overlying stroma.

(b) End stage HSV keratitis. The cornea is vascularized and opaque. Iris detail is only visible peripherally.





although a mild cellular infiltrate persisted (Metcalf and Reichert 1979). Histological studies of the primary disease in man do not exist.

b (i) Recrudescent disease. After the primary infection HSV establishes a latent infection in the dorsal root ganglion. The spectrum of ocular disease resulting from subsequent reactivation within the dorsal root ganglion includes; (i) shedding of HSV in the absence of clinical disease; (ii) epithelial disease - a dendritic ulcer (fig. 2); (iii) epithelial disease with associated stromal disease - stromal keratitis (fig. 3); (iv) and stromal and endothelial disease possibly associated with uveitis in the absence of epithelial disease - disciform keratitis (fig. 4). HSV can be isolated from all stages of recrudescent disease, although an anterior chamber tap is required to remove aqueous humour for culture in cases of disciform keratitis with an associated uveitis (Patterson et al., 1968).

The precise pathogenesis of herpetic stromal disease and herpetic disciform keratitis has been a source of controversy for many years. Many studies have been designed to determine whether the in vivo response is due to viral replication or to an immune response.

(ii) Pathology of recrudescent disease

The typical light microscopy findings were described by Hogan et al. (1964). Briefly, in dendritic ulcers, the white cell response was non-specific, but giant cells were found frequently, and intranuclear inclusion bodies were seen rarely. Stromal keratitis with associated epithelial disease often resulted in; loss of epithelial

tissue and the underlying Bowman's membrane; stromal necrosis; oedema and inflammatory cell infiltrate. Stromal necrosis could extend to Descemet's membrane. Perforation of the globe occurred in two of the ninety-nine specimens studied by Hogan et al. (1964). In disciform keratitis necrotic zones occurred in the stroma within lymphocytes and polymorphonuclear cells present. Endothelial oedema and degeneration caused endothelial cell loss and the denuded areas were sometimes replaced by a coagulated film containing fibrin and inflammatory cells.

Electron microscopy studies on human corneal discs removed in the course of treatment for herpetic keratitis revealed that HSV was present in the stroma of 5 of 19 patients at the time of graft. Four of the discs had associated epithelial defects and the epithelium was intact in the fifth (Dawson et al., 1968a and b). Additional case reports have also identified virus particles in a clinically quiescent failed corneal graft (Collin and Abelson, 1976), and in a clinically active cornea but HSV culture negative at the time of surgery (Meyers-Elliott et al., 1980a).

Iatrogenic effects on recrudescent disease

The evolution of human herpetic disease is subject to modification by medical intervention in two main areas; (i) suppression of the damaging effects of the immune response to HSV and the cornea by the anti inflammatory effect of steroids: and (ii) reduction and elimination of virus from the cornea by anti viral chemotherapy.

In animal studies the beneficial effects of steroids in

suppressing inflammation with the consequent reduction in corneal scarring and visual impairment were counterbalanced by an extended period when virus could be isolated from infected eyes. (Kimura et al., 1961; Takahashi et al., 1971; Easty et al., 1985). However steroids did not increase the growth of HSV (Cooper et al., 1978). Histopathologically, the epithelium and stroma were more widely involved in infected rabbits treated with steroids (Kimura et al, 1961).

Specific antiviral agents that inhibit the viral DNA polymerase enzyme have been developed over the past 25 years. They include; iododeoxyuridine (Kaufman, 1962); adenine arabinoside (Kaufman et al., 1970); trifluorothymidine (Wellings et al., 1972) and acycloguanosine (Schaeffer et al., 1978). All these agents are able to inhibit HSV DNA replication and thus abort primary and recrudescent herpetic infections. Field et al. (1979) showed that acycloguanosine was incapable of eradicating latent HSV infections in mice.

The role of the immune system in herpes infections

The interaction between the mammalian immune system and herpes simplex virus during primary infection and recrudescent disease is complex. It is not within the remit of this thesis to provide a detailed review, however an appreciation of the respective roles of natural resistence, the humoral system and cell mediated immunity is essential for an understanding of the disease process in man. These three systems act separately and in concert to control microbiological infections.

a Natural resistance (i) macrophages. These cells are

derived from the bone marrow and form part of the reticulo-endothelial system. They are widely distributed throughout the body. Macrophages are scavenging cells capable of phagocytosing virus, and are among the first cells encountering an invading pathogen.

(ii) natural killer cells are also derived from bone marrow stem cell percursors. The natural killer (NK) cells cause lysis of virus infected cells and require no prior sensitization to be effective. HSV infections enhance the NK cell activity (Enger et al., 1981; Armerding et al., 1981).

(iii) interferon. The interferon proteins are produced by leucocytes -alpha IFN; fibroblasts -beta IFN; and cells of the immune system -gamma IFN. They function by converting uninfected cells at risk of viral infection into resistant cells. IFN may act indirectly by inhibiting virus replication; by augmenting the efficiency of NK cells; or by activating macrophages. Seid et al. (1986) showed that macrophage activation was linked to the presence of gamma interferon released by T-cells. IFN may influence its own production by a positive feedback mechanism.

The natural resistance mechanisms play an important role in restricting virus replication very early in the infection before the humoral and cell mediated systems are primed (reviewed by Lopez, 1985).

b The humoral system. Primary herpes simplex virus infections are followed by a rise in the level of neutralizing antibodies to HSV, both in man (Buddingh et al., 1953) and laboratory animals (Darville and Blyth, 1982). Neutralizing antibodies are produced by the B

cells of the immune system. Following a herpes virus infection the level of neutralizing antibodies tends to remain constant even in the presence of recrudescent disease (Darville and Blyth, 1982). Openshaw et al. (1979), showed that neutralizing antibody was able to reduce the virus titre of primary infections in vitro and in vivo, but not eliminate acute ganglionic infections. Neutralizing antibody permits cell to cell spread of virus, but inhibits extracellular spread (Notkins, 1974). In a small experimental series using B-cell suppressed mice, Kapoor et al. (1982) demonstrated that these mice had a more florid primary infection in peripheral tissue and in the dorsal root ganglia, compared to normal mice. A higher incidence of latent infection was also noted in the B cell suppressed group.

More recent studies by Simmons and Nash (1984, 1985) using the zosteriform spread model have postulated a role for neutralizing antibody in recrudescent disease. Although zosteriform spread is not strictly recrudescence, it is similar in that centrifugal spread of virus from the ganglion is involved. Intravenously administered antibody was able to prevent zosteriform spread of HSV when given up to 60 hrs. post inoculation. After 60 hrs. HSV was present intracellularly and the effect of neutralizing antibody was negated. High levels of neutralizing antibody were required for this effect, at least five times higher than that normally found in infected mice. In view of the high antibody levels required for protection, the humoral immune system is unlikely to have a significant influence on recrudescent disease.

c Cell mediated immunity. Cell mediated immunity is effected by thymus derived lymphocytes, T cells. A range of T cells exist with different inter-related functions. T cells play roles in the elimination of virus following primary infections and in the control of recrudescent disease. Nude mice were only able to survive a primary HSV infection following transfer of immune T cells, despite previous transfer of neutralizing antibodies (Kapoor et al., 1982).

The cytotoxic T (Tc) cell response is induced by live virus (Rouse et al., 1983). The Tc lymphocytes are detectable in draining lymph nodes within 4 days of primary infection. Levels peak around day 6/7 and decline thereafter, being undetectable by day 14 (Nash et al., 1980a). The Tc lymphocyte acts against glycoproteins B, C, D and E (Eberle et al., 1981; Carter et al. 1982) the response against gC is type specific (Eberle et al, 1981). Delayed hypersensitivity T cells (T-DH) are induced by live or UV inactivated virus introduced sub-cutaneously or intra dermally. The T-DH cells are detectable in draining lymph nodes within 4 days, activity again peaks around day 6-7, and declines by day 12 (Nash et al., 1980b). T helper lymphocytes (Th cells) augment the function of herpes primed B cells causing a non-specific rise in anti herpes antibodies (Leung et al., 1984). The supressor T cell population (Ts cells) contain two cell populations affecting the delayed hypersensitivity response (a), cells blocking the activation of the delayed hypersensitivity response and (b) cells acting on the established delayed hypersensitivity response (Nash et al., 1981; Schrier et

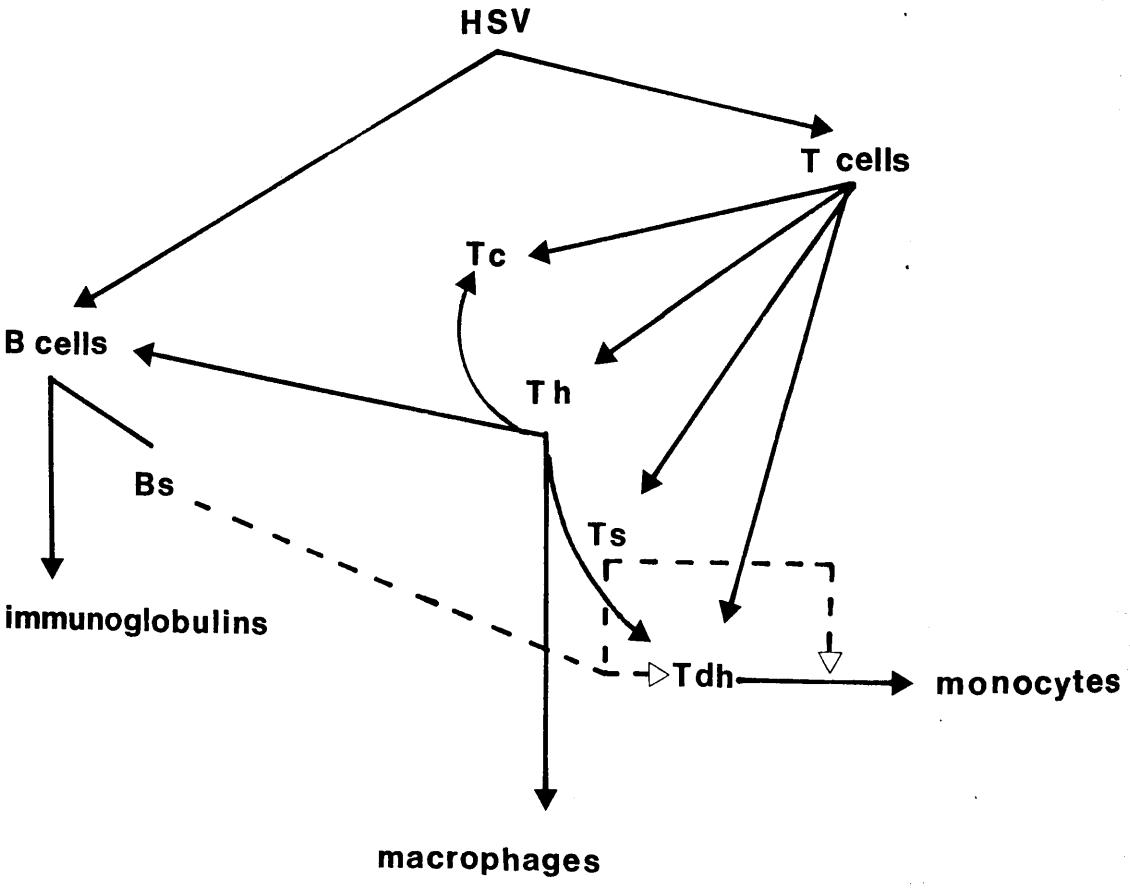

HSV
T cells
Tc
B cells
Th
Bs
Ts
immunoglobulins
Tdh
monocytes
macrophages

FIGURE 5

Immunological responses to HSV in mice from Nash *et al.* (1985).

⟶	+ve stimulation
- - - ▷	-ve stimulation
c	cytotoxic
h	helper
s	suppressor
dh	delayed hypersensitivity

al., 1983). The induction of suppressor T cells gives rise to cell populations present throughout life. This finding is unusual for suppressor cells and may be related to continued presence of antigen following recrudescent disease (Nash et al., 1985).

A further population of B cell suppressors exist, whose effect is to dampen the delayed hypersensitivity response. The exact mode of action is unclear. The memory of cell mediated immunity means that detectable levels of Tc and T-DH cells are present within 2 days of reinfection. The Ts response has been already discussed. The T cells thus act as watchmen for the immune system. The state of the immune system in mice against HSV is summarised from the review of Nash et al. (1985) (fig. 5).

d Immunopathology in herpetic stromal keratitis. The immunological response to HSV is generally protective for the body, however in the localised context of the cornea an immunological response is often detrimental to the function of the cornea. Cellular invasion leads to disruption of the normal corneal anatomy causing scarring and an inability to transmit formed images to the retina.

T cells play an important role in the pathogenesis of stromal herpetic keratitis. Experimental studies performed on euthymic and athymic mice given adoptive transfers of HSV immune and non immune spleen cells showed that the T cell lymphocyte was essential in immuno-competent mice in the development of herpetic stromal keratitis (Russell et al., 1984). Oakes et al. (1984) showed in experimental mice given whole body irradiation to depress the immune system, that T cells with the Lyt-1$^+$ surface antigen phenotype were the

dominant mediator of antiviral protection in immune spleen cell reconstituted mice. Reconstituted mice and immunosuppressed mice had similar titres of virus present within the eye and trigeminal ganglion, in the 8 days immediately post inoculation. However at 10 days post inoculation all immunosuppressed mice were dead due to an encephalitis, whereas tissues from immune reconstituted mice were free of virus. Studies using monoclonal antibodies against specific T cell markers identified the Lyt-1^+ T cell phenotype as the effector cell. Further studies showed that the Lyt-1^+ T cells caused enhanced antibody synthesis in HSV-1 infected mice.

These studies suggest that the T cells mediating delayed type hypersensitivity and/or antibody synthesis (Lyt-1^+) and not the cytotoxic T cells (Lyt-23^+) are the mediators of the immune response causing virus clearance.

e HLA antigen typing and herpetic disease. At present there is no consensus of opinion on the importance of HLA antigen type and its association with herpetic disease. In a prospective study of 260 HSV-1 herpes labialis patients compared with 606 controls, the frequency of the HLA-A_1 antigen was increased (Russell and Schlaut 1977). Zimmerman et al. (1977), in a study of 46 patients with herpetic keratitis found that the HLA-B_5 type was significantly more common. Meyers-Elliott et al. (1980b) examined 48 patients with herpetic keratitis and found a slight increase in the frequency of HLA-DRw3 whereas Jensen et al. (1984) found no significant association between HLA type and stromal and epithelial disease in a study of 50 patients. The disparity in observed frequency of HLA types can be simply explained by the

limited number of patients used in each study. A larger study with adequate controls is required to determine the importance of HLA type and its relationship to herpetic disease.

f The protective effect of HSV infection. The immune system in animals and man fails to protect against reactivation of latent virus and subsequent recrudescent disease. Asbell et al. (1984) showed that the virus strain within an individual where virus was recovered from successive infections of eyes, eyelids and mouth and then characterised by restriction enzyme analysis, was identical. A group of ten individuals was studied. This suggests that recrudescent disease is caused by reactivation of the same latent virus strain within the trigeminal ganglion. Lonsdale et al. (1979) characterised isolates recovered from superior cervical ganglia, trigeminal ganglia and the vagus ganglia in seventeen post mortem cases. Where virus was isolated from more than one site within an individual, it was found to be identical by restriction enzyme and polypeptide profiles.

Case reports have demonstrated that on occasion two HSV strains can be isolated from the same individual, even from the same site in recurrent genital infections (Buchman et al., 1979), or two different HSV-1 strains in the cerebrospinal fluid coincident with HSV-1 and HSV-2 isolates in the rectum (Heller et al., 1982), or in a larger series of eight patients with encephalitis; five patients had identical HSV strains from the brain and oral sites, but three had different strains at each site (Whitley et al., 1982). No patients had simultaneous

type 1 and type 2 infections.

These simultaneous isolations of HSV may represent shedding of the "original" latent strain plus a superinfecting strain. Centifanto-Fitzgerald et al. (1982), showed that a primary HSV infection with a relatively non pathogenic strain led to a decreased mortality and milder disease when rabbits were subsequently challenged with a virulent HSV strain. Only the HSV strain from the primary infection was recovered from ganglia, despite the presence of a prolonged superinfection in some animals. This implies that some protective effect is given against subsequent challenge with a different virus strain.

The Molecular Biology of HSV

The aim of this section is to provide background information, and not to provide a comprehensive review of a vast literature.

The Genome. HSV-1 and -2 possess a double stranded DNA genome with a molecular weight of around 100 x 10^6, and G+C base compositions of 68.3% and 69%, respectively (Kieff et al., 1971; Wilkie, 1973; McGeoch, personal communication).

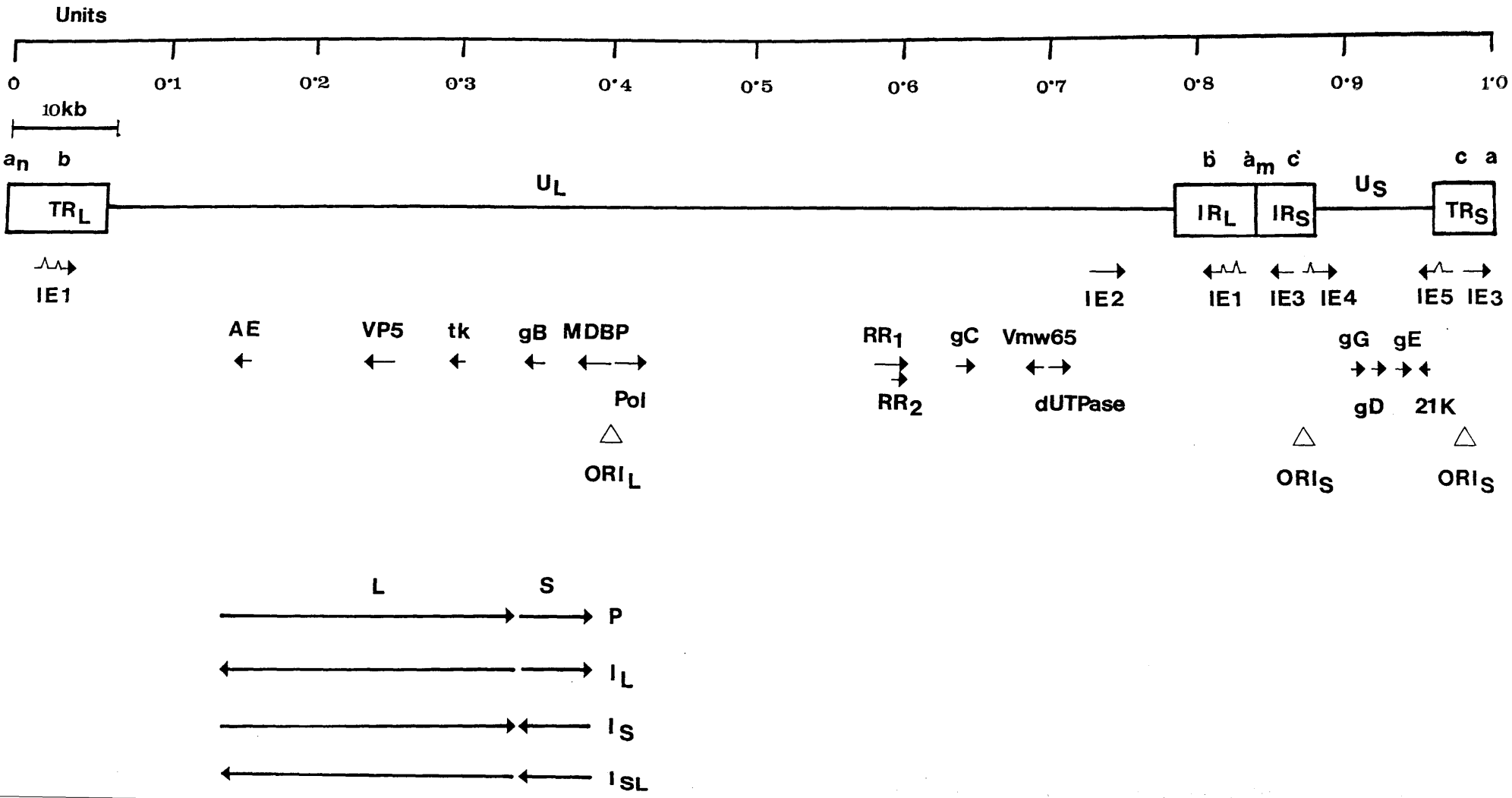
The HSV genome consists of a long component of DNA composed of largely unique sequences, designated (U_L), and flanked by inverted repeat sequences, designated terminal repeat long (TR_L) and internal repeat long (IR_L) (terminally redundant sequences of 0.5 x 10_6 were also reported, designated 'a' sequences (Sheldrick and Berthelot, 1974, and Grafstrom et al., 1975); and a short component of DNA with unique sequences, (U_S), flanked by terminal repeats, a short

terminal repeat (TR_S) and a short internal repeat (IR_S). The terminal and internal long repeats can be written ab and b'a' where the "a" sequence is the redundant sequence. Similarly the terminal and internal short repeats can be written ca and a'c'.

Sheldrick and Berthelot (1974), calculated that recombination events between the long and short terminal and internal repeats could generate four isomers of the HSV genome differing in orientation of the unique sequences, see fig. 6. Subsequent analysis of restriction endonuclease cleavage fragments confirmed their calculation and showed that the four isomers of HSV DNA were normally present at equal frequency (Clements et al., 1976; Wilkie et al., 1977).

Restriction Endonuclease Maps. Restriction endonuclease enzymes recognise specific DNA base palindromes within the HSV genome. The enzymes cleave the genome, producing DNA fragments of consistent but varying size (the number depending on the number of sites for the enzyme) for any individual HSV strain. The DNA fragments can be separated by size electrophoretically on agarose gel, giving a fingerprint characteristic to each HSV strain. The use of multiple restriction endonuclease enzymes in isolation, or a combination of two enzymes further characterises the fingerprint. Lonsdale et al. (1979) used restriction enzymes to analyse HSV isolated from cadavers, and showed that the strains isolated from each cadaver were distinct, though identical (when isolated from different sites) within the same cadaver.

HSV genomes have been selected and isolated lacking XbaI restriction endonuclease sites (Brown et al., 1984;


Units
0
0·1
0·2
0·3
0·4
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0·6
0·7
0·8
0·9
1·0
10kb
a_n b
TR_L
U_L
b' a_m c'
IR_L
IR_S
U_S
c a
TR_S
IE1
IE2
IE1 IE3 IE4
IE5 IE3
AE
VP5
tk
gB
MDBP
Pol
ORI_L
RR_1
RR_2
gC
Vmw65
dUTPase
gG
gE
gD
21K
ORI_S
ORI_S
L
S
P
I_L
I_S
I_{SL}

FIGURE 6

Organisation of the HSV-1 genome.

The HSV-1 genome is shown to scale in prototype (P) orientation. The long unique (U_L) and short unique (U_S) regions (single lines) are flanked by terminal (TR) and internal (IR) repeats (double lines). The a sequences (a) at the termini of the L and S components are represented by heavy vertical lines, and may be duplicated "n" or "m" times (a' = inverse orientation). The remainder of the long and short repeats are referred to as "b" and "c". Below the genome are mapped (i) the IE mRNAs (spliced regions are raised); (ii) E and L transcripts which specify the best known virus-encoded proteins; and (iii) the three HSV-1 origins of DNA replication (ORI). The four possible isomers of the HSV-1 genome are depicted below, where the L and S components may be inverted (I) relative to the P orientation as indicated. Genes in U_L are numbered U_L1 to U_L56 genes in U_S are numbered U_S1 to U_S12 (McGeoch, personal communication).

Abbreviations are as follows:

IE		immediate-early
E		early
L		late
AE	U_L12	alkaline exonuclease
VP5	U_L19	major capsid protein
tk	U_L23	thymidine kinase
gB	U_L27	glycoprotein B
MDBP	U_L29	major DNA binding protein
Pol	U_L30	DNA polymerase
RR	$U_L39/40$	ribonucleotide reductase
gC	U_L44	glycoprotein C
$V_{mw}65$	U_L48	IE stimulatory protein
gG	U_S4	glycoprotein G
gD	U_S6	glycoprotein D
gE	U_S8	glycoprotein E
21K	US11	gene product
ORI_S		origin of replication (short)
ORI_L		origin of replication (long)

Harland and Brown, 1985; MacLean and Brown, 1987). The isolated mutants have been characterised to elucidate the nature of the alterations in the genomes. The isolated HSV-1 variants have been used in superinfection experiments to study the HSV genome in latency (Cook and Brown, 1987).

HSV-1 Sequence Determination. The HSV genome is a large and complex genetic system by the criteria of animal virology. Many of its 70 genes are uncharacterised by function and structure, while others were recognised originally through the existence of ts mutants or by the mapping of protein species as originating from particular regions of the genome. A complete transcript map of the genome now exists (Wagner, 1985; McGeoch et al., 1985, 1987; McGeoch and Davison 1986; Rixon and McGeoch, 1984, 1985; Perry et al., 1986; McGeoch, personal communication).

Fifty-six genes were detectable in the U_L region (McGeoch, personal communication). Twelve genes were detected in the U_S region (McGeoch et al., 1985). The genes are evidently active during lytic infection of tissue culture cells as mRNAs have been detected from most of U_S. Current knowledge of the functions of the 56 U_L and the 12 U_S encoded proteins is incomplete. The following U_S encoded proteins are known; V_{mw}IE68 is the product of the immediate early gene U_S1 (Hay and Hay, 1980); a protein kinase encoded by U_S3 has been identified by comparing the predicted protein sequence of an unknown gene with protein sequences available in data banks, using computer analysis (McGeoch and Davison, 1986); glycoprotein G, is the product of the U_S4 gene, this was determined by Frame et al. (1986), by raising antisera against synthetic oligopeptides

predicted to appear in HSV encoded proteins from sequence data; gD and gE encoded by genes U_S6 and U_S8 respectively are relatively well characterised surface glycoproteins (Spear, 1976; Bauke and Spear, 1979; Hope et al., 1982); gI was identified by Longnecker et al. (1987) and Johnson et al. (1988) mapped this glycoprotein to the U_S7 open reading frame; 21k the product of the late gene U_S11 is a protein that binds to double stranded DNA (Bayliss et al., 1975; Rixon and McGeoch, 1984; and Johnson, et al. 1986); and last of all $V_{mw}IE12$ is the product of U_S12.

As yet little is known of the nature of the other five encoded proteins. Seventy nine percent of U_S is occupied by open reading frames specifying polypeptides, and a further 16% appears as untranslated 3' and 5' mRNA (Rixon and McGeoch, 1985). After allowing for transcription initiation and termination signals 5' and 3' of transcription units only a few hundred base pairs of U_S are without an obvious function, illustrating the compact sequence utilisation of the HSV genome.

The U_L, TR_L, IR_L, IR_S, TR_S and U_S fragments have been sequenced and many genes identified. The organisation of the HSV-1 genome showing the regions coding for important proteins is shown in figure 6. The organisation and characterization of specific genes was reviewed by Wagner (1985).

The HSV lytic cycle.

Assembly of virions. HSV virions are composed of an icosahedral capsid containing 150 hexagonal and 12 pentagonal capsomers. The capsids are contained in a glycoprotein and lipid envelope. The earliest steps in the lytic cycle are adsorption of the virion to the host cell

plasma membrane, and penetration into the cell. The plasma membranes contain type specific receptor sites (Vahlne and Lycke, 1978). It is unclear whether HSV virions enter cells by fusion or by pinocytosis. Membrane fusion was shown by Manservigi et al. (1977) to be related to the virus induced glycoprotein B (gB). Further work by Sarmiento et al. (1979) showed that two HSV-1 temperature sensitive mutants with defects in the gB gene, adsorbed to cells but did not penetrate. Fusion/pinocytosis removes the virion envelope and releases the Herpes simplex virion to the cytoplasm. The virus particle migrates across the cytoplasm to the nuclear membrane where dissociation of the capsid occurs releasing HSV DNA which migrates through pores in the nuclear membrane (Hummeler et al., 1969). Virion DNA is transcribed by host cell RNA polymerase II (Costanzo et al., 1977).

Temporal control of the viral transcription programme characterises the HSV-1 lytic cycle. Three groups of HSV genes, immediate early (IE), early (E) and late (L) (Clements et al., 1979) or alpha, beta and gamma (Honess and Roizman, 1974) are recognised based on the kinetics of appearance of their gene products in the presence and absence of inhibitors of protein DNA synthesis.

Immediate early gene expression. The five immediate early polypeptides synthesised in HSV-1 infections, V_{mw}IE110, V_{mw}IE63, V_{mw}IE175, V_{mw}68 and V_{mw}IE12 are defined as those encoded by genes (IE1, IE2, IE3, IE4 and IE5 respectively) which are transcribed and translated in the absence of viral protein synthesis [For simplicity the Glasgow nomenclature will be used throughout.] (Honess and Roizman, 1974; Pereira et al., 1977; and Preston et al.,

1978). The HSV-1 genes are located as follows; IE1 and IE3 are diploid genes within the TR_L/IR_L and TR_S/IR_S segments respectively; the 5' termini of IE4 and IE5 are within TR_S/IR_S regions; and the coding regions are within U_S; the IE2 is wholly within U_L (Clements et al., 1979). Transcription of DNA occurs from both strands of DNA.

Post et al. (1981) observed that a component of the virus particle could stimulate IE gene expression. Batterson and Roizman (1983) suggested that the factor may be a tegument protein and Campbell et al. (1984) identified the virion component as the major tegument protein $V_{mw}65$. The functions of the polypeptides encoded by the immediate early genes are outlined below.

(i) V_{mw}IE110. Brown et al. (1984) suggested that the IE1 gene product may be essential for lytic growth as an HSV-1 mutant with an additional XbaI site in the region of the IE1 gene in the TR_L segment did not have an identical lesion in the IR_L. This suggested that the mutation could not be tolerated in a homozygous form. However Stow and Stow (1986) constructed a recombinant virus with a deletion in both copies of the IE1 gene. [The same deletion inactivates the E gene transcription stimulatory activity of V_{mw}IE110 in a transient expression assay (Perry et al., 1986)]. The recombinant virus is able to grow with reduced efficiency which suggests that V_{mw}IE110 is not essential for lytic growth in tissue culture. The effect of the deletion is manifest primarily at low multiplicities of infection and is overcome by increasing the virus dose (Stow and Stow, 1986). Sandri-Goldin et al. (1983) confirmed that the V_{mw}IE110 was non essential, by infecting cell lines containing an anti sense V_{mw}IE110 message which reduced the

level of V_{mw}IE110 to less than 10%. Everett (1984a) used a co-transfection system with recombinant plasmids to show that V_{mw}IE110 may be involved in the control of transcription. O'Hare and Hayward (1985a) confirmed that V_{mw}IE110 plays a role in the stimulation of early promoters and suggested that the V_{mw}IE12 may also have a role along with V_{mw}IE175.

(ii) V_{mw}IE63. Sacks et al. (1985) characterised four ts mutants with lesions in IE2. Cells infected with the ts mutants at non permissive temperatures overproduced V_{mw}IE175 and V_{mw}IE63, but not V_{mw}IE110. Functional V_{mw}IE63 was not required for the synthesis of early proteins or viral DNA synthesis at non permissive temperatures, however the expression of late genes was greatly reduced. V_{mw}IE63 thus appears to be a polypeptide essential for lytic growth of HSV.

(iii) V_{mw}IE175. This polypeptide has been shown to be essential for the initial activation and continued expression of E and L genes (Preston, 1979a; Watson and Clements, 1980). Experiments using an HSV-1 ts mutant (ts k) with a mutation in IE3 showed that the mutant overproduced immediate early polypeptides but synthesised reduced or undetectable amounts of early and late proteins at the non permissive temperature. It is thought that the effect of V_{mw}IE175 is to control viral transcription by suppressing the synthesis of IE mRNA and activating E and L genes (Preston, 1979a, b; Watson and Clements, 1980). Using cloned IE genes in transient assays the level of V_{mw}IE175 was shown to determine whether IE gene expression was stimulated or inhibited (O'Hare and Hayward, 1985b; Gelman and Silverstein, 1985).

(iv) V_{mw}IE68. Post and Roizman (1981) have shown that the IE4 gene is not essential for lytic growth in Vero cells and Hep-2 cells. Sears et al. (1985a) further analysed the deletion mutant of Post and Roizman (1981) in rat cell lines and found that plating efficiency was reduced and growth was multiplicity dependent. The HSV-1 deletion mutant was able to establish latency in mice. Sears et al. (1985a) speculate that a cellular function substitutes for V_{mw}IE68 in cells infected with the HSV-1 recombinant containing a deletion in the IE4 gene, and that this function is involved in late gene expression. The host cell factor complementation is cell dependent.

(v) V_{mw}IE12. This polypeptide is non essential for lytic growth of HSV-1 and HSV-2 in tissue culture (Longnecker and Roizman, 1986; Umene, 1986; and Brown and Harland, 1987).

Early gene expression. The early group of polypeptides is diverse containing enzymes including alkaline exonuclease, thymidine kinase, DNA polymerase, ribonucleotide reductase and deoxypyrimidine triphosphotase; the major DNA binding protein V_{mw}136 and several glycoproteins i.e. gB, gD and gE. Early gene transcription peaks around 4-6 hrs. post adsorption after the appearance of functional IE protein in the cell. There is variation in the kinetics of expression of early genes. The large sub unit of ribonucleotide reductase may be expressed under IE conditions and by some mutants within the IE3 gene that do not otherwise express E gene products (DeLuca et al., 1985). gD can be detected early in infection, however viral DNA synthesis is required for its maximal synthesis (Gibson and Spear, 1983; Johnson et al., 1986).

Continued expression of V_{mw}IE175 throughout the lytic cycle was shown to be essential for the synthesis of early and late polypeptides (Watson and Clements, 1980). Much effort has been expended recently in determining the precise requirements for transcription of E genes and their subsequent translation. Everett (1984b) showed that E genes unlike IE genes, do not have enhancers upstream of promoters, thus the integrity of the whole promoter is essential for full activation of the gene. Co-transfection experiments by Sandri-Goldin et al. (1983) examined the expression of HSV-1 E and L genes in the absence of IE functions. Transcripts of 4 genes including glycoprotein B and DNA binding protein were detected, however protein synthesis was not detectable unless V_{mw}IE175 was made available. Many of the enzymes synthesised in the early stage of the lytic cycle have a role in DNA replication.

HSV glycoproteins with the exception of gC are synthesised in the early or delayed early phase of lytic infection. HSV glycoproteins are incorporated into both nuclear and cytoplasmic membranes of infected cells (Spear et al., 1970). The total number of glycoproteins specified by HSV is unknown. The existence of four major HSV glycoproteins designated gB, gC, gD and gE has been known for some time (reviewed by Spear, 1985; Marsden, 1987).

gB, already discussed, is involved in cell membrane fusion and penetration. gC has been shown to be inessential for infectivity (Peake et al., 1982), and in HSV-1 but not HSV-2 to be a receptor for complement factor C3b (Friedmann et al., 1984). gD may also be involved in virus adsorption and penetration (Johnson et al., 1984), and gE interacts with the Fc region of immunoglobulin G.

Glycoprotein G was identified (Marsden et al., 1984) in HSV-2 and its gene identified in U_S (McGeoch et al., 1987). The HSV-1 gG was identified by immunoprecipitation (Frame et al., 1986; Richman et al., 1986) and mapped to the U_S4 open reading frame confirming the prediction of McGeoch et al. (1985). Glycoprotein G-2 has been shown to be non essential in HSV-2 using deletion variants (Harland and Brown, 1988). McGeoch et al. (1985) predicted that the two open reading frames U_S5 and U_S7 may code for transmembrane glycoproteins. Longnecker et al. (1987) identified glycoprotein I and Johnson et al. (1988) ascertained that the glycoprotein was encoded by the U_S7 open reading frame. Glycoprotein H was characterised and mapped to U_L in HSV-1 (Buckmaster et al., 1984).

Centifanto-Fitzgerald et al. (1982) analysed the glycoproteins synthesised by different virus strains with defined disease patterns in rabbit corneas, i.e. epithelial or stromal disease. Strains secreting larger amounts of glycoprotein induced stromal disease rather than epithelial disease. Smeralgia et al. (1982) were able to reproduce clinical disease patterns by injecting purified glycoproteins from these strains into the corneas of immune rabbits. Thus glycoproteins clearly have a role in generating an immune response in vivo.

Late gene expression. The kinetics of late gene expression are not strict and genes can be subdivided into delayed early genes (DE), early late genes (EL), and true late genes (L). The efficient expression of L genes is dependent on viral DNA replication (Jones and Roizman, 1979), unlike the earlier DE or EL genes whose expression is reduced but not abolished in the absence of DNA synthesis

(Silver and Roizman, 1985). Synthesis of HSV-1 DNA begins around 2 hours post absorption and peaks about 8 hours. Late gene products can be detected 2-3 hours post absorption and peak by 10-16 hours; (Wilkie, 1973; Rixon et al., 1983). Johnson et al. (1986) studied a well characterised DE gene (U_S6) and its product gD; an uncharacterised L gene (U_S11) and its product, a 21k protein, preliminarily classified as a late protein; and the effects of phosphonoacetic acid, a viral DNA replication inhibitor upon them. Their results, using sensitive assays demonstrated that very low levels of the U_S11 gene product were detectable under conditions designed to eliminate DNA replication. They speculate that late genes may be transcribed early in infection, but that true late promoters may require a high copy number achieved through DNA replication, before abundant expression. Johnson et al. (1986) propose that the definition of late gene be regarded as functional.

Other late proteins synthesised by HSV include groups of DNA binding proteins, the major capsid protein UP5 (Costa et al., 1984), an assembly protein necessary for the encapsidation of DNA (Preston et al., 1983), and $V_{mw}65$ (tegument protein) (Campbell et al., 1984) which stimulates immediate early transcription.

The expression of late genes is influenced by $V_{mw}IE175$ (Preston, 1979a; Watson and Clements, 1980), by $V_{mw}IE63$ (Sacks et al., 1985), and by $V_{mw}IE110$ (O'Hare and Hayward, 1985a, b), which have been discussed previously.

DNA Replication and Encapsidation. The HSV genome contains three origins of DNA replication, one within the U_L region of the genome, map coordinates 0.407-0.429 (Spaete and

Frenkel, 1982), and two within the reiterated sequences flanking U_S close to map coordinates 0.86 (IR_S) and 0.96 (TR_S) (Stow, 1982). Deletion studies localised the cis acting sequences within TR_S and IR_S, necessary for function as an origin of replication, to a 100 base pair fragment with a 45 base pair palindromic sequence (Stow and McMonagle, 1983). DNA replication starts around 1-3 hrs. post infection and peaks at around 7-9 hrs. post infection (Rixon et al., 1983). Viral DNA is thought to circularize and then form head to tail concatemers by a rolling circle mechanism (Jacob et al., 1979) (see fig. 7). DNA replication generates four equimolar populations differing with respect to the orientation of the U_L and U_S components, see fig. 6. It is thought that at least two of the genome populations must be involved in replication. If the genome template is arranged in a circular form and both isomers replicate, see fig. 7, then an intramolecular recombination event between L/S junctions plus loss of an L/S junction would generate all four genomic isomers (Jongeneel and Bachenheimer, 1981).

The "a" sequence has been shown to be the cis-acting site responsible for inversion of the genome, and this function has been further localised to "direct repeat" sequences within the "a" sequence (Chou and Roizman, 1985). The "a" sequence also contains signals required for the encapsidation of viral DNA (Stow et al., 1983). Further experiments by Varmuza and Smiley (1985) have localised the cleavage/packaging signals to a 250 base pair sub fragment within the terminal repeat. Only genomes of approximately unit length virion DNA complete the maturation process (Vlazny et al., 1982). Preston et al. (1983) used a ts

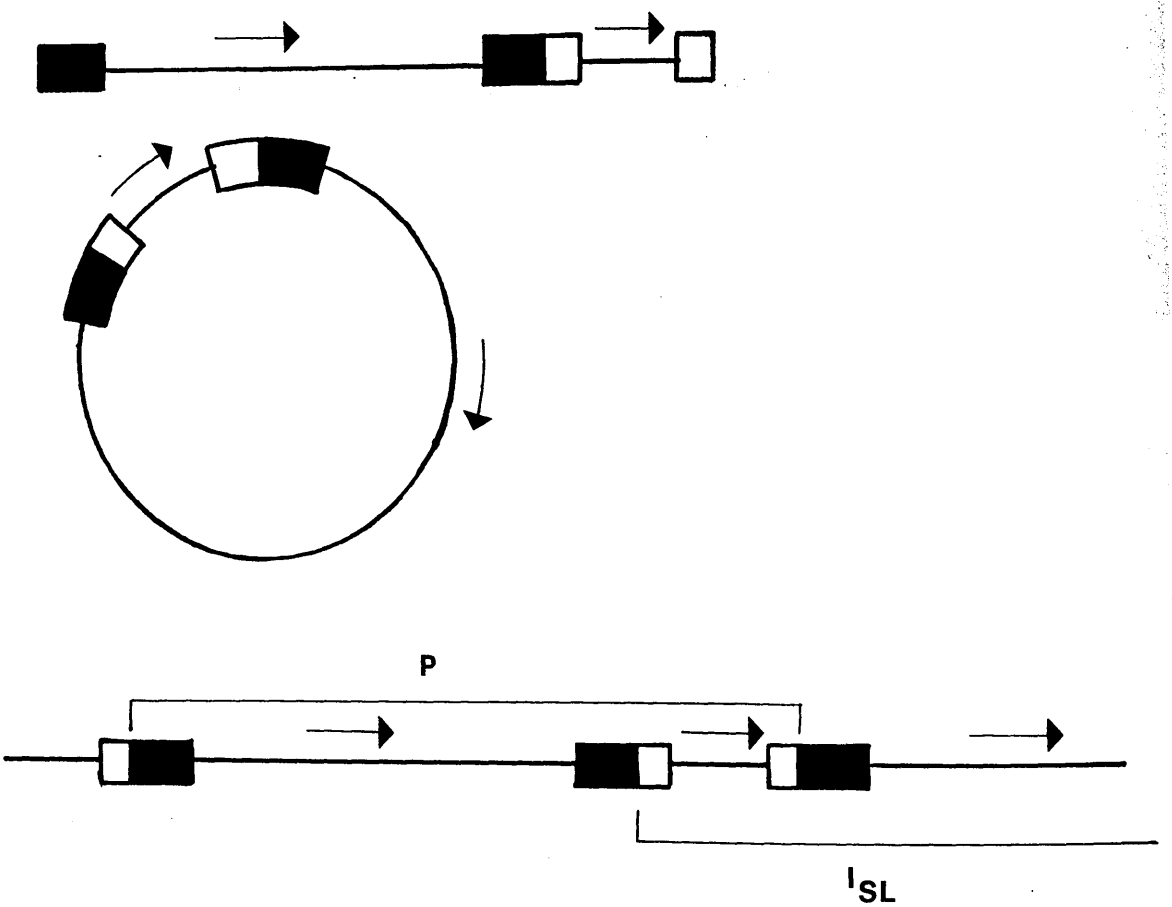

P
I_{SL}

FIGURE 7

DNA replication

Top HSV DNA in prototype orientation

Middle Circular structure of template DNA after end to ligation of L and S termini.

Bottom Head to tail concatamers generated by rolling circle replication.
Unit length genomes cleaved at appropriately oriented L-S junctions, giving P and I_{SL} isomers.
From Varmuza and Smiley (1985).

mutant to show that a late polyptpeide p40 was essential for packaging synthesised DNA into nucleocapsids. Addison *et al*. (1984) isolated *ts* mutants with lesions close to but outside the gB gene. The *ts* 1204 mutant was unable to penetrate the cell membrane, but when that defect was surmounted, and virion assembly continued, nucleocapsids were unable to package DNA. A second mutant *ts* 1208 penetrated cells normally at non permissive temperatures but, was unable to package DNA. A defect in a gene encoding a structural polypeptide was proposed.

HSV Latency in vivo

a The nervous system.

Latent herpes virus infections differ from lytic infections in that the cell and virus are able to co-exist for prolonged intervals, in the absence of reactivation. The precise mechanism of this virus/cell interaction remains unclear. Goodpasture and Teague (1923) were the first to link primary ocular HSV infections with simultaneous productive viral infections in the trigeminal ganglia of rabbits, and Goodpasture (1929) suggested that the trigeminal ganglion might be a source of latent HSV infections. Almost fifty years elapsed before Stevens and Cook (1971) showed that HSV could be recovered from the spinal ganglia of mice 3 weeks to 4 months post footpad inoculation. Virus was detected after explanted tissue had been maintained in organ culture. This work was followed by the isolation of HSV from:- the trigeminal ganglia of rabbits after ocular inoculation (Nesburn *et al*., 1972); the trigeminal ganglia of rabbits with a history of spontaneous ocular shedding of virus

(Stevens et al., 1972); human trigeminal ganglia (Bastian et al., 1972; Baringer and Swoveland., 1973); human sacral ganglia (Baringer, 1974); and animal and human autonomic ganglia (Price et al., 1975; Warren et al., 1978). Approximately 50% of the population have antibodies to HSV (Smith et al., 1967). The technique of viral superinfection was used by Brown et al. (1979), to detect non inducible genomes in explanted human trigeminal ganglia. This method raised the proportion of individuals with detectable HSV after organ culture from 53% to 80%, a figure closer to that of Buddingh's serological study.

Pseudorabies virus is transported to the sensory ganglia via neuronal axons following a productive infection at the site of inoculation (Field and Hill, 1975). Kristensson et al. (1974) calculated that HSV moves along the peripheral nerves towards the cell body at a rate of 2-8mm/hr. This calculation is similar to the figure for the transport of proteins by retrograde axonal flow (Kristensson, 1978). The route of inoculation is also important in the generation of a latent infection. Blyth et al. (1984) showed that inoculation of virus through scarified skin gave a higher incidence of latency than subcutaneous inoculation of higher titres of virus. Presumably more cutaneous nerve endings were exposed to virus. The "back-door" route described by Tullo et al. (1982a) is also a means by which latent infections can establish in the cell bodies of neurones remote from the inoculation site. Despite the spread of HSV through the central nervous system (CNS) and back to the dorsal root ganglion, the incidence of HSV latency within the CNS is

reduced compared with latency in the peripheral nervous system. Tullo et al. (1982a), reported no latent HSV within the CNS after culturing the brain stems of infected mice. However Cook and Stevens (1976), were able to isolate latent HSV from the CNS of 18% of animals, compared to an isolation rate of 82% from spinal ganglia, and Cabrera et al., (1980) produced similar figures of 5% positive from CNS culture and 95% from trigeminal ganglia following organ culture. The latter group were able to detect HSV DNA sequences in the CNS of 30% of animals following DNA-DNA hybridization techniques. It is thus conceivable that the detected DNA did not represent the entire genome, and the authors concede that the sequences detected may be an unbalanced representation of portions of the genome. Stroop et al. (1984) used in situ hybridization techniques with whole virus probes, and found that HSV-1 DNA and RNA sequences were detectable at very high frequency in the CNS of mice, up until 10 days post inoculation. Thereafter HSV-1 RNA rather than DNA was detected during the latent stages of infection up to 150 days post inoculation, suggesting that only limited transcription occurs during latency.

Experiments by McLennan and Darby (1980) used ts mutants to identify the neurone as the site of viral latency both in vivo and in vitro. Reactivation of latent virus was carried out at the permissive and non permissive temperature, viral antigens were identified by immunofluorescence and cells were identified histologically. Attempts to quantify the number of neurones harbouring latent HSV, using enzymatically dispersed dorsal root ganglia, suggested that about 1% of

neurones may harbour latent HSV (Walz et al., 1976). Experiments by Kennedy et al. (1983) using a double label immunofluorescent technique showed that the proportion of neurones harbouring latent HSV may be as low as 0.4%. The in situ hybridization techniques of Stroop et al. (1984) only located HSV-1 RNA in neurones during latent infections. The experiments quoted above were performed in different in vivo and in vitro systems.

b Peripheral tissue.

Evidence is accumulating that cells other than neurones may be capable of maintaining latent viral infections. Hoyt and Billinson (1976) reported four cases where ipsi lateral HSV labial infections occurred following blow-out fractures of the orbit. All patients had dense cutaneous infra orbital anaesthesia, and in two cases the infra orbital nerve had been severed. These findings are not compatible with the theory of centrifugal spread from the trigeminal ganglion and raise the possibility of skin latency. The guinea pig model of Scriba (1977), showed that virus was often isolated from the footpad in the absence of virus in the spinal ganglia. Hill et al. (1980) showed that 8% of mice had virus present in clinically normal skin. This virus was detected after organ culture. Approximately 3.5% of mice demonstrate spontaneous HSV disease recurrences. Hill et al. (1980) hypothesised that the 8% might be shedding virus asymptomatically from the ganglion and that a proportion of them would develop clinical disease. Shimeld et al. (1982), isolated HSV from two of three corneal discs maintained in organ culture and these findings were expanded by Tullo et al. (1985). Cook et al., (1987)

succeeded in isolating HSV-1 from rabbit corneas, however isolation of HSV-1 occurred only in rabbits infected with the HSV-1 strain McKrae, or HSV-1/HSV-2 recombinants whose genome structure is the same as that of the McKrae strain except for the sequence between 0.33 and 0.56 map units which originates from HG52 (Batra, 1987). Openshaw (1983) demonstrated that HSV could be isolated from the posterior segment of mouse eyes after organ culture, and suggested that the retina, of neural origin, may be the site of latent infection. Al-Saadi et al. (1983) also demonstrated that HSV can be isolated from the mouse footpad after organ culture. This work has been extended and the findings confirmed following neurectomy and acycloguanosine treatment (Clements and Subak-Sharpe, 1988).

c Maintenance of the latent state.

Much of the analysis of the latent state has been expressed in terms of the lytic cycle. In other words, analysis of the products of the lytic cycle detectable in tissue where HSV is presumed to be latent. A potential hazard to clarification, is the current functional definition of HSV latency, where a latent infection is only acknowledged after virus has been released from organ culture.

Temperature sensitive mutants of HSV-1 were used by Lofgren et al. (1977), and Watson et al. (1980) in an attempt to define essential viral functions necessary for the latent state. The initial report suggested that DNA replication was not essential for latency within the CNS or peripheral nervous system. Five ts mutants of HSV-1 were studied. The second report examined an additional

eight ts mutants. The results demonstrated that the mutant tsK was unable to induce a latent viral infection. This mutant has a lesion in the IE3 gene encoding V_{mw}IE175 (Preston, 1979a). Other ts mutants which do not synthesise DNA after production of immediate early proteins were latency negative. Work by Batra (1987) suggests that the ability to establish a latent infection is dependent upon the inoculating titre. Studies by Al-Saadi et al. (1983), with HSV-2 ts mutants confirmed that DNA replication was not essential for a latent viral infection either in the dorsal root ganglia of mice or in the mouse footpad. The V_{mw}IE175 polypeptide has been shown in the ganglionic neurones of latently infected rabbits using a monospecific antibody against the polypeptide. Specific antibodies against early and late proteins were negative (Green et al., 1981). However these results have not been confirmed by other workers. Stevens et al. (1987) used RNA probes covering most of the genome to make an initial assessment of HSV-1 gene expression in latently infected mice. The results showed that only genes from the terminal repeats were expressed. Finer probes covering the IE genes 1-3 were used and only genetic information from the IE1 gene was detected. Further analysis revealed that "antisense" RNA from the strand complementary to that encoding IE1 messenger RNA was the major species detected. The positive signal was localised to the nuclei of neurones. Much effort has been expended in determining whether the thymidine kinase tk gene is essential for latency. Recent experiments using HSV-1 recombinants with, immediate early, early and late regulated tk genes showed that levels of tk activity

did not correlate directly with an ability to establish latent infections (Sears et al., 1985b). Tenser and Edris (1986) questioned the validity of the tk assay used in the above experiments. They suggested that the tested HSV-1 recombinants had an intermediate level of tk activity and therefore that the relationship between the tk gene and latency remained unclear.

d The genome in the latent state.

Puga et al. (1978) were able to demonstrate viral DNA in both acute and latent infections but mRNA only in acute infections. This implies that genome expression is severely repressed or possibly blocked during latent infection. Brown et al. (1979) showed that non inducible viral genomes in human neural tissue were detectable following superinfection. Galloway et al. (1979) used DNA-RNA in situ hybridization to detect the presence of HSV mRNA in human paravertebral ganglia. Neural tissue from two of seven individuals had mRNA present, detected by non specific HSV-2 DNA probes. A further study by Galloway et al. (1982), used more specific probes to locate the areas of transcription. Transcripts of the left hand 30% of the genome were present in all positive specimens, with other regions of the long unique less represented, and no detectable transcripts from the short unique region of the genome.

Early work by Fraser et al. (1981) on HSV recovered from human brains suggested that the viral genome may be present in a non integrated and linear form. However subsequent work by Rock and Fraser (1983, 1985), on experimental mice suggested that the viral DNA is likely to exist in the latent state either in a concatameric or

episomal form. Puga et al. (1984) used viral DNA probes from the "ends" of the genome to probe DNA extracts of latently infected ganglia in mice. Their results suggested that the terminal repeats of the genome may undergo a rearrangement or perhaps an integration into cellular DNA. These results are at variance with the work of Rock and Fraser (1985), who detected 2M joint fragments but no ends, in latently infected mice. Efstathiou et al. (1986) confirmed the results of Rock and Fraser (1983, 1985) and showed in experimental mice and man that DNA loses its "ends" during latency and is thus arranged in concatameric or episomal form. In addition they found that the "endless" DNA was present in all four isomeric forms.

It is clear that the precise nature of the genome in latency is not yet understood. Most of the evidence presented tends to favour a static state for the virus where genome expression is at least partially suppressed. There is little evidence to support the alternative explanation of a dynamic state where the virus undergoes a chronic low grade (persistent) infection within latently infected tissue.

HSV Latency in vitro.

Many attempts have been made to create an in vitro system which mimics HSV latency, because of the difficulties in isolation and analysis of small quantities of viral DNA within neural tissue in vivo. By definition in vitro systems are artificial and considerable caution is required before extrapolating in vitro results to the in vivo situation.

The first *in vitro* latency system was described by O'Neill *et al*. (1972). Human embryo lung cells were pre-treated with cytosine arabinoside (ara-C) for 24 hrs. prior to infection with HSV-1, and then maintained in ara-C for up to 22 days. Ara-C did not eliminate HSV-1 from the cells, and a delay of 6-11 days post drug removal occurred before HSV-1 became detectable again.

A variety of different cell types have been used to create a more typical environment for latency. These include neuroblastoma cells (Vahlne and Lycke, 1978); transformed neural cells (Adler *et al*., 1978); rat foetal neurones (Wigdahl *et al*., 1984a); human foetal neurones (Wigdahl *et al*., 1984b); and rabbit trigeminal ganglion neurones (Dunkel *et al*., 1984).

The most common method of inducing a latent infection is to pre-treat cells with an antiviral agent prior to infection, followed by maintenance in the presence of the drug for a pre-determined time. Ara-C was used most commonly until 1982, thereafter bromovinyl deoxyuridine was used in combination with interferon (Wigdahl *et al*., 1982a, 1983, 1984a and b). Acycloguanosine has also been used (Dunkel *et al*., 1984).

Supraoptimal temperatures (greater than 37^{o}C) have been shown to greatly reduce the synthesis of viral DNA (Crouch and Rapp, 1972), and temperature elevation to 42^{o}C totally blocks the synthesis of viral DNA within cells (Marcon and Kucera, 1976). Using temperature elevation to 39.5^{o}C, O'Neill (1977) was able to extend the period of "latency" at the supraoptimal temperature for up to 120 days. Notarianni (1986) and Russell and Preston (1986) were the first to use temperature elevation as the sole means of

inducing a latent infection in human foetal lung cells.

Dunkel et al. (1984) showed that in rabbit trigeminal ganglion cells V_{mw}IE175 was detectable by immunofluorescence during the acute and desuppressed infections. Wigdahl et al. (1984a) analysed the HSV genomes present in the "latent" state by blot hybridization and found that both ends and joints were present in equimolar quantities. He suggested that the genome was thus present in unit lengths in a non integrated, non concatameric form. In this respect in vitro results are at variance with the in vivo results of Rock and Fraser (1983, 1985), and Efstathiou et al. (1986). Youssoufian et al. (1982) studied methylation of HSV DNA during a "latent" state induced by mitogens in a lymphoid cell line. Their results suggested that DNA in the "latent" state was heavily methylated, and that no methylated copies of DNA were detectable during a productive infection. Dressler et al. (1987) studied the methylation pattern of HSV-1 DNA in the CNS of latently infected mice. No extensive methylation of latent HSV-1 DNA was found in vivo. Russell et al. (1987a) have attempted to define HSV genes required for latency in vitro. By using ts mutants, insertion mutants and deletion mutants with lesions in the V_{mw}IE110 and V_{mw}IE175 polypeptides, they showed that the lack of either protein was not enough to prevent a latent infection occurring in vitro. Again the in vitro results showing that V_{mw}IE110 is non essential for the establishment of latency, contrast with the report of Stevens et al. (1987) who found that latently infected mouse ganglia contain a transcript complementary to V_{mw}IE110 mRNA in vivo. Further studies using in situ hybridisation have detected latency related RNAs in the trigeminal ganglia of rabbits,

mice and man (Rock et al. 1987a; Spivak and Fraser, 1987; and Croen, 1987). More than one latency related transcript was detected and the genes for the latency related RNAs mapped in the region of the IE1 gene which encodes the Vmw110. The latent HSV-1 RNAs were found to be transcribed in the direction opposite to that of IE1 mRNA. The "anti sense" RNA transcripts were detectable in lytically infected cells but at a level approximately one-tenth that in latently infected cells. The precise role of the latency related RNAs has yet to be elucidated. The anti sense RNA may regulate the IE1 gene or it may encode a regulatory protein capable of suppressing the HSV-1 lytic cycle or interacting with cellular transcription factors.

Reactivation of HSV from latency in vivo.

The concept of trigger factors stimulating latent virus in man is widely accepted. A variety of diverse factors are associated with recurrent herpetic disease including stress, trauma, fever, menstruation, and excessive sunlight. Two other conditions have been implicated in the past; firstly immunosuppression (already discussed), which may lead to an increased duration of herpetic disease through disruption of the immune surveillance mechanisms, although immunosuppression per se is unlikely to influence the process of reactivation; and secondly the immunosuppressant corticosteroid drugs which have been shown to have no effect on the frequency of HSV shedding in rabbit eyes (Kibrick et al., 1971) or mouse skin (Blyth et al., 1976).

Two theories have evolved to take account of the known observations regarding HSV reactivation. The "ganglion trigger" hypothesis, suggests that after reactivation within

the dorsal root ganglion the virus travels down axons to the peripheral site and there infects cells. This hypothesis is concordant with the observations of spontaneous virus shedding from HSV infected rabbit eyes in the absence of disease (Nesburn, 1967; Laibson and Kibrick, 1969; Gerdes and Smith, 1983; and Berman and Hill, 1985); spontaneous virus shedding in mice (Tullo et al., 1982a); and the induced shedding of virus following reactivation in the absence of disease (Laibson and Kibrick, 1967; Nesburn et al., 1977; Kwon et al., 1981). Wildy et al. (1982) make a distinction between virus shedding in the absence of disease - recurrence - , and virus shedding with clinical disease - recrudescence - .

Hill and Blyth (1976), formulated the alternative "skin trigger" theory. In this hypothesis, virus reactivates periodically from the dorsal root ganglion and travels to the peripheral site. There under most circumstances, the virus is eliminated by the host's immune system. However if a breach in the peripheral site is present, perhaps induced by trauma, then conditions are more favourable for virus replication. Experiments by Shimomura et al. (1985) demonstrated that epinephrine iontophoresis to the corneas of latently infected rabbits induced reactivation of HSV from the trigeminal ganglia within 24 hrs. Iontophoresis is a technique for transporting ions or charged molecules into tissues via an electrical current. Erlanger (1954) suggested that the process could be used to administer drugs to the eye in a clinical setting, but more recently iontophoresis has been used in research (Hill et al., 1978; Kwon et al., 1979). Iontophoresis of epinephrine has been shown to induce ocular shedding of HSV at high frequency

(Kwon et al., 1982). The effect of epinephrine on ganglionic reactivation is now known to be due to the laevo(-) stereo isomer of epinephrine (Hill et al., 1985). The precise role of epinephrine in triggering ganglionic reactivation in vivo remains unclear.

Further studies support the "skin trigger" theory. Mild trauma to the skin of latently infected mice was followed by a clinical recurrence of herpetic disease (erythema within 2-5 days) in approximately 30% of mice. HSV was isolated from the skin of 73% of mice with recurrent disease (Hill et al., 1978). In addition studies by Harbour et al. (1983) showed that infectious HSV was detectable within the dorsal root ganglia supplying the traumatized dermatome between days 1-5 post skin trauma. Further work by Hill et al. (1983) showed that recurrence of HSV requires an intact nerve supply. This suggests that peripheral stimuli induce reactivation within the ganglion, and that virus travels down the axon to the peripheral site which may or may not still be a favourable site for virus replication. The "ganglion trigger" hypothesis and the "skin trigger" theory are not mutually exclusive.

Cook et al. (1986) took a "latency negative" HSV-1 ts mutant tsI whose defect is expressed late in the lytic infection (Gerdes et al., 1979) (The late expression of the defect is paradoxical as current evidence suggests that only immediate early gene functions are required for latency), and repaired the genetic defect. The resultant virus was found to possess an additional ts lesion limiting its reactivation from latency, the defect was correlated with a viral replication function specific for neurones. Some caution is necessary in interpreting this result, as what

the authors see as evidence of reactivation is a productive infection in explanted tissue i.e. replication, and it is possible that reactivation per se is not involved.

Host and virus factors affecting reactivation.

In man the probability of a recurrent ocular HSV infection was estimated to be around 50% within 2 years of the initial ocular HSV infection (Carroll et al., 1967).

More recent work showed that 32% of 108 patients had one or more recurrences between two and fifteen years of the initial ocular infection (Wishart et al., 1987). Both papers presume with little justification that the initial ocular HSV infection is a primary infection, when it is more probable that the initial ocular infection represented a recurrence following an asymptomatic primary infection. Host factors including the immune system and HLA typing have been discussed. Harbour et al. (1981) showed that strain differences among groups of inbred and outbred mice were demonstrable when induced herpetic disease was considered. Intratypic variation also occurred within a mouse strain.

The effect of virus strain on spontaneous ocular shedding of HSV in rabbits was documented by Gerdes and Smith (1983). Virus strains were regarded as having high or low frequency of recurrence. Inter and intratypic strain variation was present. The biological properties of latency and recurrence were not linked. Hill et al. (1987) also demonstrated HSV-1 intratypic variation with induced viral shedding after epinephrine iontophoresis. Five viral strains gave no ocular shedding in rabbits after epinephrine iontophoresis. Co-cultivation of the trigeminal and superior cervical ganglia revealed that all ten strains

tested were able to maintain a latent infection in neural tissue.

Reactivation of HSV from latency in vitro.

Reactivation of HSV from the "latent" state in vitro can be induced by removing factors suppressing virus replication, i.e. removing the anti-viral agent (O'Neill et al., 1972; Dunkel et al., 1984); or restitution of incubation temperature to 37°C (O'Neill, 1977; Wigdahl et al., 1981, 1982a, 1983, 1984). In addition, viral superinfection using; HCMV (Colberg-Poley et al., 1979, 1981); HSV-2 ts mutants (Wigdahl et al., 1982b); an intertypic HSV strain (Nilhesen et al., 1985); and HSV-1 variants lacking XbaI sites (Cook and Brown, 1987), have all been used to reactivate "latent" virus in vitro.

An attempt has been made to determine viral genes necessary for reactivation in vitro using ts, deletion and insertion mutants in the IE1 and IE3 genes (Russell et al., 1987a). HSV-2 could be reactivated by mutants which failed to synthesise active V_{mw}IE175 but not by a mutant that failed to synthesise V_{mw}IE110. Caution is again required before drawing firm conclusions, as in vivo the dl 1403 mutant of HSV-1 which fails to produce V_{mw}IE110 (Stow and Stow, 1986), establishes latent infections after footpad inoculation, and virus can be recovered after co-cultivation of ganglia (G.B. Clements and N.D. Stow, unpublished results). This suggests that a cellular factor replaces the transactivating effect of V_{mw}IE110.

Growth and characterization of corneal cells.

The cornea consists of three distinct cell types;

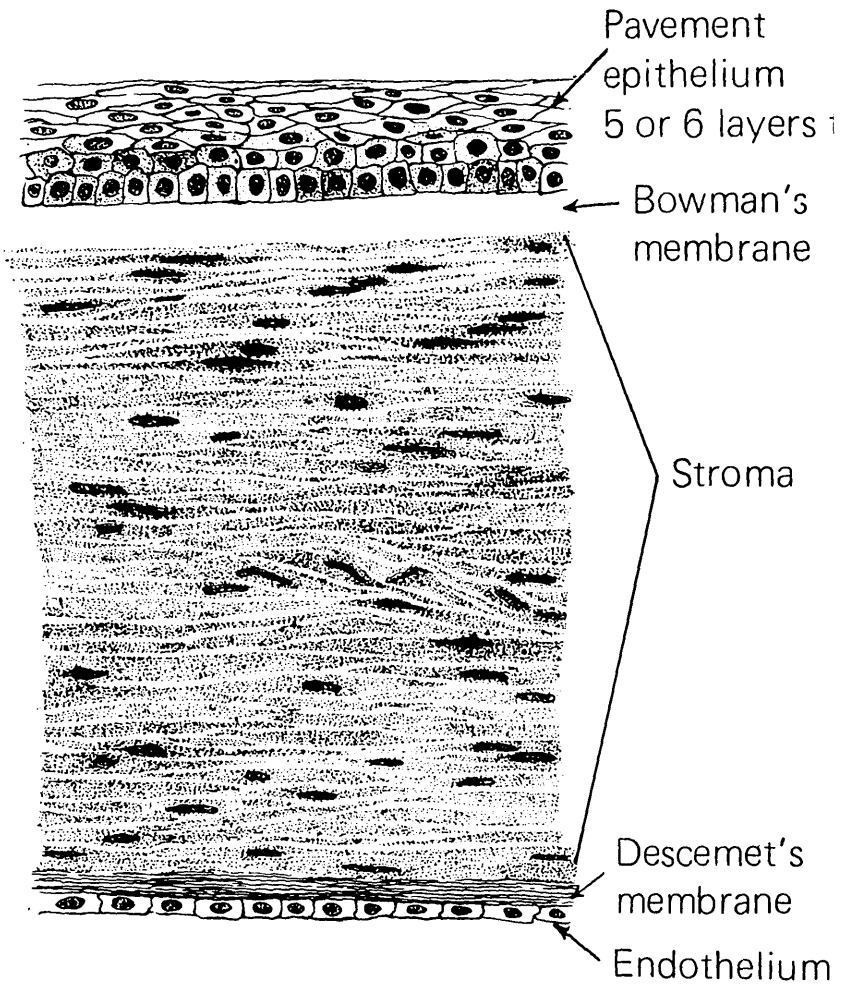
epithelial cells which make up the superficial layer of the cornea, approximately seven cells deep, and divide throughout life (Davson, 1980); keratocytes, which are contained within the connective tissue stroma of the cornea occupying about 90% of the cornea, and tend to be stable *in vivo* although retaining the potential for replication (Maumenee and Kornblueth, 1949); and endothelial cells which are found in a monolayer on Descemet's membrane in contact with the aqueous humour, and do not divide *in vivo* (Davson, 1980) (see fig. 8).

Microdissection techniques for the preparation and growth of rabbit corneal epithelial cells, keratocytes and endothelial cells were first described by Stocker *et al.* (1958) (see fig. 9). Baum *et al.* (1979) applied this method to the human cornea to obtain cultured endothelial cells. An alternative enzymatic method for preparing epithelial cell cultures was described by Gipson and Grill (1982). Once pure cell lines have been established serial passage of corneal cells quickly yields a population of homologous cells suitable for experimentation. However a potential for inadvertent cellular contamination exists with the method of Stocker *et al.* (1958), as microdissection techniques are used to separate the epithelial layers from the underlying stroma. In addition cellular morphology can alter with serial passage or in response to the environment in which cells are grown. Epithelial cells or endothelial cells grown in a fibronectin matrix will assume the morphology of fibroblasts (Hsieh and Baum, 1985). Clearly the identity and purity of cellular preparations should be established before starting experimental studies.

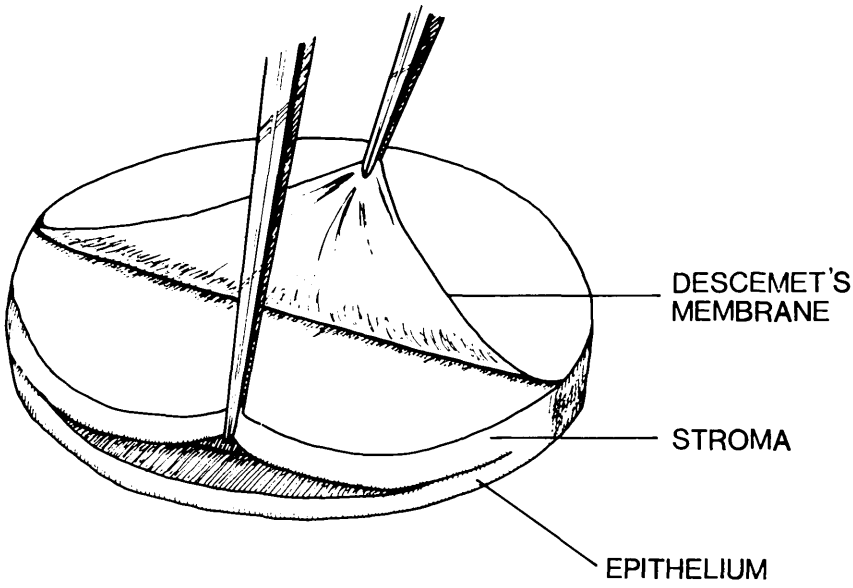
Ultrastructural differences have been described *in vivo*

FIGURE 8

Cellular layers of the cornea.


Pavement
epithelium
5 or 6 layers
Bowman's
membrane
Stroma
Descemet's
membrane
Endothelium

SEPARATION OF CORNEA INTO THREE LAYERS



(From Stocker : Am. J. Opth. 1958)

FIGURE 9

Microdissection of the cornea for preparing cell cultures.

by Jakus (1961) and Hogan et al. (1971). The three corneal cell types display recognisable morphological features such as size, shape, nuclear and cytoplasmic organelles. Cell-type-specific markers also unambiguously distinguish epithelial cells from keratocytes and endothelial cells. Keratin is found only in cells of epithelial origin (Lazarides, 1980), whereas keratocytes and endothelial cells synthesise a fibronectin matrix (Yamada and Olden, 1978; Gospodarowicz et al., 1979). Indirect immunofluorescence techniques using antibodies against keratin and fibronectin can thus identify epithelial cells, and keratocytes and endothelial cells respectively.

Cultured bovine endothelial cells have been used to assess the cytotoxic effects of pharmacological agents in vitro (Jay and Macdonald, 1978). Cell cultures of rabbit cornea have been used to study virus/cell interactions by Oh (1976), and Carter et al. (1985) who observed the lytic cycle of HSV-1 in the three distinct corneal cell types.

Cell culture permits study of virus/cell interactions in the absence of immunological mediation.

Cellular stress proteins.

By definition the endpoint of the HSV lytic cycle is cell death. In the course of this destructive virus/cell interaction host cell directed macromolecular synthesis is switched off early in the lytic cycle (Sydiskis and Roizman, 1966; Fenwick and Walker, 1978). In a latent HSV infection, the productive infection is aborted or directed to another course. The mechanism for the alteration in outcome is unknown, but clearly virus/cell interaction is

occurring. It is possible that cellular stress proteins may play a role in the induction and maintenance of the latent state, but at present the evidence for this remains circumstantial.

Heat shock has been shown to induce stress genes, manifest as chromosome puffs in Drosophila (Ritossa, 1962). Tissieres et al. (1974) showed that the appearance of the chromosome puffs was associated with the synthesis of six novel proteins detectable by SDS-PAGE. A similar system has been described in eukaryotic cells namely chick embryo fibroblasts (Hightower and Smith, 1978; Kelley and Schlessinger, 1978). Currie and White (1981) demonstrated that a cellular stress protein of molecular weight 70,000, was synthesised in vitro in rat tissue and in vivo after rats were subjected to hyperthermia. The in vivo synthesis implies that stress proteins have a physiological role. A variety of other toxic stimuli including disulfiram, sulphydryl groups, anoxia, and viruses have been shown to induce cellular stress proteins (Levinson et al., 1978, 1980; Ashburner, 1982; Nevins, 1982; Collins and Hightower, 1982 and Khandjian and Türler, 1983). The exact function of the cellular stress response is unknown, but thought to be protective for the cell.

Notarianni and Preston (1982) were the first to demonstrate that cellular stress proteins were induced in chick embryo fibroblasts by HSV using the HSV-1 tsk mutant, which has a lesion in the IE3 gene encoding the V_{mw}IE175 and overproduces the other immediate early polypeptides. The HSV-1 parental virus did not induce detectable synthesis of cellular stress proteins. Cellular stress proteins were visualised by SDS-PAGE. Further work by Russell et al.

(1987b) has shown that it is overproduction of the abnormal V_{mw}IE175 that induces the cellular stress response *in vitro*. Truncated V_{mw}IE175 or increased amounts of normal V_{mw}IE175 do not induce a stress response. LaThangue *et al*. (1984) reported that a cellular stress protein, identified by a monoclonal antibody T156, accumulated during the HSV-2 (strain 333) lytic cycle in human embryo fibroblasts and BHK21C13 cells. The cellular stress protein was also present during the cellular growth phase, in heat shocked cells and in cells treated with disulfiram. It is thus apparent that not all host cell genes are shut off following HSV infection. Kennedy *et al*. (1985) showed that the T156 monoclonal antibody identified a stress protein which accumulated during acute HSV infection, in cultured human neural cells.

Recent work by Patel *et al*. (1986) used the monoclonal antibody T156 to screen a lambda gtII library and for polysome immunoprecipitation, to isolate cDNA clones derived from the corresponding cellular stress protein gene. Their results showed that HSV is capable of transcriptionally inducing a cellular gene. The precise mechanism by which a few cellular genes are activated in the face of generalised repression of cellular function is as yet unknown. The results of Russell *et al*. (1987) who used *tsk* which overproduces abnormal V_{mw}IE175 to induce a stress response differ from work of LaThangue *et al*. (1984), Kennedy *et al*. (1985) and Patel *et al*. (1986) who used wild type HSV to induce a stress response. This suggests that more than one mechanism may induce the cellular stress response.

CHAPTER 2

IN VIVO EXPERIMENTS

Isolation of herpes simplex virus from the cornea in patients with chronic stromal keratitis.

MATERIALS

Corneas. Twelve consecutive corneal graft specimens from clinically diagnosed herpetic keratitis patients were collected within 24 hours of surgery and transported to the laboratory in balanced salt solution or EFC_{10} (v/v) (Eagle's medium 10% foetal calf serum). Corneas were received from throughout Scotland between April 1985 and June 1986. The co-operation of clinical colleagues in providing suitable tissue is gratefully acknowledged.

METHODS

PATHOLOGY

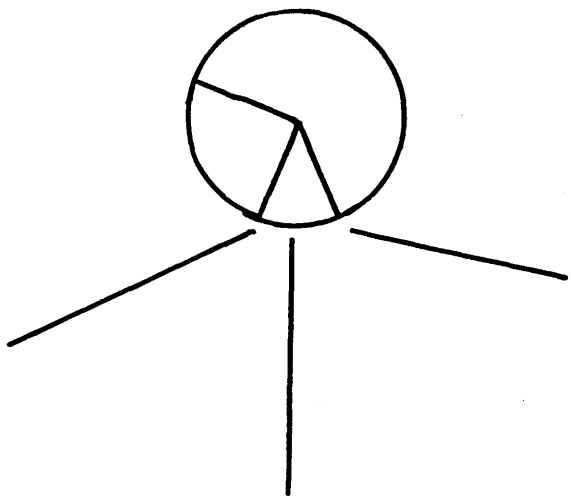
Corneas were processed as follows (Figure 10). One quarter was fixed in 2% gluteraldehyde for several hours and then processed routinely for paraffin histology to assess pathological changes by light microscopy. Sections were treated with Haemotoxylin-Eosin, Periodic Acid Schiff (P.A.S.) and the Masson-Trichrome stains. In two of the specimens (1 and 2) where inflammation was conspicuous, additional stains for bacteria and fungi (Gram-Jenson, Gram-Weigert, Methenamine-Silver) were applied (Work done by Dr. K.U. Loeffler and Professor W.R. Lee, Tennent Institute of Ophthalmology, Glasgow).

ELECTRON MICROSCOPY

Tissue for electron microscopy was fixed in

EXPERIMENTAL PROTOCOL

Corneal disc 12



Organ culture 12

Light microscopy

pre culture 12
post culture 8

Electron microscopy

pre culture 8
post culture 2

R.E. analysis
of HSV 2

FIGURE 10

Experimental protocol for the isolation of HSV from the cornea in patients with chronic stromal keratitis.

Numbers to the right denote the number of corneas undergoing that procedure. Corneal discs were divided into eight wedge shaped segments.

Pre-culture - Two eighths of 12 corneas were examined by light microscopy and not subjected to organ culture

- One eighth of 8 corneas was examined by electron microscopy and not subjected to organ culture

Post-culture - Two to five eighths of 8 corneas were examined by light microscopy after organ culture

- Five and six eighths of 2 corneas respectively were examined by electron microscopy after organ culture

RE - Restriction enzyme

summarises the clinical data.

Cases 1-5 had clinically active disease at the time of corneal grafting, as manifest by a stromal defect down to Descemet's membrane. Cases 6-12 were regarded as clinically inactive immediately pre graft. The history of herpetic disease ranged from 6 months to 30 years. All but two patients, 5 and 8, had required topical steroid preparations to the affected eye to suppress an inflammatory reaction in the course of their stromal keratitis. All patients had been treated with topical anti-viral agents at some time. These included idoxuridine, vidarabine, trifluorothymidine and acycloguanosine. Corneas were collected and placed in organ culture within 24 hours of the corneal graft. Only four specimens 3, 4, 8 and 10 were prepared outwith the first ten hours.

PATHOLOGY

A pie shaped segment (one quarter of each corneal disc) containing a representative piece of diseased tissue, was prepared and examined by conventional light microscopy. (This work was performed by Dr. K.U. Loeffler and Professor W.R. Lee). Significant inflammatory cell infiltrate was found in seven of the twelve corneas. Four of these specimens, 1-4, had descemetocoeles. However specimen 5 also with a descemetocoele had minimal inflammatory cell infiltrate. The correlation between clinical and pathological examination was poor in specimens 10-12, where the corneas were regarded as clinically quiescent but histology revealed significant inflammatory cell infiltrate (Table 1b). Specimens 1 and 2 revealed features not commonly found in herpes simplex keratitis viz, nuclear

Table 1 (a)

Patients with chronic herpetic stromal keratitis

		History pre-graft	Duration of HSV disease (years)	Steroid treatment	Anti-viral treatment	Time to culture (hours)
NS	1	Descemetocoele	30	+	+	8.5
MJ	2	Descemetocoele	4	+	+	3.0
IF	3	Descemetocoele	30	+	+	23.5
BD	4	Descemetocoele	15	+	+	20.0
DMcE	5	Descemetocoele	0.5	-	+	1.0
JR	6	Graft (6 yrs ago)	12	+	+	1.5
JG	7	Grafts (3 & 9 yrs ago)	11	+	+	7.5
ML	8	Quiescent	33	-	+	20.0
MF	9	Quiescent	28	+	+	3.5
PW	10	Quiescent	16	+	+	20.0
HW	11	Quiescent	8	+	+	9.0
IR	12	Quiescent	9	+	+	1.0

Patients 1-5 had active corneal disease at the time of surgery. Corneal ulcers were present to the level of Descemet's membrane (figure 8). All corneas had been treated with antiviral agents for some time prior to surgery. Only patient 5 did not receive topical steroid - therapy.

Patients 6-12 had no clinically active corneal disease (i.e. no ulceration, no inflamation) and were on no ocular medication at the time of surgery.

Table 1 (b)

Patients with chronic herpetic stromal keratitis

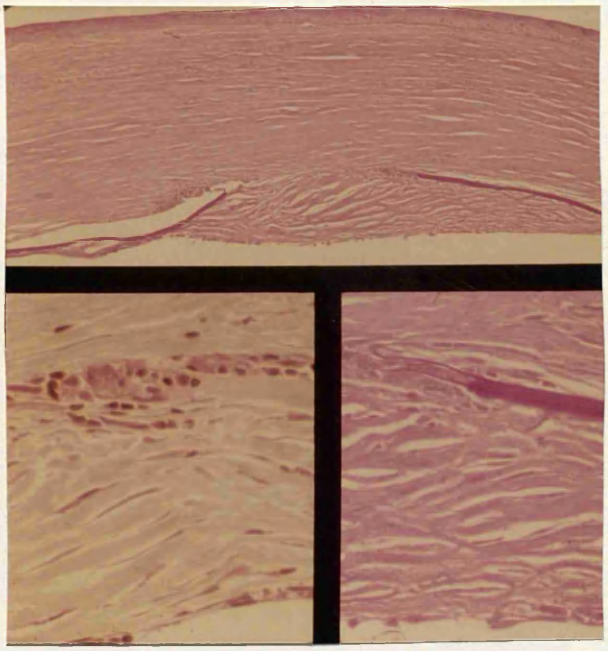
Active inflamation (path.)	HSV recovered	E.M. performed	E.M. HSV seen	Pathology post culture
+ +	-	ND	ND	+
+	-	ND	ND	+
+	-	pre	-	+
+	-	pre	-	+
-	-	pre	-	ND
-	-	ND	ND	ND
-	-	ND	ND	ND
-	-	pre	-	ND
-	+	post	+	+
+ +	+	pre post	- +	+
+	-	pre	-	-
+	-	pre	-	+

pre = pre organ culture
post = post organ culture
ND = not done

dust, cellular debris and necrosis of the corneal lamellae; bacteria or fungi could not be identified. Specimen 1 had been treated with tissue glue prior to grafting. (Tissue glue is a synthetic polymer used to "plug" a leaking cornea.) Specimens 3 and 4 showed features suggestive of active herpetic disease including inflammatory cell infiltrate, degeneration of stromal lamellae, multinucleate cells and attenuation and loss of endothelial cells on Descemet's membrane. Specimens 6 and 7, both of which were previous corneal grafts had minimal inflammatory cell infiltrate and no evidence of an immune rejection process. Specimens 8 and 9, clinically quiescent, showed evidence of previous herpetic disease; loss of Bowman's membrane, loss of stromal organisation with fibrous replacement, and in specimen 9, a break in Descemet's membrane with a granulomatous reaction at its ends causing a fan shaped splitting of its lamellae, fig. 11(a-c). This relatively unusual feature is known to be associated with longstanding herpetic keratitis (Naumann, 1980, and Hogan et al., 1964). Specimen 10 (Fig. 12(a-c) and specimen 11 showed features suggestive of active herpetic disease.

ORGAN CULTURE AND RESTRICTION ENDONUCLEASE ANALYSIS

Organ culture was performed on five/eighths or three/quarters of each cornea. Tissue was maintained in organ culture for up to 28 days. Herpes simplex virus was cultured from only two of the twelve corneas, specimens 9 and 10. Virus was isolated from three of six segments after 7 days (two segments positive) and 9 days (three segments positive) in organ culture, in specimen 9; and from one of five segments after 7 days in organ culture in



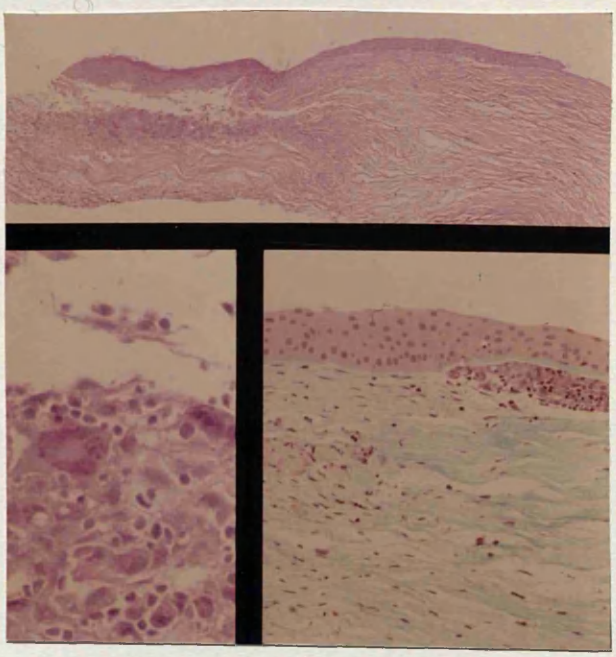


FIGURE 11

Specimen 9.

(a) gap in Descemet's membrane (PAS X 80)

(b) granulomatous reaction at the end of Descemet's membrane (Masson X 320)

(c) lamellar split in Descemet's membrane (PAS X 320)

FIGURE 12

Specimen 10.

(a) corneal epithelium, irregular in thickness and architecture (H and E X 30)

(b) multinucleate cells with swollen keratocytes (Masson X 120)

(c) numerous foamy macrophages, multinucleate cells, lymphocytes and polymorphs within the stroma (Masson X 70)

specimen 10. All other segments from these and other corneas were negative. The isolates from the two corneas were shown to be HSV-1 by restriction endonuclease analysis (see fig. 13). The EcoRI digests show that the k and l bands run together in specimen 10. The HpaI digests show that the k and l bands of specimen 9 run together. Both isolates are thus distinct from each other and from HSV-1 strain 17.

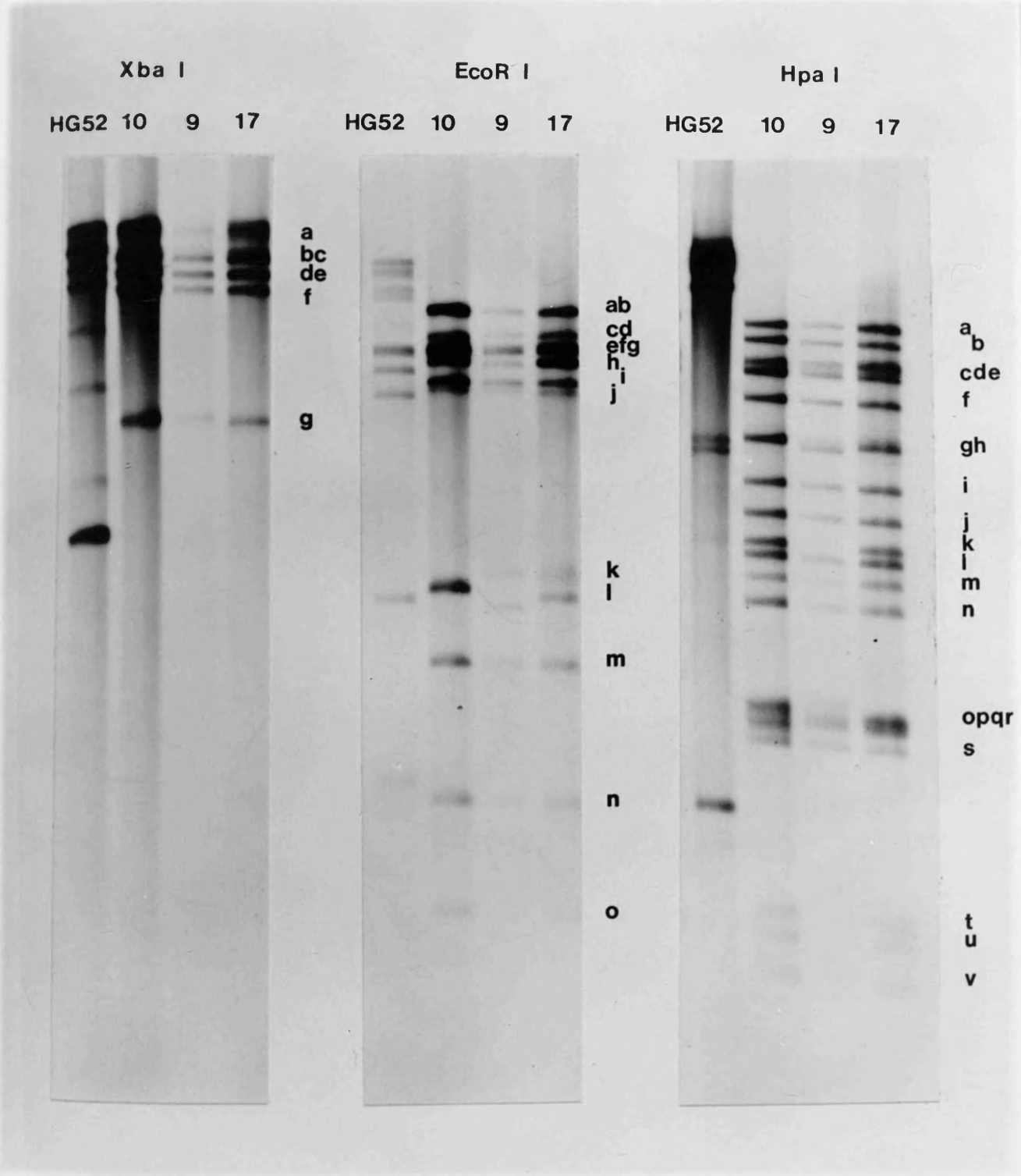
ULTRASTRUCTURAL STUDIES

Corneal tissues pre and post organ culture were examined including segments releasing and not releasing HSV in an attempt to identify HSV particles within corneal tissues. (Tissues were prepared and sectioned by Mrs. D.A. Aitken.) A total of seven specimens (one/eighth of each corneal disc) were examined ultrastructurally immediately pre-culture. These segments were not subjected to organ culture and included one segment from specimen 10, whose "co-segment" subsequently released HSV after organ culture. No HSV particles were seen in the segments not subjected to organ culture, however features typical of active inflammation were seen, including disturbance of the normal stromal architecture and macrophages engulfing dead cells (Table 1b).

Figure 14 shows a negative co-segment from specimen 10 after 14 days in organ culture. Epithelial cells are migrating down the sides of the tissue block towards the tissue culture plate, and healthy keratocytes are present within the stromal lamellae. A total of eight specimens were subjected to histological examination post organ culture. In the main, epithelial and endothelial cells

FIGURE 13

Restriction endonuclease profiles for specimens 9 and 10, compared with profiles from HSV-1 strain 17 and HSV-2 strain HG52. The enzymes XbaI, EcoRI and HpaI were used. The HSV-1 strain 17 profiles are labelled. Restriction endonuclease maps for the genome HSV-1 strain 17 are shown in figure 31.


Xba I
HG52 10 9 17
a
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HG52 10 9 17
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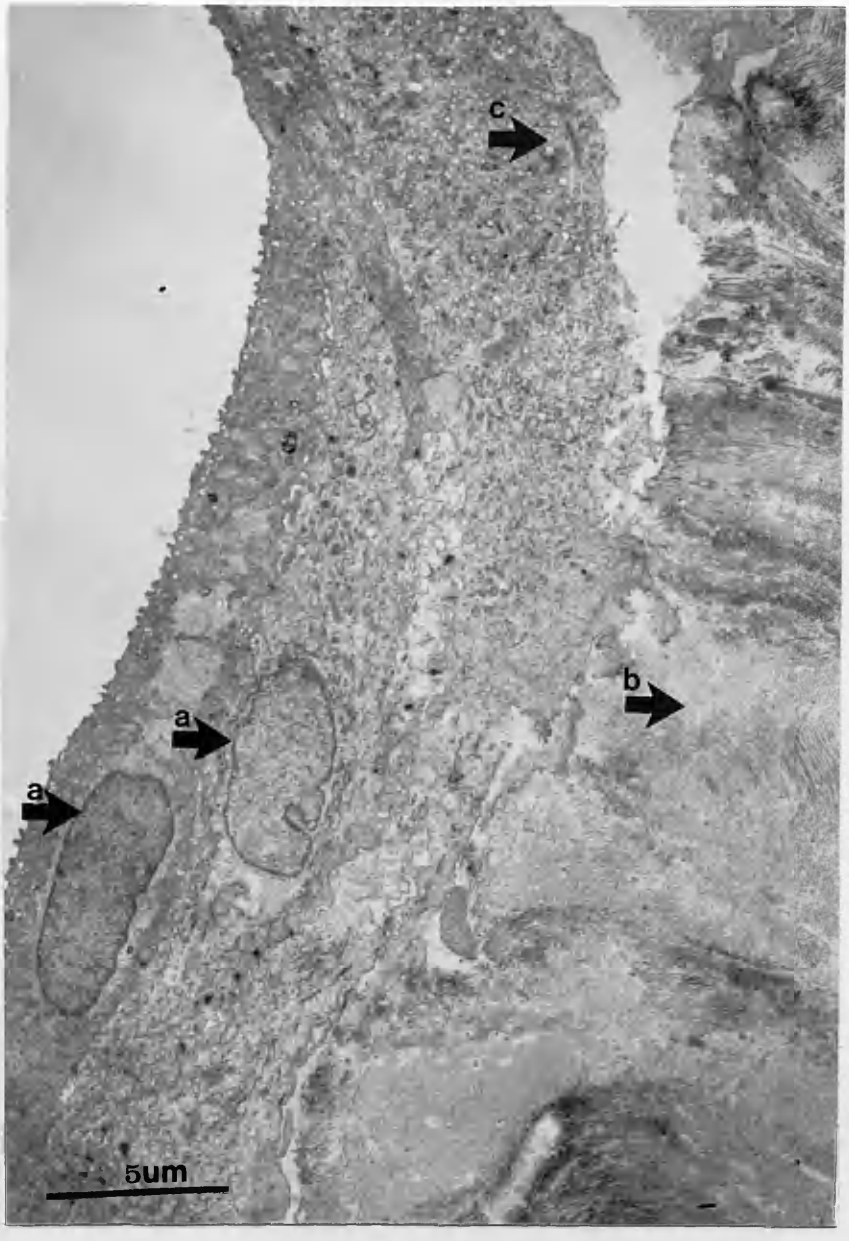
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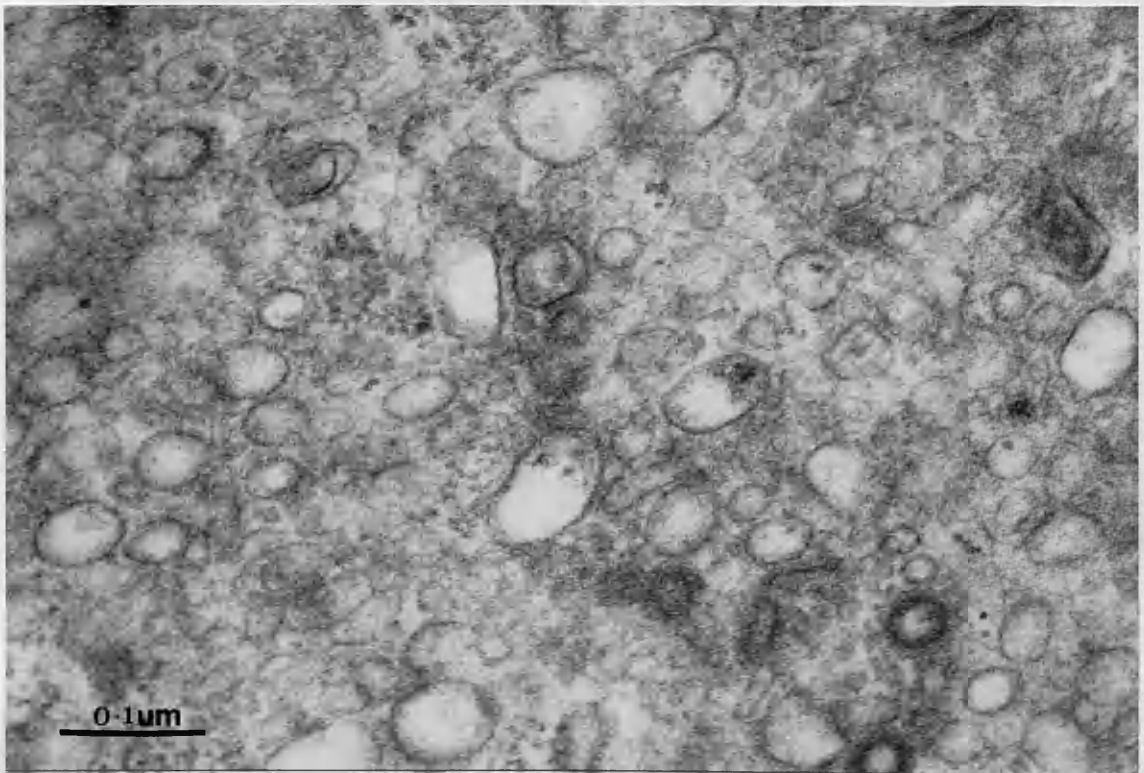
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FIGURE 14

Specimen 10.

(a) A culture negative co-segment after 14 days in organ culture. Epithelial cells are migrating down the side of the tissue towards the tissue culture plate (a). The stromal lamellae are evident (b) (E.M. x 4,500). Enlarged area shown in figure 14 (b) - (c).

(b) Vesicles within specimen 10. No viral structures are apparent (E.M. x 77,000).

were absent, and keratocytes were often well preserved. Inflammatory foci were often present, though other sections had well preserved corneal architecture. There was no evidence of active herpetic disease.

The three segments of specimen 9 which released HSV after organ culture on days 7 and 9, were subjected to detailed ultrastructural examination after 14 days in organ culture. Similar studies were performed on the culture positive segment and a culture negative segment of specimen 10, on day 11; and on the remaining three culture negative pieces of specimen 10, on day 14.

HSV particles were only seen in segments that had been organ culture positive. No epithelial cells or endothelial cells were present, although both Bowman's layer and Descemet's membrane were evident. Nearly all cells examined showed effects of viral synthesis. It was possible to observe many of the steps of viral synthesis within the corneal tissue. Electron micrographs showing stages in the formation of virions after organ culture are shown in figures 15-19. These micrographs were obtained from specimens 9 and 10.

Figure 15 shows a virion about to enter or exit a keratocyte by fusion or pinocytosis. Precise interpretation of a dynamic process in a static micrograph is impossible. The characteristic morphological changes of a host cell after transcription of virion DNA by host cell RNA polymerase II are shown in figure 16; they include margination and condensation of host cell chromatin (Nii et al., 1968). Infecting virion structural components or immediate early viral proteins (Dargan and Subak-Sharpe, 1983), or a host cellular stress response may play a role in

FIGURE 15

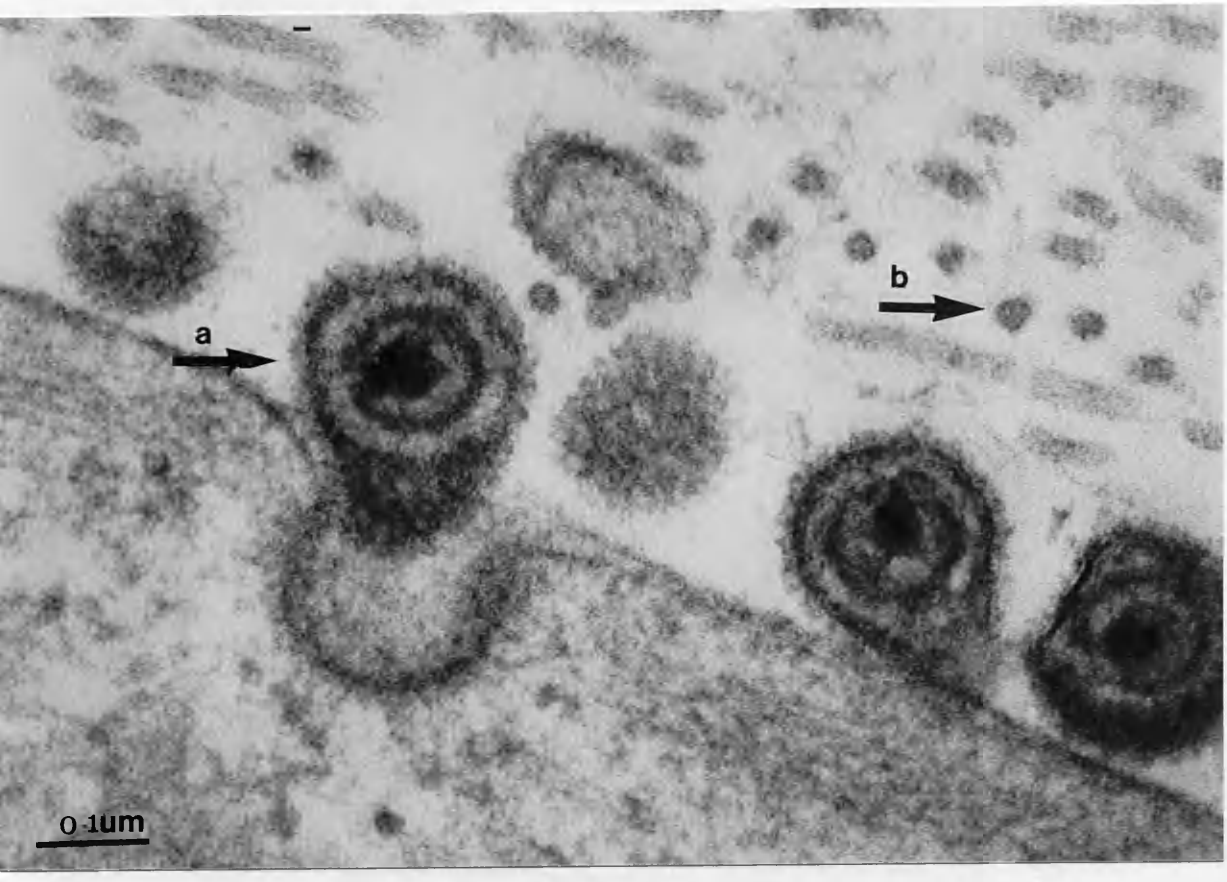
Specimen 10.

A virion (a) about to enter/exit a cell by fusion/ pinocytosis. Stromal lamellae (b) in cross section are seen superiorly (E.M. x 212,000).

FIGURE 16

Specimen 9.

A keratocyte with margination and condensation of host cell chromatin at the nuclear membrane (a). A variety of nucleocapsid structures are present within the nucleus (b) core containing nucleocapsid (c) empty nucleocapsid (E.M. x 21,000).


a
b
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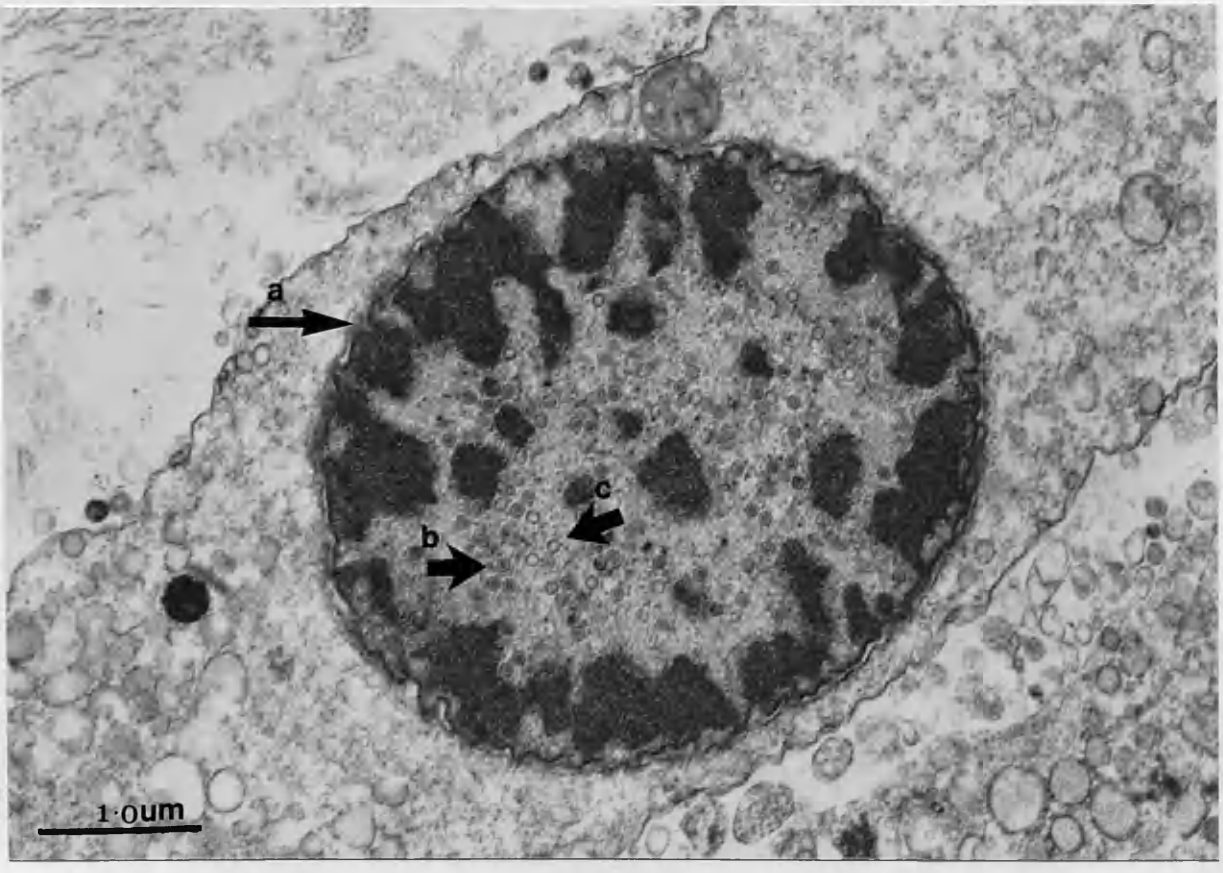
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the induction of these structures (Dewey et al., 1971). Nucleocapsid forms representing different stages of maturation are shown in figure 17. An empty capsid without core, incapable of encapsidating viral DNA is shown. Electron dense fibres appear to be penetrating the nucleocapsids, figure 18, and the fibres are thought to represent supercoiled DNA (Nii et al., 1968, Friedmann et al., 1977 and Puvion-Dutilleul et al., 1982). Envelopment of nucleocapsids occurring at the inner nuclear membrane is shown in figure 19.

DISCUSSION

HSV was isolated from two of twelve herpetic corneas subjected to organ culture. HSV was isolated from three of six segments in specimen 9, and from one of five segments in specimen 10. The HSV was typed by restriction endonuclease analysis and found to be HSV-1. The DNA of each isolate had its own distinct restriction endonuclease pattern. The isolation of HSV from herpetic corneas independently confirms the results of Shimeld et al. (1982) and Tullo et al. (1985), in Bristol, however the frequency of isolation, two out of twelve, differs markedly from the published results, six out of nine. Since 1985 the Bristol series has been expanded and current figures for HSV isolation after organ culture stand at ten positives out of forty (Shimeld, personal communication). The reduced frequency of isolation may be accounted for by better recognition and treatment of virus induced disease within the cornea in the human population, thereby reducing or eliminating virus from corneal cells; and by the fact that inclusion/exclusion criteria became less rigorous as the study progressed

FIGURE 17

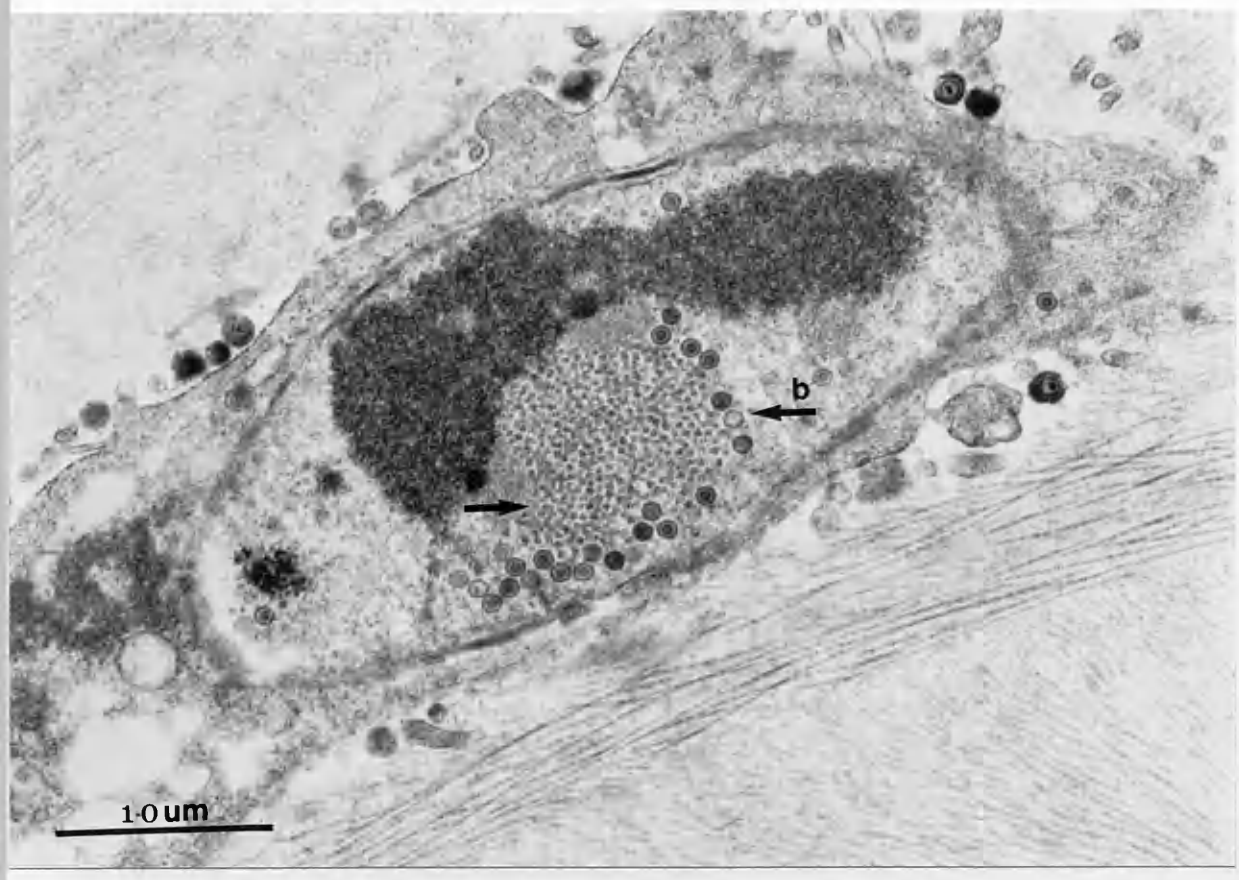
Specimen 9.

A keratocyte within the stromal lamellae. Core and capsid sub units are present within the nucleus (a). An empty capsid without a core is present (b). Stromal lamellae (c) surround the keratocyte. (E.M. x 28,000).

FIGURE 18

Specimen 9.

Electron dense fibres penetrating nucleocapsids and attached to the central core (a), this is thought to represent encapsidation (Nii et al., 1968). This appearance was found only once. (E.M. x 122,000).


b
1·0 um

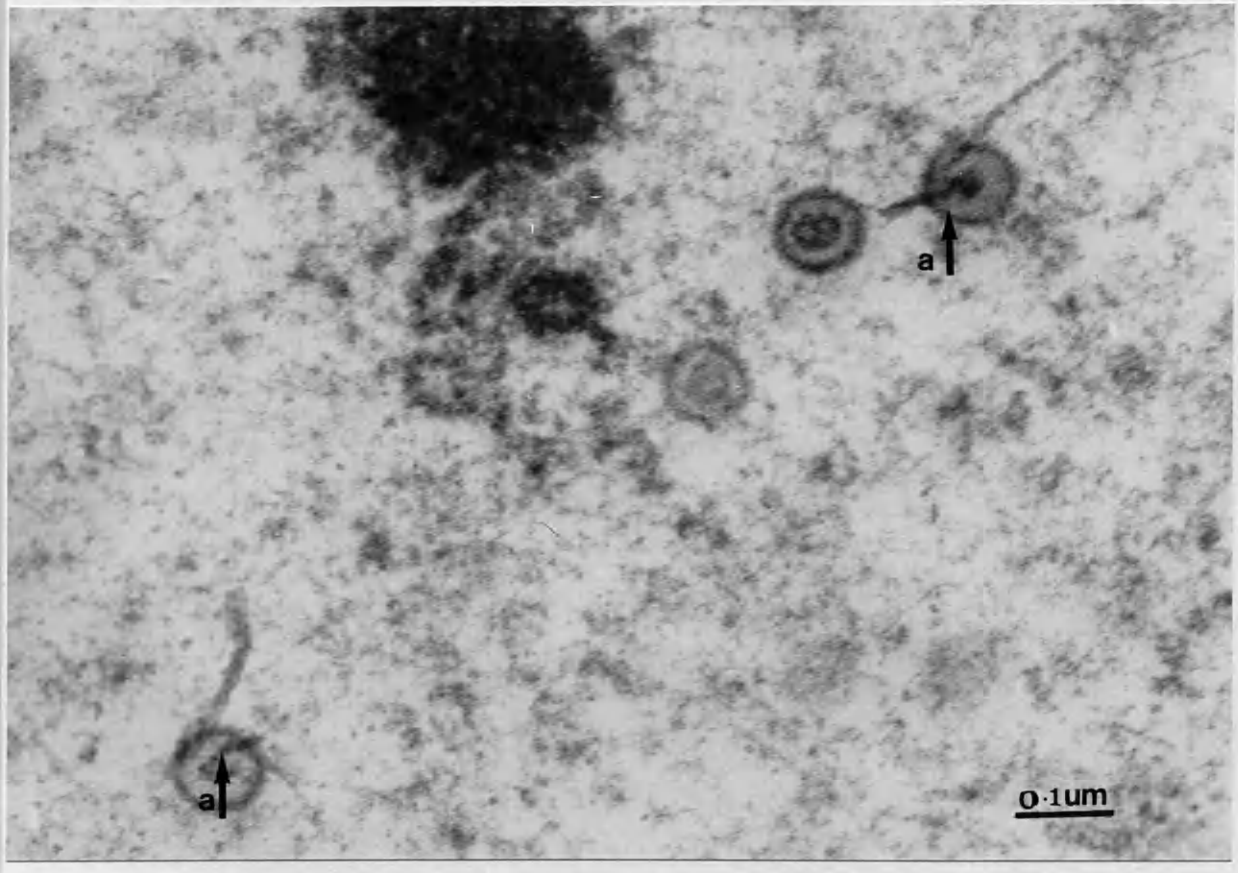

a
a
0·1um

FIGURE 19

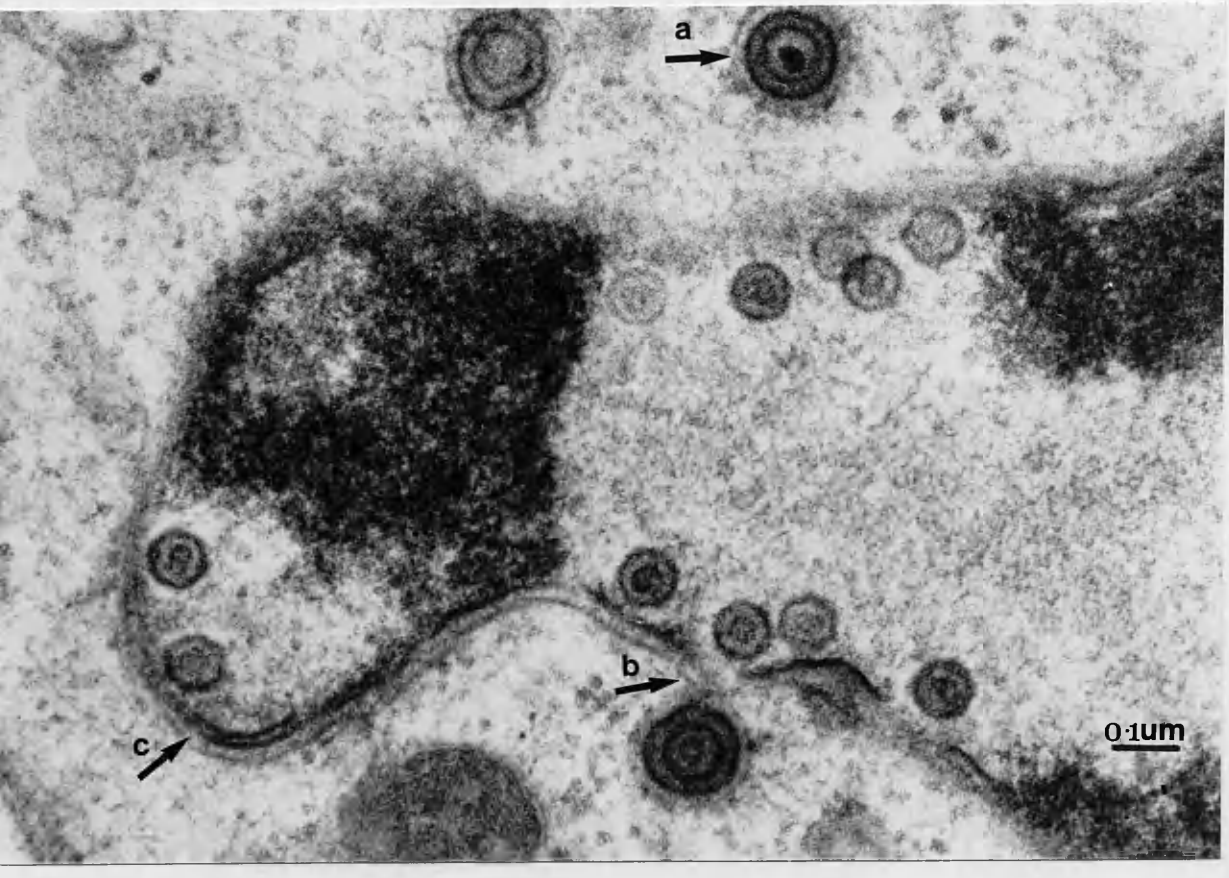
Specimen 9.

Nucleus with marginated chromatin showing:

Top right — A mature enveloped virion within the cytoplasm (a).

Bottom centre — A virion which has acquired the inner nuclear membrane and is acquiring an outer nuclear membrane (b).

Bottom left — Reduplication of the nuclear membrane (c) (E.M. x 350,000).


a
b
c
0·1um

(Shimeld, personal communication). The isolation of virus within a cornea is sporadic, in other words not every segment will release HSV within a positive cornea. Shimeld, Tullo and Easty (personal communication) observed that virus tended to be recovered from clinically diseased tissue.

The pathological responses of the human cornea to a toxic stimulus from whatever source are limited. Four corneas, specimens 3, 4, 10 and 11, showed immediately post graft, pathological changes, suggestive of an active herpetic infection. Of these only specimen 10, said to be clinically quiescent pre graft, yielded HSV on organ culture. Other specimens, notably 1 and 2 but also 12, showed evidence immediately post graft, of an active inflammatory process, which was not characteristically herpetic. Another two specimens, 8 and 9, showed evidence of long standing though inactive herpetic disease immediately post graft (specimen 9 released HSV on organ culture).

It is thus clear that the inflammatory response, *per se* does not correlate with an ability to recover HSV from organ culture. The human immune response to herpetic keratitis is complex and has been covered in the introduction, but briefly it is T cell mediated. Meyers-Elliott *et al.* (1980a) demonstrated viral antigens by immuno-perodixase electron microscopy in the cornea of a patient with active keratitis. Viral antigen was present in the keratocyte nucleus, on keratocyte nuclear membranes and in degenerating keratocytes. Henson *et al.* (1974) used human immunoglobulin G against HSV to localise viral antigens in cultured rabbit corneal cells infected with HSV. Viral

antigen was found at the nuclear membrane on areas of focal plasma thickening resembling the envelope of HSV, and on enveloped virus particles. Unenveloped virus particles did not react with labelled antibody. This suggests that the humoral immune response is mounted against HSV induced structures present after viral DNA synthesis has occurred, and thus viral replication is an important step in the evolution of chronic stromal and/or disciform keratitis. The subsequent immune response continues against HSV induced cellular antigens which may explain the presence of an immune response in the absence of infectious HSV.

HSV has been demonstrated previously in ultrastructural studies performed on corneal discs when viral disease was clinically active. Epithelial disease was generally co-existent with stromal disease but HSV was rarely isolated (Dawson et al., 1968a and b; Jones et al., 1977). Meyers-Elliott et al. (1980a) demonstrated viral antigens within a herpetic cornea but found that structurally complete enveloped viral particles were rarely seen, suggesting that an abortive non-replicating infection only occurred in keratocytes. Mature virus particles were demonstrated by Collin and Abelson (1976), in a clinically quiescent failed corneal graft, but the detection of infectious HSV was not reported.

Our ultrastructural studies link the detection of HSV by organ culture with the presence of HSV on ultrastructural examination, and show that the steps outlining most of the sequence of virion assembly were demonstrable within keratocytes. Epithelial and endothelial cells were not observed, presumably because virus replication had already occurred within them causing lysis, or because they had

migrated to the tissue culture plate during organ culture. The ultrastructural studies performed immediately post graft, on the five corneas with active disease did not reveal HSV structures, however only one eighth of each cornea was examined by this method. This contrasts with the results of Dawson et al. (1968a) who found HSV particles in the corneal stroma of five out of nineteen corneas examined by electron microscopy. Four of his five positive specimens had epithelial defects, at the time of surgery, but no virus isolates were recovered from swabs taken prior to surgery. The five positive corneas (Dawson et al. 1968a) received ocular steroids prior to surgery, however only one of the five was documented as being treated with an antiviral agent. This does not necessarily mean that the others did not receive antiviral treatment pre operatively. It is conceivable that current antiviral agents achieve better corneal penetration and are thus more able to eliminate virus deeper within the cornea. Alternatively the pre operative antiviral treatment given to patients in my series may have eliminated HSV from the patients with descemetocoeles prior to surgery. Both positive corneas in my series were regarded as clinically inactive at the time of surgery and had not been treated with antiviral agents.

The recovery of infectious HSV from human corneas after organ culture raises questions on the state of HSV within the human cornea; is it latent, persistent, or newly arrived from a neural source? The evidence cited in the introduction and this study support the hypothesis that HSV may indeed by capable of maintaining a latent infection within a non-neural cell type.

CHAPTER 3

IN VIVO EXPERIMENTS

MATERIALS

Viruses: HSV-1 Glasgow strain 17 syn^+, Brown et al. (1973), HSV-1 strain McKrae (From Dr. J.M. Hill, Louisiana State University, New Orleans, La., USA) (Williams et al., 1965) and HSV-2 strain HG52 Timbury (1971) were used in animal experiments.

Cells: $BHK_{21}C_{13}$ cells (MacPherson and Stoker, 1962) derived from a fibroblastic line of baby hamster kidney cells were used for growth of virus, and screening for virus.

Tissue culture media: $BHK_{21}C_{13}$ cells were grown in Glasgow modified Eagle's medium supplemented with 100 units/ml penicillin, 100ug/ml streptomycin, 0.2 ug/ml amphotericin and 10% calf serum, 0.002% phenol red and 10% calf serum (EC_{10}).

Versene (0.6mM EDTA in PBS, 0.002% (w/v) phenol red) and trypsin (0.25% (w/v)(Difco) in Tris saline were used for the washing and removal of cell monolayers during passaging or setting up cells for growth of viruses.

PBS 170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4 (pH 7.2).

Tris saline 140mM NaCl, 30mM KCl, 280mM Na_2HPO_4, 1mg/ml glucose, 0.0015% (w/v) phenol red, 25mM Tris HCl (pH 7.4), 100 units/ml penicillin, 100ug/ml streptomycin.

Enzymes: Restriction enzymes were obtained from Bethesda Research Laboratoris (BRL).

Radiochemicals: Radio isotopes, ^{32}P orthophosphate (carrier free) were obtained from Amersham International.

Solutions:

(i) EFC_{10} Glasgow modified Eagle's medium supplemented with antibiotics as before plus 10% (w/v) foetal calf serum.

(ii) EFC_{25} Glasgow modified Eagle's medium supplemented with antibiotics as before plus 25% (w/v) foetal calf serum.

(iii) General restriction endonuclease buffers.

	Low salt	Medium salt	High salt
Tris HCl	10mM pH 7.5	10mM pH 7.5	10mM pH 7.5
NaCl	-	50mM	100mM
$MgCl_2$	10mM	10mM	10mM
DTTl	1mM	1mM	1mM

10X stocks of these buffers were prepared, autoclaved and stored at -20°C.

Reaction volumes of 30ul contained DNA in a solution with a final concentration of 1 x reaction buffer, 100 ug/ml bovine serum albumin, and the appropriate restriction enzyme at approximately 1 unit per ug DNA.

(iv) RNase mixture. 1ml distilled H_2O and 100ug RNase A + 250 units of RNase Tl. The mixture was freshly prepared and boiled for 10 minutes prior to use.

(v) 10X E buffer. Tris 43.6g/L, $NaH_2PO_4H_2O$, 46.8g/L Na EDTA 3.7g/L, H_2O 1 litre.

(vi) Restriction enzyme stop (R.E. stop) mixture.

10ul of the following mixture was added to stop digestion of DNA; 2g Ficoll, 0.54g EDTA, 10ml 10X E buffer, 10ml H_2O, plus bromophenol blue to colour.

Animals: New Zealand white female rabbits less than six months old and between 1.5-2 kg were obtained from Hyline Rabbit Farms. Rabbits were kept apart in individual cages.

Only the left eye of any animal was used in experiments. Animals were monitored scrupulously, and any animal showing evidence of pain or systemic illness was humanely destroyed.

Iontophoresis apparatus: A system was devised by Mr. W. Davies (Department of Medical Physics, Western Infirmary, Glasgow), to administer an ionised solution of epinephrine to the eye by way of an electrical current (Figure 20). The design was based on the system of Kwon et al. (1981), such that a fixed current of 0.8 mAmps was used and a voltage of around 7V depending upon the integrity of the connection between electrode and epinephrine solution. An automatic timer was incorporated into the apparatus to allow the iontophoresis to run for 8 minutes. The circuit was arranged in series so that four animals might be treated simultaneously. Practical problems dictated that three animals were the optimum number. The circuit was arranged so that the cathode was applied to the animal's shaved trunk, and the anode was in contact with the epinephrine solution (see figure 21).

Iontophoresis eye cup: A cylindrical well with an expanded flanged end was produced by Mr. J. Macfarlane (Department of Ophthalmology, Western Infirmary, Glasgow) and copied from an original well of Kwon et al. (1981). The flanged end was bevelled to the approximate curvature of a rabbit's cornea. This arrangement permitted the well to sit on the cornea with the eyelids over the flange, thus creating a partially closed system and minimising loss of drug solution. The wells were made of nylon which allowed them to be autoclaved and re-used.

Electrodes: The anode electrode was formed by a small strip of aluminium foil. The cathode electrode was an ECG

electrode supplied by Cambmac Instruments Ltd.

Chemicals: (a) anaesthetics — Ketamine supplied by Parke Davis and Co. Xylazine supplied by Bayer UK Ltd.

(b) iontophoresis — Epinephrine (adrenalin) supplied by Sigma Chemical Co.

(c) killing animals — Expiral supplied by Ceva Ltd.

METHODS

Tissue cell culture: BHK21C13 cells were passaged in 80 oz roller bottles and kept in an atmosphere of 95% air, 5% CO_2 at 37°C.

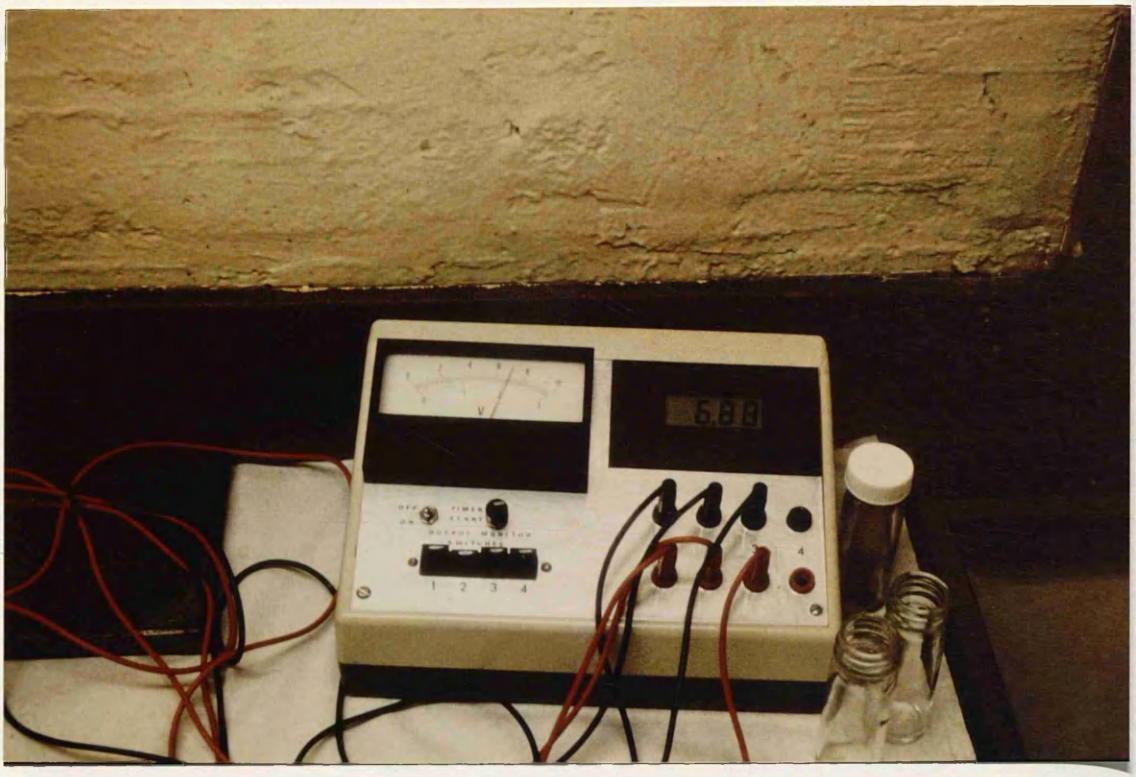
Virus preparation: $BHK_{21}C_{13}$ cells in 80 oz roller bottles were infected with HSV-1 strain 17 or strain McKrae, or HSV-2 strain HG52 at an m.o.i. of 0.003 pfu/cell (10^6 pfu per bottle) in 40 mls EC_{10}. Virus infected cells were incubated at 31°C for 3-4 days until extensive cytopathic effect (CPE) had developed. Cells were removed by shaking into the medium and pelleted (MSE Coolspin, 2,000 rpm/10min/4°C). Supernatant medium was further centrifuged (Sorvall SS34 rotor, 12K rpm/2 hr/4°C) to concentrate cell released virus (CRV), which was sonicated and stored at -70°C. The pelleted material from the low-speed centrifugation was sonicated in a small volume of supernatant medium for recovery of cell-associated virus (CAV). Cell debris was removed by further low speed centrifugation and the virus solution sonicated and centrifuged again if necessary. Supernatant containing CAV was stored at -70°C.

FIGURE 20

Iontophoresis apparatus.

FIGURE 21

Three anaesthetised animals undergoing iontophoresis of epinephrine. The cylindrical wells containing epinephrine are loosely held by clamps.


6.88
1 2 3 4

The CRV and CAV fractions were checked for sterility (on blood agar plates) and titrated on $BHK_{21}C_{13}$ monolayers in the presence of human serum, as described by Brown et al. (1973).

Inoculation: Outbred New Zealand white rabbits were inoculated with either HSV-1 strain 17 (Brown et al., 1973), HSV-1 strain McKrae (Williams et al., 1965) or HSV-2 strain HG52 (Timbury, 1971). The left eye was infected by placing 50ul of a virus suspension in the lower cul-de-sac and massaging over the closed eye for approximately 1 minute. The virus inoculum ranged from 10^5 pfu - 5×10^7 pfu.

Scarification: Prior to inoculation, as above, rabbits were anaesthetised. The cornea was traumatised by applying 10 linear incisions followed by a further 10 perpendicular to the first series, using a 26 gauge needle.

Anaesthesia: Rabbits were anaesthetised using intramuscular injections of xyalazine, 4mg/kg and ketamine 20 mg/kg in combination. When rabbits were anaesthetised on three consecutive days, a degree of tolerance often occurred by the third day. It was thus necessary to increase the dosages to achieve anaesthesia.

Sampling of the pre ocular tear film: A solution of EFC_{10} was used. Both eyes were sampled. 0.1ml of EFC_{10} was dropped into the lower cul-de-sac and massaged over a closed eye for 30 seconds approximately. 0.5ml of EFC_{10} was then poured over the cornea and allowed to collect in the lower cul-de-sac. The fluid was removed with a pipette and the process repeated twice using the same 0.5ml of EFC_{10}. This procedure was not foolproof as a rabbit might occasionally pull away causing loss of the ocular washing. In that event the procedure was repeated. The sample of pre-ocular

Table 2

Lesions Score for HSV-1 Infections in Rabbit Eyes

Corneal epithelium:

0.0	= Normal.
0.1-0.9	= Superficial punctate ulcerations.
1.0-1.9	= One or more dendritic ulcerations.
2.0-2.9	= Geographic ulcerations (less than 75% of cornea involved).
3.0-4.0	= Geographic ulcerations (more than 75% of cornea involved).

Stroma:

0.0	= Normal.
0.5	= Mild oedema; not diffuse; no haze.
1.0	= Mild oedema; slight haze.
2.0	= Oedema and haze; diffuse.
3.0	= More oedema; cloudy, can see anterior chamber.
4.0	= Severe oedema; very cloudy, cannot see anterior chamber. Add 1.0 if descemetocoele formation, perforation or neovascularization is present.

Iris:

0.0	= Normal.
0.5	= Mild hyperemia.
1.0	= Hyperemia.
2.0	= Marked hyperemia, beginning oedema, folds seen in iris.
3.0	= Marked oedema; excessive hyperemia.
4.0	= Fibrinous iritis.

Conjunctiva:

0.0	= Normal.
1.0	= Mild hyperemia.
2.0	= Severe hyperemia Add 1.0 if chemosis is present.

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tear fluid was dealt with in one of two ways.

(i) During primary infections, sonication then storage at -70°C for titration,

(ii) division of sample between two 50mm plates of $BHK21C_{13}$ cells for viral screening post iontophoresis.

Clinical scoring of rabbit eyes during primary infection: An established clinical scoring system table, devised by Kwon et al. (1979) was used to assess the appearance of the rabbit eyes (see Table 2). The tissues assessed clinically were corneal epithelium, corneal stroma, iris and conjunctiva. The rabbits were examined with a flashlight torch and fluoroscein dye to determine whether corneal ulceration was present. A magnifying loupe was used initially to examine corneas in greater detail. This technique proved difficult and was abandoned.

Iontophoresis: Rabbits were subjected to iontophoresis of a 0.01% solution of epinephrine for 8 minutes on three consecutive days. The cathode was attached to the shaved trunk of the animal and the anode was placed in the solution of epinephrine. A direct current of 0.8 mAmp, 7V was applied. The tear film of the rabbits was sampled prior to iontophoresis to determine whether spontaneous shedding of HSV was occurring and for 8 to 10 days after the first iontophoresis procedure to determine whether induced shedding was occurring.

Killing of animals: Rabbits were killed humanely by one of two methods, (i) intravenous injection of Expiral 200mg/1.5kg body weight, (ii) rabbits were stunned and then bled by severing jugular arteries and veins. This permitted dissection of the central nervous tissue in a bloodless field.

Dissection of animals: Rabbit eyes and trigeminal ganglia were removed in the animal house, then dissected in a laminar flow hood.

(i) Removal of eyes; a limbal incision was made to the conjunctiva through 360°; extra ocular muscles were isolated and divided; the optic nerve and vessels were divided freeing the globe; the globe was placed in EFC_{10} prior to dissection of the cornea.

(ii) Dissection of cornea; each cornea was removed from the globe with scissors at the limbus; the cornea was divided into six pie shaped wedges which were placed in organ culture.

(iii) Removal of trigeminal ganglia; a midline incision was made in the rabbit skin over the skull and flaps were reflected; connective tissue was divided and bone exposed; the vault of the skull was removed using a bone punch and scissors; the frontal and middle cranial fossae were cleared of cerebral tissue and nerves were divided; the middle cranial fossa was exposed and the trigeminal nerve root was divided; the trigeminal cave was opened using scissors to divide bone; the trigeminal ganglion was exposed and removed; the contralateral ganglion was treated identically.

(iv) Dissection of trigeminal ganglia; the ganglia were maintained in EFC_{10} for a short time prior to dissection; the ganglia were divided into six segments and placed in organ culture.

Screening for latent HSV by organ culture: At time of death, the rabbits showed no clinical evidence of active infection and were not shedding virus that was detectable by sampling the pre-ocular tear film. The technique of

homogenizing a portion of tissue and plating out to screen for an active infection in either corneas or trigeminal ganglia was not employed. The dissected corneas and trigeminal ganglia were maintained in EFC_{25} at 31°C or 37°C in 5% CO_2 - separate organ cultures within a 96 well microtitre plate. Each filled well was surrounded by empty wells to eliminate cross contamination. The incubated cultures of corneas and ganglia were each screened regularly post explantation by removing 100ul of supernatant medium onto semi-confluent $BHK_{21}C_{13}$ cells, incubating for 4 days at 31°C and examining for plaques or cytopathic effect after staining with Giemsa stain. Organ cultures were generally screened daily for 14 days then twice weekly up to 28 days.

Restriction endonuclease analysis: Lonsdale (1979).

$BHK_{21}C_{13}$ cells were seeded into Linbro wells at 5×10^5 cells per well in 500ul of phosphate free Glasgow modified Eagle's medium with 1% calf serum (PIC), and grown at 37°C overnight. The medium was then removed and 0.1ml of virus suspension (containing approximately 5×10^6 pfu of virus) was added to the Linbro well, and allowed to absorb at 37°C for 1 hour. Cells were then washed with 800ul PIC, and 450ul of PIC added to each well. Linbro plates were incubated for 2 hours at 31°C, before adding 50uCi ^{32}P-orthophosphate (Amersham, carrier free) to each well in 50ul PIC. The incubation was continued for 48 hours at 31°C.

When cytopathic effect was evident, DNA was harvested by adding 0.5ml 5% (w/v) sodium dodecylsulphate. The mixture was removed with Eppendorf tips and transferred to conical plastic 15ml centrifuge tubes (Falcon) and allowed to stand at 37°C for 10 minutes. Saturated phenol 1ml was then added to each tube and inverted several times. The

mixture was left for 5 minutes at room temperature and the same time on ice. The mixture was centrifuged (2,000 rpm/10 mins/4°C). The aqueous phase was removed to a glass centrifuge tube and the nucleic acids precipitated by 2 mls of ethanol. A further 10 minute spin at 2,000 rpm pelleted the DNA. The supernatant was discarded and the tubes inverted and drained. The nucleic acids were re-suspended by gentle shaking for 2 hours in a 37°C waterbath, with 200ul of H_2O containing 25ul of RNase.

The re-dissolved nucleic acids (10ul) were transferred to a 2.5cm Whatman's no. 1 filter paper disc and air dried. The discs were given three washes in 5% trichloracetic/0.1 ml sodium tetrapyrophosphate, three washes in absolute ethanol and then air dried. The discs were placed in 5% toluene PPO and counted for 1 minute in a scintillation counter. Samples containing equal counts were added to the appropriate restriction enzyme buffer mix including 1% bovine serum albumin, water and sufficient restriction enzyme to give a complete digest in 4 hours at 37°C; total volume 30ul. The reactions were stopped by the addition of 10ul R.E. stop.

Digests were analysed after overnight electrophoresis 40mV, on agarose gels of suitable strength. Gels were air dried onto glass plates and exposed to film for 12-48 hours.

RESULTS

Data is presented in tabulated form and groups of animals are identified by a series of letters and numbers to permit their course to be followed. Each group of animals did not necessarily follow an identical protocol. This was necessitated by the restriction on animal house space and

the difficulties encountered in working with small numbers of rabbits.

EXPT. (1) group A(i) 3 rabbits; infecting dose 5×10^5 pfu/eye HSV-1 strain 17.

group B(i) 3 rabbits; infecting dose 5×10^5 pfu/eye HSV-2 strain HG52.

Clinical scoring: The clinical scoring system was not in use during experiment 1, and animals were simply examined and a clinical impression of the eye was noted. The three animals inoculated with HSV-1 strain 17 [A(i)], had evidence of herpetic eye disease including dendrites and conjunctival inflammation, which was maximal around day 7, and almost completely resolved by day 13. The group B(i) animals had no clinically discernible evidence of ocular herpetic infection.

Titration: Group A(i); the average titration from the animals on days 1-10 post inoculation is presented in figure 22.

Group B(i); a few viral plaques were detected from 2 rabbits on days 1 and 2 respectively post inoculation. No other virus was detected from this group.

Mortality: group A(i); one animal at 21 days post inoculation. This was quite unexpected and not attributed, by us, to the herpetic infection.

Group B(i); none.

Spontaneous shedding: days 40-60

Group A; none

Group B; none.

Iontophoresis: days 61-63 (inclusive). No HSV shedding was induced in either group A(i) or B(i) after iontophoresis

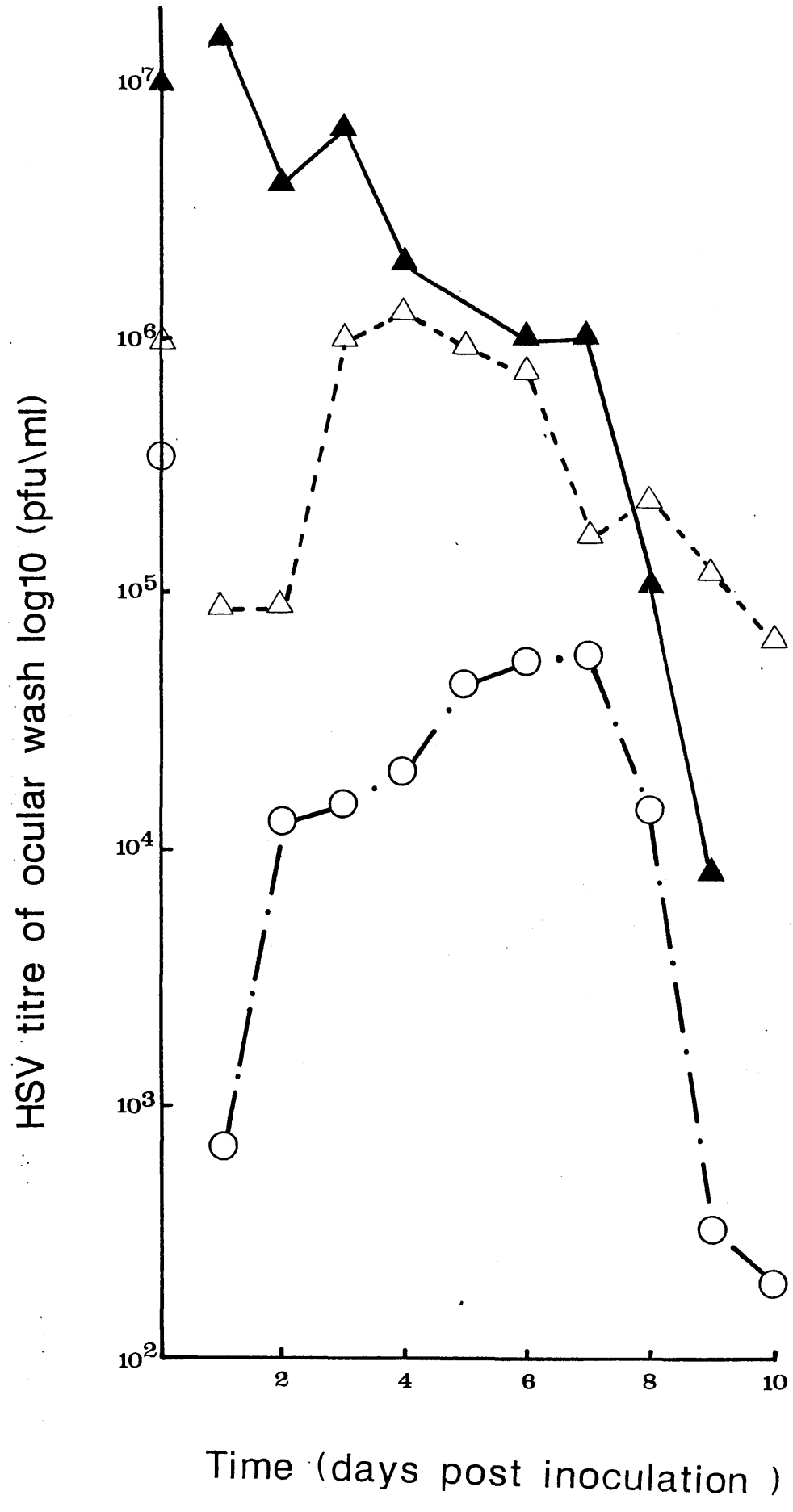
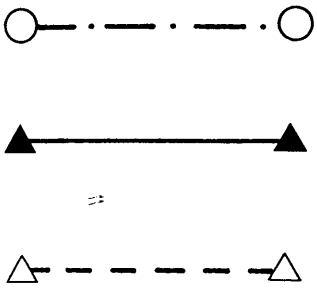

HSV titre of ocular wash log10 (pfu\ml)
10^7
10^6
10^5
10^4
10^3
10^2
2
4
6
8
10
Time (days post inoculation)

FIGURE 22

Average titration of primary infection with HSV-1 strain 17.



group A (i) infecting dose 5×10^5 pfu/eye

group A (ii) infecting dose 10^7 pfu/eye

group A (iii) infecting dose 10^6 pfu/eye

Symbols on the Y axis indicate the infecting dose. Rabbits that died within 10 days of inoculation in groups A (ii) and (iii) are not included in the average titrations.

of epinephrine.

Latency: One animal of group A(i) released virus from the left trigeminal ganglion after 3 days of organ culture and from the right trigeminal ganglion after 6 days of organ culture. The other surviving animal in this group did not release latent HSV. (It is likely that inexpert dissection was to blame!) The group B(i) animals were not dissected as it was thought unlikely at that time that there would be latent infection. Similarly corneas were not organ cultured for HSV.

EXPT. (2) group A(ii) 8 rabbits; infecting dose 10^7 pfu/eye HSV-1 strain 17.

Clinical scoring: The average clinical score from the animals on days 1-10 is shown in figure 23. The clinical scores from the rabbits destroyed on day 8 because of encephalitis and excoriation are not included in the average clinical scores but shown separately.

Titration: The average titration of HSV-1 from the left eyes of animals is shown in figure 22. All animals had detectable virus in the right eye within the 10 days post-inoculation. The amount of virus was minimal, except in three animals; the animal that died on day 13 from presumed encephalitis had HSV shedding from the right eye at a titre of 10^5 pfu/ml between days 8 and 10; the animal with the excoriated ear destroyed on day 8 shed virus from the right eye on days 7 and 8 at a titre of 7×10^3 pfu/ml; and the animal destroyed on day 30, had shed HSV at a titre of 10^3-10^4 pfu/ml between days 6 and 10. The rabbits destroyed on day 8 because of encephalitis and excoriation are not included in the average titrations.

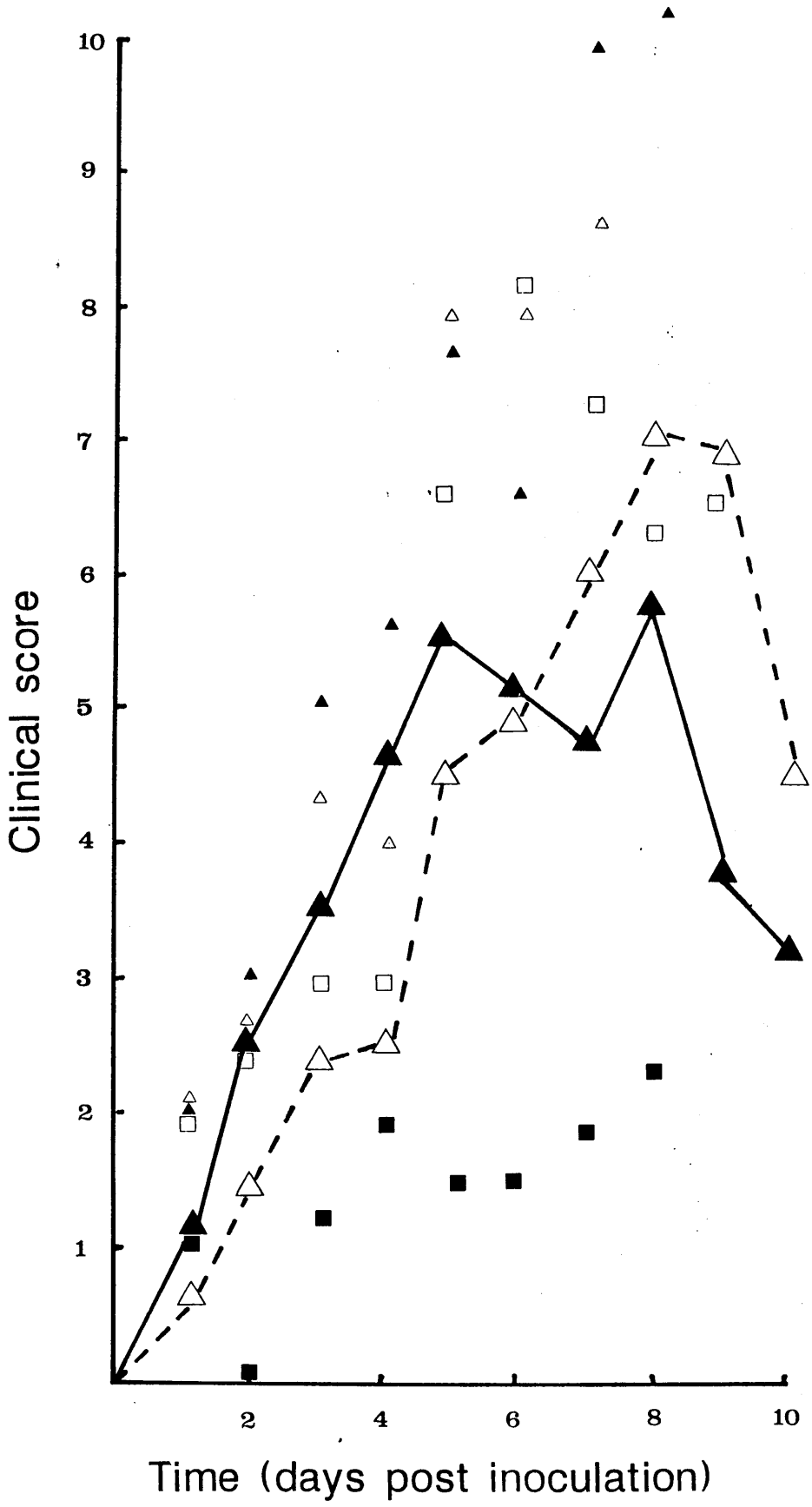
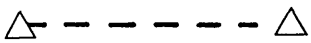

Clinical score
10
9
8
7
6
5
4
3
2
1
2
4
6
8
10
Time (days post inoculation)

FIGURE 23

Average clinical score of primary infection with HSV-1 strain 17.

group A (ii) infecting dose 10^7 pfu/eye



group A (iii) infecting dose 10^6 pfu/eye

Group A (ii) The rabbit destroyed on day 8 (encephalitis) is not included in the average scores but is shown separately▲ The rabbit destroyed on day 8 (excoriation) is not included in the average score but is shown separately■

Group A (iii) The rabbit destroyed on day 8 (encephalitis) is not included in the averge scores but is shown separately△ The rabbit destroyed on day 10 (excoriation) is not included in the average score but is shown separately□

Mortality: Five out of eight rabbits in this group died or were killed because of severe disease within 1 month of inoculation. One animal was destroyed because of an encephalitis 8 days post inoculation, and another was found dead 13 days post inoculation presumably due to encephalitis. Three other animals were destroyed because of severe ocular disease with adjacent lid, skin or ear infection and excoriation.

Spontaneous shedding: The three surviving animals were monitored for spontaneous shedding for 4 weeks, 5 days out of 7, from days 21-46. One animal shed virus spontaneously for 3 days between days 23-25, from the left eye.

Iontophoresis: Applied days 85-87 post inoculation. No rabbits shed virus after iontophoresis including the spontaneous shedder. Further iontophoresis treatments were performed on days 126-128 post inoculation with negative results, and on days 147-149 post inoculation when an animal that had not shed virus spontaneously, had detectable HSV in the ocular washings on the 4th and 6th day after the first iontophoresis treatment, highest titre 2.8×10^2 pfu/ml.

EXPT. (3) group A(iii) 8 rabbits; infecting dose 10^6 pfu/eye HSV-1 strain 17.

Clinical scoring: The average clinical score from the animals on days 1-10 post inoculation is shown in figure 23.

Titration: The average titration from the left eyes of animals is shown in figure 22. The rabbit destroyed because of encephalitis on day 8, shed HSV-1 from the right eye in significant amounts (not titrated but extensive c.p.e. on culture plates) between days 5 and 7.

Mortality: One rabbit was destroyed on day 8 because of an

encephalitis, another was destroyed on day 10 because of severe ocular disease with lid excoriation.

Spontaneous shedding: Animals were sampled for 20 days, as in experiment 2, between days 18 and 43. No rabbits shed virus spontaneously.

Iontophoresis: Two animals shed HSV after iontophoresis; the first animal within 24 hours of the first treatment, detected on one day only, 10^2 pfu/ml; the second animal within 48 hours of the first treatment for five consecutive days, peak titre on day 3 of shedding 2×10^3 pfu/ml. Iontophoresis was applied on days 48-50 inclusive. Further iontophoresis treatments were repeated on days 59-61 and days 80-82 with negative results.

EXPT. (4) group C(iv) 7 rabbits; infecting dose 10^5 pfu/eye HSV-1 strain McKrae.

Clinical scoring: The average clinical score from the animals on days 1-10 post inoculation is shown in figure 24.

Titration: The average titration from the left eye of animals is shown in figure 25.

Mortality: Two animals were found dead in their cages on days 10 and 18 post inoculation, respectively. One animal was destroyed on day 8 with an encephalitis, and another was destroyed on day 20 post inoculation because of its poor condition. A fifth animal was destroyed on day 53 post inoculation because of severe ocular disease.

Spontaneous shedding: One of the three surviving rabbits shed virus spontaneously for one day within the 20 day period between days 19 and 44.

Iontophoresis: The two surviving animals had iontophoresis of epinephrine between days 65 and 67 inclusive. Both

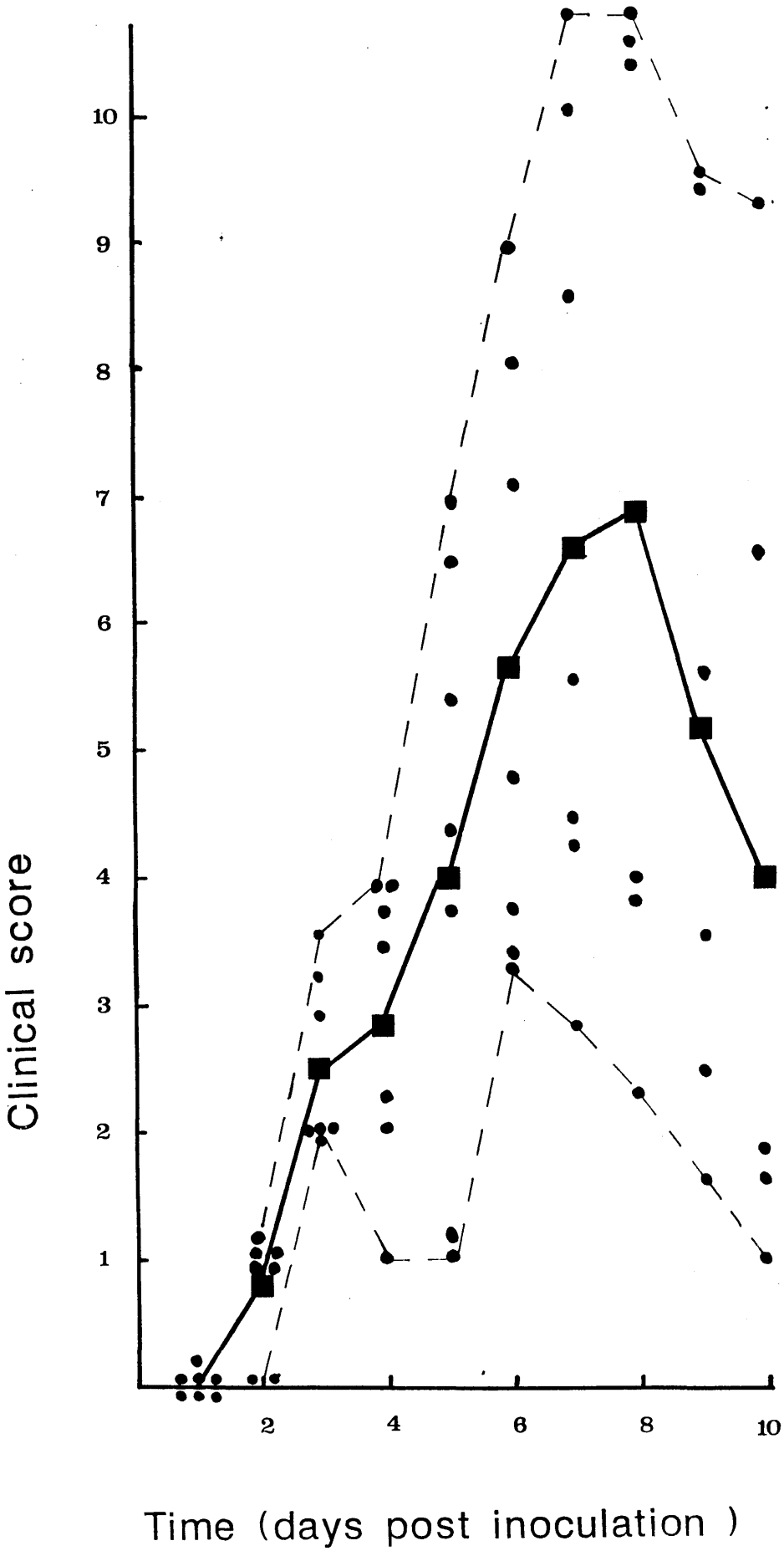
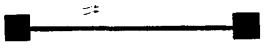

Clinical score
10
9
8
7
6
5
4
3
2
1
2
4
6
8
10
Time (days post inoculation)

FIGURE 24

Average clinical score of primary infection with HSV-1 strain McKrae.



group C (iv) infecting dose 10^5 pfu/eye

The clinical score of each animal is indicated on each day by a ●. Considerable variation in the clinical score exists among animals. The maximum and minimum ranges are indicated by dashed lines.

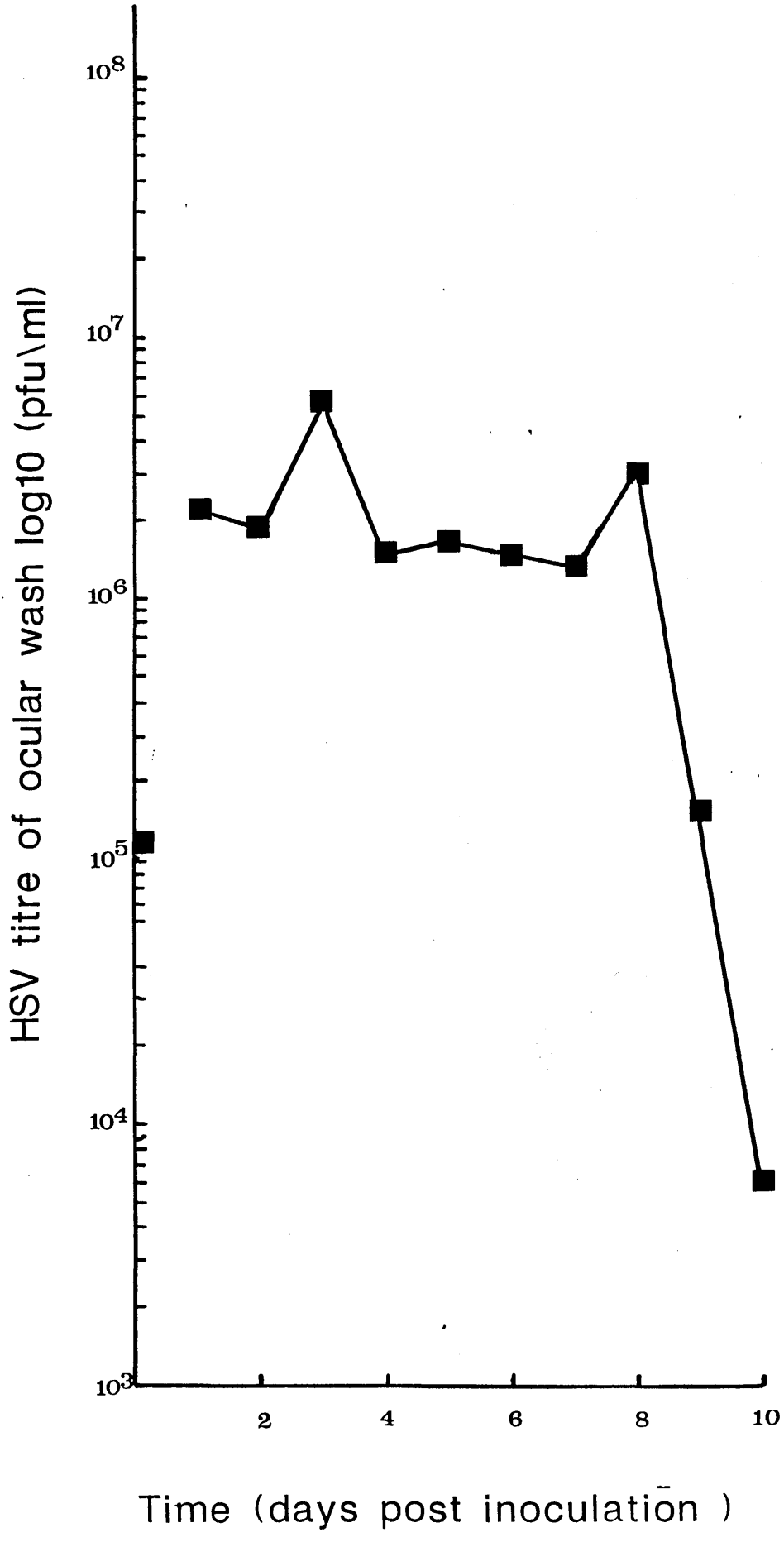

HSV titre of ocular wash log10 (pfu\ml)
10^8
10^7
10^6
10^5
10^4
10^3
2
4
6
8
10
Time (days post inoculation)

FIGURE 25

Average titration of primary infection with HSV-1 strain McKrae.

group C (iv) infecting dose 10^5 pfu/eye

Symbol on the Y axis indicates the infecting dose.

animals shed HSV-1 for 48 hours on the fourth day after first iontophoresis. The titres were 5×10^2 pfu/ml and $>10^4$ pfu/ml.

EXPT. (5) group B(v)a 3 rabbits; infecting dose 5×10^6 pfu/eye HSV-2 strain HG52 (unscarified)

group B(v)b 3 rabbits; infecting dose 5×10^7 pfu/eye HSV-2 strain HG52 (unscarified)

group B(v)c 3 rabbits; infecting dose 5×10^6 pfu/eye HSV-2 strain HG52 (scarified)

group B(v)d 3 rabbits; infecting dose 5×10^7 pfu/eye HSV-2 (scarified)

Clinical scoring: The average clinical score from each group of animals on days 1-10 post inoculation is shown in figure 26.

Titration: The average titration from the left eyes of the animals in each group is shown in figure 27.

Mortality: A rabbit from group B(v)d was found dead 18 days post inoculation.

Spontaneous shedding: Animals were not screened for spontaneous shedding.

Iontophoresis: Eleven surviving animals had iontophoresis with epinephrine between days 27-29. One rabbit from group B(v)c shed detectable virus 72 hours after first iontophoresis. One rabbit from group B(v)d shed detectable virus 72 hours after first iontophoresis, which was detectable for the following 72 hours.

Latency: All rabbits from group B(v)a, and two of the three

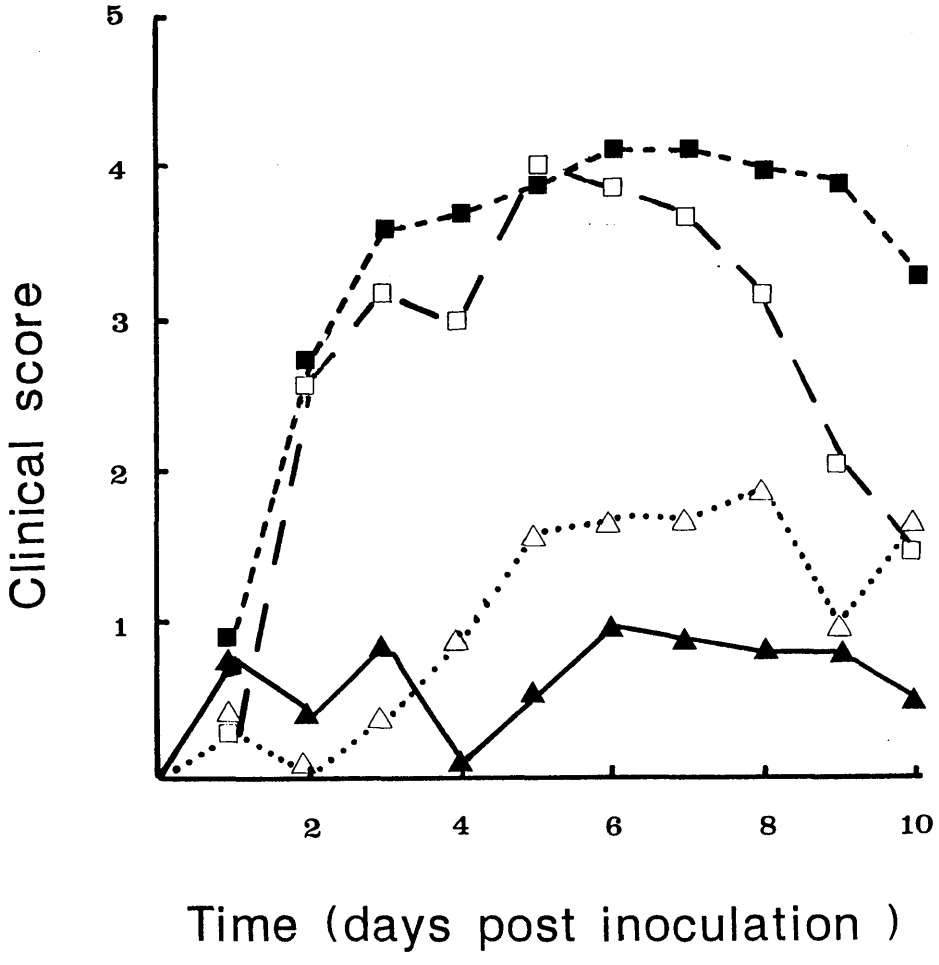
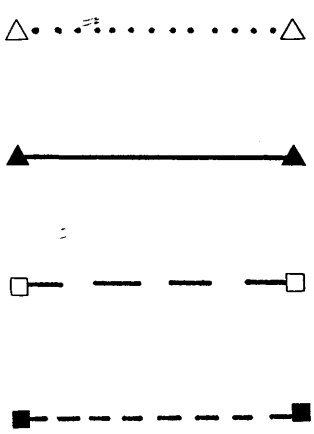

Clinical score
5
4
3
2
1
2
4
6
8
10
Time (days post inoculation)

FIGURE 26

Average clinical score of primary infection with HSV-2 strain HG52.



△.....△ group B (v) a infecting dose 5×10^6 pfu/eye (u)

▲——▲ group B (v) b infecting dose 5×10^7 pfu/eye (u)

□— — — —□ group B (v) c infecting dose 5×10^6 pfu/eye (s)

■- - - - - -■ group B (v) d infecting dose 5×10^7 pfu/eye (s)

(u) - unscarified

(s) - scarified

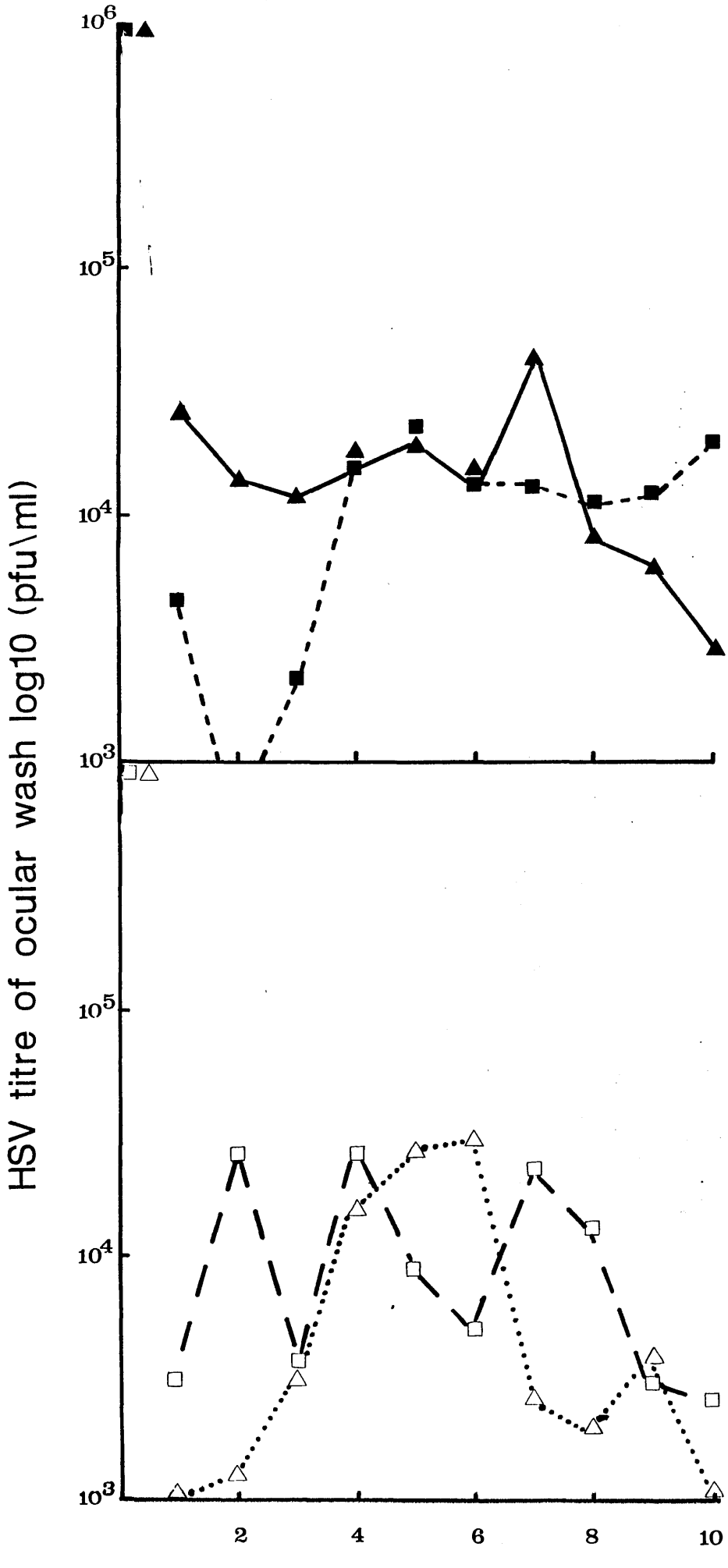
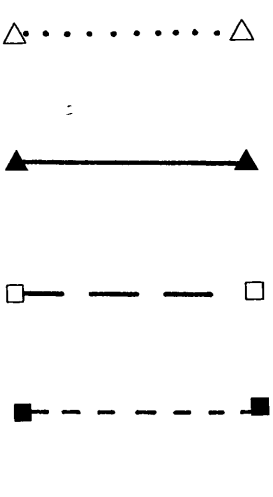

HSV titre of ocular wash log10 (pfu\ml)
10^6
10^5
10^4
10^3
10^5
10^4
10^3
2
4
6
8
10
Time (days post inoculation)

FIGURE 27

Average titration of primary infection with HSV-2 strain HG52.



group B (v) a	infecting dose 5×10^6 pfu/eye (u)
group B (v) b	infecting dose 5×10^7 pfu/eye (u)
group B (v) c	infecting dose 5×10^6 pfu/eye (s)
group B (v) d	infecting dose 5×10^7 pfu/eye (s)

(u) - unscarified

(s) - scarified

Symbols on the Y axis indicate the infecting dose.

rabbits from group B(v)b were sacrificed between days 43 and 45 post inoculation. The third rabbit from group B(v)b was sacrificed on day 58 post inoculation. Trigeminal ganglia and corneas were explanted, randomly divided and screened for latent HSV. No HSV was detected. This result from the trigeminal ganglia was very surprising, and suggested that dissection was inadequate. The technique was therefore re-demonstrated. It appeared that trigeminal nerve root rather than ganglion was being explanted.

The five remaining animals in groups B(v)c and d were sacrificed and tissues explanted for screening on day 59. Left trigeminal ganglia from animals in group B(v)c shed virus for the first time on days 8, 11 and 18 respectively. No right ganglia shed virus. No corneas shed virus. The left ganglia from both animals in group B(v)d shed virus on day 8 post explantation. No right ganglia or corneas shed virus.

EXPT (6) group A(vi) 4 rabbits; infecting dose 5×10^6 pfu/eye HSV-1 strain 17 (unscarified)

group B(vi) 3 rabbits; infecting dose 5×10^7 pfu/eye HSV-2 strain HG52 (unscarified)

group C(vi) 4 rabbits; infecting dose 5×10^5 pfu/eye HSV-1 strain McKrae (unscarified)

Clinical scoring: The average clinical score from each group of animals on days 1-10 post inoculation is shown in figure 28.

Titration: The course of the primary infection was not

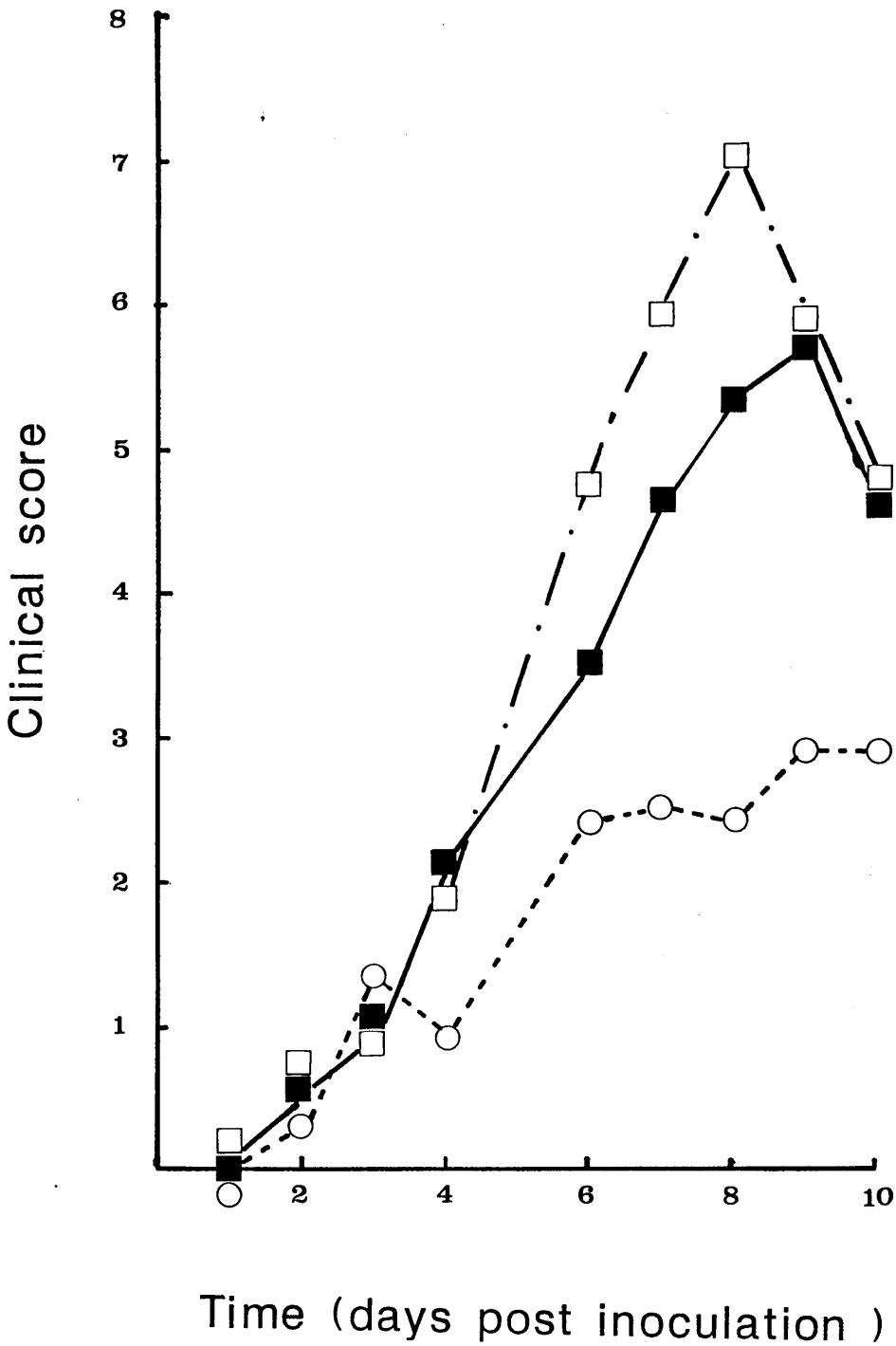
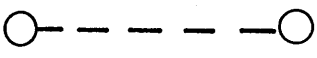

Clinical score
8
7
6
5
4
3
2
1
2
4
6
8
10
Time (days post inoculation)

FIGURE 28

Average clinical score of primary infection with HSV-1 strains 17 and McKrae and HSV-2 strain HG52.

group A (vi) infecting dose 5×10^6 pfu/eye HSV-1 strain 17



group B (vi) infecting dose 5×10^7 pfu/eye HSV-2 strain HG52

group C (vi) infecting dose 5×10^5 pfu/eye HSV-1 strain McKrae

titrated. Virus was present in the ocular washings of all animals on day 7, post inoculation.

Mortality: An animal from group A(vi) was put down on day 16 post inoculation because of severe excoriation and scratching of the left lower lid. A rabbit from group C(vi) was put down on day 31 because of a necrotising keratitis.

Spontaneous shedding: Animals were sampled for spontaneous shedding between days 20-29 post inoculation. Spontaneous shedding of HSV occurred, in one animal of group A(vi) and one animal of group B(vi) both on day 27 post inoculation and for one day only.

Iontophoresis: Animals were subjected to iontophoresis of epinephrine on days 29-31. No rabbits from groups A(vi) or B(vi) including the spontaneous shedder, shed HSV after iontophoresis. The three surviving animals in group C(vi) all shed HSV 72 hours after first iontophoresis for a further 72 hours, 24 hours and 48 of 96 hours respectively. Iontophoresis of epinephrine was repeated on days 67-69 on the three animals inoculated with the HSV-1 strain McKrae. Two of the three animals shed ocular HSV 96 hours post iontophoresis, one for 72 hours, the other only on one day. No ocular shedding of virus was detected in the third rabbit. The technique of iontophoresis of epinephrine thus gave partially repeatable induced reactivation (two out of three) and ocular shedding with the HSV-1 strain McKrae.

Latency: Group A(vi); two animals shed virus from the explanted left trigeminal ganglion on days 6 and 10 post explantation. Another animal shed HSV from the right trigeminal ganglion on day 12 and the left ganglion on day 22 post explantation. The fourth animal in the group first

shed from the left on day 10 and the right on day 28 post explantation. All corneas were negative.

Group B(vi); virus was shed from the left ganglion on days 15 and 22 in two rabbits, and from the left on day 7 and the right on day 13 in the third animal. All corneas were negative.

Group C(vi); see table 3.
Virus was shed from all left ganglia and three right ganglia. Virus was also detected from the left cornea of animal 3. This animal was sacrificed on day 31 because of a necrotising keratitis. However ocular washings for the 2 days prior to death were negative for HSV.

EXPT (7) group C(vii) 14 rabbits; infecting dose 5×10^5 pfu/eye HSV-1 strain McKrae (unscarified)

Clinical scoring: Animals were examined on days 5 and 10 post inoculation for evidence of clinical ocular herpetic disease. All animals had clinical evidence of herpetic disease on days 5 and 10.

Titration: Primary infections were not titrated. Clinical disease was regarded as positive proof of infection.

Mortality: Seven of the fourteen animals died within 14 days of the primary infection, six of them from encephalitis. The seventh had severe excoriation of the lower lid and was put down. An eighth animal was moribund on day 27 post inoculation and was put down.

Spontaneous shedding: Animals were sampled for 15 of 17 consecutive days. All animals shed virus on at least two separate occasions, often for more than one day. Animals were sampled between days 24 and 41 post inoculation.

Table 3

Group C(vi):

Detection of latent virus after organ culture (days post-explantation)

		ANIMAL			
		1	2	3	4
S	(L)TG	8	10	6	17
I	(R)TG	28	24	-	10
T	(L)C	-	-	24	-
E	(R)C	-	-	-	-

L = left
R = right
TG = trigeminal ganglion
C = cornea

Iontophoresis: Animals were subjected to iontophoresis between days 46-48 post inoculation. One shed HSV within 48 hours of first iontophoresis, the other five animals shed virus within 72 hours of first iontophoresis.

Latency: All left trigeminal ganglia released HSV-1 into the supernatant medium between 4 and 7 days after explantation. Two right trigeminal ganglia released HSV-1, 5 and 7 days after explantation, but no corneas released HSV-1 after explantation.

RESTRICTION ENDONUCLEASE ANALYSIS

The HSV-1 strain 17 and strain McKrae were analysed simultaneously using seven restriction endonuclease enzymes, namely; HindIII, EcoRI, HpaI, BamHI, PvuII and XbaI. The method of Lonsdale (1979) was used throughout. The XbaI, HindIII, EcoRI and PvuII digests were run on a 0.6% agarose gel. The BamHI, HpaI and KpnI digests were run on a 1% gel. The results are presented in figures 29 and 30. Restriction endonuclease maps for the above enzymes and HSV-1 strain 17 are shown in figure 31.

Analysis:- XbaI profiles, no sites lost and no discernable fragment size differences.

HindIII, the m/n site in McKrae compared to 17 is lost from the U_S region.

The following bands are missing:

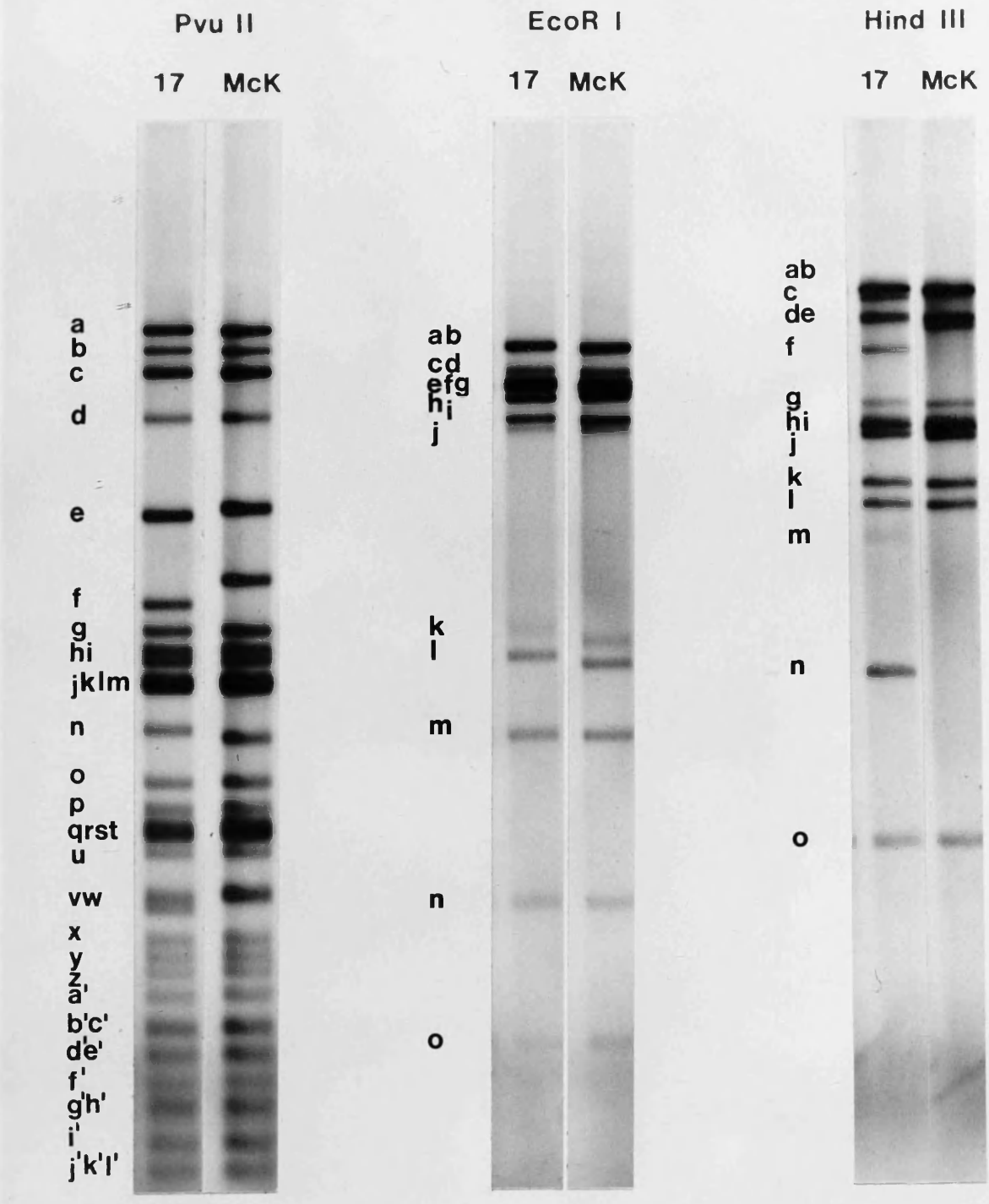
m	U_S	0.83 - 0.88 map units
n	U_S	0.88 - 0.914 map units
f (i+m)	$TR_L + IR_S$	
c (d+m)	$IR_L + IR_S$	

The following new bands are formed:

m(4.5) + n(2.9) total 7.4 band running with h/i

FIGURE 29

Restriction endonuclease analysis of the genomes of HSV-1 strain 17 and strain McKrae using HindIII, PvuII and EcoRI enzymes. Maps for the genome of the HSV-1 strain 17 are shown in figure 31.


Pvu II
17
McK
a
b
c
d
e
f
g
hi
jklm
n
o
p
qrst
u
vw
x
y
z
a'
b'c'
d'e'
f'
g'h'
i'
j'k'l'
EcoR I
17
McK
ab
cd
efg
hi
j
k
l
m
n
o
Hind III
17
McK
ab
c
de
f
g
hi
j
k
l
m
n
o

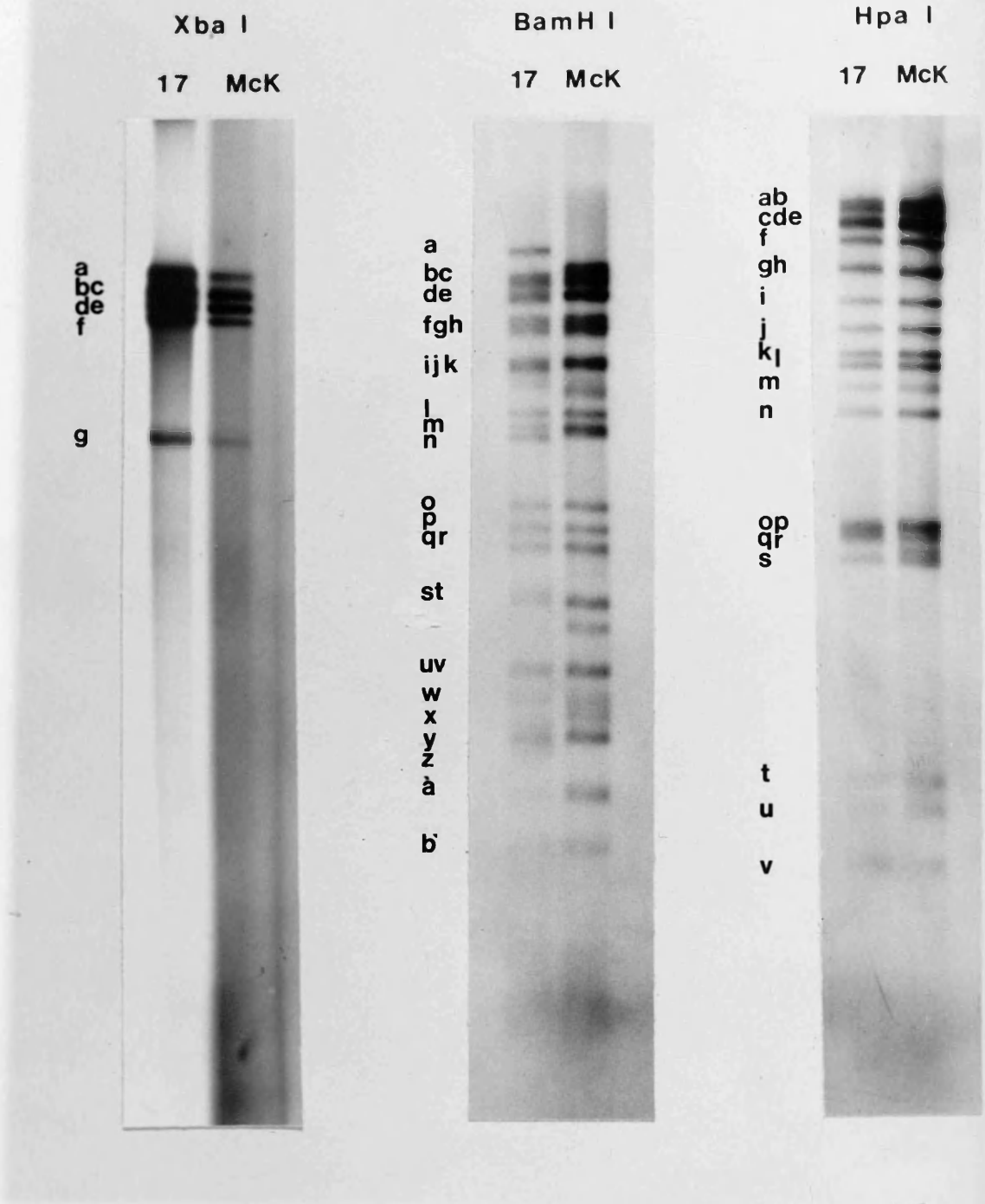
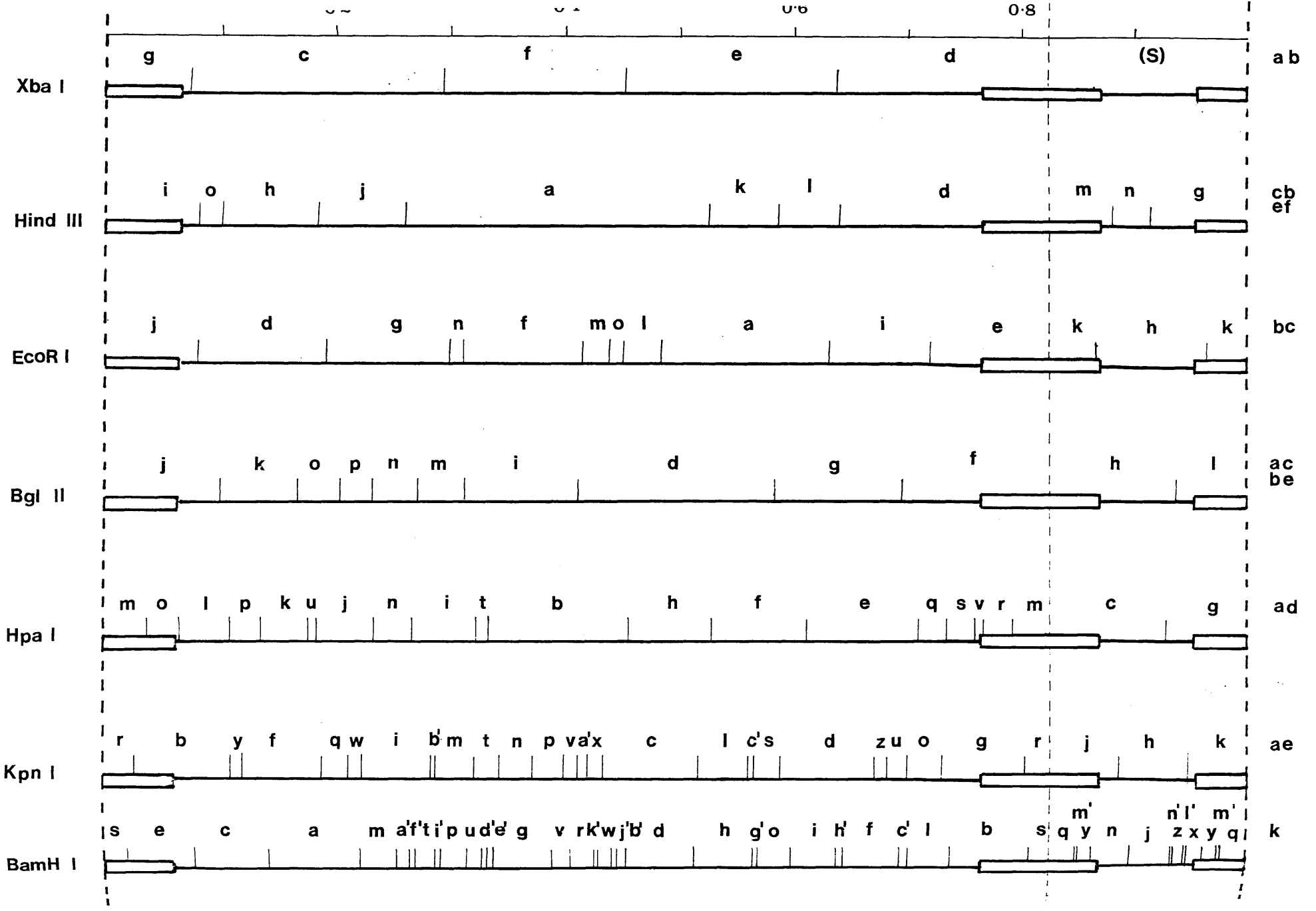

Xba I
17 McK
a
bc
de
f
g
BamH I
17 McK
a
bc
de
fgh
ijk
l
m
n
o
p
qr
st
uv
w
x
y
z
à
b
Hpa I
17 McK
ab
cde
f
gh
i
j
k
l
m
n
op
qr
s
t
u
v

FIGURE 30

Restriction endonuclease analysis of the genomes of HSV-1 strain 17 and strain McKrae using XbaI, BamHI and HpaI enzymes. Maps for the genome of the HSV-1 strain 17 are shown in figure 31.


0·6
0·8
Xba I
g c f e d (S)
a b
Hind III
i o h j a k l d m n g
cb
ef
EcoR I
j d g n f m o l a i e k h k
bc
Bgl II
j k o p n m i d g f h l
ac
be
Hpa I
m o l p k u j n i t b h f e q s v r m c g
ad
Kpn I
r b y f q w i b' m t n p v a' x c l c' s d z u o g r j h k
ae
BamH I
s e c a m a' f' t' i' p u d' e' g v r k' w j' b' d h g' o i h' f c' l b s m' q y n j n' l' m' z x y q
k

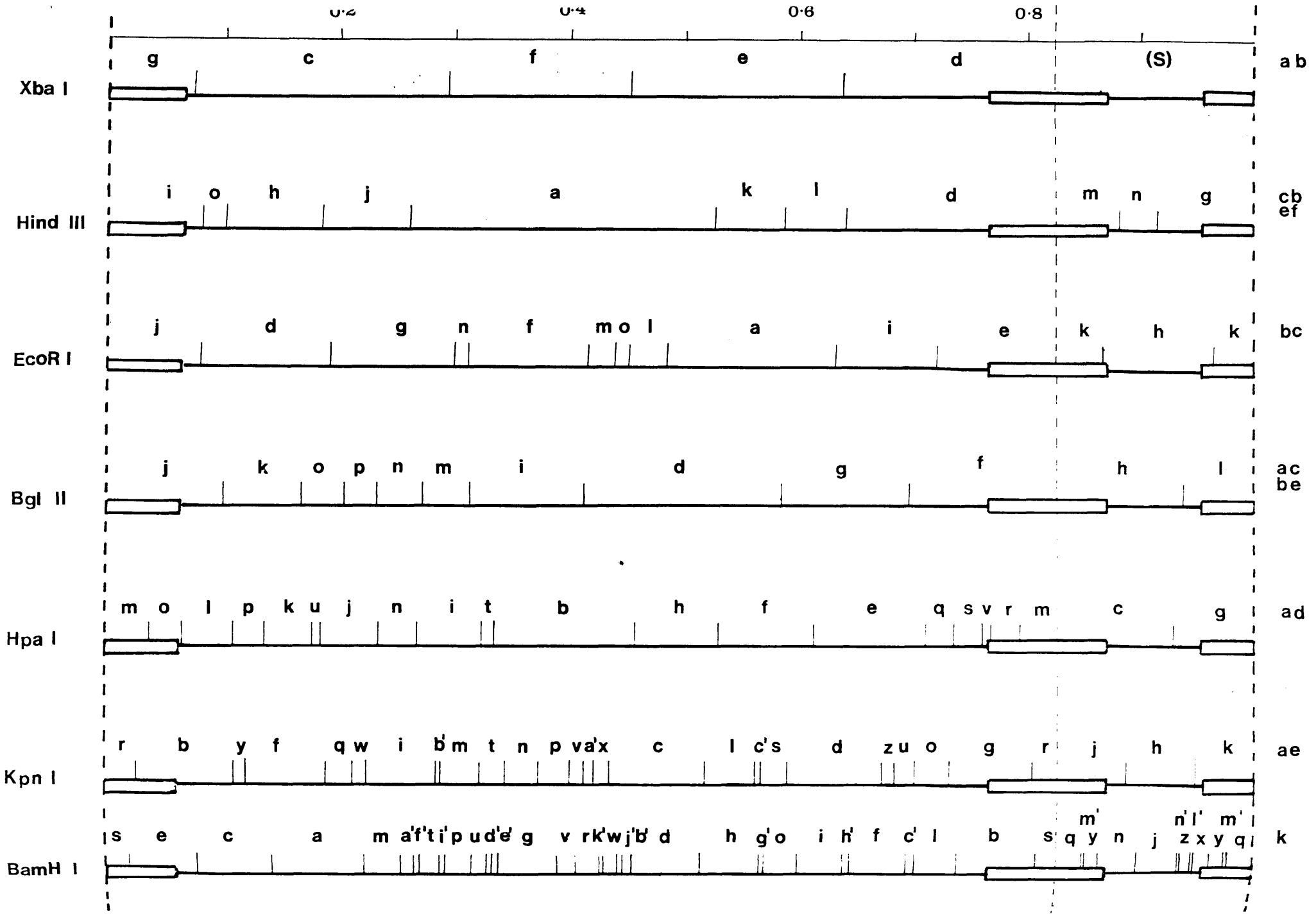

0·2
0·4
0·6
0·8
Xba I
g c f e d (S)
a b
Hind III
i o h j a k l d m n g
cb
ef
EcoR I
j d g n f m o l a i e k h k
bc
Bgl II
j k o p n m i d g f h l
ac
be
Hpa I
m o l p k u j n i t b h f e q s v r m c g
ad
Kpn I
r b y f q w i b' m t n p v a' x c l c' s d z u o g r j h k
ae
BamH I
s e c a m a' f' t' i' p u d' e' g v r k' w j' b' d h g' o i h' f c' l b s q m' y n j n' l' z x m' y q
k

FIGURE 31

Restriction endonuclease maps for the genome of HSV-1 strain 17 using the enzymes XbaI, HindIII, EcoRI, BglII, HpaI, KpnI and BamHI.

m+n + l(7.0)	total 14.4	band runs below d/e	
m+n + d(18)	total 25.4	bands runs with a/b	

EcoRI, no sites lost.

Fragment size differences:

l	U_L	smaller	approx. 0.1×10^6
k	IR_S	smaller	approx. 0.1×10^6
n	U_S	larger	approx. $0.1\text{-}0.2 \times 10^6$

PvuII, no sites lost

Fragment size differences:

e	U_S	larger	approx. 0.1×10^6
f	U_L	larger	approx. $0.2\text{-}0.3 \times 10^6$
n	U_L	smaller	approx. 0.1×10^6
w	IR_S	larger	approx. 0.1×10^6

BamHI, no sites lost.

Fragment size difference:

a	U_L	smaller	approx. 0.5×10^6
n	IR_S+U_S	larger	approx. 0.1×10^6
s or t	IR_L	smaller	approx. $0.1\text{-}0.2 \times 10^6$
y or z	IR_S	larger	approx. 0.1×10^6

HpaI, no sites lost.

Fragment size differences:

Some size alterations in the opqrs group, probably the changes are in the i, o, or r terminal fragments.

pqrs	U_L
o	TR_L

DISCUSSION

Primary infections

(i) Clinical scores: The three strains of HSV used in experiments are all capable of inducing ocular herpetic disease. The HSV-1 strain 17 induced ocular disease at the

lowest m.o.i., 5 x 10^5 pfu/eye, expt. 1. The ocular signs were maximal on day 7 and almost completely resolved by day 13. When the m.o.i. was increased to 10^6 pfu/eye, expt. 2, and 10^7 pfu/eye, expt. 3, the ocular signs were maximal on day 8, and diminishing thereafter (fig. 23). This figure also shows that the higher m.o.i. did not lead to more severe ocular disease, confirming the results of Wander et al. (1980). The HSV-2 strain HG52 induced no discernible ocular disease at 5 x 10^5 pfu/eye, expt. 1. When higher m.o.i.'s were used, expt. 5, ocular disease occurred, however there was minimal difference in the clinical appearance of eyes infected at 5 x 10^6 pfu/eye or 5 x 10^7 pfu/eye (fig. 26). The severity of ocular disease was increased when HSV-2 strain HG52 was inoculated onto a scarified cornea. Scarifying the cornea presumably allows virus to adsorb to a greater number of cells, and also increases the initial depth of viral penetration within the cornea. Infection thus becomes established, superficially and deeply. The clinical course of eyes infected with the HSV-1 strain McKrae at a m.o.i. of 10^5 pfu/eye, expt. 4, is shown in figure 24. The signs of infection were maximal on day 7 and diminish thereafter. This was broadly similar to the results with the HSV-1 strain 17. Within an experimental group of animals the clinical score varied widely. This is demonstrated in figure 23 where animals that died of encephalitis or were destroyed because of excoriation were recorded individually. The clinical scores of all animals in experiment 4 are shown in figure 24. A considerable range existed between the minimum and maximum clinical score after day 3 post inoculation. Larger numbers of animals would be required to determine

significant trends.

When the three strains of virus were used at m.o.i.'s capable of inducing ocular disease and minimising mortality, expt. 6, the HSV-1 strains gave a similar disease score. The HSV-2 strain HG52 had a much reduced clinical score despite the m.o.i. being 1 log greater than the HSV-1 strain 17 and 2 logs greater than the HSV-1 strain McKrae (fig. 28). The ability of the HSV-2 strain HG52 to cause ocular disease was limited in comparison with the two HSV-1 strains, however HG52 is known to be less pathogenic than most HSV-2 strains.

Intertypic and intratypic strain variation is well documented. Oh and Stevens (1973) have suggested that HSV-2 strains were more pathogenic to the eye. This contrasts with the findings above.

ii) Titrations: The average ocular titration for each group of animals during the first 10 days post-inoculation in experiments 1-3 for HSV-1 strain 17 is shown in figure 22. In experiments 1 and 3 at the lower m.o.i.'s, virus appears to be present in the ocular washings in increasing amounts after a lag phase of 24-48 hours. At the highest m.o.i. 10^7 pfu/eye, expt. 2, the amount of virus recovered from the eye decreases steadily after inoculation. Figure 27 shows the average titration of virus in the first 10 days post inoculation with HSV-2 strain HG52, expt. 5. The average titration for the McKrae strain during primary infection is shown in figure 25. Two peaks in the McKrae curve are obvious on days 3 and 8; these were caused by a high titre of virus being found in the eye of one animal, which caused the average to be raised. No relationship between m.o.i., and/or scarification is obvious using the

HSV-2 strain HG52.

As the infection progresses, the clinical score correlates poorly with the virus recoverable from ocular washings. The clinical score takes account of more widespread disease within the corneal stroma and the iris, whereas ocular washings only collect virus produced superficially by corneal and conjunctival epithelium.

Mortality: The mortality of animals inoculated with HSV-1 strain 17 does not increase in relation to the multiplicity of infection, Table 4. For these purposes mortality refers to death from encephalitis within 30 days post inoculation. The HSV-2 strain HG52 has a low incidence of mortality irrespective of m.o.i. The mortality rate for rabbits infected with HSV-1 strain McKrae ranged from 0-50%. The infecting doses used were 10^5 and 5×10^5 pfu/eye. The mortality rates for the McKrae strain are similar to the results of Berman and Hill (1985). Encephalitis was occasionally preceded by the shedding of virus from the non inoculated right eye, expts. 2 and 3. A total of nine animals out of 66 were put down humanely because of severe excoriation around the lower lid. Scratching may have been caused by discharge from the eye (although care was always taken to keep animals clean) or perhaps by spread of virus to the surrounding skin via zosteriform spread (Simmonds and Nash 1984).

Spontaneous shedding: Spontaneous shedding of HSV in the tear film of rabbit eyes has been documented (Nesburn et al., 1967, Laibson and Kibrick 1969, and Berman and Hill 1985). The largest study of spontaneous ocular shedding of HSV in rabbits was performed by Gerdes and Smith (1983). Cumulative data from experiments 1-7 is presented in Table

Table 4

Cumulative Data: Experiments 1-7

Mortality within one month of inoculation due to encephalitis.

		Viral Strain		
		A ($17ts^{+}syn^{+}$)	B (HG52)	C (McKrae)
Multiplicity of Infection	10^5			$3/_7$* (iv)$
	$5x10^5$	0/3 (i)	0/3 (i)	0/4 (vi) 7/14 (vii)
	10^6	$1/_8$ (iii)		
pfu/eye	$5x10^6$	0/4 (vi)	0/6 (v)	
	10^7	$2/_8$ (ii)		
	$5x10^7$		1/6 (v) 0/3 (vi)	

* Number of deaths/number of animals inoculated.

$ Experiment number

5. The small number of animals used in experiments, and the infrequent samplings for spontaneous shedding do not permit any valid comparison with the results of Gerdes and Smith (1983). Their results suggested that both the McKrae strain and the 17 strain had high frequencies of spontaneous shedding with 5.3% and 2.4% respectively of positive cultures for the total number of cultures taken. The HG52 strain had a very low frequency of spontaneous recurrence at 0.15%. The spontaneous shedding results from my experiments (1-6) give a frequency of spontaneous shedding for the 17 and McKrae strains considerably less than that of Gerdes and Smith (1983). The spontaneous shedding result from experiment 7 give a considerably higher frequency. No valid conclusions can be drawn from this series of experiments. The importance of spontaneous ocular shedding of HSV was noted by Shimomura et al. (1983) who observed that latently infected rabbits that shed virus spontaneously could be induced to shed virus at a much higher frequency and for a longer duration than animals that had not shed virus spontaneously. Spontaneous shedding proves that both HSV latency and reactivation mechanisms are operating.

Induction of virus shedding after iontophoresis: The induction of ocular shedding of HSV-1 following iontophoresis of epinephrine into the rabbit eye was described by Kwon et al. (1981). The iontophoresis of epinephrine reactivates latent HSV within the trigeminal ganglion, and infectious virus can be quantified from cell free homogenates of the ganglion after iontophoresis (Shimomura et al., 1985).

The cumulative results from experiments 1-7, Table 6, suggest the HSV-1 strain 17 is capable of induced

Table 5

Cumulative Data: Experiments 1-7

Spontaneous viral shedding.

		Viral Strain		
		A ($17ts^{+}syn^{+}$)	B (HG52)	C (McKrae)
Multiplicity of Infection	10^5			$^1/_{60}$* (iv)$
	$5x10^5$	0/40 (i)	0/60 (i)	30/90 (vii)
	10^6	$^0/_{120}$ (iii)		
pfu/eye	$5x10^6$	1/30 (vi)		
	10^7	$^3/_{60}$ (ii)		
	$5x10^7$		1/30 (vi)	

* Number of days viral shedding/number of days sampled.

$ Experiment number

Table 6

Cumulative Data: Experiments 1-7

Iontophoresis induced viral shedding.

		Viral Strain		
		A ($17ts^{+}syn^{+}$)	B (HG52)	C (McKrae)
Multiplicity of Infection	10^5			2/2* 4$
	$5x10^5$	0/3 (i)	0/3 (i)	3/3 (vi) 2/3 (vi) 6/6 (vii)
	10^6	$^2/_6$ (iii)		
pfu/eye	$5x10^6$	0/3 (vi)	1/6 (v)	
	10^7	$^1/_3$ (ii)		
	$5x10^7$		1/5 (v) 0/3 (vi)	

* Number of animals shedding virus post iontophoresis/ number of animals undergoing iontophoresis.

$ Experiment number

reactivation and ocular shedding following iontophoresis of epinephrine. However the results were not repeatable as subsequent treatments failed to induce ocular shedding of virus. The rabbits of the 17 strain that shed virus spontaneously did not have iontophoresis induced virus shedding. The picture for the HSV-2 strain HG52 is similar, but the frequency of induced reactivation is lower.

In contrast the HSV-1 strain McKrae had a 100% frequency of induced reactivation in the three groups of rabbits subjected to iontophoresis. The process was repeatable in two out of three rabbits, experiment 6. Induced shedding of ocular HSV-1 was therefore produced in some animals that failed to shed virus spontaneously.

A recent study by Hill et al. (1987) concluded that the 17 strain had a low frequency of spontaneous reactivation and shedding, 3.3%, but a high frequency of induced shedding, 67%. The study was based on six survivors from twenty animals bilaterally inoculated through a scarified cornea.

Latency in neural and extra neural tissue: HSV was always recovered from all left trigeminal ganglia explanted and then screened for latent HSV, irrespective of whether or not the animal had a spontaneous or induced reactivation of ocular virus shedding during life, Table 7. This implies that HSV was always present in the ganglia during life, and thus potentially available for reactivation. Failure to spontaneously shed virus or failure to shed virus after the iontophoresis of epinephrine, suggests that epinephrine is not the sole stimulus to reactivation, and that viral genetic differences are involved in view of the intra- and intertypic strain variation. Virus from right trigeminal

Table 7

Cumulative Data: Experiments 1-7

Virus latent in left trigeminal ganglion

Multiplicity of Infection	Viral Strain		
	A ($17ts^{+}syn^{+}$)	B (HG52)	C (McKrae)
10^5			$^4/_4$*o (vi)$
$5x10^5$	1/1 (i)		6/6 (vii)
10^6			
$5x10^6$	3/3 (vi)	3/3 (v)	
10^7			
$5x10^7$		2/2 (v) 3/3 (vi)	

* Number of positive left trigeminal ganglia/number of animals.

$ Experiment number

o Not included are negatives which occurred as a result of inexpert dissection. Details available in results section.

ganglia was also detected from animals in group A(i) one rabbit; group A(vi) two rabbits; group B(vi) one rabbit; group C(vi) three rabbits and group C(vii) two rabbits. Animals were not inoculated via the right eye. It is likely that HSV reached the right trigeminal ganglion during the course of the primary infection, with virus spreading from the left eye to the left trigeminal ganglion, through the central nervous system to the right trigeminal ganglion. The spread of virus may stop there, or it may continue to the right eye as happened with several rabbits who had an encephalitis, expts. 2 and 3. A similar route of spread was postulated by Tullo et al. (1982a and b), to explain latent HSV in non ophthalmic divisions of the trigeminal ganglion after ocular inoculation. Two other explanations have to be considered for spread to the right trigeminal ganglia; the animals may cross-infect between right and left eyes by scratching; and the mucous membranes of the eyes are in continuity via the nasolacrimal tear passages and nasopharynx.

The McKrae strain of HSV-1 was also recovered from the left cornea of one rabbit, expt. 6, after 24 days in organ culture. This animal had a severe necrotising keratitis and was put down because of this. However ocular washings for the two days prior to death were negative. In addition virus was recovered from the left trigeminal ganglion after only 6 days of organ culture. HSV has been recovered from other non neural sites after explantation and culture, namely mouse skin (Hill et al., 1983), mouse footpads (Al-Saadi et al., 1983), and ocular posterior segments in mice (Openshaw, 1983) in latently infected animals. HSV has also been recovered from human corneas after

explantation and culture (Shimeld et al., 1982, Tullo et al., 1985 and Cook et al., 1986). Batra (1987) confirmed that HSV-1 strain McKrae was recoverable from the corneas of latently infected rabbits. Virus was always recovered later from the corneal explants than from the trigeminal ganglion explants. The observation has been repeated using an HSV-1 McKrae/HSV-2 HG52 recombinant whose genome structure is McKrae except for the sequence between 0.33 and 0.56 map units (Cook et al., 1987).

In summary the HSV-2 strain HG52 is not pathogenic to rabbit eyes, and is not neuropathogenic. It has a low frequency of spontaneous reactivation and ocular shedding for a HSV-2 virus, and a very low frequency of induced reactivation after iontophoresis of epinephrine. The HSV-2 strain HG52 is able to maintain latent infections within the trigeminal ganglion. The HSV-1 strain 17 is pathogenic to rabbit eyes, and is neuropathogenic. It has a low frequency of spontaneous reactivation, and an intermediate frequency of induced viral reactivation, which is unreliable in terms of repeatability. Latency is established within the trigeminal ganglion. The HSV-1 strain McKrae is pathogenic to rabbit eyes and particularly neuropathogenic, even at the lowest m.o.i. tested (10^5 pfu/eye). It has a high frequency of both spontaneous and induced viral reactivation. Induced reactivation is repeatable. The McKrae strain is able to maintain latent trigeminal ganglion infections. The McKrae strain appears to differ in that it has a limited ability to maintain a latent infection within the cornea. These three viruses, particularly the HSV-1 strain McKrae and the HSV-2 strain HG52 with distinct biological properties will provide

useful tools in determining regions of the genome responsible for reactivation and neuropathogenic. Batra (1987) has constructed McKrae XHG52 recombinants to study latency reactivation differential. Thompson et al. (1983, 1985) utilized intertypic recombinants from the HSV-1 strain 17 and HSV-2 strain HG52, to locate the neuro-virulence region of the genome to between 0.71 and 0.74 map units in the HSV DNA sequence. Recent work by Halliburton et al. (1987) using 31 different HSV-1 X HSV-2 intertypic recombinants is less definite about the location of the genes for neurovirulence and suggests that this biological function may be under multigenic control.

Restriction endonuclease analysis: Only limited mapping data is available for the HSV-1 strain McKrae from Gerdes and Smith (1983), who reported the loss of the following 17 strain defined sites in the McKrae strain: HindIII m/n, KpnI c/x and a HpaI k site. My restriction endonuclease results confirm the loss of the HindIII m/n site in the short unique section but show a similar HpaI map with no site losses. No sites are lost with the other enzymes tested, EcoRI, PvuII, BamHI and XbaI, although fragment size variation exists. Batra (1987) has made a more detailed examination of the KpnI site differences and confirmed the loss of the KpnI c/x site and the KpnI d/z site. The McKrae strain also had an additional KpnI site in TR_L and IR_L.

Variation in restriction maps between HSV-1 strains is predictable. Correlating different biological properties and relating them to virus genes, and DNA sequences represents the work of a lifetime.

CHAPTER 4

IN VITRO EXPERIMENTS

Growth and characterization of rabbit corneal cells in vitro

MATERIALS

Rabbits: Young female animals 3-6 months old and weighing between 1.5-2kg were obtained from Hyline Rabbit Farms. Outbred New Zealand white rabbits were used.

Dissection of the cornea was performed using a Griffin dissecting microscope.

Media: Epithelial cells were grown in Glasgow modified Eagle's medium plus 10% (v/v) foetal calf serum, supplemented with penicillin/streptomycin 100 units/ml, and L-glutamine 1% (v/v)(EFC_{10}). Keratocytes and endothelial cells were grown in 20 mM Hepes buffered Dulbecco's medium plus 10% (v/v) foetal calf serum supplemented with penicillin/streptomycin 100 units/ml, and L-glutamine 1% (v/v)(DFC_{10}). Epithelial cells were stored in Eagle's medium 65%, supplemented as above with penicillin/ streptomycin and L-glutamine, foetal calf serum 25% (v/v) and glycerol 10% (v/v). Keratocytes were stored in Dulbecco's medium 65% (v/v), supplemented as above with penicillin/streptomycin and L-glutamine, foetal calf serum 25% (v/v) and glycerol 10% (v/v).

Glasswear: Cells were grown in 25cm^2, 80cm^2 and 175cm^2 plastic tissue culture flasks supplied by Nunclon.

Antisera : Monospecific rabbit antiserum against human plasma fibronectin was obtained from Bethesda Research Laboratories. Polyspecific guinea pig anti keratin antiserum was obtained from Miles Scientific. Antibody binding was visualised with sheep anti rabbit IgG,

conjugated to fluoroscein, supplied by Wellcome, and rabbit anti guinea pig IgG conjugated to fluoroscein, supplied by Miles Scientific.

Electron microscopes: A Jeol T200 scanning electron microscope and a Philips 301 transmission electron microscope were used (Work done by Mrs. D.A. Aitken, Tennent Institute of Ophthalmology).

METHOD

Cells: Rabbit corneal cells were prepared by the microdissection method described by Stocker et al. (1958). Briefly, the eyes of freshly killed rabbits were ennucleated. Corneal explant cultures were prepared within a laminar flow hood. The cornea was separated from the globe by a circumferential incision 1mm within the limbus and then anchored to a sterile dissecting block, epithelial surface face down. Descemet's membrane with its attached endothelial cells was peeled from the underlying stroma, divided and placed in a $25cm^2$ tissue culture flask containing medium. Endothelial cells grew from Descemet's membrane. A cleavage plane was formed in the stroma close to the epithelium, and the superior stromal tissue containing keratocytes was removed, divided and placed in medium. The remaining tissue which consisted largely of epithelium was treated similarly, fig. 9. Secondary cultures were obtained by splitting confluent primary cultures 1:2 with trypsin/versene. Cultures were grown at 37^oC in a humidified incubator with 5% CO_2.

Growth kinetics of cells: Suspensions of rabbit corneal epithelial cells, keratocytes or endothelial cells at passage no. 3, were seeded at 10^5 cells onto 35mm plastic

tissue culture plates (Nunclon). Cells were grown at 37°C in a humidified incubator with 5% CO_2, and harvested after 1, 3 or 6 days in culture. Cells were counted on a haemocytometer.

Storage and recovery of cells: Secondary cultures were prepared for storage by harvesting a large tissue culture flask (175cm^2), and suspending the cells 5 x 10^7 approximately, in 5mls of EFC_{10} or DFC_{10}. The suspension was spun at 2,000 rpm for 5 minutes and the cells resuspended in 1ml of storage medium. Cells were then frozen slowly in an insulated box at -70°C for 24 hours before transfer to -170°C. Cells were recovered by rapid thawing in a 37°C water bath. The cells were then resuspended in appropriate medium and grown at 37°C.

Indirect immunofluorescence: Indirect immunofluorescence was performed following methods described by Kennedy et al. (1983). Cultures of the three corneal cell types grown on 12mm glass coverslips were studied. Cells were fixed in methanol for 10 minutes at -20°C before incubation with antikeratin antiserum. Coverslips were washed three times in phosphate buffered saline (PBS) then incubated either with monospecific rabbit anti fibronectin antiserum (diluted 1:30) or polyspecific guinea pig anti keratin antiserum (diluted 1:5) for 30 minutes at 37°C. Coverslips were washed another three times in PBS, and then exposed to the appropriate fluoroscein labelled secondary antibody, sheep anti rabbit IgG (diluted 1:20) and rabbit anti guinea pig IgG (diluted 1:20) for 30 minutes. Coverslips were washed a further three times in PBS then once in distilled water. Coverslips incubated with anti fibronectin antiserum were then fixed in 5% glacial acetic acid with 95% ethanol

(acid-alcohol). Appropriate controls including omission of the primary antibody and the labelled secondary antibody were employed. The fluorescent staining of cells was assessed when coverslips were examined under a Leitz Ortholux fluorescent microscope equipped with epi-illumination and phase contrast optics.

Electron microscopy of corneal cells: Epithelial cells, keratocytes and endothelial cells were seeded onto 35mm plastic tissue culture plates containing 12mm glass coverslips. The coverslips were processed for scanning electron microscopy, critical point dried, gold coated and examined in a Jeol T200 scanning electron microscope. A total of 40 coverslips were examined by scanning electron microscopy (SEM). The plastic plates were prepared for transmission electron microscopy (TEM). After dehydration the monolayer was removed by dissolving the plate in propylene oxide prior to embedding in araldite. Sections were examined using a Philips 301 transmission electron microscope. A total of 20 tissue culture plates were prepared and each cell type was represented a least six times. Sections of 80nm were cut at 20um intervals through the tissue blocks for TEM.

RESULTS

Establishing explant cultures and cellular growth kinetics: Epithelial cells migrated rapidly from the explanted tissue and began to divide on the surface of the tissue culture flask. Only a few explants failed to adhere to the flasks and establish cellular growth within a few days. By contrast stromal cultures were slow to establish as stromal tissue was poorly adherent to the tissue culture flask.

However when secondary cultures were established, the doubling time (time taken for cells to increase their number 2-fold) for epithelial cells and keratocytes was 18 hours. The adherence of Descemet's membrane with attached endothelial cells, to the tissue culture flask was intermediate. Endothelial cells migrated rapidly from Descemet's membrane onto tissue culture flasks, but the doubling time was prolonged to 5 days, and with successive passage, cellular contact became less and cells increased in size. Cellular growth kinetic experiments were performed in duplicate, fig. 32 (endothelial cells excepted).

In my hands, epithelial cells and keratocytes survived up to 12 serial passages, and were successfully recovered after 9 months in storage. Endothelial cells survived eight serial passages, but were not stored.

Electron microscopy of corneal cells: (Electron micrographs were prepared by Mrs. D.A. Aitken.) Cells were prepared for SEM or TEM after 2 days in tissue culture. The preparations showed marked homology within a cell type, and the morphological features were consistent in the large number of sections viewed both by SEM and TEM. Epithelial cells on SEM were small (25um) spindle shaped cells lacking contact inhibition. The nuclear and cellular outlines were poorly defined, figure 33a. By TEM the cells were cuboidal and stratified, figure 33b. Small cytoplasmic processes were present in some cells forming microplicae, figure 33c. The cytoplasm contained small mitochondria, rough endoplasmic reticulum and numerous vesicles which are a common feature of cultured cells. Keratin filaments condensed to form desmosomes at sites of cell contact, figure 33d.

FIGURE 32

Average cellular growth curves.

Top	epithelial cells
Middle	keratocytes
Bottom	endothelial cells

Suspensions of cells at passage number 3 were seeded at 10^5 cells onto 35mm tissue culture plates. Cells were grown at 37°C in a humidified incubator with 5% CO_2 and harvested after 1, 3 or 6 days in culture. Cells were counted on a haemocytometer. Experiments were run in duplicate (endothelial cells excepted).

Symbols indicate the results▲

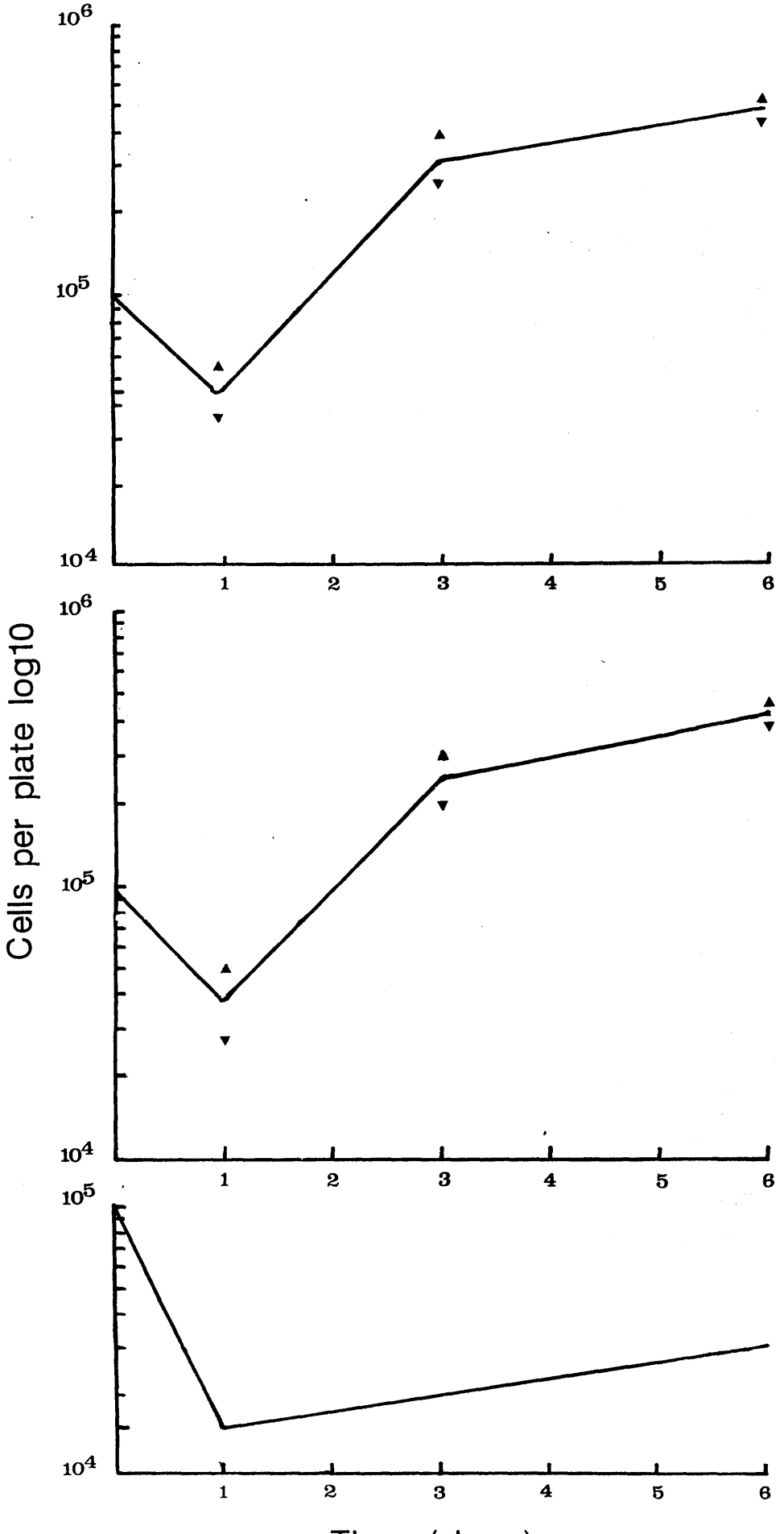
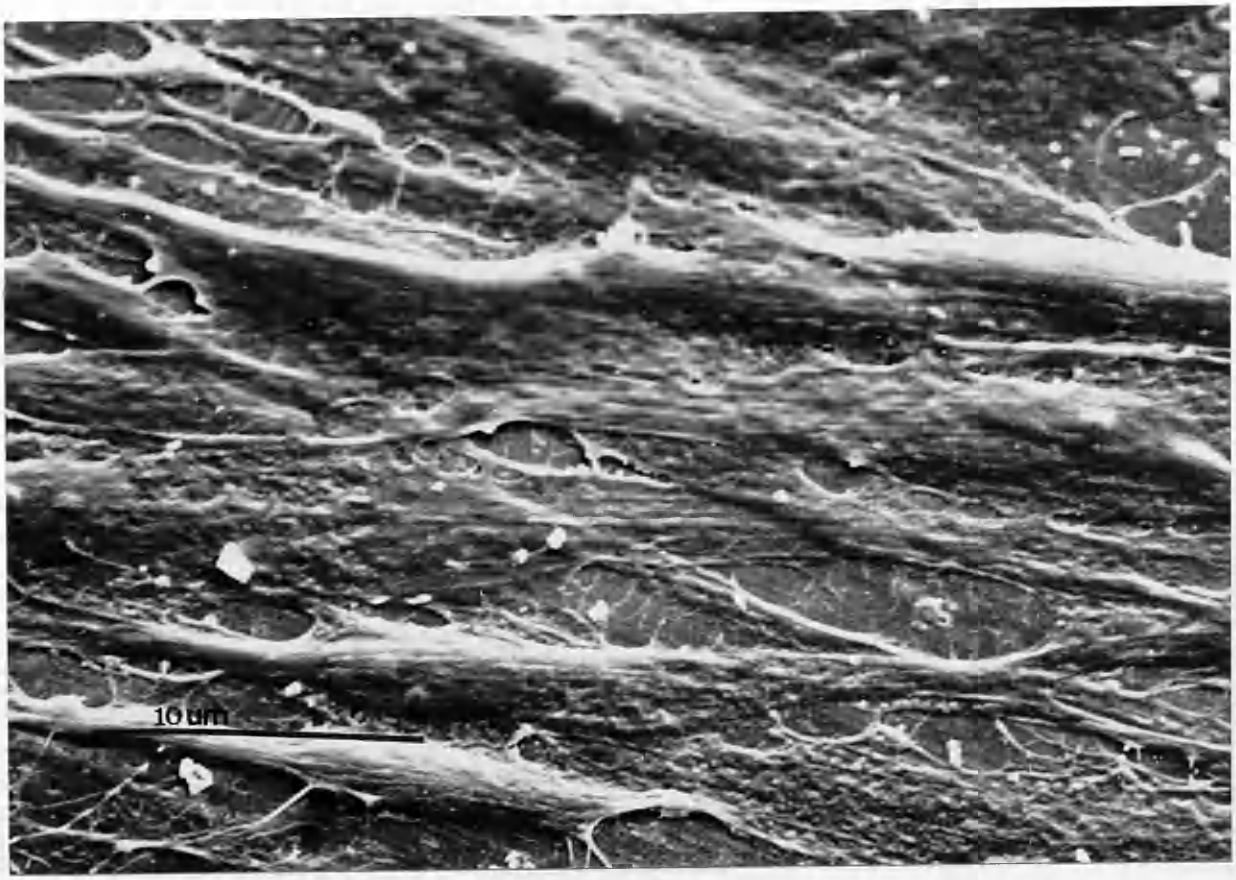

Cells per plate log10
10^6
10^5
10^4
1
2
3
4
5
6
Time (days)

FIGURE 33

Epithelial cells.

(a) Top left	Small spindle shaped cells lacking contact inhibition (SEM x 3,800)
(b) Top right	Stratification of cuboidal cells (TEM x 5,000)
(c) Bottom left	Abundant vesicles (a) and formation of microplicae (b) (TEM x 11,800)
(d) Bottom right	Aggregation of keratin filaments forming desmosomes (a) (TEM x 11,800)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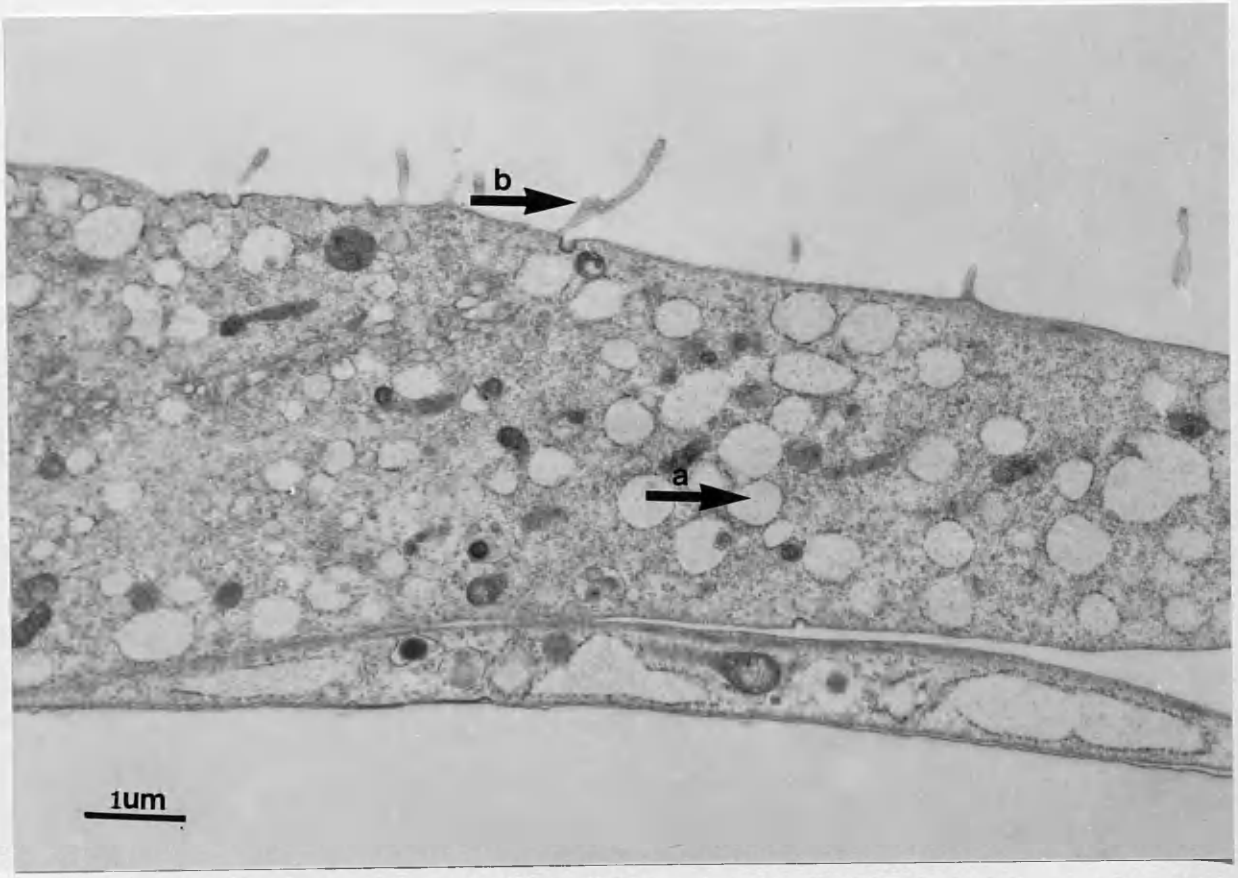
b
a
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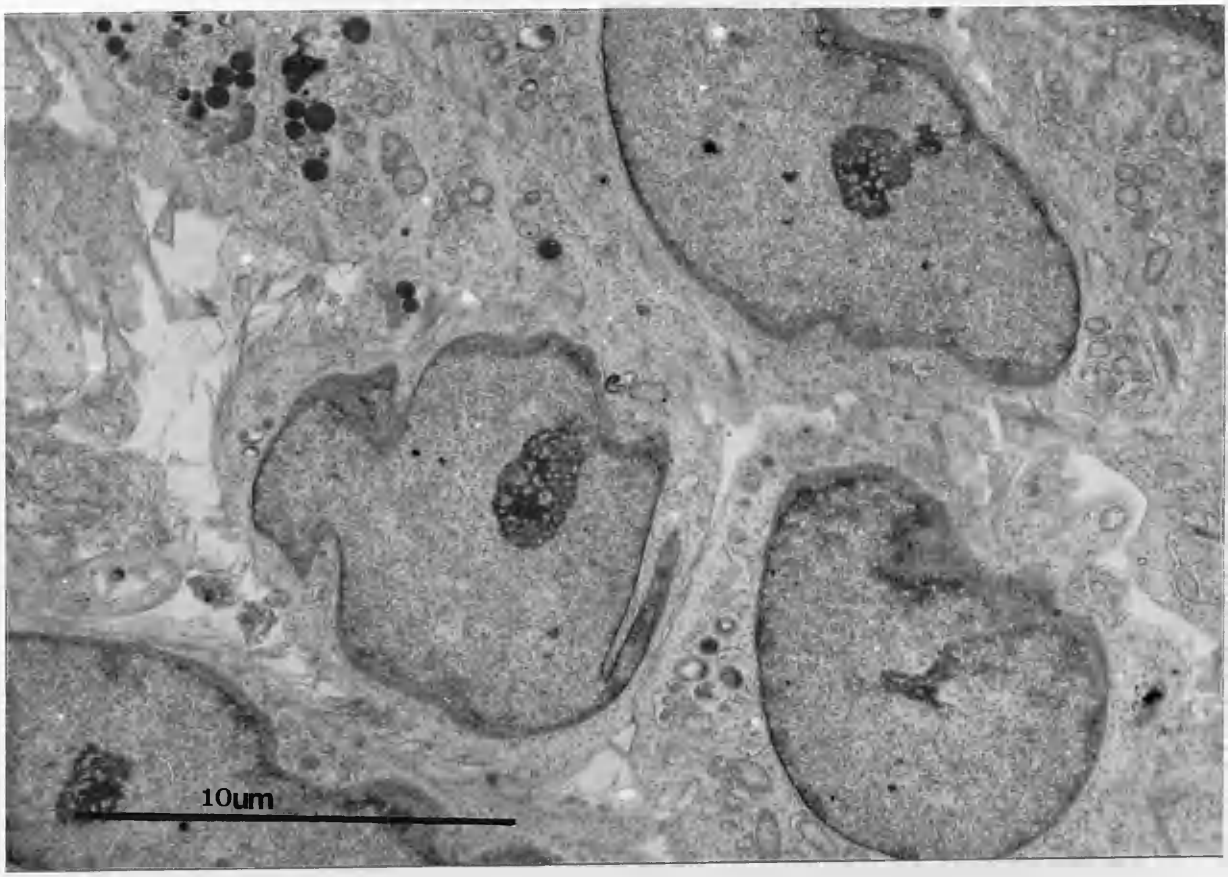
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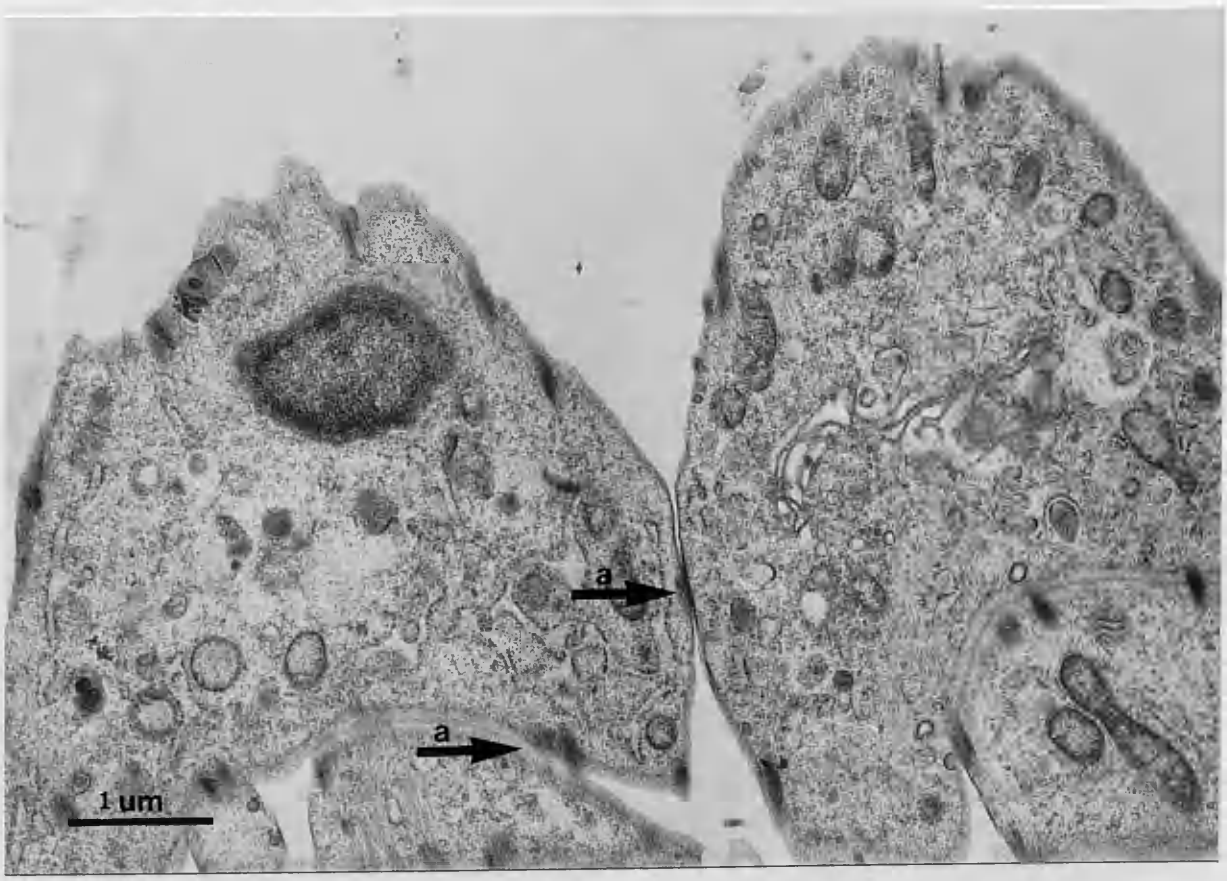
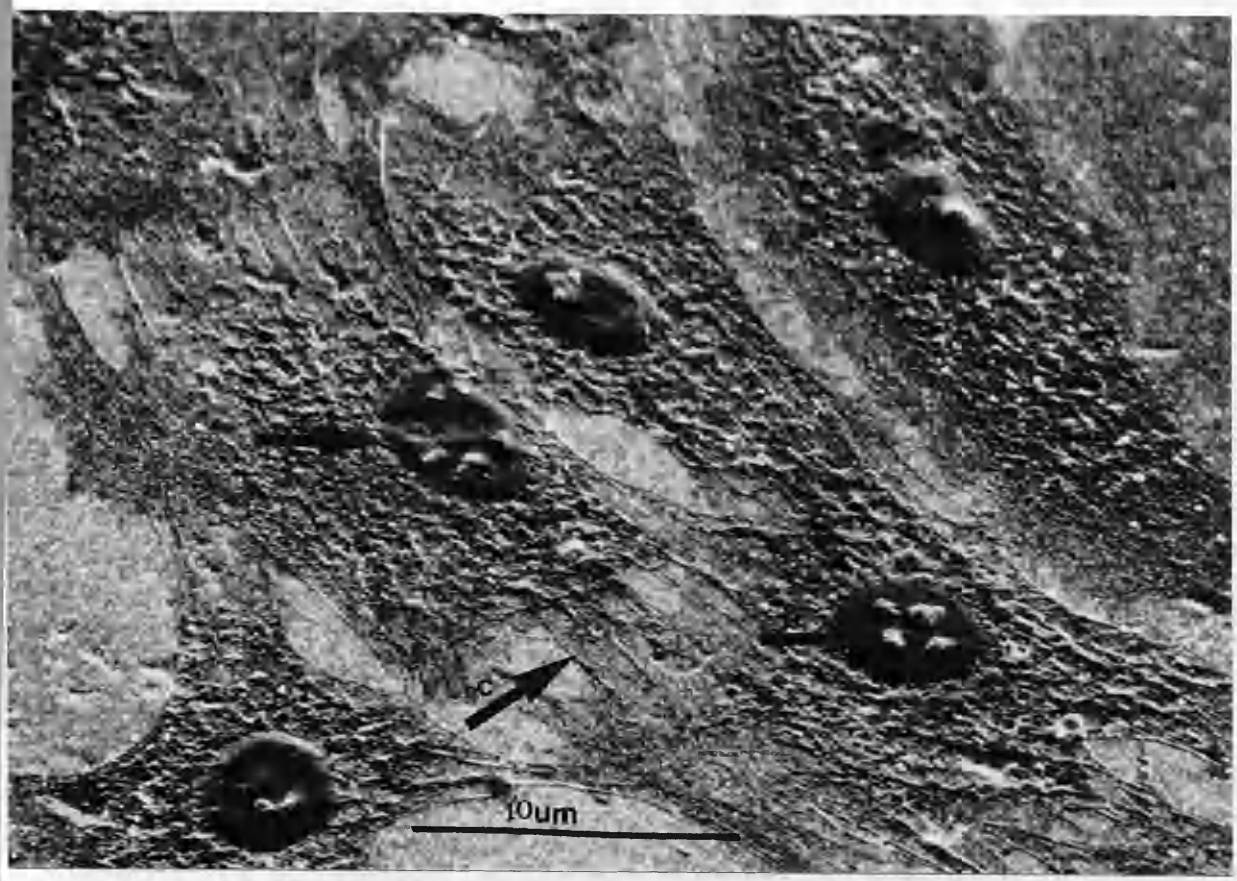

a
a
1 um

FIGURE 34

Keratocytes.

(a) Top left	Cells showing prominent nuclei (a) with nucleoli (b) and overlapping cytoplasmic processes (c) (SEM x 3,000)
(b) Top right	Two cells at right angles (TEM x 6,000)
(c) Bottom left	Dilated profiles of rough endoplasmic reticulum (arrows) (TEM x 40,000)
(d) Bottom right	Nucleoli (a) within prominent nucleus (b) (TEM x 9,500)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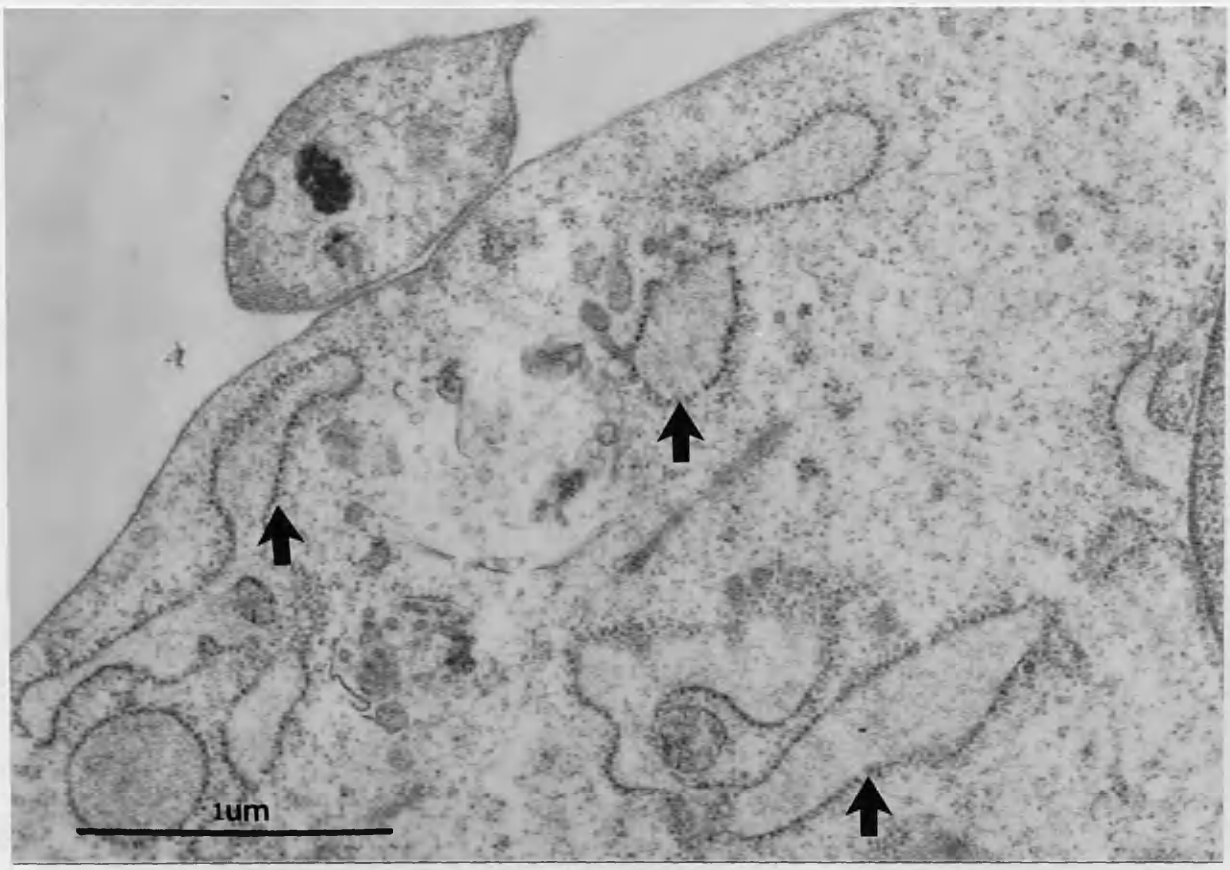
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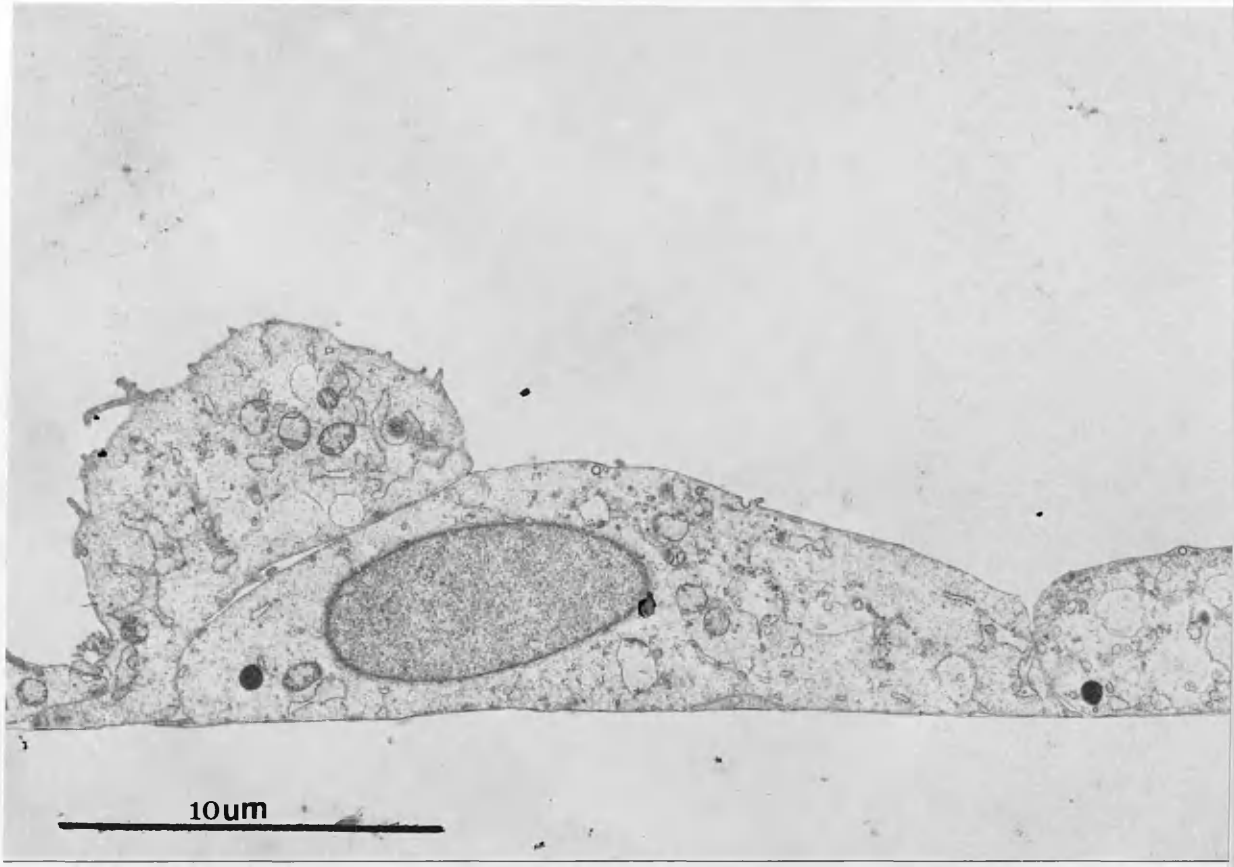
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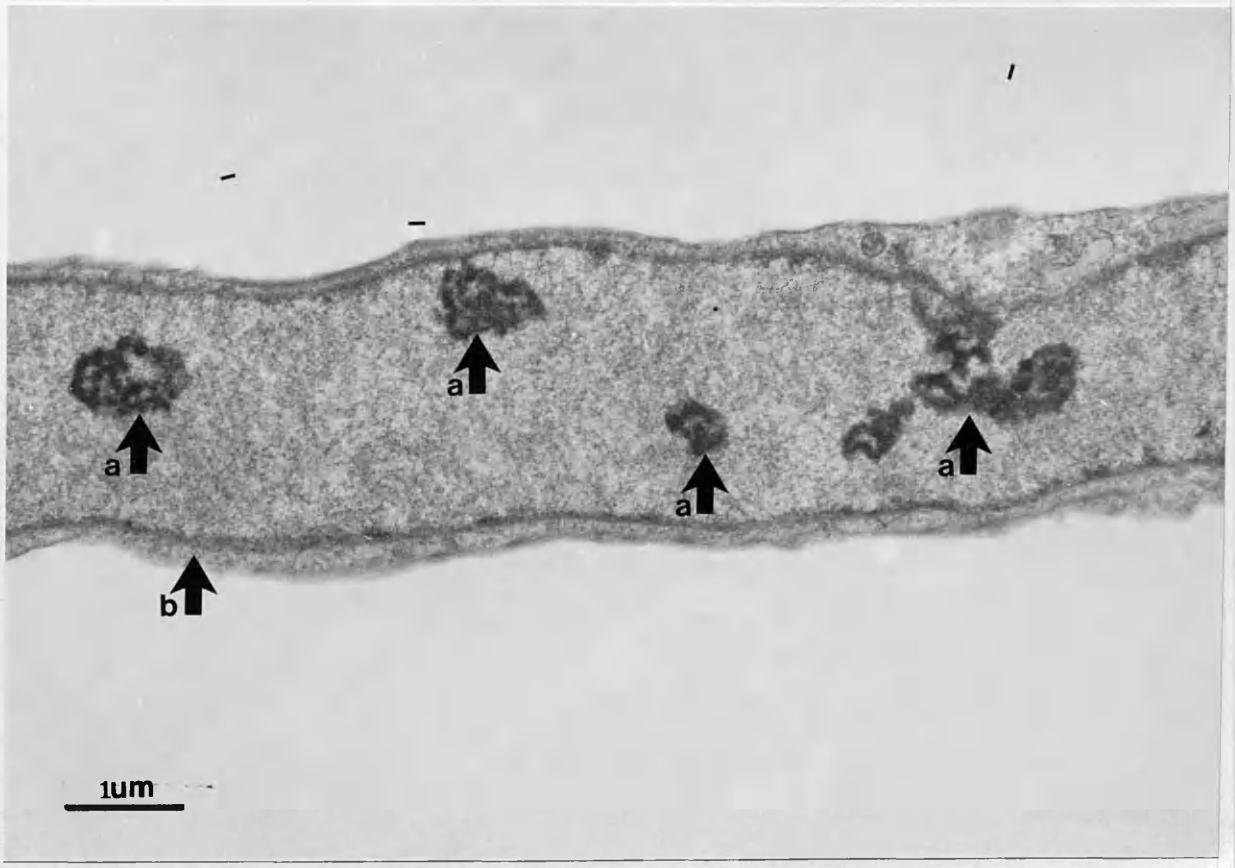
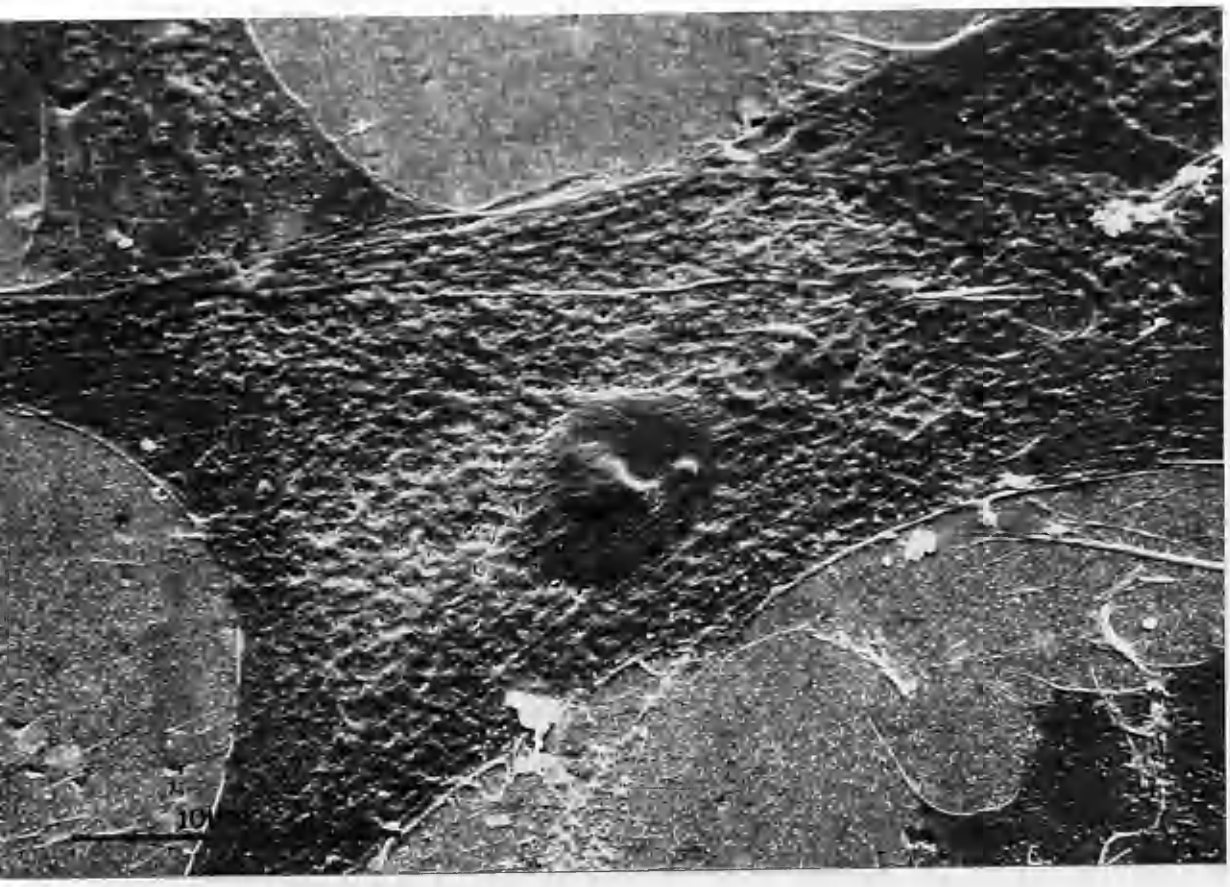
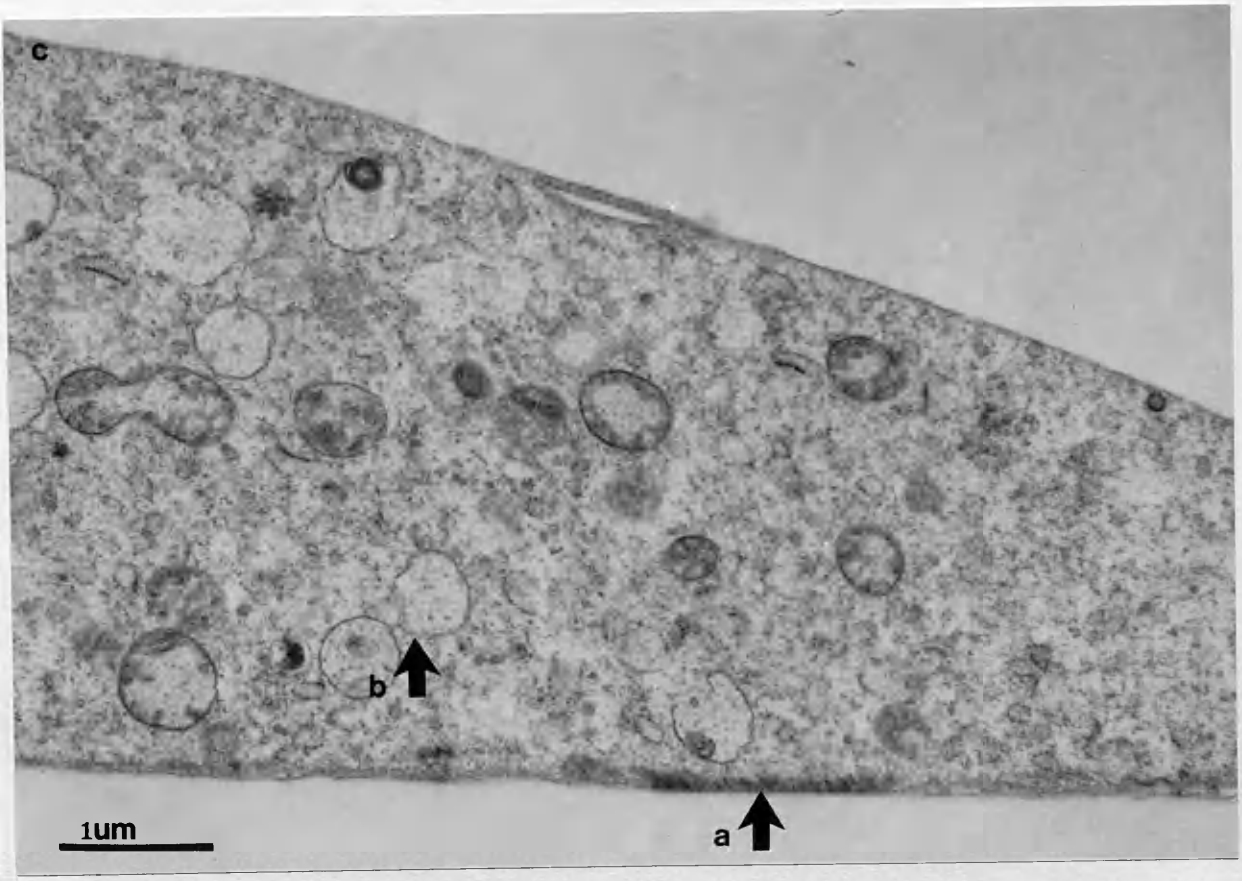

a
a
a
a
b
1um

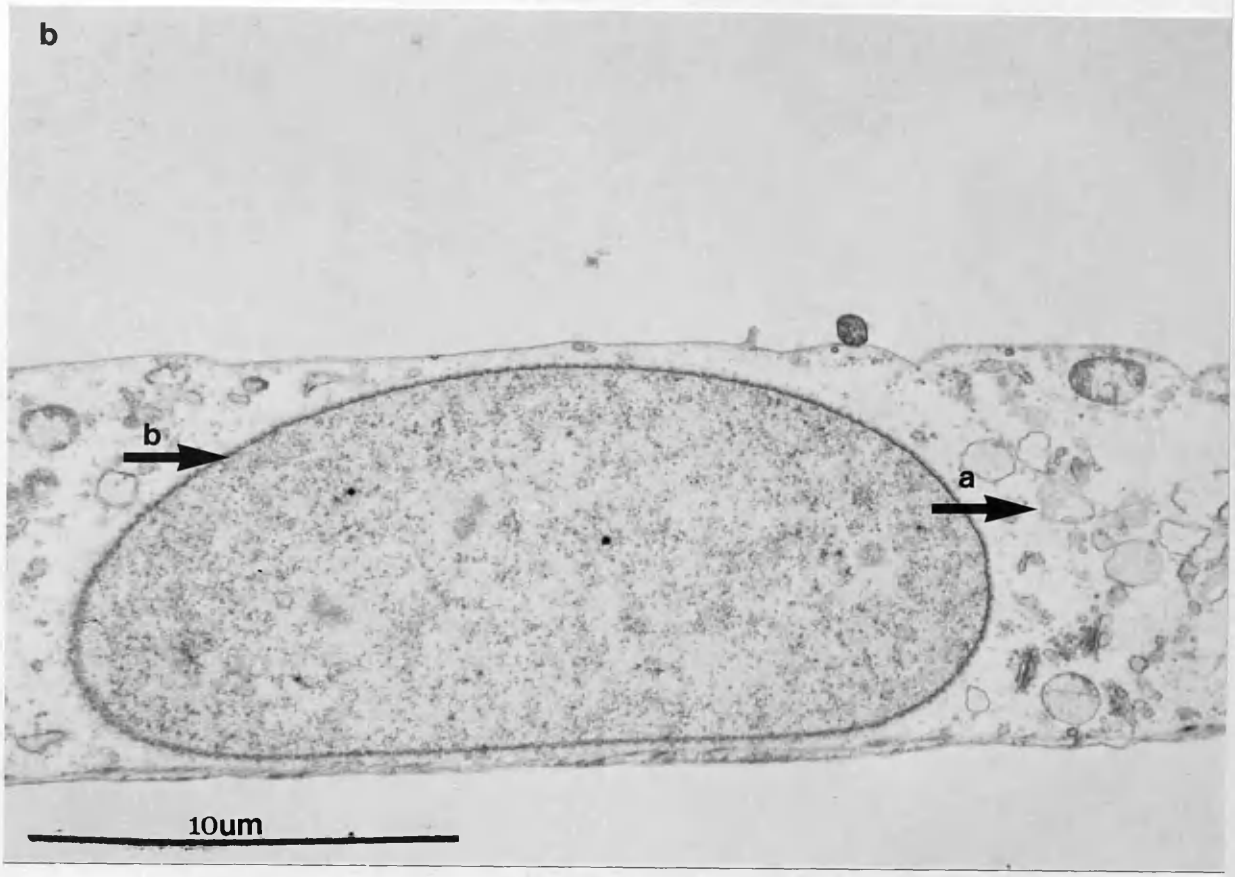
FIGURE 35

Endothelial cells.

(a) Top left	Portion of a large endothelial cell (SEM x 3,000)
(b) Top right	Small Golgi (a) surrounding large central nucleus (b) (TEM x 6,000)
(c) Bottom left	Dense fibriller matrix forming thickened basement membrane (a) and micropinocytotic vesicles (b) (TEM x 15,000)
(d) Bottom right	Higher magnification of matrix, intracellular and extracellular component (arrows) (TEM x 22,500)




c
b
a
1um



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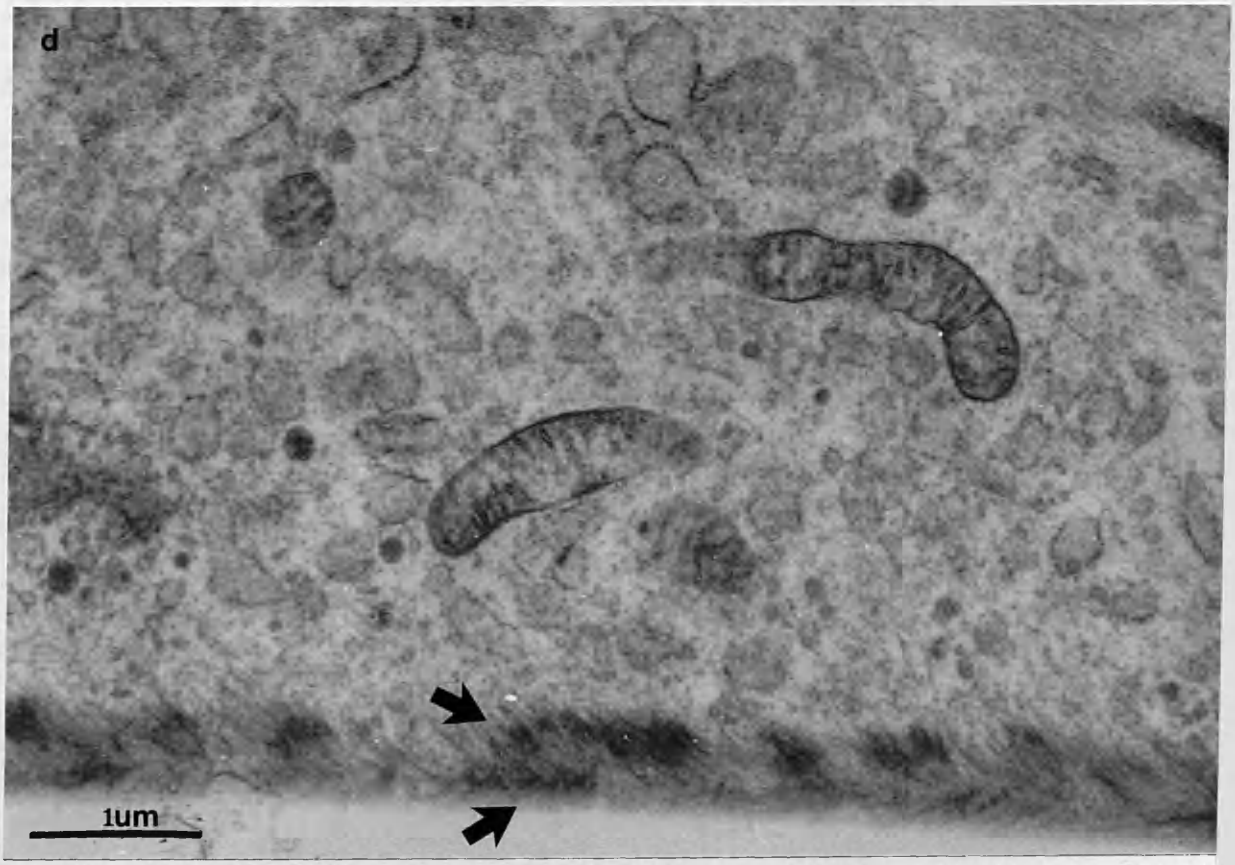


On SEM keratocytes (35um) were flattened. The nuclear outline was clearly visible, and nuclei contained several prominent nucleoli, fig. 34a, and TEM fig. 34d. Cellular processes radiated in an irregular manner, fig. 34a. By TEM the keratocytes were orientated at right angles to each other and cellular processes were abundant, fig. 34b. Cytoplasmic organelles were sparse, consisting mainly of large profiles of rough endoplasmic reticulum and small mitochondria with swollen cristae, fig. 34b, c.

Endothelial cells were large and polygonal on SEM. The cell size was variable (60-100um), fig. 35a. Contact between cells was rare. Organelles consisted of a few mitochondria, some rough endoplasmic reticulum, small Golgi close to the nucleus and micropinocytotic vesicles, fig. 35b, c. A dense matrix was deposited at the basal aspect of the cells. This appearance was peculiar to the endothelial cells and probably represented primitive Descemet's membrane, fig. 35b, c. d.

Indirect immunofluorescence studies: Epithelial cells and keratocytes at passage no. 8, and endothelial cells at passage no. 4, were seeded onto glass coverslips 2 days prior to examination. The use of cell specific markers allowed the screening of larger numbers of corneal cells than was possible by electron microscopy. The appropriate controls using primary antibody and secondary antibody in isolation; and primary antibody and secondary antibody together against the inappropriate cell types were performed to ensure that there was no cross reactivity (data not shown). Epithelial cells (keratin+) showed perinuclear fluorescent staining corresponding to the cytoplasmic location of keratin filament bundles (Cook et al., 1987).

Around 75% of epithelial cells were weakly positive or at background fluorescence. However no fluorescent staining above background was detected when epithelial cells were tested with antisera and conjugate against fibronectin.

Approximately 80% of keratocytes and endothelial cells were strongly positive using indirect immunofluorescence against fibronectin. The fibronectin matrices of the keratocytes and endothelial cells were identified by the cell surface fibronectin, figs. 36 and 37. The endothelial cells were sparse on the coverslips and no intercellular matrices were demonstrated. Neither the keratocytes nor endothelial cells reacted positively with anti keratin antibodies and conjugate.

DISCUSSION

Other authors have described techniques for the isolation of corneal epithelial cells, keratocytes and endothelial cells. The identity of their cell cultures was ascertained either by light microscopy or by ultrastructural studies on a single cell type (Stocker et al., 1958; Baum et al., 1979 and Gipson and Grill, 1982). Light microscopy distinguished endothelial cells from epithelial cells and keratocytes morphologically, but differences between epithelial cells and keratocytes are harder to establish. The dissection technique of Stocker et al. (1958) has the potential for inadvertent contamination of a cell line when separating stroma with its keratocytes, from epithelium.

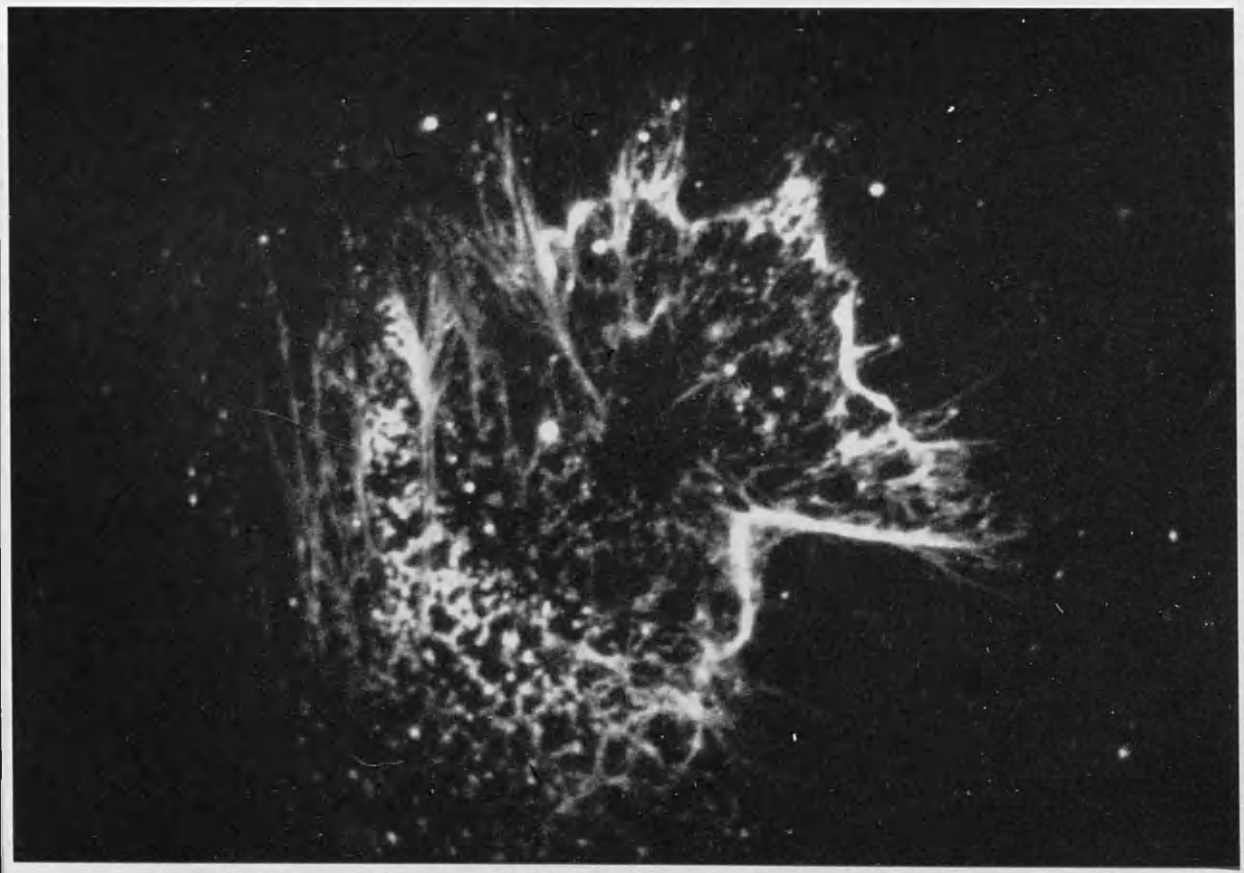
The identification of the rabbit corneal cell lines was performed in two ways. Ultrastructural examination showed microplicae and desmosomes in epithelial cells (Gipson and Grill, 1982) and demonstrated stratification and a lack of

FIGURE 36

Fibronectin^{+} keratocytes with fluorescent staining of cell walls and intercellular matrix (oil immersion X 1,200).

FIGURE 37

Fibronectin^{+} endothelial cells showing cell surface fluorescence (oil immersion X 1,200).



contact inhibition (Newsome et al., 1974). Keratocytes showed dilated cisternae of endoplasmic reticulum and prominent nucleoli (Newsome et al., 1974). The endothelial cells were polygonal and covered a large surface area (Perlman and Baum, 1974). Ultrastructural studies permit only a small proportion of cells to be examined. Indirect immunofluorescence techniques were used to screen larger numbers of cells. Antibodies against keratin and fibronectin identified epithelial cells, and keratocytes and endothelial cells respectively. The purity of the cell lines was confirmed by this method.

The cellular doubling times for the three corneal cell types grown without extracellular matrices or growth factors were similar to the results of other groups (Carter et al., 1985; Hsieh and Baum, 1985). The slow growth of endothelial cells prevented the accumulation of sufficient cells for storage, but epithelial cells and keratocytes were successfully stored at -170°C.

CHAPTER 5

IN VITRO EXPERIMENTS

HSV-1 persistence and latency in rabbit corneal cells in vitro

MATERIALS

Growth of viruses: Virus stocks were grown and titrated as before. Strains of HSV-1 used were HSV-1 strain 17 (Brown et al., 1973), and the X2 variant of strain 17 which lacks the XbaI sites at 0.07 map units and 0.63 map units (Brown et al., 1984). The HSV-1 strain 17 particle:pfu was 10:1. The X2 variant particle:pfu was 5:1.

Cells: Rabbit corneal epithelial cells, keratocytes and endothelial cells whose purity and morphology were confirmed by indirect immunofluorescence and electron microscopy, as described before, were used.

Acycloguanosine: Acycloguanosine (Schaeffer et al., 1978) is phosphorylated by the herpes specified thymidine kinase. The triphosphate of acycloguanosine is inhibitory to viral DNA polymerase. Acycloguanosine was used in 10uM concentrations to treat cell cultures, and eliminate lytic infection. Acycloguanosine was supplied by the Wellcome Medical Division.

Radiochemicals: Radiochemicals were obtained from Amersham International. L-[^{35}S] methionine was used to label proteins; the specific activity was variable with storage, fresh stocks were usually about 1300 Ci/m mol. [^{32}P]-dCTP and dGTP (3000 Ci/m mol) were used to label nick-translated viral DNA probes.

Gels: (i) polyacrylamide gels were prepared using the method of Marsden et al. (1976). The recipes for the 7.5% single concentration gel and the 5% stacking gel are given below.

	7.5%	5%
30% acrylamide/bisacrylamide	12 mls	4 mls
resolving gel buffer/stacking gel buffer	12 mls	6 mls
distilled water	24 mls	14 mls
10% ammonium persulphate	300 ul	200 ul
Temed	20 ul	10 ul

Solutions:

(i) Polyacrylamide gel buffers; tank buffer 6.32g of Tris HCl, 4g of glycine and 1g of SDS made to 1 litre with deionised water.

Resolving gel buffer; 18.15g of Tris HCl and 0.4g of SDS made to 100ml and pH 8.9.

Stacking gel buffer; 5.9g of Tris HCl and 0.4g of SDS made to 100ml and pH 6.7.

(ii) Polyacrylamide gel fix/stain; a mixture of methanol, water and acetic acid in the ratio of 50/43/7 (w/v) containing 0.2% w/v Coomassie brilliant blue.

(iii) Polyacrylamide gel destain; a mixture of methanol, water and acetic acid in the ratio of 50/880/70.

(iv) Acrylamide; 30% acrylamide solution (+ 5% linker) - 28.5g acrylamide and 1.5g N-N' methylene biacrylamide. These were made up to 100ml and filtered through a Whatman number 1 filter prior to use.

(v) Boiling mixture; 1ml stacking gel buffer, 0.8ml 25% SDS, 0.5ml 2-B mercaptoethanol, 1ml glycerol and 0.025ml of a saturated solution of Bromophenol blue. This was diluted 1 part in 3 parts with distilled

water and if necessary the pH adjusted by the addition of NaOH to give a blue colour.

(vi) Gel soak I; 200mM NaOH, 600mM NaCl.

(vii) Gel soak II; 1M Tris HCl (pH 8.0), 0.6M NaCl.

(viii) SSC; 0.15M NaCl, 0.015M trisodium citrate (pH 7.5).

(ix) Denhardt's buffer (50X); 1% Ficoll (w/v), 1% solid BSA (w/v), 1% polyvinyl pyrrolidone (w/v).

(x) Hybridization buffer; 15% 20X SSC (v/v), 10% 50X Denhardt's buffer(v/v), 1% 100X salmon sperm DNA (v/v), 1% 1M Tris HCl (pH 7.5) and 73% H_2O.

METHODS

One step growth experiment: Confluent plates of $BHK_{21}C_{13}$ cells (10^5 cells), corneal epithelial cells (10^5 cells), keratocytes (10^5 cells), and corneal endothelial cells (5×10^4 cell), were infected with HSV-1 strain 17 at a multiplicity of infection (m.o.i.) of 10 plaque forming units (pfu)/cell. After absorption for 1 hour at 37°C the cultures were washed twice in PBS plus 5% (v/v) calf serum (PBSC5) and overlaid with EC_{10}. At various time intervals between 0 and 24 hours the infected cells were harvested into 1 ml of medium and virus released by sonication. Samples were stored at -70°C and virus titres measured by plaque assay on $BHK_{21}C_{13}$ cells.

Lytic infection in corneal cells and acycloguanosine treatment: Four confluent cultures of epithelial cells at passage number 6 and keratocytes at passage number 8 (approximately 10^6 cells/flask), were each infected at a m.o.i. of 1 pfu/cell. Virus was absorbed for 1 hour at 37°C then cultures were washed twice with PBS. Epithelial cells were incubated at 37°C for 24 hours; with medium

containing 10uM acycloguanosine (ACG), with medium containing ACG pre-incubated at 42°C for 48 hours; with control medium, or with control medium pre-incubated at 42°C for 48 hours. Keratocyte cultures were treated similarly. The cultures were harvested after 24 hours and the yield of cell associated virus was determined by titration on $BHK_{21}C_{13}$ cells.

Non productive infections in corneal cells I: Twenty-four confluent cultures of each corneal cell type were grown in 25cm^2 flasks (approximately 10^6 cells/flask) at 37°C. The cells ranged from passage nos. 5-8 (epithelium); passage no. 12 (keratocytes); or passage nos. 5-8 (endothelium). Cultures were maintained at 41.5°C for 24 hours prior to inoculation. Eight flasks of each of the three cell types were inoculated at m.o.i.'s of 0.01, 0.1 and 1.0 pfu/cell in 100ul of medium. Virus was absorbed for 45 minutes at 41.5°C. The cultures were then overlaid with the appropriate medium which was changed every third day. At weekly intervals after inoculation two cell cultures at each m.o.i. and of each cell type were transferred to 37°C. The medium was supplemented with 5% (v/v) human serum for the first five days at 37°C. Human serum was added to neutralize any residual extracellular virus.

Assay for infectious virus I: The presence of infectious virus was determined by two methods; (1) observation of viral plaques on corneal cell cultures; (2) assay of 0.5ml corneal cell supernatant on $BHK_{21}C_{13}$ cells. After a 45 minute absorption period at 37°C the cell monolayer was overlaid with EC_{10} and incubated for 48 hours at 37°C. Cultures were stained with Giemsa and examined for viral cytopathic effect. Cultures were scored as positive for

virus (+) or negative (-). The amount of virus released was not quantified. The assays were performed daily for two days after inoculation, and then every third day when the medium was changed.

Non productive infection in corneal cells II; the effect of ACG treatment: Forty-four confluent cultures each of epithelial cells, passage no. 8, and keratocytes, passage no. 7, were grown in 25cm^2 flasks (approximately 10^6 cells/flask) at 37^{o}C. Cultures were maintained at 42^{o}C for 24 hours prior to HSV-1 inoculation. (As 42^{o}C was not detrimental to cell viability it was considered a more stringent temperature to eliminate virus replication.) The cultures of each cell type were divided into two groups of 22 flasks; A - treated with 10uM ACG, and B - ACG free control. Flasks 1-10 were infected at a m.o.i. of 0.0005 pfu/cell, flasks 11-20 were infected at a m.o.i. of 0.001 pfu/cell, while flasks 21 and 22 were not infected with virus (MI).

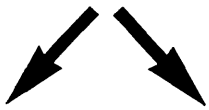
From day 0, (day of inoculation) the medium was changed every second day and assayed for infectious virus (using method 2 only) until the experiment was concluded. On day 10 post infection the 22 flasks of group A of each cell type had ACG added to a concentration of 10uM. Fresh ACG was added with each change of medium between day 10 and 15 to the group A flasks. On day 15, flasks 1 and 11 from groups A and B were superinfected at 37^{o}C. The remaining twenty flasks were transferred to 37^{o}C where medium was changed and assayed every second day until day 29 (see fig. 38).

Superinfection: The X2 variant of HSV-1 strain 17 (Brown et al., 1984) was used for superinfection. Both ACG treated and untreated cultures were superinfected either on day 15,

FIGURE 38

Experimental protocol for non productive infection in corneal cells II; the effect of ACG treatment.

Figure 38



44 CELL CULTURES

A (ACG) 22 CELL CULTURES

B (no ACG) 22 CELL CULTURES

10 (0.0005 pfu/cell) 10 (0.001 pfu/cell) 2(M.I.)

Day -1	Cells to 42°C
Day 0	Infection
Day 10-14	ACG to A
Day 15	Superinfection (2 cell cultures)
	Transfer to 37°C
Day 29	Superinfection
	DNA extraction

the day of transfer from 42°C to 37°C, or on day 29 after a fourteen day incubation at 37°C. The cultures were superinfected with X2 at a m.o.i. of 0.005 pfu/cell or 0.01 pfu/cell (10 times the original m.o.i. with strain 17). After absorption for 1 hour and removal of unabsorbed X2 virus, incubation was continued at 37°C for 24 hrs. The cultures were harvested, virus was released by sonication and samples were stored at -70°C.

Restriction endonuclease analysis of progeny virus from superinfection experiments: Virus samples from superinfected cultures were titrated on $BHK_{21}C_{13}$ cells with the overlay medium containing human serum. A large number of single, well separated plaques were picked individually into 0.5ml PBS 5% calf serum and sonicated. 0.2ml of the single plaque samples was used to infect 10^6 $BHK_{21}C_{13}$ cells. Progeny virus was harvested from these cultures after 48 hrs. at 37°C and used for restriction endonuclease analysis.

Restriction enzyme analysis was carried out using the Linbro well method described by Lonsdale (1979). Confluent cells were infected in the presence of ^{32}P at an approximate m.o.i. of 10 pfu/cell and incubated at 31°C for 48 hrs. ^{32}P labelled viral DNA was treated with restriction endonucleases at concentrations sufficient to give complete digestion in 4 hrs. at 37°C. The generated DNA fragments were separated by overnight electrophoresis on agarose gels of appropriate concentration and after drying the gels were exposed to XS1 film. HSV-1 strain 17 DNA and the X2 variant DNA were used as controls.

DNA-DNA hybridization:

(i) DNA extraction. DNA was extracted from half the cultures which had failed to shed infectious virus

after 14 days at 37°C. The method used was as described by Stow et al. (1983).

Medium was removed from the cultures and the cells lysed with buffer (0.6% SDS, 1mM EDTA, 10mM Tris pH 7.5) plus 500ug/ml pronase for 4 hrs. at 37°C. NaCl was added to a final concentration of 200mM. After sequential extraction with phenol, phenol + chloroform and chloroform the DNA was precipitated with ethanol at -20°C. The DNA was redissolved in Tris/EDTA containing 10ug/ml RNase/T1 and digested for 2 hrs. at 37°C. Samples of DNA were digested with the BamHI or HpaI restriction endonuclease. Digested samples were run overnight on agarose gels of appropriate concentration in 1XE buffer. Following gel electrophoresis DNA was visualized by staining in ethidium bromide (1ug/ml) prior to examination under UV light.

(ii) Transfer of DNA to nitrocellulose. The transfer of DNA fragments from agarose gels to nitrocellulose membranes was performed by the method described by Southern (1975). After electrophoresis the DNA was denatured by soaking the gel for 45 mins. in Gel Soak I, and then neutralized by soaking in Gel Soak II for 45 minutes. The gel was transferred to a glass plate wrapped in 2 sheets of Whatman 3MM paper moistened with 10XSSC. The plate was supported in a tray of 2 litres of 10XSSC. Care was taken to ensure that no air bubbles were present between the 3MM paper and either the plate or the gel. A sheet of nitrocellulose paper was floated on the surface of the 10XSSC until it soaked from beneath, then it was

applied to the gel surface. All air bubbles between the gel and filter were removed. Two moistened sheets of 3MM paper cut to the same size as the filter were placed on top of the nitrocellulose followed by a weighted stack of paper towels. DNA fragments transferred from the gel to the nitrocellulose overnight maintaining their original spatial arrangement. The filter was dried at room temperature and baked in a vacuum oven for 2 hours.

(iii) Nick translation of DNA. In vitro labelling of DNA with ^{32}P deoxyribonucleoside triphosphates was done essentially as described by Rigby et al. (1977). Nick translated DNA was made from total HSV-1 DNA, from recombinant plasmid pGX8 containing HSV-1 BamHI k (Davison and Wilkie, 1981) and from recombinant plasmids pGX121, 122, 123 and 134 containing the HSV-1 KpnI b, c, d and m fragments respectively.
Nick translation reactions were set up on ice and typically contained 0.5ug DNA in a volume of 30ul of the following solution: 1ul BSA, 3ul 10XNT buffer, 2.25ul 0.2mM dATP, 2.25ul 0.2mM dTTP, 2ul ^{32}P-dCTP, 2ul ^{32}P-dGTP, 1ul DNase 10^{-4}mg/ml, 1ul DNA polymerase 1 and H_2O to a final volume of 30ul. The reaction mixture was incubated at 15°C for 90 minutes. After incubation the mixture was made up to 50ul with distilled water and the labelled DNA precipitated twice with 0.1 volume 3M NaAc and 0.75 volume isopropanol on dry ice for a minimum of 30 minutes. The radioactivity of the probe was monitored using a portable beta counter.

(iv) Hybridization. Nitrocellulose strips were pre-soaked

in sealed polythene bags with 10mls of hybridization buffer for 2 hours at 75°C. Radioactively labelled probes were used to detect homologous sequences in the single stranded DNA immobilized on the nitrocellulose. Nick translated probe DNA was denatured by heating at 100°C for 2-3 mins. in 80% (v/v) formamide. The bags containing the pre-incubated nitrocellulose strips were drained and the denatured probes added in 10mls of fresh hybridization buffer. The nitrocellulose strips were hybridized for 48 hours at 75°C in a shaking water bath. The nitrocellulose filters were then extensively washed for 2 hours at 60°C in a shaking water bath in 10% 20XSSC, 10% SDS and 0.5% 1.0M Na_2HPO_4 pH 7. The sheets were air dried and autoradiographed.

Preparation of corneal cell protein gels:

(a) Radiolabelling of cells. Growth medium was removed from confluent 35mm plates of epithelial and keratocyte cells and the cellular monolayers washed twice with PBS. Methionine free Eagle's medium plus 2% (v/v) calf serum was added. After 4 hours 10u Ci of ^{35}S methionine was added to each culture for a further two hours. Control cultures infected with HSV-1 strain 17 at a m.o.i. of 20 pfu/cell were labelled with ^{35}S methionine for 24 hours. Disulfiram 0.3uM was added to the cell growth medium at 37°C to obtain radiolabelled stress protein markers (Levinson et al., 1978). After incubation, labelling medium was removed, monolayers were washed with ice cold PBS and cells disrupted with 0.25mls of boiling mix diluted 1 in 3 with distilled water. The infected cells were then solubilised by incubation at 60°C for 10 minutes. The sample (0.01ml) was

then spotted onto a Whatman number 1 filter disc, which was washed twice in ice cold TCA (10 minutes each) and rinsed with absolute alcohol. The amount of radioactivity in the air dried disc was estimated in the usual way. The radioactive content in each sample was standardized using diluted boiling mix as diluent so that each contained the same amount of radioactivity per unit volume.

(b) Electrophoresis. Samples of cell extracts were loaded into preformed wells in the stacking gel. Electrophoresis was started at 40mAmps and left for 3-4 hrs., until the Bromophenol blue dye front emerged from the end of the gel. At this time the gel was removed and fixed/stained for 1 hr. at room temperature, following which they were destained and rehydrated by the addition of several changes of destain over a 24 hr. period. The gel was then dried onto paper using a BioRad gel slab dryer, operating under a vacuum of 30mm Hg. The dried gel was sandwiched between glass plates against Agfa-Gevaert Xray films. The autoradiograph was then developed in an X-omat industrial film processor.

RESULTS

One step growth kinetics of HSV-1 in three corneal cell types: Single cycle growth experiments were performed in epithelial cells, keratocytes and endothelial cells using $BHK_{21}C_{13}$ cells as a control. The experiments were run in duplicate. The results are presented in fig. 39. HSV-1 strain 17 replicated efficiently in the three corneal cell types, however distinct differences were observed. In $BHK_{21}C_{13}$ cells viral growth showed a four-hour eclipse phase, a plateau of virus production within 24 hrs. and a yield of around 100 pfu/cell. The viral growth curve was

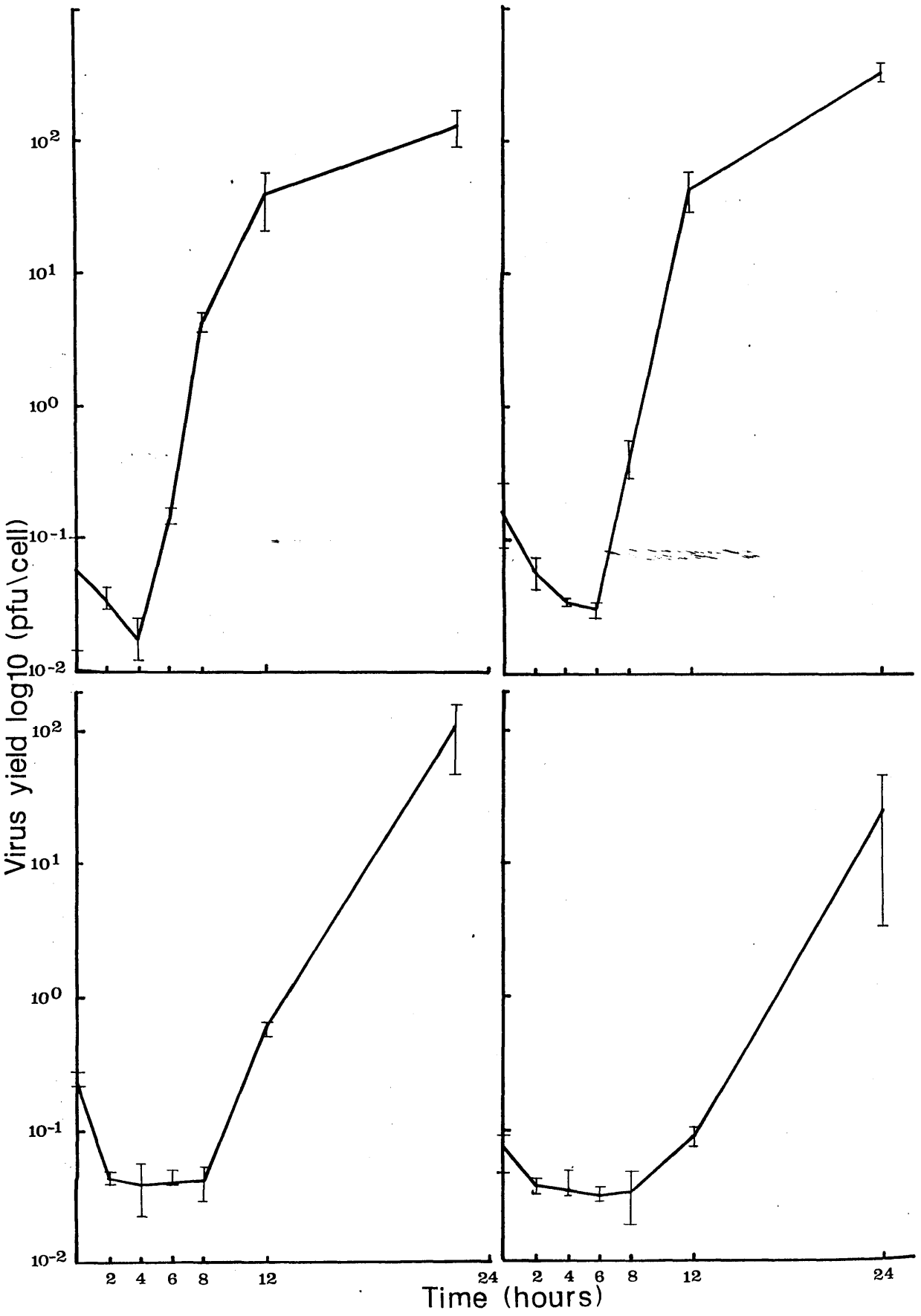
FIGURE 39

One step growth curves of HSV-1 strain 17 in corneal cells.

Top left	BHK21C13 cells
Top right	epithelial cells
Bottom left	keratocytes
Bottom right	endothelial cells

Cells were infected at m.o.i. 10 pfu/cell, after absorption for 1 hour at 37°C the monolayers were washed twice with PBS C5, overlaid with EC10 and incubated at 37°C. Cultures were harvested at the times indicated and the virus titre measured by plaque assay on BHK21 C13 cells.

Horizontal bars indicate the range of values.


Virus yield log10 (pfu\cell)
10^2
10^1
10^0
10^{-1}
10^{-2}
2 4 6 8 12 24
Time (hours)

similar in epithelial cells but the eclipse phase was prolonged to six hours. Virus growth in keratocyte and endothelial cells showed a prolonged eclipse phase of eight hours, and a slower rate of growth. Both curves were still in the logarithmic phase at 24 hours suggesting that virus production had yet to reach a maximum.

Non productive infections in corneal cells I: Two assay methods (see Methods) were used to determine the presence of infectious virus. There was 87%, 79% and 77% correlation between the two assay methods in the epithelial cells, keratocytes and endothelial cells respectively. The results presented are based on method (2) - the corneal cell culture supernatant assay on $BHK_{21}C_{13}$ cells.

The three corneal cell types were infected with HSV-1 strain 17 at various low m.o.i.'s at 41.5°C. At weekly time intervals up to 28 days cultures were transferred to 37°C. At the start of the experiment there were twenty-four cultures of each cell type. Cells were able to divide at 41.5°C, so when cells became over-confluent the cultures were split 1:2. The progeny cultures continued in the experiment increasing the original number of cultures (Table 8a-c).

Infectious virus was detected in all cultures on the first day post inoculation and in most cultures on the second day post inoculation. Focal cytopathic effect, observed by rounding of cells was seen to some extent in all cultures after HSV-1 infection. At higher m.o.i.'s (0.1 and 1.0 pfu/cell) cell death was sometimes overwhelming by the seventh day, leaving no surviving cells. This was marked in keratocyte and endothelial cells, but was less noticeable in epithelial cells where a small number of

Course of non productive infections in corneal cells.

TABLE 8(a) EPITHELIUM

Culture number	Culture death	No. of days at 41.5°C	PI	LI	Reactivation failure	Time of virus release after HS removal at 37°C (days)
1*		7		+		3
2		7		+		3
3		14		+		2
4		14		+		2
5		21		+		6
6		21		+		9
7		28	+			
8		28		+		2>
9A+$		7		+		3
9B		7		+		3
10		7		+		3
11		14	+			
12		14	+			
13		21		+		3
14		21	+			
15		28	+			
16		28		+		2
17‡	C<	7				
18		7		+		3
19		14		+		4
20		14		+		4>
21		21		+		9
22		21		+		3
23		28		+		2
24		28		+		2

PI = Persistent infection
LI = Latent infection
HS = Human serum

Course of non productive infections in corneal cells.

TABLE 8(b) KERATOCYTES

Culture number	Culture death	No. of days at 41.5°C	PI	LI	Reactivation failure	Time of virus release after HS removal at 37°C (days)
1A*$		7		+		3
1B		7		+		3
2A		7		+		3
2B		7		+		3
3A	+	14				
3B	+	14				
4A	+	14				
4B	+	14				
5A		21			+	
5B		21		+		6
6A		21			+	
6B		21		+		6
7A		28			+	
7B		28			+	
8A		28			+	
8B		28		+		5
9+		7		+		6
10		7		+		11
11		14		+		13
12		14		+		2>
13	+	21				
14	+	21				
15	+	28				
16	+	28				
17‡		7		+		9
18		7		+		23
19		14		+		7
20	+	14				
21	+	21				
22		21		+		1>
23		28		+		>
24	+	28				

PI = Persistent infection
LI = Latent infection
HS = Human serum

Course of non productive infections in corneal cells.

TABLE 8(c) ENDOTHELIUM

Culture number	Culture death	No. of days at 41.5°C	PI	LI	Reactivation failure	Time of virus release after HS removal at 37°C (days)
1*		7		+		9
2A$		7		+		3
2B		7		+		3
3		14			+	
4		14		+		2
5		21		+		6
6		21		+		9
7		28		+		2
8		28			+	
9A+		7		+		3
9B		7		+		3
10		7		+		3
11	C<	14				
12		14		+		2
13A		21			+	
13B	C	21				
14		21		+		3
15		28		+		2
16A	+	28				
16B	+	28				
17‡	C	7				
18		7		+		9
19		14		+		7
20	+	14				
21	+	21				
22		21		+		3
23		28		+		5
24		28		+		2

PI = Persistent infection
LI = Latent infection
HS = Human serum

Footnotes: * Cultures 1-8, m.o.i. 0.01 pfu/cell
+ Cultures 9-16, m.o.i. 0.1 pfu/cell
‡ Cultures 17-24, m.o.i. 1.0 pfu/cell
$ A or B: Cultures were split 1:2 on day 13
< C: cell death occurred due to contamination
> Cultures shed virus at 37°C in the presence of human serum; keratocyte culture 23 did not shed any other virus at 37°C

surviving cells were able to divide and re-form confluent monolayers. Over confluent cell monolayers were sub-cultured 1:2 on day 13 post inoculation. An anomalous finding was cell death in keratocyte cultures 3A, 3B, 4A and 4B. Their parent cultures 3 and 4, inoculated at the lowest m.o.i. (0.01 pfu/cell) were sub-cultured on day 13. On day 14 the progeny cultures were transferred to 37°C and medium was replaced by medium supplemented with 5% human serum. The cells had not settled onto the culture flasks by day 14 and were removed with the medium. Other keratocyte cultures 5A-8B, sub-cultured on day 13, but left undisturbed at 41.5°C until day 16, settled and ultimately became confluent (Table 8b).

Persistent infection, defined as the presence of infectious virus in the supernatant medium at 41.5°C, after the immediate period post infection, was detected in one epithelial cell culture infected at 0.01 pfu/cell, and four epithelial cell cultures infected at 0.1 pfu/cell. The infection appeared to be partially suppressed in that it did not succeed in destroying cellular monolayers until cells were transferred to 37°C. No infectious virus was detected in surviving keratocyte and endothelial cell cultures maintained at 41.5°C for up to 28 days post infection (Table 8a-c).

Latent infection was defined as absence of infectious virus in the supernatant medium at 41.5°C, but detectable infectious virus in the supernatant medium after transfer to 37°C. All cell types proved capable of releasing infectious HSV after human serum was removed at 37°C.

By day 40 post infection, all surviving epithelial cells had released infectious virus, but five keratocyte and

three endothelial cell cultures at 37°C had failed to release virus up to 20 days after human serum removal. The eight cultures that failed to release infectious virus at 37°C were defined as reactivation failures (Table 8b and c). The release of infectious virus was quicker from epithelial and endothelial cells than from keratocytes after human serum was removed.

Superinfection I: An attempt was made to recover non inducible virus genomes from the reactivation failures by superinfection. The X2 variant was used at a m.o.i. of 5 pfu/cell with the intention of assuring that every cell in the flask was infected. Cultures were harvested after 48 hours. All cultures were contaminated at 48 hrs., and were therefore Arcton sterilized. The sterilized stocks were then titrated and 50 plaques picked from each stock. A total of 189 plaques from the eight reactivation failures were analysed by XbaI restriction endonuclease analysis. (50 plaques from keratocyte culture 5A; generally 20 plaques from the other flasks.) All analysed plaques showed the X2 profile. This line of investigation was not pursued for three reasons; firstly despite Arcton sterilization post-contamination, the possibility remained that novel bands might be from the contaminating organism; secondly the superinfection m.o.i. was too high resulting in a ratio of superinfecting virus to input virus of 500 or 50 to 1; and thirdly harvesting after 48 hours probably permitted multiple rounds of virus replication by the superinfecting strain, thereby overwhelming the original input virus. These factors were all corrected in the subsequent series of superinfection experiments.

Cellular stress proteins: Supra-optimal temperatures induce

the synthesis of cellular stress proteins. Other agents including Disulfiram, transition series metals and sulphydryl reagents induce the synthesis of cellular stress proteins (Levinson et al., 1978, 1980). Figure 40 shows the effect of temperature elevation on protein synthesis in rabbit corneal epithelial cells and keratocytes. Disulfiram 0.3uM was added to the cell growth medium at 37°C to obtain radiolabelled stress protein markers. In mock infected keratocytes and epithelial cells, incubated at 41.5°C there was increased synthesis of proteins having approximate molecular weights of 90,000, 75,000 and 43,000. Novel proteins, having approximate molecular weights 70,000, 63,000, 53,000 and 45,000 also appear to have been synthesised.

Many characteristic herpes simplex virus proteins were detected after 24 hours in both keratocytes and epithelial cells infected at 20 pfu/cell and maintained at 37°C; however these were absent at 41.5°C. The herpes simplex virus infected cells at 41.5°C had similar protein profiles to the mock infected cells at 41.5°C.

Lytic infection in corneal cells and ACG treatment:
Epithelial cell cultures and keratocyte cultures gave virus yields of 3.5×10^8 pfu/flask and 2.0×10^8 pfu/flask in control medium. After 24 hours no cell associated virus was detected from the cultures incubated with 10uM ACG. Similarly epithelial cell cultures and keratocyte cultures gave no virus yield when treated with medium containing 10uM ACG that had been pre-incubated at 42°C for 48 hrs. prior to the lytic infection at 37°C. The control epithelial cell culture and keratocyte virus yields were 2×10^9 pfu/flask and 10^9 pfu/flask respectively.

FIGURE 40

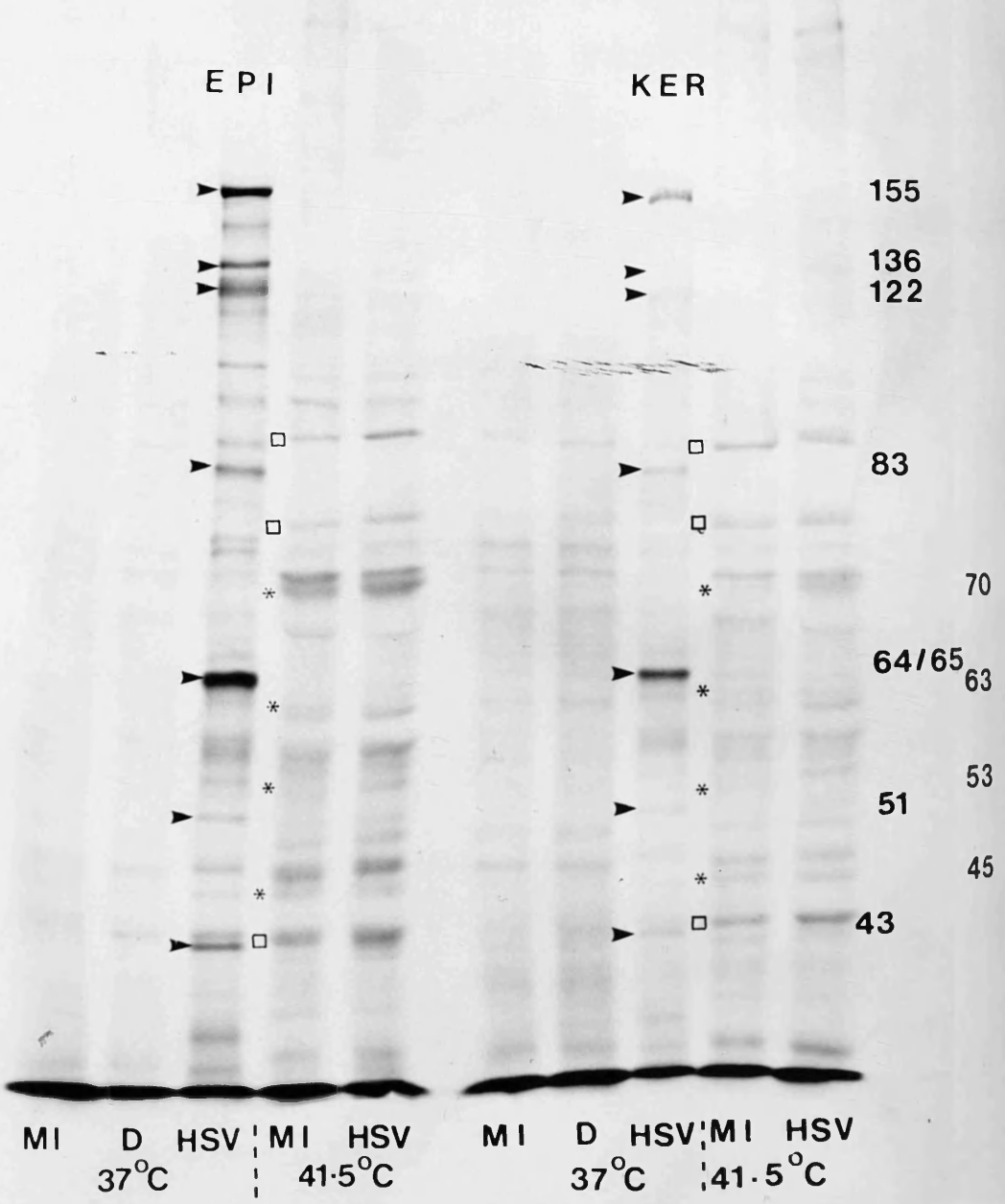
Protein synthesis in rabbit corneal epithelial cells and keratocytes. Cultures were mock infected (MI), treated with 0.3uM disulfiram (D), or infected with herpes simplex virus at 20 pfu/cell (HSV). Cultures were maintained at 37°C or 41.5°C.

□ Increased synthesis of cellular stress proteins

✱ Synthesis of novel stress proteins

▶ Synthesis of HSV induced proteins

Approximate molecular weights of HSV proteins are given on the near right of the last track. Approximate molecular weights of stress proteins are given on the far right.


EPI
KER
155
136
122
83
70
64/65
63
53
51
45
43
MI
D
HSV
MI
HSV
37°C
41·5°C
MI
D
HSV
MI
HSV
37°C
41·5°C

Non productive infections in corneal cells II: The experimental protocol is summarised in figure 38. The essential differences from the series I experiments were; (1) lower m.o.i's were used; (2) medium was changed and assayed for infectious virus every second day; (3) an incubation temperature of 42°C was used to induce the non productive infections (other experiments, see series III, had indicated the importance of maintaining the incubation temperature; where the temperature had dropped to between 40°C and 41°C a low grade infection had occurred); (4) ACG was applied to half the cell cultures.

The results of the infection in epithelial cells are shown in Table 9a and b, and in keratocytes in Table 10a and b. The tables allow the course of infection within an individual flask to be followed. The cumulative information is presented in graphic form in figures 41 and 42. In both groups A and B of epithelial cells and keratocytes, infectious virus was detected on the first and second day post infection and in one case on the fourth day. In four of the epithelial cell cultures no infectious virus was detectable on the days immediately post infection and in three of these none was detected subsequently.

Virus was not detected in the culture medium of any of the infected cultures between day 4 and day 15. On transfer to 37°C on day 15, cultures 1 and 11 in each group were immediately superinfected. The remainder were monitored for infectious virus up to day 29. Of the group A epithelial cells 8 cultures released virus at 37°C and 10 remained negative. Of the group B epithelial cultures, 11 released virus and 7 did not. Similarly in keratocytes group A, 11 became positive while 7 remained

Table 9a. Assay for HSV in ACG treated epithelial cells.

Days post infection		0	1	2	4	6	8	10	12	14	15	17	19	21	23	25	27	29
		<----------42°C---------->										<----------37°C---------->						
Flask No.	1		+	-	-	-	-	-	-	-	-*							
	2		-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	-
	3		+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-x
	4		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M.O.I.	5		+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	6		+	-	-	-	-	-	-	-	-	+	+	+	+	———		
$5x10^{-4}$	7		-	-	-	-	-	-	-	-	-	-	-	+	+	+	———	
	8		+	-	-	-	-	-	-	-	-	-	+	+	+	———		
	9		+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	10		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	11		+	+	-	-	-	-	-	-	-*							
	12		+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-x
	13		+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	14		-	+	-	-	-	-	-	-	-	+	+	+	+	———		
M.O.I.	15		+	+	-	-	-	-	-	-	-	-	+	+	+	———		
	16		+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-x
10^{-3}	17		+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	18		+	+	+	-	-	-	-	-	-	+	+	+	+	———		
	19		+	-	-	-	-	-	-	-	-	-	+	+	+	———		
	20		+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	21		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MI	22		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* Flask superinfected on day 15
x Flask superinfected on day 29
——— Cell sheet destroyed by reactivated virus
\+ c.p.e. detected

Table 9b. Assay for HSV in epithelial cells not treated with ACG.

Days post infection		0	1	2	4	6	8	10	12	14	15	17	19	21	23	25	27	29
		<----------42°C---------->									<----------37°C---------->							
Flask No.	1		+	+	-	-	-	-	-	-	-*							
	2		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-x
	3		+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-x
	4		+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
M.O.I.	5		-	+	-	-	-	-	-	-	-	-	+	+	+	+	———	
	6		+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$5x10^{-4}$	7		-	+	-	-	-	-	-	-	-	+	+	+	+	———		
	8		+	-	-	-	-	-	-	-	-	+	+	+	+	———		
	9		+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	10		+	-	-	-	-	-	-	-	-	+	+	+	+	———		
	11		+	+	-	-	-	-	-	-	-*							
	12		+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-x
	13		-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-
	14		+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-
M.O.I.	15		-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	-
	16		+	+	-	-	-	-	-	-	-	-	+	+	+	———		
10^{-3}	17		+	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-
	18		+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	19		+	+	-	-	-	-	-	-	-	+	+	+	+	———		
	20		+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MI	21		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	22		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* Flask superinfected on day 15
x Flask superinfected on day 29
——— Cell sheet destroyed by reactivated virus
\+ c.p.e. detected

FIGURE 41

Epithelial cells; non productive infections II.

□- - - -□ ACG treated cells

■———■ non ACG treated cells

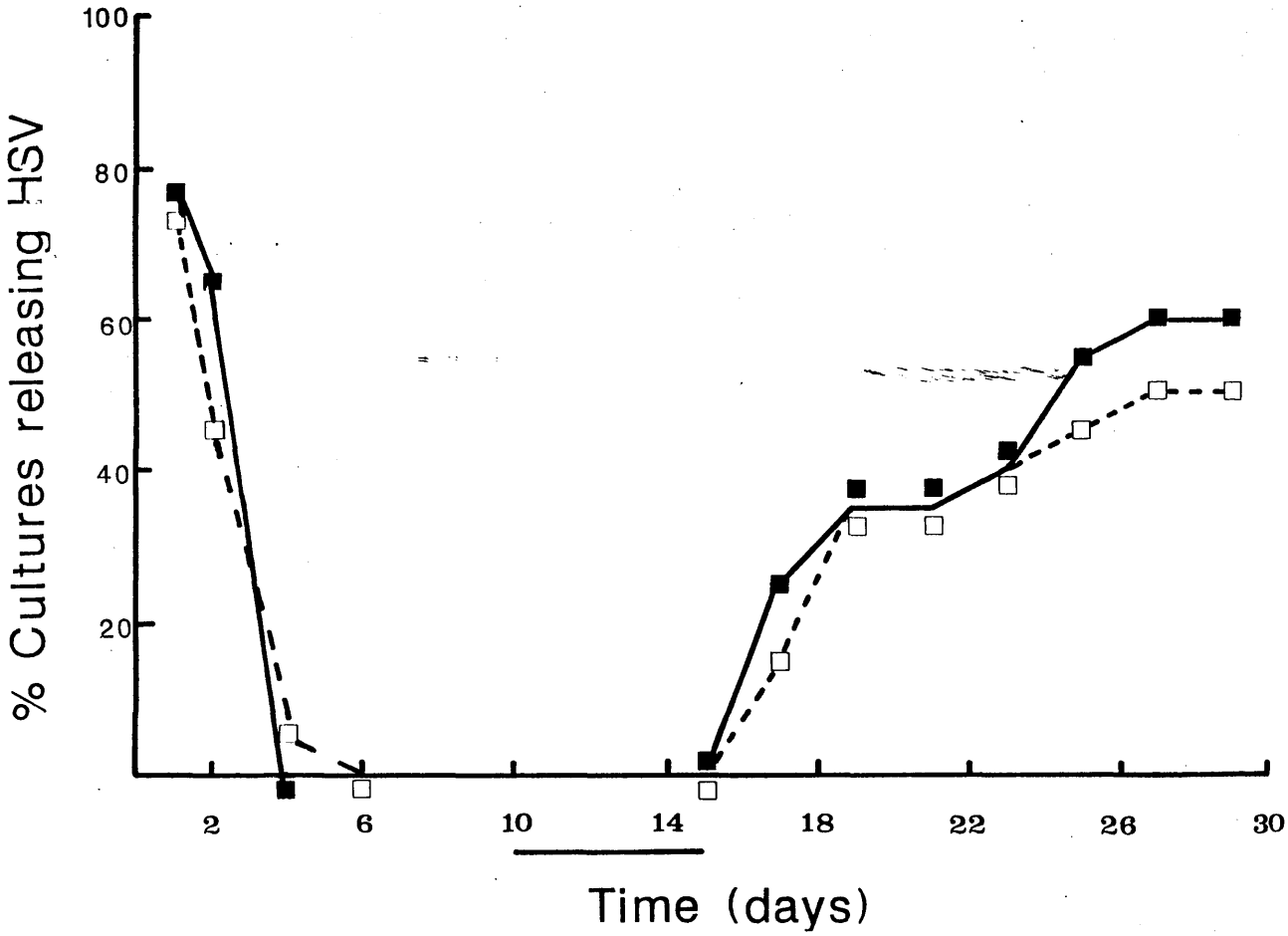

% Cultures releasing HSV
100
80
60
40
20
2
6
10
14
18
22
26
30
Time (days)

Table 10a. Assay for HSV in ACG treated keratocytes.

Days post infection		<----42°C---->								<----37°C---->							
		0	1	2	4	6	8	10	12	14	15	17	19	21	23	25	27	29
Flask No.	1		+	+	-	-	-	-	-	-	-*							
	2		+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-x
	3		+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-x
	4		+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M.O.I.	5		+	-	-	-	-	-	-	-	-	-	+	+	+	+	———	
	6		+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
$5x10^{-4}$	7		+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
	8		+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
	9		+	-	-	-	-	-	-	-	-	-	+	+	+	+	———	
	10		+	-	-	-	-	-	-	-	-	-	+	+	+	+	———	
	11		+	-	-	-	-	-	-	-	-*							
	12		+	+	-	-	-	-	-	-	-	-	+	+	+	———		
	13		+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-
	14		+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-x
M.O.I.	15		+	-	-	-	-	-	-	-	-	+	+	+	+	———		
	16		+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-x
10^{-3}	17		+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	18		+	-	-	-	-	-	-	-	-	-	+	+	+	———		
	19		+	+	-	-	-	-	-	-	-	-	+	+	+	———		
	20		+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MI	21		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	22		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* Flask superinfected on day 15
x Flask superinfected on day 29
——— Cell sheet destroyed by reactivated virus
\+ c.p.e. detected

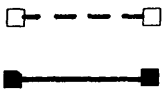
Table 10b. Assay for HSV in keratocytes not treated with ACG.

Days post infection		<----42°C---->								<----37°C---->							
		0	1	2	4	6	8	10	12	14	15	17	19	21	23	25	27	29
Flask No.	1		+	-	-	-	-	-	-	-	-*							
	2		+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-x
	3		+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-x
	4		+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M.O.I.	5		+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-
	6		+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+
$5x10^{-4}$	7		+	-	-	-	-	-	-	-	-	+	+	+	+	+	———	
	8		+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-
	9		+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
	10		+	-	-	-	-	-	-	-	-	+	+	+	+	———		
	11		+	-	-	-	-	-	-	-	-*							
	12		+	+	-	-	-	-	-	-	-	+	+	+	+	———		
	13		+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
	14		+	-	-	-	-	-	-	-	-	+	+	+	+	———		
M.O.I.	15		+	-	-	-	-	-	-	-	-	+	+	+	+	———		
	16		+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-x
10^{-3}	17		+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-x
	18		+	+	-	-	-	-	-	-	-	-	-	+	+	+	———	
	19		+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	20		+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MI	21		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	22		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* Flask superinfected on day 15
x Flask superinfected on day 29
——+ Cell sheet destroyed by reactivated virus c.p.e. detected

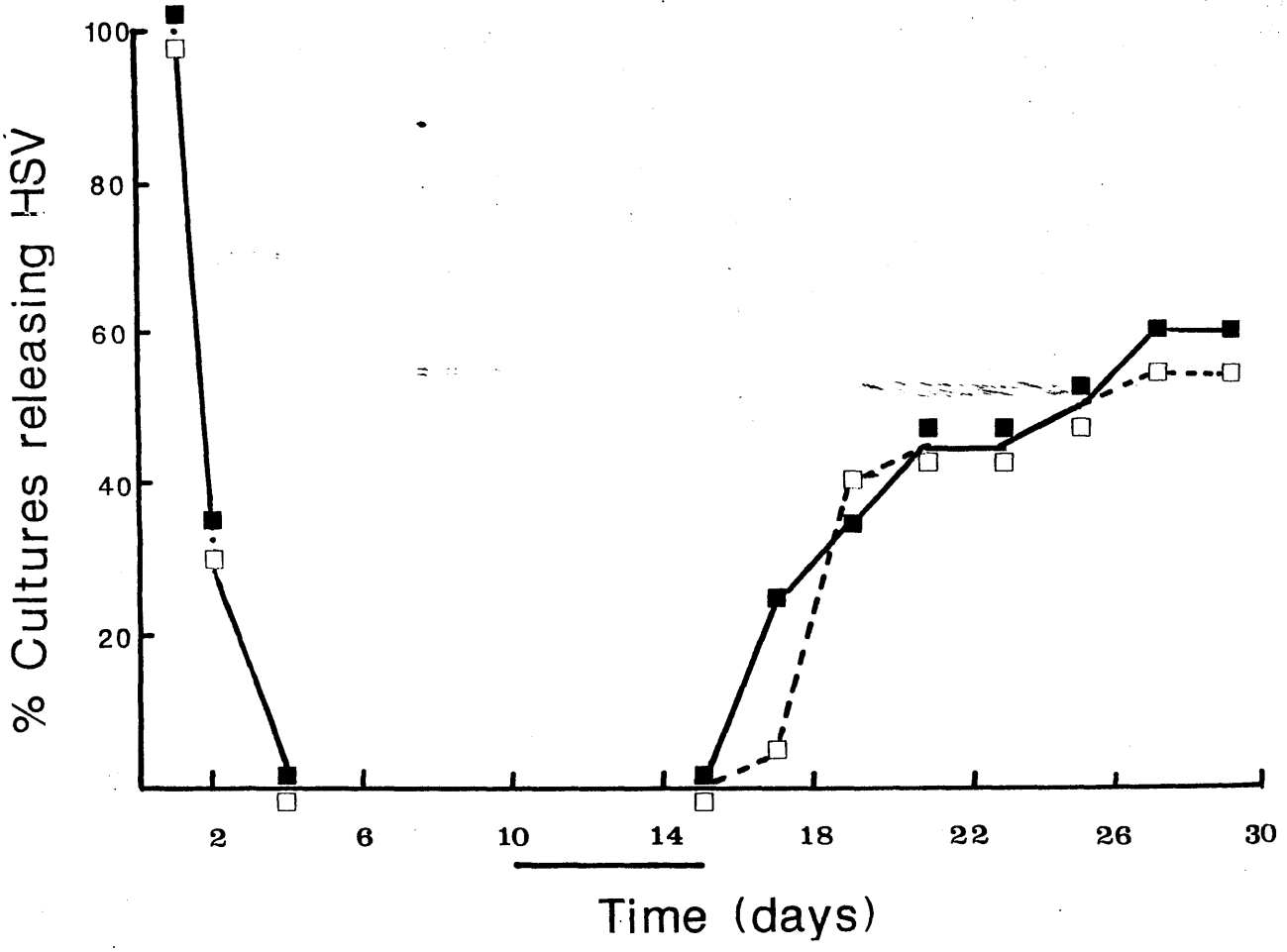
FIGURE 42

Keratocytes; non productive infections II.



ACG treated cells

non ACG treated cells


% Cultures releasing HSV
100
80
60
40
20
2
6
10
14
18
22
26
30
Time (days)

negative and in group B, 12 were positive and 6 negative. The range of first detection of virus was day 17 to 27. Some cultures shed virus for several consecutive days until the cell sheet was totally destroyed e.g. culture 19 epithelial cells B. Others shed detectable virus on one occasion only e.g. culture 13 keratocytes A.

Treatment of the infected cells with 10uM ACG for the 5 days at 42°C prior to transfer to 37°C did not significantly change either the frequency or rate of spontaneous reactivation at 37°C although reactivation seemed to occur slightly earlier in the cells that had not been treated with ACG.

Superinfection II - the isolation of latent wild type genomes and recombinants: Cultures 1 and 11 of groups A and B of both keratocytes and epithelial cells were superinfected with the X2 mutant virus on day 15 immediately after transfer from 42°C to 37°C. The XbaI and six other restriction endonuclease maps of Glasgow strain 17 are shown in Fig. 31. Superinfection was at a m.o.i of either 0.005 pfu/cell or 0.01 pfu/cell, such that the m.o.i. of superinfection was 10 times that of the initial infection. The superinfected cultures were harvested after a further 24 hrs. incubation at 37°C and the progeny virus released by sonication and then titrated. From each of the progeny virus samples 48 single plaques were isolated (making a total of 384), grown into small plate stocks and their DNA analysed using the restriction endonuclease XbaI. All 48 plaques from epithelial flask A1 had the XbaI profile of the superinfecting mutant X2 (Fig. 43, track 4). From epithelial flask A11, 1 of the 48 plaques had a novel profile (Fig. 43, track 3). The 48 plaques from epithelial

FIGURE 43

Autoradiographs of XbaI restriction digests of viral DNA labelled with ^{32}P. Glasgow strain 17 (lane 1); recombinant 3 (lane 2); recombinant 1 (lane 3); X2 (lane 4); Strain 17 (lane 5); recombinant 2 (lane 6).

▶ positions of missing fragments

● position of novel fragments

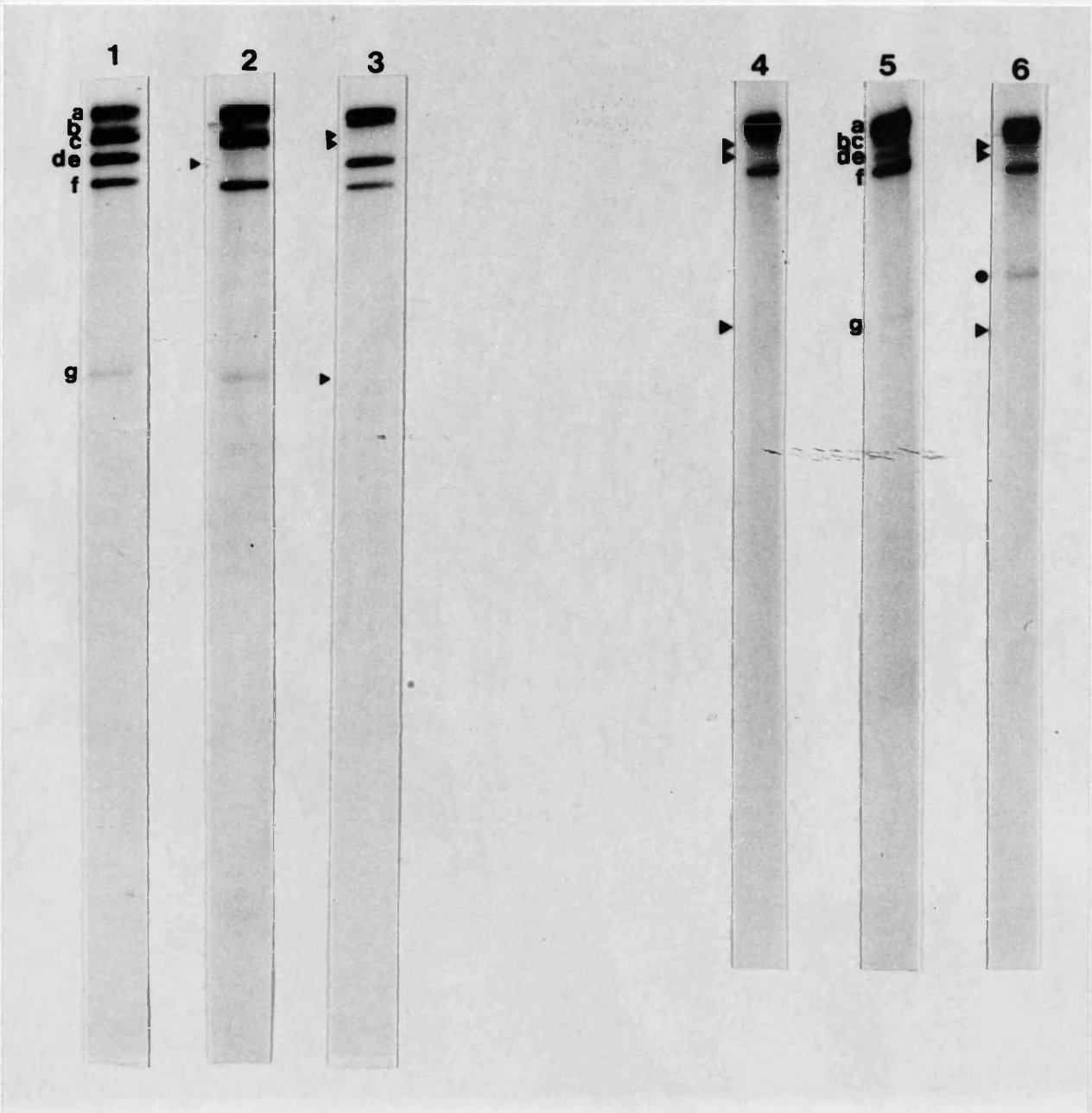

1
2
3
4
5
6
a
b
c
d
e
f
g

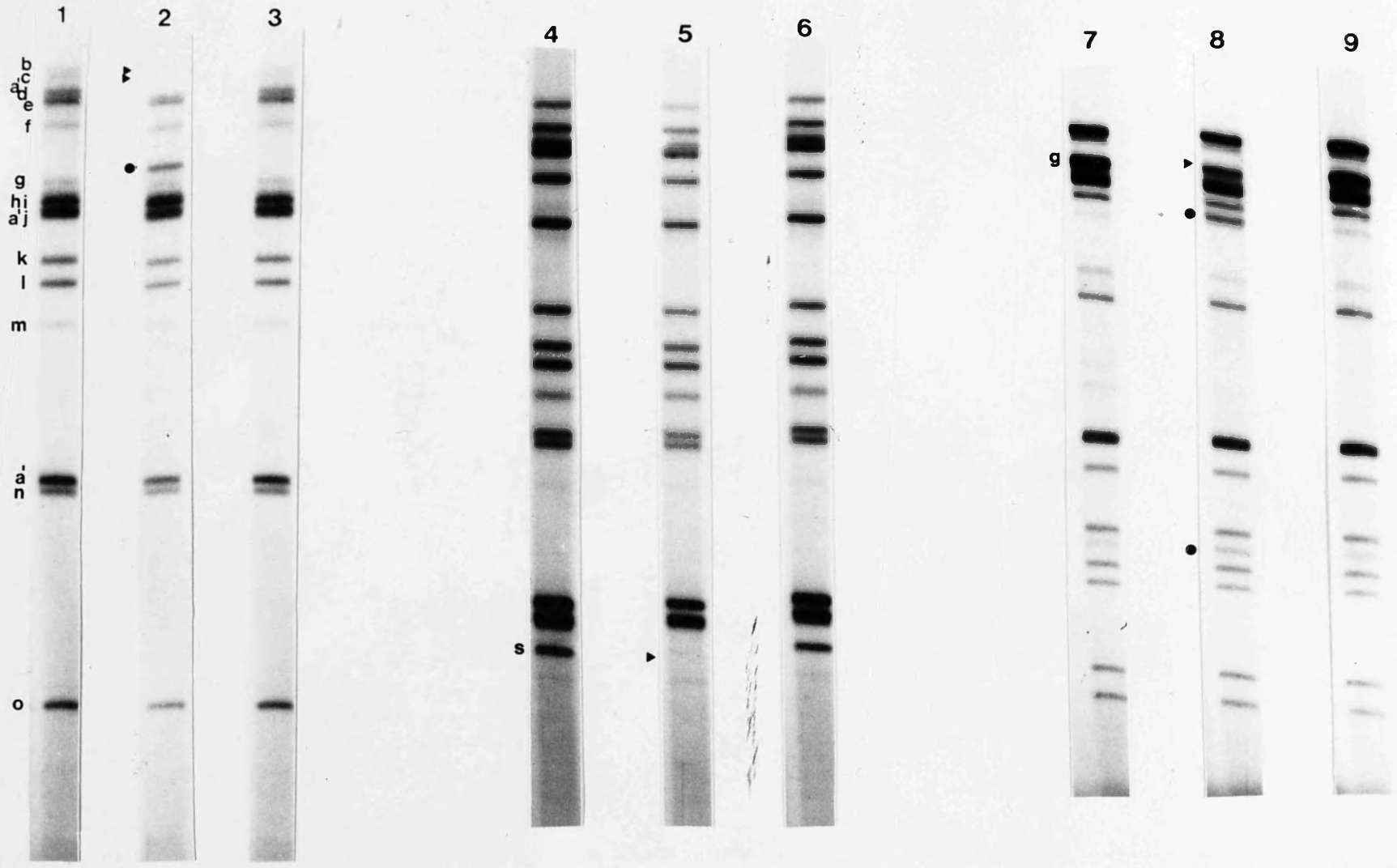
FIGURE 44

Autoradiographs of restriction digests of viral DNA labelled with ^{32}P: lanes 1-3 XbaI/HindIII; lanes 4-6 XbaI/HpaI; lanes 7-9 XbaI/KpnI.

Glasgow strain 17 (lanes 1, 4, 7); recombinant 2 (lanes 2, 5, 8); X2 (lanes 3, 6, 9).

▶ positions of missing fragments

● position of novel fragments


1
2
3
4
5
6
7
8
9
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
s

flask B1 had the X2, XbaI profile but from flask B11, 6 isolates had XbaI profiles different from X2. Three had the strain 17 wild type XbaI profile (Fig. 43, tracks 1 and 5), 2 had the same profile as shown in Fig. 43, track 3, and one had a second novel profile (Fig. 43, track 6). The 192 plaques from the keratocyte cultures showed XbaI profiles of the superinfecting X2 virus.

This shows that superinfection of the epithelial cells allowed retrieval of the original input 17^+ wild type virus genome and in addition that recombination occurred between the resident genomes and the superinfecting genomes. The XbaI profile in Fig. 43, track 3 shows missing b, c and g bands and the regained d and e bands. This means that in A11 and B11 recombinants were generated containing the XbaI site at 0.63 map units but still missing the 0.07 map unit site (Recombinant 1). The profile in Fig. 43, track 6 shows a new band running between XbaI g and XbaI f. To characterise this isolate further, XbaI/HindIII, XbaI/HpaI and XbaI/KpnI double digests were carried out. For reference the HindIII, HpaI and KpnI restriction endonuclease maps are given in Fig. 31. Figure 44 shows the HindIII/XbaI digest in which the new isolate (Recombinant 2) is compared to wild type strain 17^+ and the superinfecting X2 virus. The XbaI sites at 0.29 and 0.45 map units cut the HindIII a fragment to give three new a' fragments. The other XbaI sites do not affect the HindIII profile. It can be seen in Fig. 44, track 2 that in Recombinant 2 the HindIII d band is missing and the joints containing d, i.e. b and c are also missing. Three new bands can be seen: a 1.5M band running above g, a 0.25M band running with f and a 0.5M band running with d and e.

This is due to a new XbaI site at approximately 0.74 map units giving rise to 1M and 0.5M fragments of approximately the same size i.e. 10 x 10^6 mol. wt. and the new joints of mol. wt. 18.5 x 10^6 and 14.5 x 10^6. The XbaI/HpaI digest, Fig. 44, track 5 shows a missing s band and confirms the position of the new XbaI site. The two expected new bands are too small to be seen on this gel. As expected the XbaI/KpnI digest of Recombinant 2 shows a missing g fragment and two new fragments of mol. wt. 6 x 10^6 and 1 x 10^6 formed by the new XbaI site (Fig. 44, track 8).

Superinfection with X2 has therefore generated a genome containing the 0.29 and 0.45 map unit sites and a novel XbaI site at 0.74 map units (Recombinant 2).

Flasks which had survived to day 29 without shedding virus were also superinfected with the X2 mutant of strain 17 at 37°C. These were epithelial A flasks 3, 4, 12 and 16 epithelial B flasks 2, 3 and 12; keratocyte A, flasks 2, 3, 14 and 16 and keratocyte B, flasks 2, 3, 16 and 17. The DNAs from 48 progeny plaques each from epithelial flasks A12 and B12; and 96 plaques each from keratocyte flasks A14 and B16 were subjected to restriction endonuclease analysis by XbaI.

From the epithelial A12 progeny 45 plaques showed the X2, XbaI profile, 2 showed a wild type profile and 1 a profile indicating a recombinant with the XbaI d/e site at 0.63 map units regained (Fig. 43, track 3). From B12, 47 of the progeny plaques had the superinfecting X2 profile and one had a profile indicating a further recombinant (Fig. 43, track 2). It can be seen that the b and g bands have been regained. The profile denotes a recombinant with a regained 0.07 XbaI site, but with the 0.63 site still

missing (Recombinant 3).

Each of the 192 plaques isolated from the keratocyte cultures showed a DNA profile equivalent to the superinfecting X2 virus. Neither recombinants nor reactivated input virus was detected.

DNA-DNA hybridization I: In addition to the superinfection experiments, cells which had failed to shed infectious virus up to 29 days post infection were probed for viral DNA using the Southern blotting technique. Total DNA was extracted as described in materials and methods from the epithelial A flasks 9, 10, 17 and 20; epithelial B flasks 9 and 20; keratocyte A flasks 4 and 20 and keratocyte B flasks 4, 9 and 20. Reconstruction experiments were set up using the epithelial cells and the keratocytes infected at m.o.i.'s of 5, 1 and 0.001 pfu/cell and the DNA extracted after 24 hrs. incubation at 37°C. The probes used were ^{32}P labelled nick translated total strain 17^{+} wild type DNA and the BamHI k joint fragment containing the s and q end fragments (Fig. 31). The results for the keratocyte B group which are representative are shown in Fig. 45. Using both the total HSV-1 DNA and the BamHI k fragment, viral DNA could be detected in the reconstruction controls of epithelial cells and keratocytes infected at m.o.i.'s of 5 and 1 pfu/cell but not at 0.001 pfu/cell. No viral DNA could be detected in any of the other epithelial and keratocyte culture DNAs tested.

DNA-DNA hybridization II: Further experiments were performed to determine the origin of the novel XbaI site located at 0.74 map units. The probes used were ^{32}P labelled nick translated total wild type DNA from the strain 17; the KpnI b fragment containing the 0.07 XbaI site; the

KpnI m fragment containing the 0.29 XbaI site; the KpnI c fragment containing the 0.45 XbaI site and the KpnI d fragment containing the 0.63 XbaI site. The results are shown in figure 46.

The KpnI b fragment detects the HpaI l, m, o and r fragments; the KpnI m fragment detects the HpaI i fragment; the KpnI c fragment detects the HpaI b and h fragments and the KpnI d fragment detects the HpaI e and f fragments. All probes hybridize to their expected sites, which suggests that the new XbaI site arose because of a point mutation, or perhaps because of a small deletion/insertion. There is no evidence to support a recombination event involving one of the pre-existing XbaI sites.

FIGURE 45

Autoradiograph of nitrocellulose blot strips containing BamHI restriction fragments of strain 17 DNA to which DNA probes have been hybridized. The probes were total HSV-1 DNA (lanes 1-3) and the BamHI k fragment (pGX8) (lanes 4-11). Whole virus joint and end fragments were detected in lytically infected cells (5 and 1 pfu/cell) using the above probes. Viral DNA was not detected in latently infected cells.

Lane 1 KA21 Keratocyte flask lytic infection m.o.i. 5 pfu/cell

Lane 2 17 strain HSV-1 wild type (lug DNA)

Lane 3 KB4 latent viral infection

Lane 4 As lane 2

Lane 5 As lane 3

Lane 6 KB9 latent viral infection

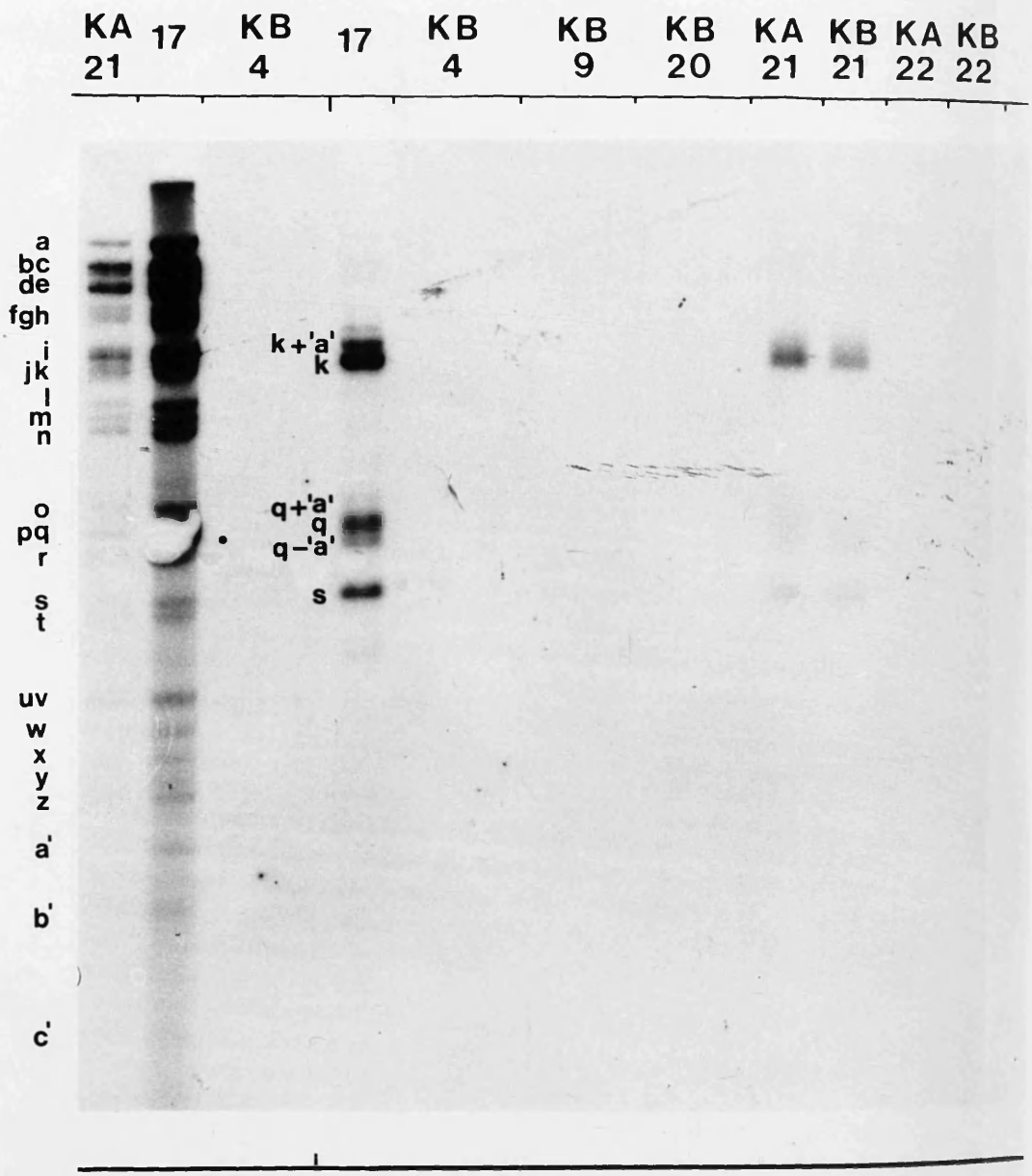
Lane 7 KB20 latent viral infection

Lane 8 As lane 1

Lane 9 KB21 keratocyte flask lytic infection m.o.i. 1 pfu/cell

Lane 10 KA22 keratocyte flask lytic infection m.o.i. 0.001 pfu/cell

Lane 11 KB22 keratocyte flask mock infection


KA 21
17
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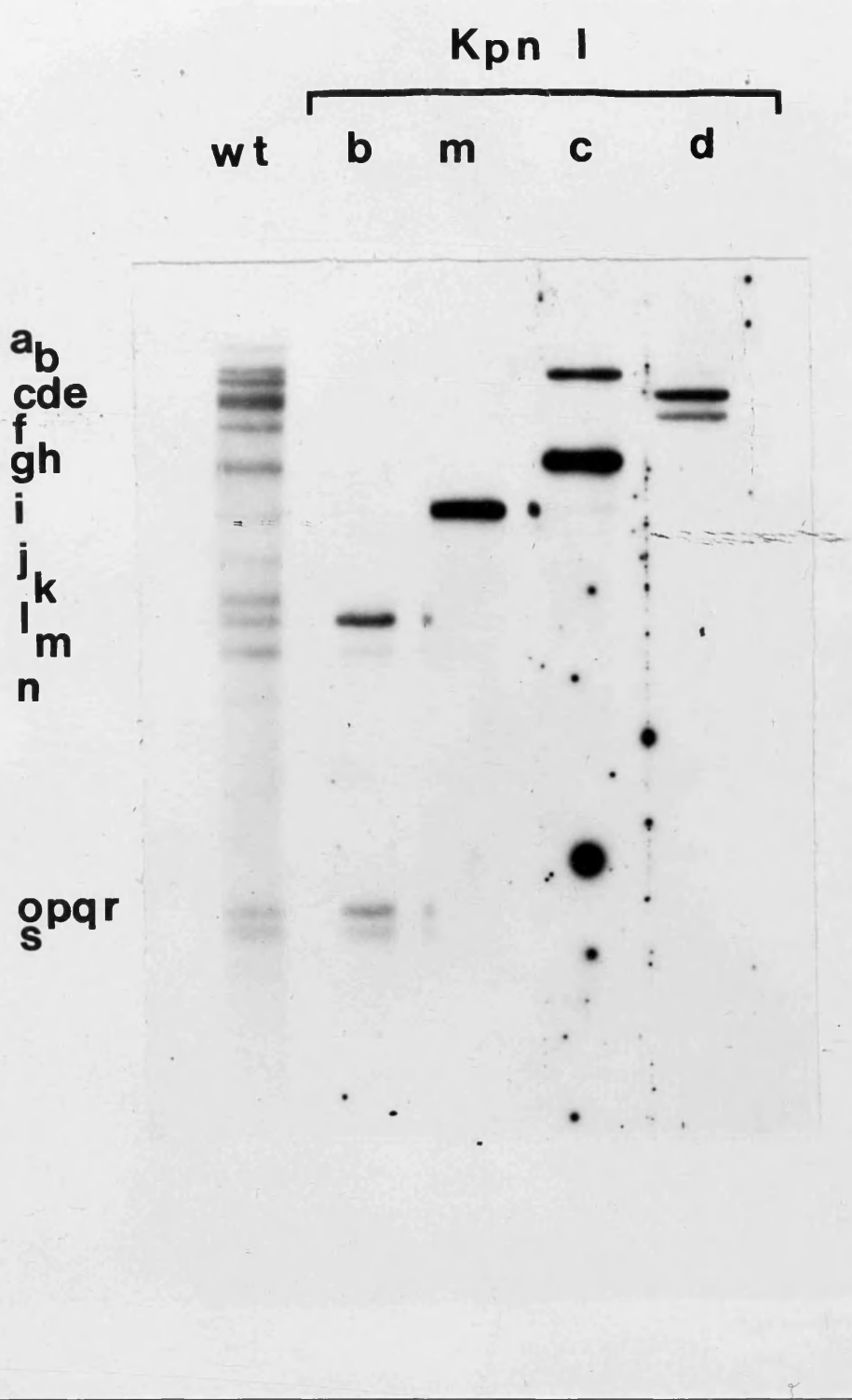
Kpn I
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FIGURE 46

Autoradiograph of nitrocellulose blot strips containing HpaI restriction fragments of strain 17 (lane 1) and recombinant 2 (lanes 2-5). The probes were HSV-1 DNA (lane 1); the KpnI b fragment (lane 2); the KphI m fragment (lane 3); the KpnI c fragment (lane 4); the KpnI d fragment (lane 5).

DISCUSSION

The isolation of herpes simplex virus from explanted human corneas (Shimeld *et al.*, 1982; Tullo *et al.*, 1985; Cook *et al.*, 1986) raises the question of whether the cell types found in the cornea are capable of supporting a latent or persistent viral infection. Separate cultures of rabbit corneal epithelial cells, keratocytes and endothelial cells whose purity was defined both morphologically and by cell specific markers were used to analyse the relationship between corneal cells and HSV. The three cell types were used initially to study the lytic cycle of HSV-1 and secondly to establish an *in vitro* latency system in cells where the virus is known to occur *in vivo*.

Previous *in vitro* latency models have used HEL-F cells and cytosine arabinoside (O'Neill *et al.*, 1972), rat foetal neurones and BVdUR plus interferon (Wigdahl *et al.*, 1983), and human foetal neurones and BVdUR plus interferon (Wigdahl *et al.*, 1982a) to examine virus and cell specific functions related to latency. All these systems employed viral inhibitors. Darai *et al.* (1975) and Marcon and Kucera (1976) showed that detectable HSV-2 DNA synthesis was blocked at the supraoptimal temperature of 42°C. Temperature elevation alone has been shown to induce latent viral infection in HEL-F cells (Russell and Preston, 1986; Russell *et al.*, 1987a). A permissive temperature of 37°C was chosen, the corneal temperature of the rabbit is 35°C *in vivo*. The *in vitro* latency system described in this thesis is solely dependent on manipulation of temperature to induce and reactivate latent HSV-1 infections. The *in vitro* latency system was used to determine whether peripheral cells, from the cornea which is a known focus of recurrent

disease in man, are capable of supporting a latent HSV infection.

During the lytic cycle, the virus growth rate was fastest in epithelial cells and closely resembled that in control BHK21C13 cells. The growth rate was intermediate in keratocyte cells and slowest in endothelial cells. Experiments by Carter et al. (1985) using corneal cells infected with HSV at lower m.o.i.s suggested that virus titres reached their maximum in 24 hours and remained relatively constant until 72 hours. It can be concluded that corneal epithelial, keratocyte and endothelial cells are capable of efficiently supporting HSV replication.

The three corneal cell types were capable of replication under heat shock conditions. Subculture infected cultures 1:2 at 41.5°C did not induce detectable reactivation. The multiplicity of infection was found to be critical in the in vitro latency system; when 0.1, 1.0 pfu/cell was used, a very large proportion of cells were killed in the seven days immediately post infection. This effect was overwhelming in some keratocyte and endothelial cell cultures leaving no surviving cells. The findings were comparable with the results of O'Neill et al. (1972) who found that the inoculating m.o.i. was critical to ensure surviving cells. Wigdahl et al. (1982a) successfully increased the m.o.i. using a combination of the antiviral drug BVdUR and interferon to treat cells prior to infection with HSV.

Epithelial cells were the only cell type to exhibit persistence, defined as a detectable chronic low grade viral infection, insufficient to destroy the cellular monolayer at 41.5°C. The three corneal cell types were able to support

a latent HSV infection, defined by absence of infectious virus in supernatant medium at 41.5°C assayed on BHK21C13 cells, but presence of infectious virus in supernatant medium at 37°C assayed on BHK21C13 cells. Virus was obtained more quickly from epithelial cells and endothelial cells than keratocytes at 37°C.

Corneal cell types have different properties of replication *in vivo*; epithelial cells divide and are shed throughout life whereas keratocytes have replicative potential but tend to be stable, and endothelial cells have limited if any replicative ability *in vivo* (Davson, 1980; Maumenee and Kornblueth, 1949). It is established that HSV latency occurs in non dividing neurones, *in vivo*, both in man (Bastian *et al.*, 1972; Baringer and Swoveland, 1973); and in animals (Stevens and Cook, 1971; Stevens *et al.*, 1972). Wigdahl *et al.* (1984a) showed in an *in vitro* system that neurones were capable of supporting more latent HSV genomes, per haploid cell, than HEL-F cells.

In our *in vitro* system, keratocytes and endothelial cells which are non dividing *in vivo* were capable of maintaining a latent HSV infection. Epithelial cells were also capable of maintaining an *in vitro* latent HSV-1 infection, but in addition epithelial cells demonstrated some persistent HSV infection at 41.5°C. No surviving epithelial cell cultures failed to release infectious HSV upon transfer to 37°C, in contrast to keratocyte and endothelial cell cultures where eight cultures in total did not release infectious virus at 37°C. It is possible that non inducible herpes simplex virus was present in these cultures and might have been detected by viral superinfection experiments. It is evident that the

phenotype of the cell affects the outcome of both lytic and latent infection of corneal epithelial cells, keratocytes and endothelial cells *in vitro*.

Only temperature elevation or reduction was used to induce latency or trigger reactivation in our *in vitro* system. Heat shock induces stress proteins (Tissieres *et al.*, 1974). Notarianni and Preston (1982) and Russell *et al.* (1987b) have shown that stress proteins are induced by the immediate early proteins of HSV. In our system most cellular stress proteins were induced by heat shock. It was not possible to ascertain whether additional proteins were induced in any of the three cell types infected at low m.o.i. with HSV. However it is clear that cellular stress proteins were present at 41.5°C when HSV was latent and were much reduced in intensity at 37°C when virus was able to reactivate.

Acycloguanosine (ACG) was used to distinguish latent and persistent viral infection in the second series of experiments. The nucleoside analogue 9-(2-hydroxy ethoxy methyl) guanine (acycloguanosine) has been shown to be an effective and extremely selective antiviral agent blocking the replication of several herpes viruses both *in vitro* and *in vivo* in a number of animal models (Elion *et al.*, 1977; Schaeffer *et al.*, 1978). If low levels of replicating virus were present in corneal cell cultures at 42°C, incubation in the presence of ACG would eliminate such virus and upon transfer to 37°C there would be no release of infectious persistent virus. However if the virus genomes were present in a latent form inaccessible to the drug ACG, treatment would be expected to have no effect and upon transfer to the permissive temperature there would be

release or reactivation of virus.

It has been shown by Schaeffer et al. (1978) that the ID_{50} of ACG on the ICI strain of HSV-1 as measured by plaque reduction on Vero cells was 0.1uM. ACG (10uM) was effective in blocking the lytic cycle of HSV at 37°C in rabbit corneal epithelial cells and keratocytes. Incubation of ACG at 42°C for 48 hours did not inactivate the effect of ACG on a subsequent lytic infection at 37°C. The time interval between medium changes in the in vitro latency system was 48 hours. Compared to Schaeffer et al. (1978), we used a 100 fold higher concentration of 10uM as being optimal for virus inactivation without a cytotoxic effect. The presence of this high ACG concentration for 5 days prior to transfer to 37°C did not affect the number of cultures shedding virus, nor did it significantly alter the onset of first virus release or the duration of shedding. This finding applied to both epithelial cells and keratocytes and to both infecting multiplicities. It appears therefore that rabbit corneal keratocytes and epithelial cells are both capable of supporting a latent infection. The latent infection was induced and maintained by incubating cells at 42°C. At 42°C the virus genomes are probably non-replicating and therefore inaccessible to the action of ACG but they must retain the potential to initiate replication within 48 hrs. of shift to 37°C.

Superinfection experiments were performed to determine whether it was possible to reactivate virus from latently infected cultures before spontaneous virus release. To maximise the chance of both reactivation of resident HSV from the corneal cultures and recombination between resident and superinfecting genomes, we chose to superinfect the

latently infected cells with the X2 mutant of HSV-1 strain 17 (Brown et al., 1984). The X2 mutant has lost the XbaI sites at 0.07 and 0.63 map units and was constructed by recombination between two viruses each lacking a single site. The viruses with the individual site losses were isolated by enrichment selection with XbaI. The removal of the site at 0.07 map units is due to a base change or deletion or insertion of less than 150 base pairs. The site loss at 0.63 map units is due to a 150bp deletion.

XbaI analysis of the progeny from cells superinfected on day 15 immediately after transfer to 37°C showed that of 192 plaques analysed from the epithelial cultures, 185 had the profile of the superinfecting virus and 7 had XbaI profiles differing from the superinfecting virus. Three had the XbaI profile of the original infecting virus and were either true reactivants or recombinants and 4 had profiles indicative of recombinant virus. Three of the four recombinants had regained the XbaI site at 0.63 but still lacked the site at 0.07 map units. The simplest generation of this recombinant would have been by a crossover anywhere between 0.07 and 0.63 map units. Two of the three recombinants could have been clonally related as they came from the same superinfected flask. The third came from a separate epithelial culture which had been treated with ACG. The fourth recombinant isolated from epithelial cells had gained a new XbaI site at 0.74 map units. This may have been generated by illegitimate recombination and the insertion of an existing XbaI site from one of four places into a novel position at 0.74 map units. To determine whether there has been insertion of sequences from other parts of the genome appropriate

experiments were carried out. The results indicated that the new site had not arisen from one of the existing XbaI sites unless it was within an insert of less than 150 base pairs. Other possibilities are that there has either been a spontaneous mutation generating a new XbaI site or that the site is derived from a host insert. There is no evidence so far in HSV of host insertion. The new XbaI site lies within the BamHI b fragment (0.735-0.81 map units). From the 0.735 map unit end of BamHI b to the 0.81 map unit end there are 10 potential sequences in which a single base change could generate an XbaI site. However in the course of isolating deletion mutants of HSV-1 and HSV-2 the DNA of over 10^4 single plaques has been analysed (Brown, Harland, MacLean, personal communication), and only one new XbaI site at 0.16 map units has been identified in a plaque stock of HG52 (M.Y. Taha, personal communication).

It was possible that by superinfecting immediately on transfer to 37°C, virus was being reactivated which would have been released spontaneously and that superinfection was merely speeding up the process. To determine whether cultures which had failed to shed virus after 14 days at 37°C contained virus genomes, similar superinfection experiments were carried out on day 29 post infection. As a random sample the DNAs from 96 single plaques from ACG and non ACG treated epithelial cultures and 96 from equivalent keratocyte cultures were analysed. The superinfecting X2 profile was present in 92 of the plaques from epithelial cells, 2 had a wild type profile, 1 had a recombinant profile with the 0.63 site regained and one had a recombinant profile with the 0.07 site regained. A single crossover between the resident and superinfecting genomes

would generate a recombinant containing the 0.07 site. The two recombinants, with the 0.07 or 0.63 map unit sites regained are generated by reciprocal recombination and could have been generated by a single crossover event. The 96 plaques analysed from the keratocyte cultures, all had the superinfecting X2 profile.

It is evident therefore that after removal from the conditions inducing latency, corneal epithelial cells are capable of continuing to harbour HSV-1 in a latent form for at least 14 days without shedding infectious virus. The genomes cannot be present as persistent intact infectious virus but the DNA must be in a state where it can readily make infectious particles as evidenced by the isolation of virus with the wild type profile, at least a proportion of which is assumed to be reactivated input virus. In addition the isolation of recombinants suggests that the resident DNA readily interacts with the superinfecting genomes. The way in which the single plaques have been isolated and their DNA analysed precludes the generation of recombinants outwith the corneal cells. The data does not distinguish between the HSV-1 DNA being in the cells as episomes, concatamers or integrated into the host chromosomes.

It could be argued that the regaining of the sites at 0.07 and 0.63 is due to spontanous reversion at these sites. The loss of the 0.63 site is due to a 150 base pair deletion so spontaneous reversion does not come into question. The 0.07 site could be regained by a base change but the DNA analysis of several thousand single plaque stocks of X2 has not brought to light a single revertant for the 0.07 site (A. MacLean - personal communication).

It is apparent that ACG has no effect on the superinfection experiment. Resident and recombinant genomes were isolated from treated and untreated cultures.

Intertypic recombinants have been isolated from rat embryo transformed cells (Park et al., 1980) but this is the first isolation of recombinants from latently infected cultured cells. The use of intratypic R.E. site deletion mutants greatly facilitated the study. Plaques were picked at random and identification of reactivants or recombinants was based on subsequent screening for unselected markers. Infecting at a m.o.i. of 0.001 pfu/cell, the maximum number of cells infected would be 10^3. Superinfecting at a m.o.i. of 0.01 pfu/cell would mean that 1 in 10^5 cells would be doubly infected. The calculation is theoretical and does not take into account the actual number of cells which would be infected initially or on superinfection; the particle:pfu ratios of the stocks; or any virus replication taking place at 42°C. In 10^6 cells, 10 at maximum would be doubly infected. For every 1 doubly infected cell 10^3 will be infected with X2. Resident input virus i.e. wild type for XbaI sites has been isolated at a frequency of 2% in epithelial cells. This is 20 fold higher than expected. There are several possible explanations for the relatively high frequency of reactivation.

(1) The ts+ virus has a growth advantage over X2. This is unlikely as one step growth experiments have shown that X2 and its parental virus have equivalent growth rates (Brown et al., 1984).

(2) Reactivation of the wild type virus does not require the superinfecting virus to be in the same cell but is dependent on a viral product synthesised by the superinfecting virus

in surrounding cells.

(3) Assuming that both resident and superinfecting virus require to be in the same cell for reactivation to occur there has to be limited replication of the original input virus at 42°C such that the number of doubly infected cells is 20 fold higher than calculated from the initial m.o.i. The frequency of isolation of recombinant virus was also about 2%. For recombination to occur both genomes must be in the same cell. It would appear therefore that there has been limited replication of the original infecting virus at 42°C.

(4) It has been shown that non-infectious particles make a contribution to complementation in ts mutant infections (L.I. Messer, PhD thesis 1978). It is possible therefore that the particle:pfu ratios of the virus stocks, may affect the number of cells both initially infected and superinfected. Our strain 17 stock had a particle:pfu ratio of 10:1 and the X2 stock a particle:pfu ratio of 35:1. The contribution made by non infectious particles could be important.

(5) If the original infecting genome is integrated into the host chromosome or is episomal and there is some cell division then the number of cells containing HSV would be higher than expected. Corneal epithelial cells divide and we have shown previously that they are capable of division at 41.5°C (Cook and Brown, 1986). Credence could be given to this argument by the fact that we have been unable to demonstrate reactivation or recombination from keratocyte cultures. The fact that keratocytes behave as epithelial cells in being able to shed virus after 14 days at 42°C suggests that the original genomes should be present.

If the original genomes are integrated, there must be rapid release fom integration to be available for recombination.
(6) After reactivation there is time for a round of viral replication within 24 hours.
(7) Another explanation would be that variation in the physiological and metabolic state of individual cells determines differences in the rate of virus release.

Southern blotting with whole HSV-1 genomes or with joint fragments failed to detect HSV information in latently infected cells. In reconstruction experiments HSV was detectable at input m.o.i.s of 5 and 1 pfu/cell but not at 0.001 pfu/cell. The Southern blotting experiments were not as sensitive as superinfection in detecting resident genomes. This contrasts with the results of Wigdahl et al. (1984a) who were able to detect HSV DNA in neurones in an in vitro latency system. Several important differences are present between their system and the corneal cell latency system. The m.o.i. used by Wigdahl et al. (1982a, 1984a) was considerably higher (2.5 pfu/cell). Interferon and antiviral agents were used to induce the latent infection and temperature elevation was used to maintain the latent state.

Other workers have found the limit of sensitivity of Southern blotting to be around 0.01-0.1 copies of viral DNA/cell (Efstathiou et al., 1986). This level of sensitivity is inadequate to detect viral genomes present in our system where cells are infected at a very low m.o.i.

The ability of rabbit corneal epithelial cells to support a latent infection gives credence to the possibility that in man recurrent episodes of herpes keratitis may be due to reactivation of virus from a latent state in the

cornea itself in addition to reactivation from dorsal root ganglia. In conjunction with evidence from the mouse model system where HSV has been shown to be latent in the skin of the footpad (Al-Saadi et al., 1983) this work supports the hypothesis that HSV does not require cells of neurological origin in order to establish and maintain a latent infection.

FUTURE PROSPECTS

Few of the variable biological characteristics of HSV with the notable exceptions of neurovirulence, which has been mapped to the region of the genome between 0.71 and 0.74 map units (Thompson et al., 1983, 1985) and the property of reactivation which has been excluded from the regions of the genome between 0.35-0.56 and/or 0.82-1.0 map units (Batra, 1987), have been mapped. Accurate characterization of HSV types with differences in biological properties, like the clinical signs of primary disease and/or frequency of induced reactivation e.g. HSV-1 strain McKrae and HSV-2 strain HG52, will allow the construction of intertypic recombinants. The novel recombinants can be tested and compared to the parental strains in animal experiments. By this painstaking technique biological properties may be assigned to a region of the genome. Pitfalls can be easily foreseen as a single gene may not be totally responsible for reactivation. Other biological variables i.e. primary disease may be similarly controlled. Further caution in the interpretation of results will be required as no virus strain is likely to be absolutely negative for a biological characteristic.

Recent in situ hybridization results have detected latency related HSV-1 RNAs in the trigeminal ganglia of rabbits, mice and man (Rock et al., 1987a; Spivak and Fraser, 1987; and Croen et al., 1987). More than one latency related RNA transcript was detected, and the genes for the latency related RNAs mapped in the region of the IE1 gene which encodes the polypeptide V_{mw}EIl10. The latent HSV-1 RNAs were found to be transcribed in the direction opposite to that of IE1mRNA. These results confirmed the

work of Stevens et al. (1987).

Rock et al. (1987b) performed similar in situ hybridization experiments using bovine herpes virus type 1, and found that the latency related RNAs mapped to 0.734-0.748 map units of the viral genome. This area is abundantly transcribed at immediate early times in infection, but precise mapping data is not available.

The precise role of the latency related RNAs has yet to be elucidated, the anti-sense RNA may regulate the IE1 gene or it may encode a regulatory protein capable of suppressing the HSV-1 lytic cycle or interacting with cellular transcription factors.

Within the confines of this thesis future work could determine whether a similar pattern of RNA expression occurs in latent infections in vitro. Does the in vitro latency system have any relevance to the in vivo situation? Just how essential is the IE1 gene for latency? G.B. Clements and N.D. Stow (unpublished results) have shown that the dl 1403 HSV-1 mutant can establish a latent infection in mice which can be reactivated after co-cultivation. The mutant synthesises a truncated form of V_{mw}IE110 and it is possible that the truncated RNA is adequate for the establishment and maintenance of latency.

Further confirmation of the cornea as a site of peripheral latency could be obtained by in situ hybridization experiments on human and animal corneas, after herpetic keratitis.

Current evidence points to viral DNA being present during latency in concatameric or circularized forms. However it remains unknown whether the DNA undergoes rearrangements or is integrated with cellular DNA or exists

in an episomal form.

In conclusion medical practitioners have potent antiviral agents which permit acute and recurrent infections to be treated. However treatment is unable to prevent the establishment or reactivation of latent infections. Until such times as the mechanisms of latency and reactivation are understood, medical therapy will be directed towards containment of disease rather than prevention.

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