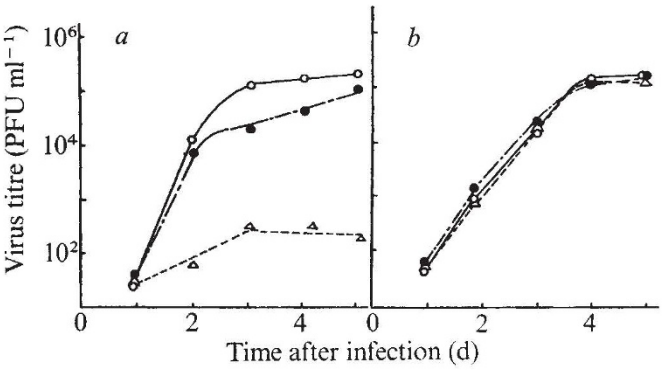

Fig. 1 Cultures of A31-714 and SA31 inoculated with HR-101, Wt, HR-101 plus SV40 (777 strain) or WT plus SV40. The multiplicities of infection of HR-101 and WT were 0.03 and that of SV40 was 60. After adsorption at 37 °C for 6 h, cultures were washed three times with medium, covered with 5 ml of Eagle's MEM medium containing 10% calf serum and incubated at 37 °C at 24 h intervals, cultures were collected and stored frozen. Cells and media were sonicated and their virus titres were assayed by plaque formation on cultures of SA31 cells. Growth of HR-101 (*a*) and WT (*b*) in SA31 cells (○), in A31-714 cells without SV40 (△) and in A31-714 cells with SV40 (●).

was detectable after 2 d and reached a maximum after 3–4 d (Fig. 1*a*). In contrast, the wild type multiplied equally well in A31-714 cells and SA31 cells (Fig. 1*b*).

To determine whether the change in susceptibility of A31-714 cells to infection by the mutant was caused by SV40, the helper function of SV40 in multiplication of the mutant in A31-714 cells was also examined. A31-714 cells were co-infected with HR-101 (multiplicity of infection (m.o.i.), 0.03) and SV40 (m.o.i., 60) and the yields of HR-101 were followed by the method described above.

The yield of HR-101 in A31 cells was enhanced approximately 60-fold by co-infection with SV40 (Fig. 1*a*).

When SV40 samples were treated with anti-SV40 rabbit serum, their ability to enhance the replication of HR-101 disappeared completely (Table 2). The viral characters of HR-86 were similar to those of HR-101 described above.

In preliminary experiments, cells in primary cultures from embryos of a BALB/c mouse also showed low susceptibility to host range mutants and co-infected SV40 enhanced the growth of these mutants in the primary cells (data not shown).

These results suggest that SV40 converts the susceptibility of mouse cells to infection with these mutants from a restrictive to a non-restrictive state. Although the role of SV40 in this conversion of cellular susceptibility has not yet been elucidated, the restrictive point to the infection of HR-101 in A31-714 cells may be considered not before the penetrating step but after the uncoating step, because DNA from HR-101 was also considerably less infectious to A31-714 cells than to SA31 cells (Table 3). The defect in virus growth of the mutants may be helped either by the direct action of a SV40 viral gene product(s) present in both SV40-transformed cells and mouse cells abortively infected with SV40, or by the indirect action in which a SV40 genome in these cells alters the expression of cellular genes. In any case, an 'early' event(s) of SV40 infection may be concerned with change in susceptibility to these host range mutants because SV40 did not produce 'late' gene products in mouse cells on either infection or transformation[12,13].

Table 2 Effect of anti-SV40 serum on the helper function of SV40

Type of infection	Type of cells	Virus titre per ml*	Enhancement ratio
HR-101	A31-714	7.8×10^2	1.0
HR-101+SV40	A31-714	4.5×10^4	57.7
HR-101+(SV40)†	A31-714	2.8×10^2	0.4
HR-101	SA31	1.0×10^5	128.2

Cultures of A31-714 and SA31 containing 3.2×10^5 cells per 4-cm plastic dish were inoculated with HR-101 at a multiplicity of 0.03. Some cultures of A31-714 were inoculated with both HR-101 and SV40 (m.o.i., 10) or HR-101 and SV40 which had been treated with anti-SV40 serum. After adsorption at 37 °C for 6 h, cultures were washed three times with medium and covered with 5 ml of Eagle's MEM medium containing 10% calf serum. These cultures were collected 72 h later. The cells and media were sonicated and virus infectivity in the lysates was assayed by plaque formation on cultures of SA31 cells.

*72 h post-infection.

†Treated with anti-SV40 serum.

Table 3 Virus yields after infection with viral DNA

DNA	DNase treatment (20 μg ml⁻¹)	Yield (PFU per culture) A31	SA31	Ratio SA31 : A31
Wt	−	1.5×10^3	1.8×10^3	1.2
	+	< 10	< 10	—
HR-101	−	5.0×10^1	9.0×10^3	180.0
	+	< 10	< 10	—

A confluent monolayer of A31-714 cells or SA31 cells in a 6-cm Petri dish was washed once with 5 ml of phosphate-buffered saline and then infected with 0.1 ml of a mixture containing wild-type DNA (7.2×10^2 PFU: assayed on SA31 cells) or HR-DNA (2.8×10^3 PFU: assayed on SA31 cells), DEAE–dextran (500 μg ml⁻¹) and Tris-HCl (*p*H 7.5, 0.05 M). After incubation at room temperature for 30 min, the cells were washed once and then 5 ml of growth medium (MEM+10% calf serum) were added. After 3 d at 37 °C, cells and fluids were collected, sonicated and assayed on SA31 cells to determine virus yield.

Further experiments on the characteristics of host range mutants and on the relation between the gene functions of polyoma virus and SV40 are in progress.

I thank Dr K. Toyoshima for discussions and Mrs Y. Fukushima for assistance. This work was supported by the Ministry of Education, Science and Culture, Japan.

AKIRA HAKURA

Department of Tumor Viruses,
Research Institute for Microbial Diseases,
Osaka University,
Suita, Osaka, Japan

Received 21 September 1976; accepted 5 April 1977.

1 Fried, M. *Virology* **25**, 669–671 (1965).
2 Eckhalt, W. *Virology* **38**, 120–125 (1969).
3 di Mayorca, G., Callender, J., Martin, G. & Giordano, R. *Virology* **38**, 126–133 (1969).
4 Hakura, A. *Biken J.* **17**, 117–119 (1974).
5 Kit, S., Tokuno, S., Nakajima, K., Trkula, D. & Dubbs, D. R. *J. Virol.* **6**, 286–294 (1970).
6 Tegtmeyer, P. & Ozer, H. L. *J. Virol.* **8**, 516–524 (1971).
7 Robb, J. A. & Martin, R. G. *J. Virol.* **9**, 956–968 (1972).
8 Kimura, G. & Dulbecco, R. *Virology* **49**, 394–403 (1972).
9 Benjamin, T. L. *Proc. natn. Acad. Sci. U.S.A.* **67**, 394–399 (1970).
10 Goldman, E. & Benjamin, T. L. *Virology* **66**, 372–384 (1975).
11 Kakunaga, T. *Int. J. Cancer* **12**, 463–473 (1973).
12 Kit, S., Dubbs, D. R., Piekarski, L. J., Detorres, R. A. & Melnick, J. L. *Proc. natn. Acad. Sci. U.S.A.* **56**, 463–470 (1966).
13 Black, P. H. *Virology* **28**, 760–763 (1966).

Extraneural localisation of herpes simplex virus in latently infected guinea pigs

CHRONIC latent herpes simplex virus (HSV) infections are associated with tissues of the nervous system in experimentally infected mice and rabbits[1,2] as well as in humans[3–5]. Cook and Stevens demonstrated, that, after inoculation into the skin, HSV migrates via the subserving nerves to the corresponding sensory ganglia[6]. The virus establishes there a latent infection which can be detected by methods of *in vitro* cultivation of the ganglia[1–5]. A consideration of the data obtained so far on the behaviour of latent HSV in mice, rabbits and men led Cook and Stevens as well as Baringer to the postulate, that latent HSV infections were restricted to tissues of the nervous system[7,8]. The failures to detect HSV in the skin during latent infections are con-

Table 1 Recovery of HSV type 2 during the chronic latent infection

Animal	Day after infection	Virus recovered from: Footpad	Sciatic nerve	Dorsal root ganglia (L_4–S_3)	Lumbosacral spinal cord	Number of recurrent eruptions	Day after infection when last recurrence developed
261	97	+	+	+	ND	None	—
885	97	+	+	+	ND	1	41
298	97	+	−	−	ND	None	—
274	133	+	−	+	−	2	73
839	136	+	−	+	−	2	80
569	136	+	−	+	−	4	104
272	143	+	−	+	−	None	—
241	143	−	+	−	−	None	—
775	194	+	−	−	+	2	125
88	194	+	−	+	−	3	38
230	208	+	+	−	+	1	38
757	208	+	?	−	+	1	73
799	210	+	+	−	ND	4	110
883	210	+	+	+	ND	2	95
603	210	+	+	−	ND	2	58

ND, not done. ?, Cultures contaminated.

sistent with this hypothesis[9,10]. Recurrent HSV infections are thought to arise by activation of the latent infection within the ganglia and the subsequent migration of the reactivated virus into the skin via the corresponding nerve fibres[8,11]. I have described recently experimental infections of guinea pigs with HSV type 2, which led to chronic latent infections of the animals with frequent spontaneously recurring lesions at the sites of initial inoculation[12,13]. In this report I present evidence that during the latent phase of the infection HSV can be recovered not only from nervous tissues but also from the skin at the site of the primary infection.

Fifteen albino guinea pigs were inoculated with 10^4 plaque-forming units of HSV type 2, strain 72, subcutaneously into the left hind footpad. They were observed for clinical signs of primary and recurrent herpetic lesions three times a week. The animals were sacrificed between 3 and 7 months after infection at times when they did not show any clinical sign of recurrent infection. Pertinent tissues were taken from exsanguinated animals and assayed for latent virus by explantation on monolayers of primary rabbit kidney (PRK) cells as described previously[11]. All isolates were passaged once on PRK cells and identified as HSV by neutralisation with an anti-HSV hyperimmune serum, prepared in rabbits.

The data (Table 1) show, that all 15 animals harboured latent HSV, irrespective of whether they had developed previous recurrent infections or not. Surprisingly, however, from 14 of the animals virus was isolated from the skin of the initially infected footpad, whereas only eight animals were shown to harbour virus in their lumbosacral dorsal root ganglia and three animals in the lumbosacral part of the spinal cord. In addition, HSV was recovered from the sciatic nerve trunks of seven animals.

To test whether chronic latent infections of guinea pigs could be established with HSV type 1 as well and if so, where the virus would be located, a group of animals was similarly infected with HSV type 1, strain McIntyre. This strain does not induce recurrent infections in guinea pigs (unpublished). Chronic latent infection of the skin of the footpad was detected in six out of eight animals (Table 2). None of the eight guinea pigs, however, could be shown to harbour latent virus in their nervous system.

The surprising finding that HSV could be recovered preferentially from the skin of the footpad during the clinically quiescent phase of the infection offers two alternative interpretations. First, in contrast to the well documented restriction of latent HSV infections to tissues of the nervous system in mice and rabbits, guinea pigs may harbour latent HSV type 2 preferentially and latent HSV type 1 exclusively in the skin at the site of the primary inoculation. Alternatively, HSV infections of guinea pigs may be no exceptions to the rule that latent virus resides exclusively in nervous tissues. Such latent virus may, however, become frequently or even consistently reactivated in the ganglia to spread via peripheral nerves into the skin, although overt clinical disease may be only rarely induced. The latently infected ganglia may harbour only small amounts of virus, frequently escaping detection in our assay, whereas the skin, and in some animals also the nerve trunks, may contain enough reactivated virus to be more easily recovered.

The second explanation seems more likely as it is essentially consistent with data, obtained in other species. In addition, it could support a hypothesis on the pathogenesis of recurrent herpes infections, suggested recently by Hill and Blyth[14]. According to this theory, virus is frequently activated in ganglia, travels to the skin, and induces microfoci of infected epidermal cells, which are usually eliminated by the host's defence mechanisms. Visible lesions develop only under some additional alteration of the local environment. If this theory is correct, virus should be frequently detectable in the skin. The previous failures to recover virus from skin of mice and men[9,10] using organ culture may be due to the comparatively rare spontaneous reactivation of the latent virus within the ganglia in these species.

Further experiments in neurectomised guinea pigs should help to determine which of these alternative interpretations is correct. Evidence was given in a previous report[12], that HSV travels along the sciatic nerve from skin to ganglia and vice versa. Dissection of the nerve before infections should therefore prevent the establishment of latent infection in ganglia. Consequently, in such animals neither

Table 2 Recovery of virus from animals infected with HSV type 1

Day after infection	Virus recovered (animals positive/animals tested) from: Footpad	Sciatic nerve	Dorsal root ganglia (L_4–S_3)	Lumbosacral spinal cord
79	0/2	0/2	0/2	0/2
198	2/2	0/2	0/2	0/2
313	4/4	0/4	0/4	0/4

recurrent lesions nor latent virus in the skin of the footpad should be found, if the second explanation holds. Such experiments are currently under way.

I thank J. Botto, A. Danielopol and E. Moser for technical assistance.

MARIANNE SCRIBA

Sandoz Forschungsinstitut, A-1235 Vienna, Austria

Received 20 December 1976; accepted 25 April 1977.

1 Stevens, J. G. & Cook, M. L. *Science* **173**, 843–845 (1971).
2 Stevens, J. G., Nesburn, A. B. & Cook, M. L. *Nature new Biol.* **235**, 216–217 (1972).
3 Bastian, F. O., Rabson, A. S., Lee, C. L. & Tralka, T. S. *Science* **178**, 306–307 (1972).
4 Baringer, J. R. & Swoveland, P. *New Engl. J. Med.* **288**, 648–650 (1973).
5 Baringer, J. R. *New Engl. J. Med.* **291**, 828–830 (1974).
6 Cook, M. L. & Stevens, J. G. *Infect. Immun.* **7**, 272–288 (1973).
7 Cook, M. L. & Stevens, J. G. *J. gen. Virol.* **31**, 75–80 (1976).
8 Baringer, J. R. *Prog. med. Virol.* **20**, 1–26 (1975).
9 Rustigian, R., Smulow, J. B., Tye, M., Gibson, W. A. & Shindell, E. *J. inves Derm.* **47**, 218–221 (1966).
10 Wohlenberg, C. R., Walz, M. A. & Notkins, A. L. *Infect. Immun.* **13**, 1519–1521 (1976).
11 Stevens, J. G. *Curr. Top. microbiol. Immunol.* **70**, 29–50 (1975).
12 Scriba, M. *Infect. Immun.* **12**, 162–165 (1975).
13 Scriba, M. *Med. microbiol. Immunol.* **162**, 201–208 (1976).
14 Hill, T. J. & Blyth, W. A. *Lancet* **i**, 397–399 (1976).

Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture

WHEN chondrocytes from sternal or articular cartilage are kept in monolayer culture at low density, they eventually lose their cartilage phenotype[1–4]. Within four passages or approximately 1 month in culture they change from a polygonal or round to a flattened, amoeboid-like shape[5–7], and instead of cartilage collagen (type II collagen[8]) they synthesise the genetically different type I collagen. It is not known whether there is a strict correlation between the occurrence of cell flattening and the change in collagen synthesis within individual cells. We have reported that preferentially flattened, fibroblast-like cells at the edge of cartilage colonies synthesise type I collagen, whereas round or polygonal chondrocytes generally synthesise type II collagen[1–3]. The change is nearly complete in a culture at a time when excessive flattening is observed[4]. Using an immunofluorescence double staining technique[9,10], we have now found that there is no strict correlation between cell morphology and type of collagen synthesised.

We used chondrocytes from 16-day embryonic chick sternal cartilage, which were released by treatment with trypsin and collagenase[1–3] and cultured on tissue culture plastic dishes at a density of 10^4 cells per cm^2 in Ham's F12 medium and 10% foetal calf serum. In these conditions the change from type II to type I collagen synthesis was nearly complete within 1 month.

The type of collagen synthesised by individual cells was determined at various stages of dedifferentiation by immunofluorescence using specific antibodies to type I and type II collagen[9]. This facilitated the comparison of cell shape and the type of collagen synthesised. The double staining procedure enabled us to determine whether a single cell could synthesise both types of collagen simultaneously[10]. During the first day of culture the chondroyctes remained almost round; staining with rabbit anti-type II collagen antibodies revealed strong fluorescence of the cell membrane and of numerous filopodia (Fig. 1*a*). Because of strong membrane-bound fluorescence no intracellular type II collagen or procallagen could be detected. No positive reaction with antibodies to type I collagen was obtained at this stage. After 2 d most chondroyctes had settled on the surface, flattened and retracted their filopodia. These cells showed little membrane bound fluorescence, for the nucleus appeared dark after staining with anti-type II collagen antibodies, but strong intracellular fluorescence (Fig. 1*b*). This was probably due to type II procollagen, the intracellular form of collagen[11], which seemed to be packed in

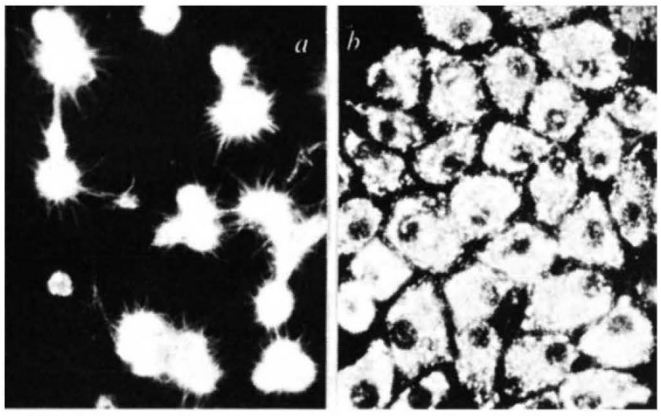

Fig. 1 Immunofluorescent staining of chondrocytes from 16-day embryonic chick sterna with antibodies against type II collagen after 24 h in culture. Chondrocytes were released from sterna by treatment with 0.25% trypsin and 0.1% crude collagenase for 90 min at 37 °C, washed with F12 medium plus 10% foetal calf serum, and plated on Falcon plastic dishes No. 3001 at cell densities of 10^4 cells per cm^2 in Ham's F12 medium and 10% foetal calf serum[1–3]. After 24 h most cells had attached to the surface, but none were flattened; they showed strong membrane-bound fluorescence and numerous filopodia. For immunofluorescent staining, cells were washed with saline and fixed with 70% ethanol followed by 98% ethanol–ether (1:1, v/v). The air dried cells were reacted with monospecific rabbit antibodies to chick type II collagen (0.12 mg ml^{-1}) for 30 min at room temperature, washed and counterstained with fluorescein conjugated goat anti-rabbit γ globulin as described earlier[9]. Preparation and specificity of the antibodies was described previously[9]. Cells did not stain with antibodies to type I collagen at this time in culture. × 260. *b*, Immunofluorescent staining (see Fig. 1) of a chondrocyte colony after 48 hours in culture. Cells have flattened and retracted their filopodia. Fluorescence with antibodies to type II collagen is located mostly intracellularly as indicated by the weak staining of the membrane above the nuclear area. × 260.

large vacuoles in some cells (Fig. 2). These vacuoles appeared dark in the phase contrast microscope after fixation and antibody staining (Fig. 2*b*) but were not visible in living cells. It cannot be ruled out that they were an artefact of the fixation and drying procedure, but we assume that they are secretory granuoles filled with procollagen[12].

After 2 d in culture the first cells started to produce type I collagen. Biochemical determinations have shown that after 2 weeks about 50% of the newly synthesised collagen was type I collagen[1–3]. Double staining with antibodies to type I and type II collagen revealed that most cells synthesised either type I or type II collagen (Fig. 3). Only in about 1 out 100 cells was fluorescence observed with both antibodies. There was no signficant difference in the morphologic appearance of type I and type II collagen synthesising cells. We observed cells of nearly identical shape in close proximity, which may have originated from the same mother cell, one synthesising type I and the other type II collagen (Fig. 3, arrows).

Type I as well as type II collagen synthesising binucleated cells were observed in increasing amount with prolonged

Fig. 2 Immunofluorescence (*a*) and phase contrast micrograph (*b*) of the same cells of a 12-day chondrocyte culture after fixation and staining with antibodies to type II collagen. Fluorescence seems to focus in large intracellular vacuoles (arrows) which may be secretory granuoles containing type II procollagen. The phase contrast is low because the cells were fixed and air dried. This kind of vacuole was not seen in phase contrast microscopy of living cells. (× 478.5).