

Growth and Other Characteristics of a Cell Line (SP-8) Established from Shope Virus-Induced Cutaneous Papilloma of Domestic Rabbit* (32931)

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Attempts have recently been carried out in our laboratory to assess the problem of virus-cell relationship in the Shope papilloma-carcinoma complex utilizing an *in vitro* system (1, 2). In a previous communication, we reported the cultivation and immunofluorescent studies of Shope virus-induced papillomas of domestic rabbits. The primary cultures derived from the neoplastic tissue were composed of cells of two different types both of which exhibited a positive fluorescent reaction against anti-Shope papilloma virus antiserum (1). The present paper reports the establishment of a continuously growing cell line, designated as SP-8, from the cells derived from the Shope papilloma after some 10 serial attempts. Growth characteristics, karyotype analysis, and other findings on the SP-8 cells will also be described.

Materials and Methods. Cell culture and media. The method of preparing primary cultures of cells from Shope papilloma tissue has been described in a previous paper (1). For serial subcultivation, cells were treated with 0.2% trypsin solution and incubated with YLE medium (Earle's balanced salt solution containing 0.1% yeast extract and 0.5% lactalbumin hydrolyzate) plus 20% calf serum and 10% tryptose phosphate broth. Cultivation was carried out at 37°C in a stationary position throughout the study.

Total cell count. The total number of cells in the cultures was determined by counting the cell nuclei after applying hypotonic 0.1 M citric acid to the specimens (3).

Immunofluorescent staining procedure. The

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indirect staining method was used. Immune sera were prepared by repeated injection of partially purified Shope papilloma virus (SPV) into domestic rabbits. The animals were bled 1 week after the final injection of antigen. Fluorescein isothiocyanate (FITC)-conjugated antirabbit γ -globulin goat serum was provided from the Institute of Medical Science, University of Tokyo. Details of the immunofluorescent technique employed have been described in previous papers (1,2). The stained preparations were examined by a Nikon fluorescence microscope with a Corning No. 5840 or a Schott BG 12 exciter filter. The light source was an Osram HBO 200 lamp.

Chromosome analysis. Chromosome studies were made on cells at the fifth, eleventh and twentieth passage. Metaphasic cells were arrested by colchicine in a final concentration of 2 μ g/ml of culture media for 2-4 hours at 37°C. The air-drying method of Ford and Woolam (4) was employed for subsequent procedures in making chromosome preparations with a slight modification (5).

Arginase assay. Cell homogenates were prepared by a Potter-Elvehjem type homogenizer and arginase activity in the homogenates was determined by the diacetylmoxime method (6) and α -isonitrosopropiophenone method (7). For comparison, the 20,000 rpm supernatant fractions of the 3 lots of Shope papillomas at 31 days after inoculation of SPV were also assayed for arginase activity.

Results. Establishment of cell line SP-8. As previously reported, two different types of cells, polygonal and columnar, grew out of papilloma explants after approximately 3 weeks of incubation. These two types of cells showed a random growth pattern, forming a mixed population in primary cultures (1). The primary cultures were transferred after



FIG. 1. Photomicrograph of the secondary culture of SP-8 cells. Note the predominance of the "columnar cells." May-Grunwald Giemsa stain; $\times 400$.

28 days of incubation. In the first subcultivation, however, the columnar cells began to overgrow the polygonal cells and subsequently the cultures were dominated by the former type of cells (Fig. 1). From the second to third generation, the cells grew at a relatively slow rate. They were transferred at intervals of 12 and 8 days in their second and third passages, respectively. By the fourth generation, i.e., 48 days after cultivation *in vitro*, the cells began to show marked proliferation. After several succeeding transfers, establishment of a cell line became evident. The SP-8 cells have been successively passaged every 5 days for 55 generations to date (October 10, 1967).

Growth characteristics. The rate of growth of SP-8 cells has been almost consistent after the cell line was established. A representative growth curve of the SP-8 cells in its thirty-eighth generation is illustrated in Fig. 2. The doubling time of the cells was around 28

hours and the cell population showed approximately a 10-fold increase over that of the inoculum in a week.

Cell morphology. After establishing the cell line, the SP-8 cell cultures consisted exclusively of columnar cells without any association of round or polygonal cells. Under a phase-contrast microscope they were observed growing in a monolayer with an irregular pattern (Fig. 3). The cytological features of SP-8 cells in general resembled those of fibroblastic cells. These characteristics have remained constant to date.

Chromosome studies. Chromosomes of well-spread metaphasic cells were counted for analyses of the distribution of chromosome number, and detailed karyotype analyses were made microphotographically.

As shown in Fig. 4, diploid was predominant and 44 was the modal number of chromosomes in each passage, while tetraploid was submodal. A wide distribution of chromosome number deviating around the

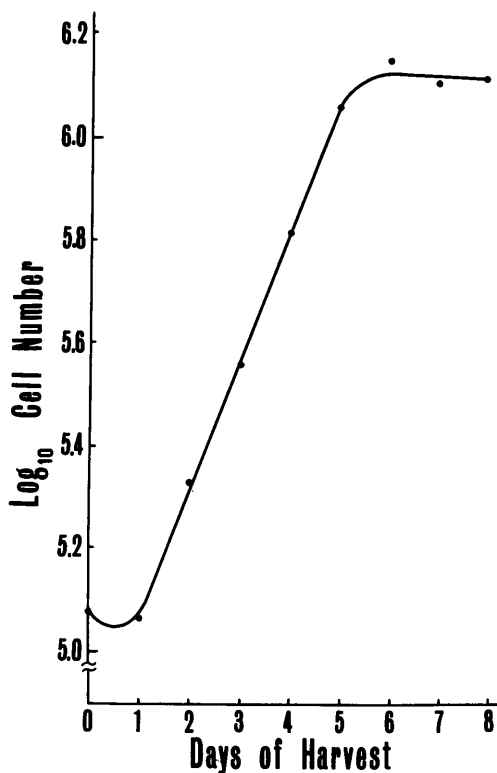


FIG. 2. Growth curve of the SP-8 cells (thirty-eighth generation).

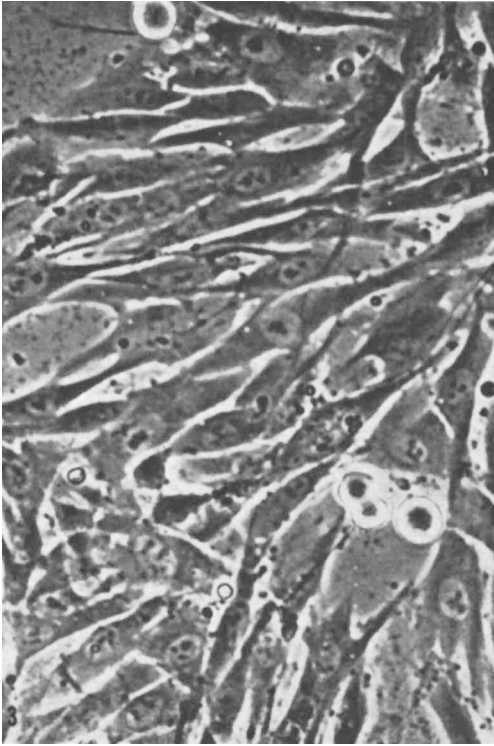


FIG. 3. Phase-contrast photomicrograph of SP-8 cells (fifty-fourth generation).

modal number was observed in each passage, though such frequency was low. The frequency of cells in the tetraploid range (27.5%) in the twentieth passage was higher than that in the fifth (13.2%) and in the eleventh (12.0%).

Based on the rabbit idiogram presented by Nichols *et al.* (8), chromosome complements were classified into 5 groups: metacentrics (nos. 1–5), submetacentrics (nos. 6–11), subtelocentrics (nos. 12–17), telocentrics (nos. 18–21), and sex chromosomes, and were arranged in descending order of length in each group. The pair no. 7 in this study seemed to be identical with the pair no. 3 in the idiogram made by Nichols *et al.* (8). Karyotype analyses revealed that the SP-8 cells had the karyotype of a male rabbit: 5 pairs of metacentrics, 6 pairs of submetacentrics, 6 pairs of subtelocentrics, 4 pairs of telocentrics, an X and a Y chromosome. The karyotype of SP-8 cells had no apparent difference from the normal rabbit karyotype so far

examined, as representatively shown in Fig. 5.

Immunofluorescence of the SP-8 cells. As described in a previous paper, both of the morphologically different primary cultured cells exhibited a specific fluorescent reaction in the nucleus (1). However, the specific fluorescence gradually decreased as the columnar cells dominated the subcultures. Subsequently, such specific nuclear fluorescence disappeared completely in the fifth generation and since then has not been found in the SP-8 cell cultures.

Inoculation of SP-8 cells into rabbits. The SP-8 cells of the fifteenth generation were inoculated subcutaneously into the dorsal part of the ears of young domestic rabbits approximately 4 weeks old. Two groups of animals were employed, i.e., four animals were conditioned by pretreatment with hydrocortisone (9), and the other four animals were untreated. Each rabbit received approximately 2×10^6 cells per site. Small cutaneous nodules were observed at all of the sites of inoculation of SP-8 cells on the third day. These disappeared within a week or so. Tissue sections made from the biopsied specimens

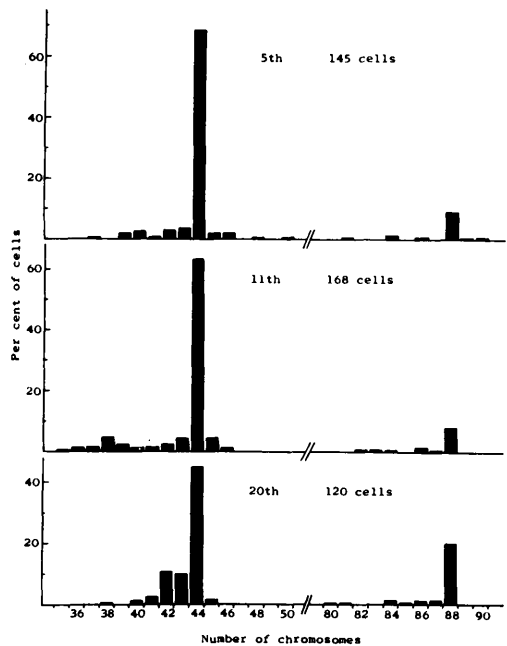


FIG. 4. Distribution of chromosome numbers observed in the SP-8 cells at fifth, eleventh and twentieth passage in tissue culture.

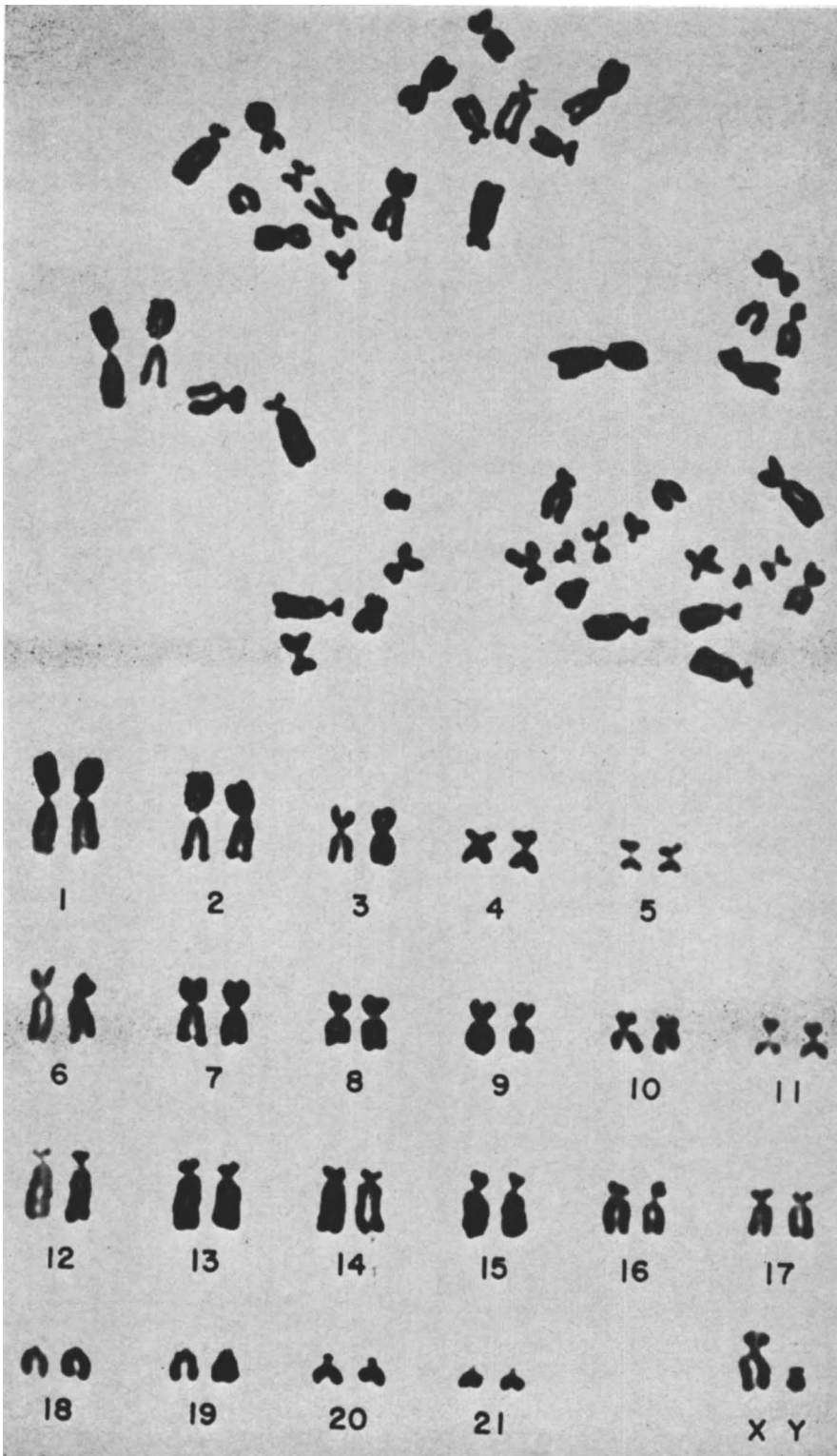


FIG. 5. Karyotype of SP-8 cells with modal chromosome number of 44 at the fifth passage. In appearance, little characteristic difference from normal karyotype is noticeable.

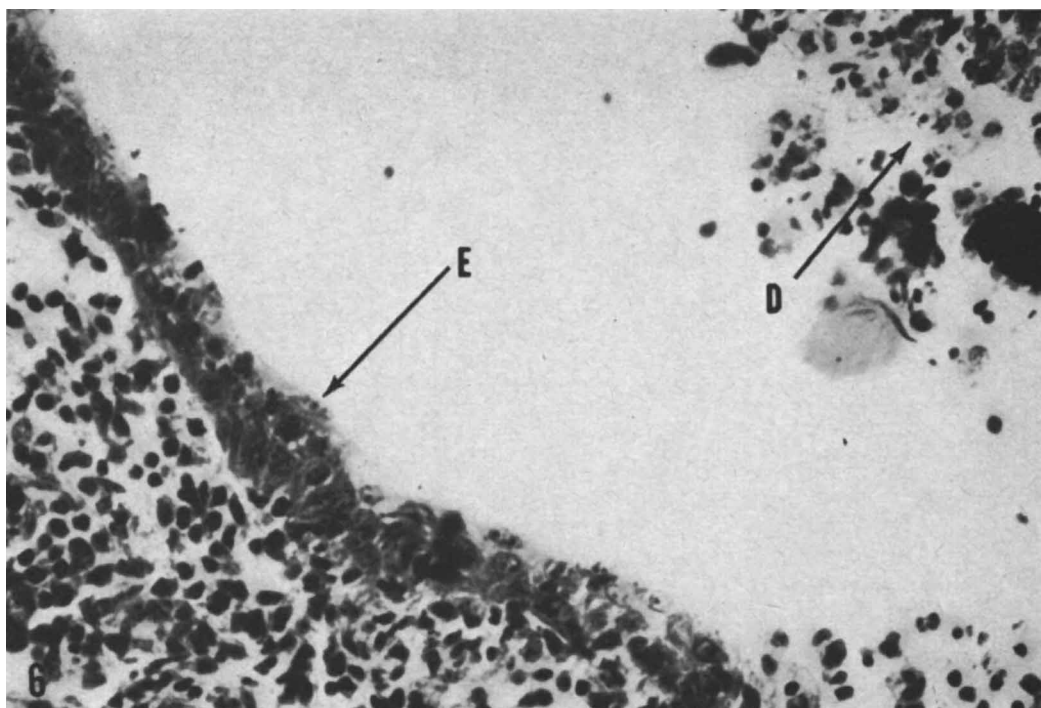


FIG. 6. Histological section of biopsied specimen taken from a "cutaneous nodule" which was temporarily seen at the site of inoculation of SP-8 cells after 4 days. Epithelial arrangement (E) and degenerating debris (D) of the inoculated cells are illustrated. Hematoxylin and eosin stain; $\times 200$.

taken on the fourth day showed that the general histological picture of the lesion was that of a degenerated granuloma. Among the degenerating mass of inoculated cells, however, a pattern reminiscent of epithelial arrangement of the cellular component was occasionally seen lining the outer wall of the lesion (Fig. 6).

Arginase activity of the SP-8 cells. Increased level of arginase in the SPV-induced papillomas has been reported by Rogers (10). However, the problem of whether the enzymatic activity is a direct consequence of the functioning viral genome in the transformed cells or is a mere enhanced expression of the constitutive function of the host cells remains equivocal (11). Nevertheless it was interesting to see whether or not such arginase activity is detectable in the culture of SP-8 cells. As shown in Table I, no significant amount of arginase could be detected in the cell homogenate prepared from the SP-8 cell culture, whereas the Shope

papilloma tissue specimens taken as a control gave positive activity.

TABLE I. Arginase Activity in Crude Extract of Virus-Induced Shope Papillomas and Cell Homogenates of SP-8 Cells.

Sample	Specific activity ^a	Evaluation
Shope papilloma ^b		
No. 1793	4.55	Pos
No. 1803	4.80	Pos
No. 1807	4.55	Pos
SP-8 cells ^c		
Lot no. 3-a ^d	<0.01 ^e	Neg
3-b ^e	<0.01	Neg
3-c ^f	<0.01	Neg

^a μ moles of urea formed/10 min/mg protein.

^b A 20,000 rpm supernatant fraction.

^c Cells pooled from forty-first and forty-second generation.

^d Activated enzyme and 15-min reaction.

^e Nonactivated enzyme and 30-min reaction.

^f Nonactivated enzyme and 15-min reaction.

^g Optical density reading not significant.

Discussion. In the present study, the establishment of a continuously growing cell line, SP-8, from the Shope virus-induced cutaneous papilloma of domestic rabbit has apparently been shown.

Although the morphology of the SP-8 cells is fibroblastic in appearance, the behavior of their growth *in vivo* is suggestive of their epithelial origin. The failure of the SP-8 cells to form tumors in noninbred animals such as rabbits does not rule out the possibility that these cells are neoplastic in nature. It has been shown by our experience that homo-transplantation of the Shope papillomas *per se* is rarely successful among the domestic animals.

It has been clearly demonstrated by Noyes and Mellors (15) in their histological sections of Shope papilloma that the viral antigen is detectable in the cell nuclei of superficial keratinized layers and not in the deeper basal or parabasal layers. In our previous paper, a hypothetical idea was proposed that the strongly fluorescing polygonal cells could have been derived from the higher layers and the weakly reacting columnar cells from the deeper layers of the papilloma. The present observation that the columnar cells overgrew the polygonal cells and finally replaced the entire culture as the transfer generation proceeded give rise to the possibility that the SP-8 cells could be of basal or parabasal layer origin.

Although elevated levels of arginase activity are demonstrable in papillomatous tissues induced by SPV in domestic rabbits (10), the problem of whether or not the enzymatic activity is related directly to the functional expression of the viral genome remains undecided. Van Scott has reported that epidermal arginase activity is found in prickle and granular cell layers but not in the basal cell layers (16). Provided the origin of the SP-8 cells is derived from the basal or parabasal cell layer as discussed above, the absence of arginase activity in the homogenate of the cells could also be explained.

Palmer observed predominant diploid and hyperdiploid cells and rare incidence of cells in the tetraploid range in the rabbit papilloma (12). Upon examination of chromosomes in

benign papilloma at different stages of tumor development, McMichael *et al.* reported that the incidence of abnormal, additional and/or missing chromosomes was not consistent from tumor to tumor (13). Translocations, dicentric chromosomes and trisomy of acrocentric chromosomes were found by Prunieras *et al.* in rabbit cells *in vitro* after inoculation with Shope papilloma virus (14). In this study of SP-8 cells, however, no chromosome aberrations such as translocation, dicentrics, breaks, or any marker chromosome were found as examined to date.

Although the karyotype of SP-8 cells with modal number of 44 had neither marker chromosomes nor chromosome aberrations and had no difference from normal karyotype in appearance, a possibility that these cells were "intrinsically" abnormal could not be neglected, because such an alteration of cellular genes evoked by possible integration of viral genes into chromosomes could not be detected under the light microscope.

Thus, the main issue pertaining to the SP-8 cells will be focused on the problem of whether the cells still harbor the viral genome. Our sequential observation has shown that the number of cells exhibiting specific intranuclear fluorescence gradually decreased as the subculture was repeated and no positive fluorescent reaction was seen among the cells in the fifth to fifty-fifth transfer generations. This could be due either to the complete loss or "inactivation" of the viral genome in the cells or to a predominance of cells with a "nonfunctioning genome." A possibility also exists that the major cellular constituent of the SP-8 cells is composed of cells primarily free of any SPV-related substance since even in the primary culture less than 10% of the columnar cells exhibited positive immunofluorescent reaction.

Recently, however, we have been able to observe the "reappearance" of SPV antigen in the SP-8 cells by lowering the temperature of cultivation to 30°C. This experimental evidence suggests the possibility that the viral genome is persisting in the SP-8 cells in a "nonfunctioning state." These findings will be reported elsewhere.

Summary. The cutaneous papilloma of

domestic rabbit induced by the Shope papilloma virus was cultured *in vitro* and a continuously growing cell line, designated as SP-8, was established. Among the two types of cells, i.e., polygonal and columnar cells, which grew in the primary culture and which both showed the presence of SPV antigen by immunofluorescence as previously reported, the latter type (columnar cells) grew predominantly even in the first subcultivation and subsequently composed the main cellular constituent of the SP-8 cell line. The morphological feature of the SP-8 cells was fibroblast-like in appearance. By inoculation into rabbits, however, the cells were occasionally observed to form epithelial arrangements. The modal number of chromosomes in SP-8 cells was found to be 44 and those cells had a characteristic male rabbit karyotype which showed no difference in appearance. Deviation of chromosome numbers from the modal number was observed. The incidence of tetraploid cells at the twentieth passage was higher than in the earlier passages. Although the SP-8 cells exhibited neither specific fluorescence for SPV antigen nor elevated arginase activity, the possibility of persisting viral genome in these cells was discussed.

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Evidence for the Release of Ovulating Hormone in PMS-Treated Immature Rats* (32932)

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In 1962, Strauss and Meyer (1) demonstrated that a critical time period for ovulation existed in immature rats induced to precocious puberty with pregnant mare serum gonadotropin (PMS). On the basis of their experiments involving carefully-timed hy-

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pophysectomy or injection of barbiturates, they hypothesized, as Everett and Sawyer (2) had done years earlier, that the 2-4 p.m. "critical period" represented the time during which sufficient pituitary ovulating hormone (OH)² was released to induce ovulation. In 1964, Schwartz (3) demonstrated that an

² The term ovulating hormone will be used to represent whatever gonadotropin(s) is (are) released to cause ovulation.