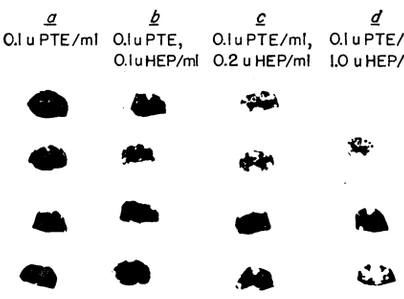

Fig. 2. Effect of different concentrations of heparin (*HEP*) when used in combination with a suboptimal concentration of parathyroid extract (*PTE*); the bone was cultured for 12 days and then stained by the Von Kossa reaction. Note the definite enhancement of resorption when 0.2 unit (*u*) of heparin per milliliter was used (*c*), and the more extensive resorption when 1.0 unit of heparin per milliliter was used (*d*).

observations were made daily on the living cultures (three to four specimens per group) and the extent of resorption observed was scored. The experiments were terminated between 7 and 14 days, at which time the bone samples, still attached to the coverslips, were removed from the tubes, fixed overnight in 10-percent neutral formalin, and then subjected to the Von Kossa reaction which stained the remaining mineral portion of the bone black. The stained calvariae of each experimental group were mounted on separate microscopic slides, and the groups to be compared were photographed together by means of transmitted light. In general, there was excellent correlation between the microscopic scoring of the living calvariae and the Von Kossa-treated bones.

Figure 1 shows that the addition to the medium of heparin sodium (Lilly) alone (10 units/ml) or parathyroid extract (Lilly) alone (0.1 unit/ml) was ineffective (Fig. 1, *b* and *c*), the stained bones being comparable to those in the unsupplemented control group (Fig. 1*a*). However, the combination of 10 units of heparin and 0.1 unit of parathyroid extract per milliliter resulted in extensive resorption (Fig. 1*d*), equivalent to that ordinarily obtained with 0.5 unit of parathyroid extract per milliliter alone. Although no clear-cut effect was demonstrable with parathyroid extract in combination with 0.1 unit of heparin (Fig. 2, *a* and *b*), definite enhancement was obtained with as little as 0.2 unit of heparin per milliliter (Fig. 2*c*). Very good enhancement was obtained with 1.0 unit of heparin per milliliter (Fig. 2*d*). The finding that a synthetic polysaccharide-sulfuric ester (Treburon) (*2*) similar in structure to heparin (*3*) was effective in the same dose range as heparin (0.01 to 0.1 mg/ml) rules out the possibility that the commercial heparin preparation contained contaminants responsible for the effect.

Other acid mucopolysaccharides were tested to determine whether they also would enhance the effectiveness of parathyroid extract. Preliminary studies indicate that neither hyaluronic acid nor chondroitin sulfate are effective. On the basis of experiments in which a five- to tenfold enhancing effect could be demonstrated with heparin in combination with suboptimal concentrations of other factors which stimulate bone resorption (such as crystalline vitamins A or D_2), it is suggested that heparin be considered a bone-resorption-stimulating "cofactor." Although the mechanism of this action of heparin is not yet known, it is conceivable that the mast cell, the only known source of heparin in tissues, might play an important role in physiological or pathological bone resorption. Along this line it should be noted that rats maintained for 15 weeks on a diet low in calcium and vitamin D and high in phosphorus developed a combination of moderate rickets, osteoporosis, and osteitis fibrosa as well as a marked increase in bone mast cells (*4*). Furthermore, in rabbits kept on a high fat diet and injected repeatedly over a long period with heparin, there were frequent occurrences of spontaneous fractures in various bones (*5*). Whether patients on long-term heparin therapy develop an unusual amount of rarefying bone disease remains to be seen.

PAUL GOLDHABER

Department of Oral Histopathology and Periodontology, Harvard School of Dental Medicine, Boston, Massachusetts

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Simian Virus 40: Isolation of Two Plaque Types

Abstract. *Two different morphological plaque types of simian virus 40 were isolated from three of six strains. Increased uptake of neutral red gave one type of plaque a uniform red appearance, whereas the center of the other remained unstained. The viruses showed stability of plaque morphology on passage in tissue culture; cross neutralization studies identified both as simian virus 40.*

During an investigation of the infectivity of simian virus 40 (SV40) by the plaque technique two plaques of differing morphology were noted. The first plaques obtained were similar in appearance to those described by Stinebaugh and Melnick (*1*), although somewhat larger, but, in later attempts to plaque virus at different passage levels, a larger "red" mutant plaque was observed.

The strain of cells used in our study was the BS-C-1 continuous passage line of grivet monkey kidney cells (*2*), grown in medium 199 containing fetal bovine serum (10 percent), and maintained in medium 199 with the serum concentration reduced to 2 percent. The strain of SV40, designated VA 45-54, was obtained from M. Hilleman.

The cells were seeded in plastic petri dishes (60 mm) and were incubated in an atmosphere containing a mixture of 5 percent CO_2 and 95 percent air until a monolayer had formed. Dilutions of the virus were made in medium 199 containing fetal bovine serum (5 percent), and 0.4 ml of each dilution was inoculated onto each monolayer after the removal of the growth medium. A 2-hour absorption period at 37°C sufficed to obtain the maximum number of plaques. The inoculum was then removed, and 5 ml of medium 199 containing fetal bovine serum (5 percent) and purified agar (1.5 percent) (*3*) was added. When the agar had solidified, 4 ml of medium 199 containing fetal bovine serum (5 percent) was added as an overlay, and the plates were incubated at 37°C for 13 days in the CO_2 atmosphere already described. The liquid overlay was then removed, and 4 ml of medium 199 containing neutral red (1:10,000) was added, and the plates were incubated overnight. The neutral red overlay was then removed, and the number

and the morphology of the plaques were noted.

Representative plaques were picked from each morphological type and passaged as plaques two additional times to insure a purified stock of virus. These stocks of virus were then cultured two more times in the BS-C-1 line of cells, and re-plaqued to determine their morphological differences and their stability on passage.

Before purification of the VA 45-54 strain of virus most of the plaques showed a morphology similar to that described by Stinebaugh and Melnick (*1*) with an average diameter of 2.6 mm (range 0.9 to 4.5 mm). The cells within the center of the plaque were completely clear (white), indicating no uptake of neutral red, or were completely lysed and not evident. The cells at the edges of the plaques stained intensely, producing a thin rim of red color, perhaps six to eight cells thick (Fig. 1). We have designated the virus producing this morphological plaque type as SV40/W.

The plaque of differing morphology resembled the "negative plaques" of adenovirus type 2 (*4*) or the Newcastle disease virus plaque mutant (*5*), in which, after treatment with neutral red, the cells in the plaque were more deeply stained than were the normal cells and appeared as a red plaque. These plaques had an average diameter of 4.4 mm (range 2.3 mm to 6.4 mm), and in the center of the larger plaques a thin, lacelike network of intensely stained cells remained. The smaller plaques showed no obvious loss of cells, and intensely stained cells remained attached in the center of the plaque (Fig. 1). We have designated the virus producing this plaque as SV40/R.

The maximum titer obtained on passage of the individual plaque-forming strains differed by approximately tenfold, the SV40/W giving a titer of approximately 4.4 to 9.3 $\times$ 10^7 plaque-forming units (PFU) per milliliter, and the SV40/R mutant giving a titer of 1.75 to 3.58 $\times$ 10^8 PFU per milliliter.

Each virus, which was plaque-passaged three times and passed two more times in bottle cultures of the BS-C-1 line of cells, showed no reversion in plaque morphology. In counting over 500 plaques of each individual type, no red plaque mutant was observed in plates which had been seeded with SV40/W, and conversely no white plaques were observed in plates seeded with SV40/R.

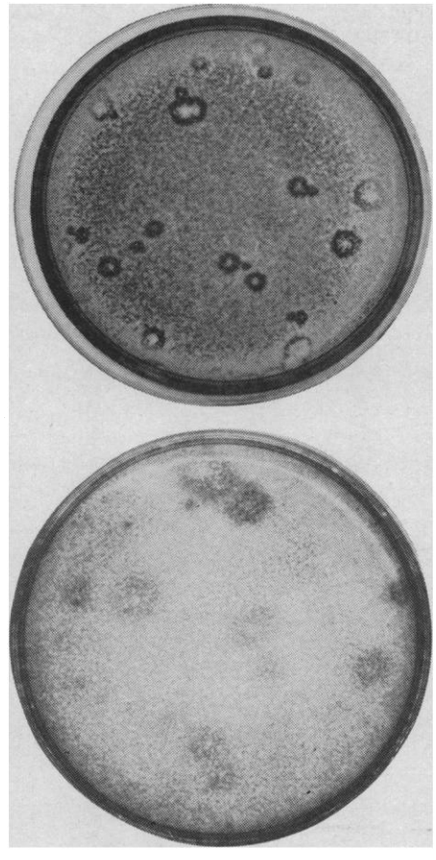

Fig. 1. Morphology of plaque types. (Top) Plaques formed by wild type virus (SV40/W). (Bottom) Plaques formed by mutant virus (SV40/R).

Serological studies indicate a complete cross between the two viruses. Antiserum prepared against SV40/W neutralizes to the same degree both SV40/W and SV40/R, as measured by the reduction of 50 percent of the plaques. Antiserum prepared against SV40/R also neutralizes both viruses to approximately the same degree. No differences in the type of cytopathic effect have been noted; both viruses produce vacuolation of the cells with subsequent complete degeneration.

Several different strains of SV40 were examined to determine whether the red plaque mutant was present. Included among these was strain 777 (*6*), and four different strains designated as follows: KSV40, PA57, 776 (BSC), and 776 (primary) (*7*). These viruses were subjected to passage once or twice in the BS-C-1 cell line and then tested for plaque-forming ability. In two strains, 777 and 776 (BSC), the red mutant appeared among a dominant population of white plaques, whereas the others yielded the white plaque type only. These results would appear to exclude the possibility that the red plaque mutant originated as a consequence of passages in this laboratory.

Preliminary experiments with SV40/W indicate that this virus is oncogenic in newborn hamsters and also can "transform" kidney cells from newborn hamsters into different morphological types that retain their capacity to multiply upon passage in tissue culture. In eight experiments in which attempts were made to transform diploid strains of human embryonic kidney cells with SV40/W, positive results were obtained. The cells showed an increased mitotic index and an increased growth rate, and they changed their morphological type.

Although experiments with SV40/R have only recently been started, the oncogenic potential of this virus is evident. Upon subcutaneous inoculation of the virus into newborn hamsters, tumors at the site of injection are readily obtained. In one out of four attempts to produce transformation in kidney cells from newborn hamsters, positive results were obtained. The morphology of such transformed cells is indistinguishable from that produced by SV40/W. One attempt to produce transformation in human fetal diploid kidney cells has failed. Whether there is a possible temporal relationship of the appearance of transformation in this type of cell with the type of virus used is not yet known.

JOHN L. RIGGS
EDWIN H. LENNETTE
Viral and Rickettsial Disease Laboratory, California State Department of Public Health, Berkeley 4

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