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"Induction" of Viral Antigen in Established Cell Line (SP-8) Derived from Shope Virus-induced Cutaneous Papilloma of Rabbits* (33501)

OSAMU SHIRATORI[1], TOYORO OSATO[2] AND YOHEI ITO
Laboratory of Viral Oncology, Research Institute, Aichi Cancer Center, Nagoya, Japan

In a previous report from this laboratory, the establishment of a cell line (SP-8) from Shope papilloma virus (SPV)-induced papillomas of rabbit skin was described (1). The presence of SPV antigen was clearly demonstrated as the specific nuclear immunofluorescence in the primary culture of the SP-8 cells (2). However, the number of fluorescent cells decreased at each subcultivation level and eventually disappeared completely at the fourth transfer generation. Since then, fluorescent cells have not been observed to date. The present communication describes findings on the attempts to "induce" the synthesis of SPV antigen in these nonreacting SP-8 cells by lowering the temperature of incubation from 37 to 30°

Materials and methods. The SP-8 cells successively passaged for over 80 generations at 37° as a stationary culture were used for the present experiment. The SP-8 cells were grown in monolayer cultures employing YLE medium (Earle's balanced salt solution containing 0.1% yeast extract and 0.5% lactalbumin hydrolysate) plus 20% calf serum and 10% tryptose phosphate broth as growth medium. Maintenance medium was composed of YLE medium plus 2% calf serum. For the immunofluorescent studies, SP-8 cells were grown for 3 days on a coverslip in Leighton tubes. After the monolayer was formed, they were washed three times with maintenance medium. Subsequently, the maintenance medium was added to the washed cell cultures and the cultivation was resumed either at 37° or at 30° for the duration of the experiment.

The indirect staining method was used for the immunofluorescent studies (1, 2). Cov-

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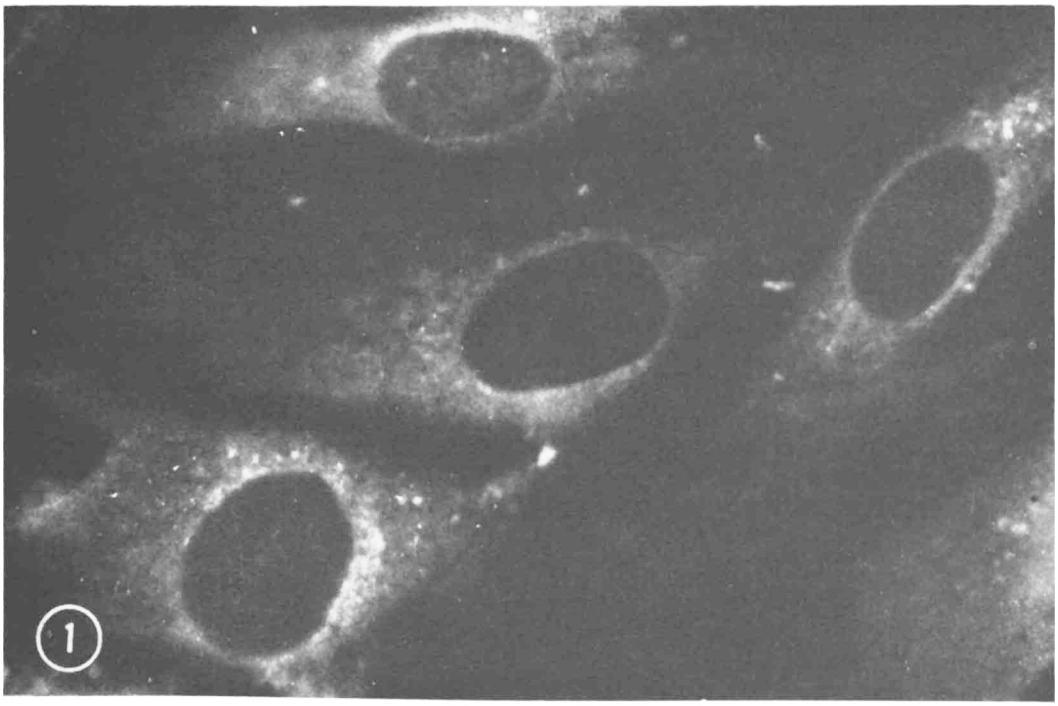
[1] On leave from Shionogi Research Laboratory, Osaka.

[2] Present address: Laboratory of Virology, Cancer Institute, Hokkaido University School of Medicine, Sapporo, Japan.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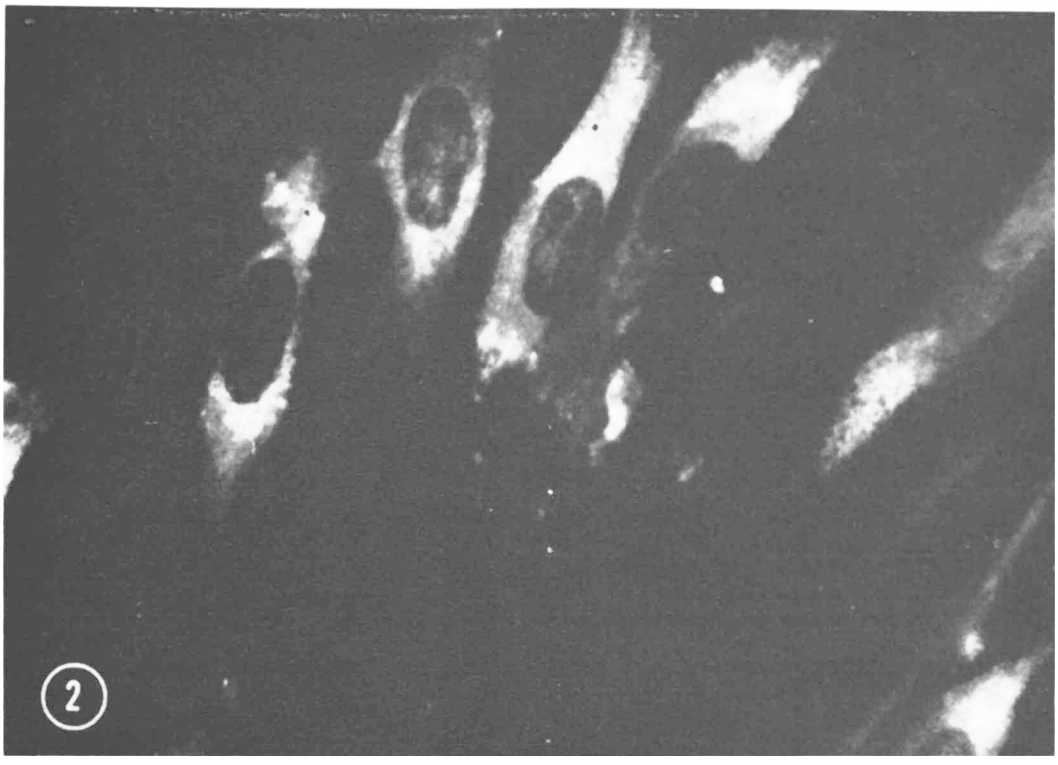
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FIG. 1. Fluorescence photomicrograph of the SP-8 cells at 7 days after transfer to 30°. Note the perinuclear fluorescence; ×400.

FIG. 2. "Reappearance" of the SPV antigen in SP-8 cells by lowering the temperature of cultivation to 30°. Photographed on day 9 after transfer to 30°; the specific fluorescence is distributed throughout the cytoplasm; ×250.

erslip cultures were dried and fixed in carbon tetrachloride for 20 min at room temperature. Immune serum which was prepared by repeated injection of partially purified SPV into domestic rabbit was placed on the coverslip with fixed cells and allowed to stand for 50 min at 37°. After washing thoroughly with phosphate buffered saline (PBS), fluorescein isothiocyanate (FITC)-conjugated antirabbit γ-globulin goat serum kindly supplied by Dr. Y. Kawamura of Institute for Medical Science, Tokyo University, was applied onto the coverslips. Then the coverslips were rinsed and mounted in 20% buffered glycerin. The specimens thus prepared were examined under 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.

In the present experiment, the specificity of the immunofluorescence observed at 30° was reconfirmed from the following points: (i) Neither normal domestic rabbit kidney cells nor normal cottontail rabbit kidney cells exhibited specific immunofluorescent staining by the similar treatment. (ii) No such immunofluorescent reaction was observed when SP-8 cells were treated with normal rabbit serum followed by treatment with the FITC-conjugated antirabbit γ-globulin goat serum. (iii) No positive reaction was seen in SP-8 cells covered with antirabbit kidney vacuolating virus (3) antiserum followed by treatment with the FITC-conjugated antirabbit γ-globulin goat serum.

Results. As shown in previous paper (1), specific nuclear immunofluorescence was detectable in the cells of the primary culture but these specific immunofluorescence decreased as cultivation elapsed. No fluorescence has been detectable since last observed in the fourth generation. However, when the cultures of SP-8 cells in which SPV antigen were not detectable any more by immunofluorescent technique were transferred to the lowered temperature of 30°, "reappearances" of SPV virion antigen in SP-8 cells were always observed when tested at 55, 56, 57, 58, 59, 65, 70, and 75 generations, respectively. The procedure employed for the detection of the viral antigen was as follows. The cultures of SP-8 cells were separated into two parts. One was continuously incubated at 37°. The other was transferred to 30° and incubation was resumed. On the seventh day after the transfer of the cultures to 30°, specific immunofluorescence first appeared in about 1% of the cells. The reaction was limited to the perinuclear area of the cytoplasm at this time (Fig. 1). As the incubation was continued, more cells became stained specifically, gradually involving the entire cytoplasm (Fig. 2). At day 11 the fluorescence was seen distributed diffusely throughout the cytoplasm of the cells (Fig. 3). The number of fluorescence positive cells progressively increased as the incubation was further continued, and the ratio of positive cells reached the maximum of 20–80% at 13 days. The pattern of increase in number of the fluorescent cells in a typical experiment carried out under "ideal" conditions is illustrated in Fig. 4. There was no incidence of intranuclear fluorescence in any of the cells examined during the experiment.

No fluorescent reaction was detectable in control SP-8 cells cultures at 37° throughout the observation period.

It has been shown in our previous immunofluorescent study that the cottontail rabbit kidney (CRK) cell cultures are susceptible to SPV (4). It was of interest, therefore, to investigate whether the CRK cell cultures could be infected by the extracts from SP-8 cells cultures at 30° and whether the subsequent synthesis of SPV virion antigen can occur in these cells. Sixteen TD-40 flasks of SP-8 cells, which were transferred to the lowered temperature of the 30° and cultured for 2 weeks, were scraped off from the glass sur-

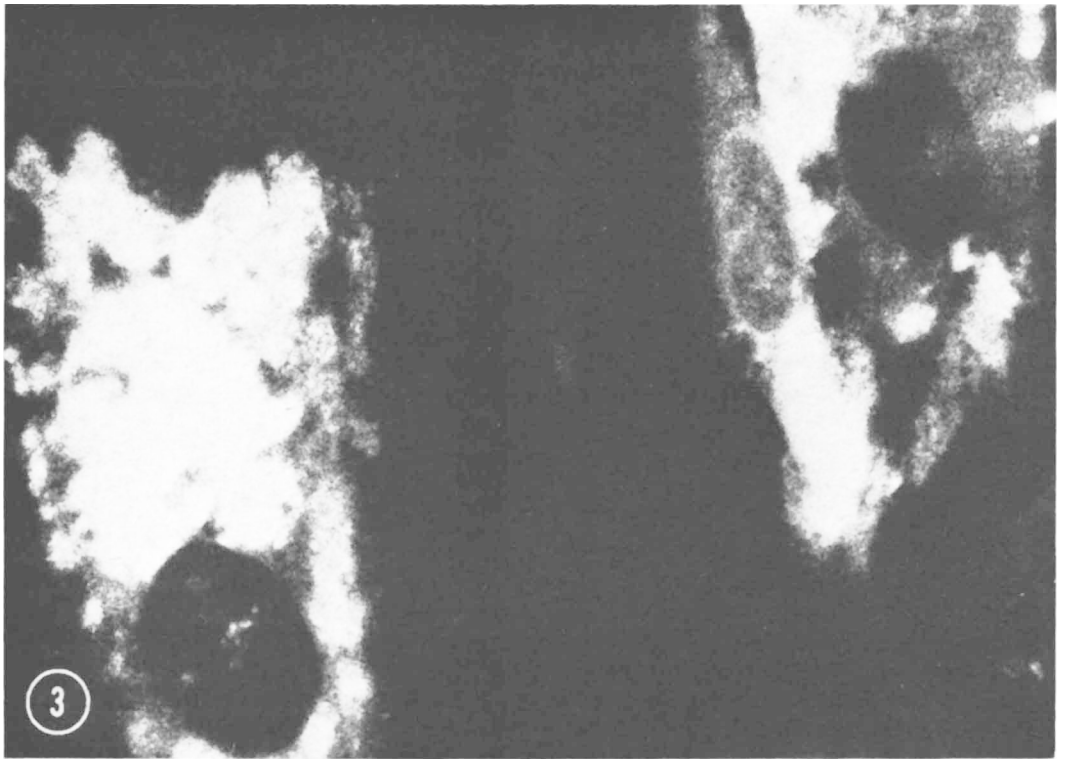


FIG. 3. SP-8 cells loaded with SPV antigen in their cytoplasm. Nuclei remain free of staining; photographed on day 11 after transfer to 30°; ×400.

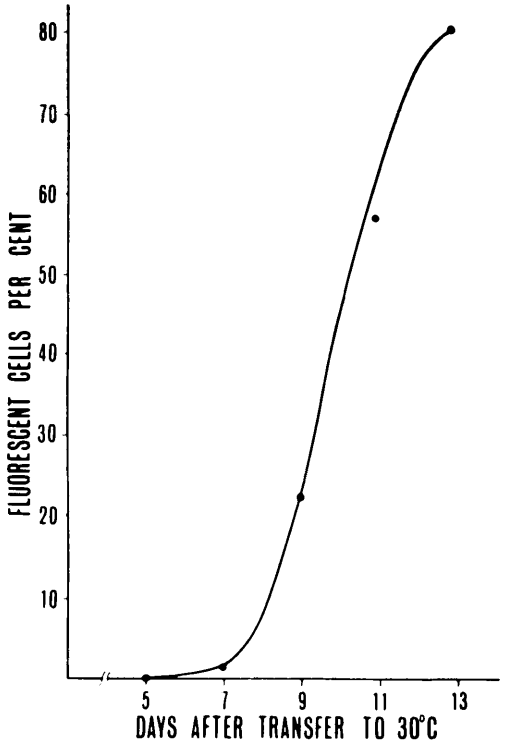
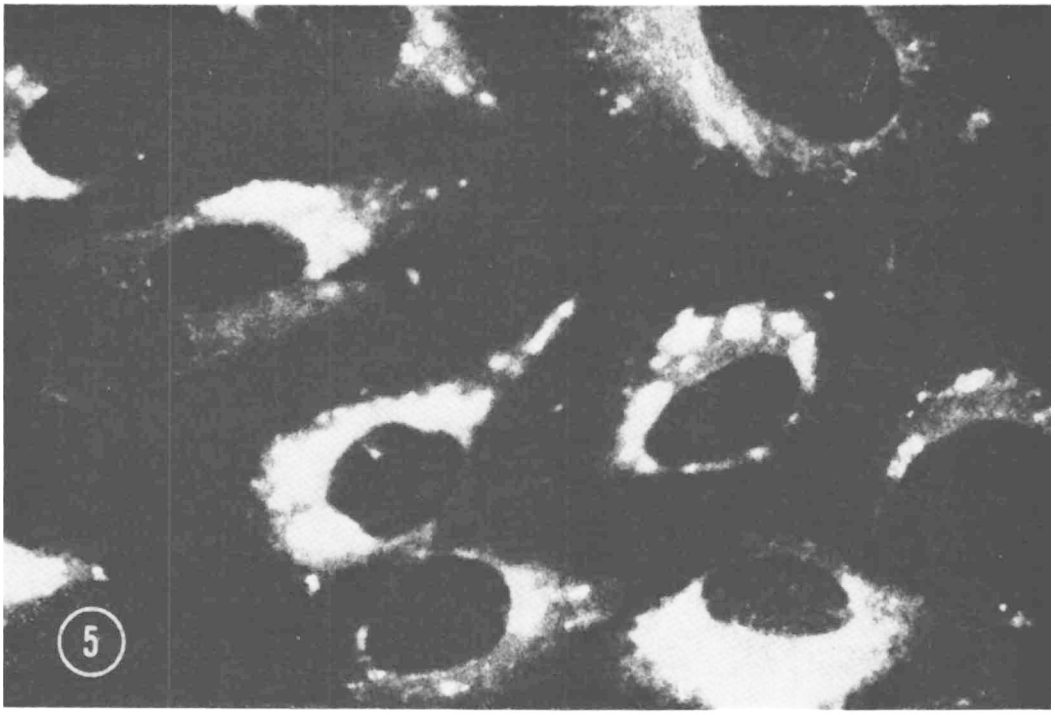


FIG. 4. Reappearance of the SPV antigen in SP-8 cells after transfer to 30°.

face with a rubber policeman and were collected by centrifugation. The pellet of cells was suspended in 2 ml of Eagle's minimum essential medium (MEM). The suspension of SP-8 cells was frozen and thawed five times and then centrifuged for 20 min at 2000 rpm. The supernatant of the SP-8 cells was pooled together and stocked at —70° till the time of use. The extract of the SP-8 cells thus prepared was used as the inoculum for the CRK cells.

The cell cultures which were to receive the inocula were the secondary cultures of CRK grown as coverslip cultures in Leighton tubes and fed with MEM plus 20% fetal calf serum. After the cell sheet was formed, these cultures were exposed to 0.2 ml of the SP-8 cell extract and incubated at 37° for 2 hr. Then the cellular extract was removed and the coverslip cultures were washed three times with MEM. Subsequently, 1 ml of maintenance medium consisting of 5% fetal calf serum in MEM was added to the cultures. The cultures were incubated at 37° and were examined by immunofluorescent technique at intervals of 2 days.



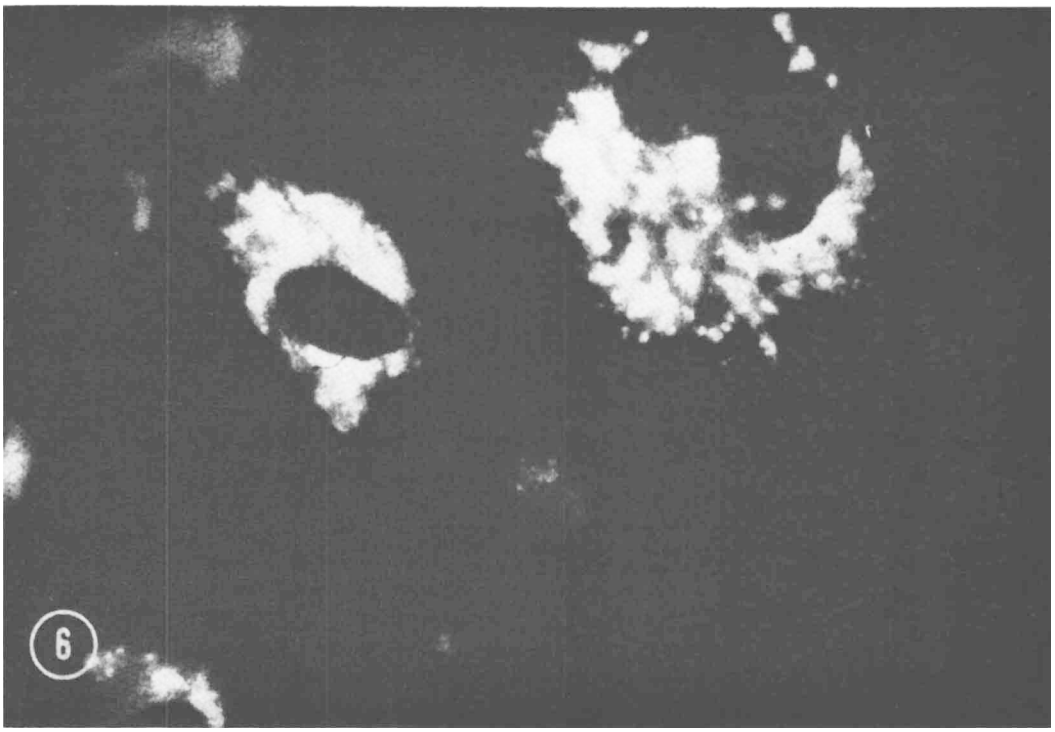


FIG. 5. Fluorescence photomicrographs of the CRK cells at 12 days after inoculation with the cellular extract prepared from the SP-8 cells cultured at 30° for 14 days. Note the intense cytoplasmic fluorescence; ×400.

FIG. 6. The CRK cells harboring the SPV antigen in their cytoplasm. Photographed from a different field of the same preparation as illustrated in Fig. 5; ×400.

Specific fluorescence was first detected in a small fraction of CRK cells 8 days after inoculation with the SP-8 cell extract. The immunofluorescent reaction was localized in the cytoplasm. At 12 days after inoculation, approximately 3% of the cells showed intracytoplasmic fluorescence (Figs. 5 and 6) and no further increase of the ratio of fluorescence positive cells was noticed in the rest of the observation period up to 16 days. The fluorescence was not observed in the nucleus of the CRK cells.

The specificity of the immunofluorescence was confirmed by several controls previously described (4). As additional control, the cellular extract of SP-8, which was cultured at 37°, was examined with the same method as mentioned above. The results were completely negative.

Discussion. The mechanism of reappearance of the SPV antigen when the culture is shifted to the lower temperature is not clear at the present time. One might speculate, however, that the procedure of lowering the temperature from 37 to 30° will provide a favorable condition for the functional expression of the SPV genome possibly persisting in the SP-8 cell in a "nonfunctioning" or "repressed" state and that the synthesis of the specific virion antigen of SPV could be "induced" or "derepressed." An alternative interpretation is that the transfer of the cultures to the lowered temperature will result in the slowdown of cellular activity, thereby leading to the "accumulation" of the SPV antigen which also may be produced in the nonfluorescent SP-8 cells cultures at 37° but only in a meager amount below the sensitivity of the immunofluorescent reaction. Such "induction" has also been reported in the "carrier culture" of cottontail rabbit kidney cells infected with SPV (4).

The previous studies by other workers (5, 6) have shown that certain cell lines of hamster origin could produce small amounts of infectious virus when these nonvirus-producing transformed cells were seeded together with SV40-susceptible African green monkey kidney cells. Furthermore, it has also been confirmed by the application of techniques of cell-fusion or heterokaryonization that the "induction" of infectious virus does occur when the nonvirus-producing transformed cells and the virus-susceptible cells are fused together (7–11). However, the cell-free extracts from the tumor cells have consistently failed to yield infectious viruses when tested in the cultures of susceptible indicator cells (5, 6). The results of the present study have clearly demonstrated that the SPV antigen could be detected by immunofluorescent techniques in the CRK cell cultures inoculated with the cell-free extracts prepared from the SP-8 cells cultured at 30° and harvested at their peak of reactivity against the SPV antiserum as checked by immunofluorescence. This experimental evidence indicates that apparent infection of CRK cells has taken place with the inoculation of the cellular extracts obtained from the SP-8 cells cultured at 30° and further suggest the possibility that not only the synthesis of the viral structural protein but also of some infectious entities, i.e., viral nucleic acid or complete virus, could be taking place in the SP-8 cell cultures at lowered temperature. These findings together with the data by others on SV40 and polyoma virus as stated above, points to the fact that the complete viral genome rather than a portion of it is possibly persisting in the nonvirus-producing transformed cells. Further experiments are planned to elucidate these state of events from the change in synthetic pattern of macromolecules in the SP-8 cells when shifted to the lower temperature of 30°.

Summary. In the SP-8 cells established from Shope papilloma virus-induced cutaneous tumors of domestic rabbits, intranuclear synthesis of the SPV antigen was clearly demonstrated by immunofluorescent technique up to fourth transfer generation. Since then, such demonstrable viral antigen disappeared completely from the cells. When the cultures of the SP-8 cells were transferred from the ordinary temperature of 37–30°, the SPV antigen reappeared in the cells after about a week of incubation and appeared in approximately 80% of the cells in the culture after 13 days. The immunofluorescence was exclusively seen in the cytoplasm. Furthermore, the cellular extract prepared from the

SP-8 cells cultures at the lowered temperature exhibited capacity to infect CRK cells as determined by the immunofluorescent technique. These findings were interpreted as an evidence supporting the view that the SPV genome, possibly a complete set of it, is persisting in these SP-8 cells.

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Vagal Blocking Action of Phentolamine and Dibenzyline Studied by Direct Perfusion of the Sinus Node* (33502)

KLAUS F. LANG[1] AND THOMAS N. JAMES[2]

Section of Cardiovascular Research, Henry Ford Hospital, Detroit, Michigan

Phentolamine and phenoxybenzamine (Dibenzyline) are considered characteristic alpha adrenergic blocking agents, but they are also known to alter responses to serotonin, histamine and acetylcholine (1). Since the chronotropic responses of the heart to vagal and sympathetic stimuli are in opposite directions and simple to observe and record, this study was undertaken to examine the effects of selective perfusion of the sinus node with phentolamine or Dibenzyline on its reponse to cholinergic stimuli.

Methods. Twenty-seven dogs were anesthetized with sodium pentobarbital (30 mg/kg intravenously) and the tracheae were intubated for mechanical ventilation with room air. Through a right thoracotomy the pericardium was opened and the right coronary artery was dissected free in the atrioventricular sulcus. The branch supplying the sinus node (2) was isolated and a small polythylene cannula was introduced into this artery and ligated in place. Details of the technic have been reported previously (3). Ligation of the canine sinus node artery has no significant effect on the pacemaking rate because of the extensive arterial anastomoses in the region. Injections of 2-ml volume were delivered from a hand syringe into a stopcock attached to the proximal end of the cannula in the sinus node artery, perfusing selectively the sinus node and a small amount of adjacent right atrial myocardium. Duration of this injection (perfusion) was 5 to 10 sec. Because of the small volume employed, there was no significant extracardiac effect by the injectate on recirculation.

In all experiments the right atrial (central venous) pressure was monitored with a cannula placed via the jugular vein, and the central aortic pressure via a cannula placed in the femoral artery. A tachogram was constantly recorded from a small analog computer monitoring successive R waves of the

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[1] National Heart Institute Research Fellow (HE-34,601-01). Present address: 65 Mainz, Schillerplatz 8, Germany.

[2] Present address: University of Alabama Medical Center, Birmingham, Alabama 35233.