THE ETIOLOGIC AGENTS OF VARICELLA AND HERPES ZOSTER

Isolation, Propagation, and Cultural Characteristics in Vitro*

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Attempts at the serial propagation of the etiological agents of varicella and of herpes zoster, have, until recently, been unsuccessful. Evidence that a single passage of the agent of varicella could be achieved by the intratesticular inoculation of monkeys was presented by Rivers (1, 2) who demonstrated intranuclear inclusions in the inoculated tissues. Similarly, the development of inclusions in inoculated fragments of human skin grafted on the chorioallantoic membrane of the chick embryo suggested that Goodpasture and Anderson (3) had transmitted the agent of herpes zoster for one passage; an observation later confirmed by Blank, Coriell, and Scott (4). In 1948 we began to utilize suspended cell cultures of human embryonic tissues for the attempted isolation of virus from varicella vesicle fluid materials. In a series of experiments, histologic examination of tissue fragments from inoculated cultures revealed focal collections of cells containing eosinophilic intranuclear inclusion bodies (5). The attempts at serial propagation of an agent in this kind of culture failed. The inclusions observed, however, were of the type first described by Tyzzer (6) in the skin lesions of varicella.

Attention was then given to the use of roller tube cultures of human tissues. As noted in a preliminary report (7), the introduction into such cultures of vesicle fluid materials derived from cases of varicella or from cases of herpes zoster was followed by specific focal cytopathic changes. The involved cells characteristically contained intranuclear inclusions. Serial propagation *in vitro* could be achieved only if the inocula contained cells obtained from the pre-

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ceding cultures. This exceptional behavior of the agents prevented immediate application of the serological procedures used as routine in the study of viruses in tissue culture. Immunologic evidence indicating that the agents responsible for varicella and herpes zoster had been serially propagated was first derived by means of Coons's fluorescent antibody technique (8). The results of these experiments also revealed a close, if not identical, antigenic relationship between the viruses from the two clinical entities. Subsequently confirmatory reports describing cultivation *in vitro* of the agents of varicella and zoster have appeared (9, 10).

Since publication of the preliminary paper on serial propagation of the varicella-zoster viruses *in vitro*, experiments on the cultivation of the agents have been continued. Further evidence of the antigenic similarity of the viruses has been obtained following the development of appropriate procedures for the detection of complement-fixing and neutralizing antibodies.[1] In the course of these studies it has become evident that the persistent focal cytopathogenicity manifest *in vitro* by the varicella-zoster viruses (referred to hereafter as "V" and "Z" virus) poses problems of practical import and is, *per se*, an interesting example of virus-host cell interrelationships. In this paper experimental observations are presented on the isolation, cultivation, and cytopathogenicity of the viruses. The results of serologic studies are described in an accompanying paper (11).

Materials and Methods

Collection and Handling of Specimens from Patients.—

Vesicle fluids from cases of varicella or of herpes zoster were collected, diluted with 0.5 to 2.0 ml. sterile, neutralized, fat-free milk, and, if not used at once, stored in sealed glass ampoules in the dry ice cabinet as previously described (5).

Tissue Culture Techniques.—

The basic techniques were similar to those employed in our studies with the poliomyelitis viruses (12, 13) and, therefore, are only summarized here. Modifications or additions thereto are detailed.

1. Equipment: For the greater portion of the study glassware was washed in hot Ivory soap solution. More recently, a detergent[2] has been used. For roller cultures, 20 x 150 mm. tubes or 250 ml. centrifuge bottles were used. Slanted cultures of trypsinized cells were prepared in 16 x 150 mm. tubes.

2. Constituents and preparation of cultures: Roller cultures of a variety of tissues were prepared utilizing a substrate of chicken plasma, subsequently clotted with chick embryo extract. The nutrient added in 1.5 to 2.0 ml. amounts per culture was the bovine amniotic fluid medium of Enders (14) containing beef embryo extract (5 per cent), inactivated horse serum (5 per cent), and soybean trypsin inhibitor (0.05 mg. per ml.),[3] antibiotics, and phenol red

[1] Certain of these findings have been briefly reported in a Harvey Lecture (*Harvey Lectures*, 1956–57, **52**, 228.)

[2] Micro-solv; Microbiological Associates, Inc., Bethesda, Maryland.

[3] Soybean trypsin inhibitor, cryst., Worthington Biochemical Corp., Freehold, New Jersey.

as previously employed (12). Originally amniotic fluid comprised 90 per cent of the medium; later, this was reduced to 45 per cent by replacement with Hanks's balanced salt solution. Roller cultures were incubated at 35°C., except as noted, and the nutrient fluids were replaced at 3- to 5-day intervals. Using the same medium, cultures of the slant tube type were prepared from trypsin-dispersed human and monkey kidney cells (15) or human amnion cells (16). Following establishment of a sheet of cells, such cultures were usually transferred to a roller machine prior to inoculation. All manipulations were performed in tissue culture "hoods."

3. Inoculation and observation of cultures: The inocula consisted as routine of 0.05 to 0.2 ml. amounts of vesicle fluid suspension in skimmed milk. It is to be emphasized that cultures were inoculated when tissue growth was well established and actively continuing. Inoculated cultures were observed at intervals of 2 to 7 days with a compound microscope, 10 × oculars, and a 3.5 × or 10 × objective. At each observation an effort was made to survey the area of cellular outgrowth, and the number of focal cytopathic lesions therein was recorded. As the focal areas of degeneration increased in size and number, estimates were made of the per cent of available tissue involved by the cytopathic process. In a few instances the evolution of a cytopathic focus was recorded by camera lucida drawings; the marginal outline could then be measured with a map measurer.

4. Maintenance of infected cultures and technique of subculture: In view of the interest in the question of the relationship of the V and Z agents, precautions were taken to minimize the possibility of cross-contamination. Originally, all manipulations of the V strains, including refeeding and subculture, were carried out in a different room from those in which the Z strains were maintained. Later, separate hoods in the same room were reserved for maintaining strains of virus from the two clinical entities. At no time were manipulations performed concurrently on the viruses from the two sources.

For maintenance of strains, at least two "control" and usually four "infected" cultures were prepared in each passage group. As it early was noted that subculture of the agents could be accomplished consistently only when the tissue phase of the culture was incorporated in the inoculum, the following technique of subculture became routine:—

To control the possibility that contaminating viruses might derive from the biologic components of the culture system, material from one or more "control" cultures was prepared first, and new "control" subcultures inoculated. The cell sheet was freed from the culture tube, using a stainless steel tissue scraper, (fabricated by affixing a half-moon shaped piece of sheet metal (17 mm. diameter) on the end of a rod 22 cm. in length). The tissue fragments were suspended in the medium, the whole transferred to a porcelain mortar, and ground by hand for 2 to 3 minutes. The resulting crudely ground suspension comprised the inoculum and was transferred to new control cultures in 0.1 or 0.2 ml. amounts. Infected cultures next were subcultured in the same manner; if the material was derived from two or more tubes, the suspension of scraped tissue fragments was pooled prior to grinding.

5. Histologic techniques: For histologic studies, tissues were grown on coverslips inserted into the roller tubes, and the coverslip then fixed, stained, and handled as a "toto" mount or else sheets of cells were removed from the roller cultures by use of the collodion embedding method. Fixation was with Zenker's or Bouin's fixatives. Harris's hematoxylin and alcoholic eosin were used as routine stains. Irregular staining of the intranuclear inclusions observed with certain eosins was avoided by the use of eosin according to the suggestion of the late Dr. A. M. Pappenheimer as follows: To a dilute aqueous solution of yellow eosin (Grübler), 10 per cent HCl was added dropwise until no further precipitate formed. The precipitate was collected by filtration using double thickness filter paper and washed repeatedly with distilled H_2O. A saturated stock solution of the dried precipitate was made in 95 per cent ethyl alcohol; this was diluted 1:4 in 95 per cent alcohol for use. Following counterstaining with eosin in 95 per cent alcohol, dehydration was "completed" without the use of absolute alcohol to

minimize loss of stain from the inclusions. At this stage coverslip preparations were transferred through two changes of acetone, for 1 and 15 minutes respectively, before clearing in xylol. The collodion sheets were transferred after eosin staining to oil of *Origanum* as employed by Cheatham (17).

EXPERIMENTAL

Introductory remarks.—

In summarizing experimental findings, the information has been organized to present, first, data on the isolation of agents from varicella and zoster cases as accomplished by the procedure adopted as routine, namely the use of cultures of human embryonic skin-muscle or of foreskin tissues (termed hereafter "SM" or "FS" cultures). The strains were maintained by serial passage in cultures of these tissues, and this experience is next presented, together with a description of the cytopathic process. Consideration is then given to the susceptibility of other human tissues and of non-human tissues to infection by the V-Z viruses under *in vitro* conditions. The failure of infectious materials to appear in the fluid phase of infected cultures led to a series of unsuccessful attempts to dissociate the agent from the infected cell or alternatively to explain the phenomenon on the basis of inactivation of the infectious particles as released. Finally, it is pertinent to record observations of practical import on the long term serial cultivation of viruses that manifest a slowly progressive focal cytopathogenic effect. Although the strains of virus are identified according to the clinical entity from which they were derived, no differences in cultural behavior were noted between strains isolated from cases of varicella and those from cases of herpes zoster; nor has evidence of an alteration in cytopathic activity yet been observed on prolonged propagation. Therefore, the examples selected for illustration are representative of the behavior of V-Z viruses under the experimental conditions employed.

Isolation of Viruses.—

Pertinent data concerning the isolation, or reisolation, *in vitro*, of the V strains that have been propagated serially and studied more or less intensively are summarized in Table I; similar data concerning Z strains from eight individuals are presented in Table II. Occasional reference will be made to additional strains that were not serially propagated.

Characteristic cytopathic changes have almost uniformly followed the introduction into FS or SM cultures of the minute amount of clear fluid obtainable from the early vesicular cutaneous lesion of varicella. During the first 3 days of the exanthem, if early unbroken lesions were present, virus could usually be recovered. Failures to isolate virus have been encountered, however, as early as the 2nd day of the eruption if the patient did not have intact young lesions. In the presence of certain concurrent diseases, patients with varicella

TABLE I

Data on Isolation of Varicella Strains from Vesicle Fluid Materials in Cultures of Human Tissues

Strain*	Age	Day illness (from onset exanthem)	Remarks on clinical picture	Interval vesicle fluid stored in frozen state	Type of culture and cytopathic response		
					Tissue	No. cult. + / No. cult. inoc.	Day cytopathic changes first noted
	yrs.						
Wel.	5	2	Typical; siblings ill	22 mos.	SM	3/4	8
McEl.	23	4	Varicella contact; atopic eczema; extensive new lesions	24 "	SM	3/3	8
Rud.	7	2	Varicella in patient with leukemia	9 "	SM	3/4	6
And.	8	2	Typical; hospital case	1 "	SM	2/4	5
Dru. A	15	3	Typical; hospital case	2 wk.	SM	3/4	6
B				5 mos.	Uterus	2/4	8
C				5 "	Testis	4/4	7
Pat. A	4	1	Typical; hospital case	1 wk.	SM	1/4	12
B				6 mos.	FS	2/4	7
Ket.	21	3	Typical; hospital case	5 "	FS	2/2	8
Cic. A	4	10	Neuroblastoma patient on	3 "	SM	3/3	8
B			therapy; large atypical	14 "	FS	3/3	7
C			lesions continuing to appear	24 "	FS	3/3	7
Cam.	15	3	Typical case	37 "	SM	2/4	11
Orc.	7	4	Autopsy material; varicella complicating rheumatic fever; on cortisone therapy	0	SM	3/3	7
Rob.	31	3	Typical case	17 mos.	SM	4/4	4
Wil.	12	2	Typical case	24 "	FS	3/3	6
Ent.	2	7	Atypical; Letterer-Siwe's disease; on cortisone therapy	0	FS	3/3	4
Jen.	2	3	Typical case	24 mos.	FS	3/3	7

* Letters indicate reisolations from aliquots of the same material.

show the continuing appearance of new vesicles or the unusual persistence thereof. Because of the heightened clinical interest in such situations, strains of virus were isolated and studied from atypical cases of varicella, as manifested by patients with eczema and malignancy, or in the course of cortisone

TABLE II

Data on Isolation of Zoster Strains from Vesicle Fluid Materials in Cultures of Human Tissues

Strain*	Age	Day illness (from onset exanthem)	Remarks on clinical picture	Interval vesicle fluid stored in frozen state	Type of culture and cytopathic response		
					Tissue	No. cult. + / No. cult. inoc.	Day cytopathic changes first noted
	yrs.						
Sto. A	87	1	Thoracic lesions	21 mos.	SM	2/2	6
B				57 "	FS	2/2	12
Pie.	44	1	Trigeminal lesions; had varicella at 4 yrs.	0	FS	3/4	5
Blu. A	37	3	Lumbar lesions	0	FS	2/4	9
B				30 mos.	FS	2/2	9
Bag. A	60	2	Thoracic lesions	0	FS	3/3	3
B			Varicella as child	26 mos.	FS	1/1	12
Fer.	25	1	Thoracic; good new vesicles; varicella at age 19 yrs.	0	FS	3/3	6
Poo.	70	7	Thoracic; new vesicles appearing; no memory of varicella	0	FS	2/3	9
Szu.	35	2	Thoracic lesions; no varicella history	0	SM	3/3	6
Fit.	17	2	Thoracic; on cortisone therapy	0	SM	3/3	6

* Letters indicate reisolations from aliquots of the same materials.

therapy. Five strains from atypical cases are included in Table I; clinical-pathological descriptions of patients "Cic" and "Orc" have been presented elsewhere (18). These strains did not differ in respect to *in vitro* behavior from agents derived from classical cases of varicella. With a single exception, noted below, Z strains were obtained from patients with vesicular lesions of less than 4 days' duration. Failures were encountered in two instances when the inoculum consisted of material collected from mature vesicles of 4 days' duration.

As indicated in Tables I and II, vesicle fluid materials were infective after periods of storage in the CO_2 cabinet for as long as 57 months. Reisolation of virus from frozen samples of vesicle fluid known to be infective was usually accomplished without difficulty. Little quantitative information was obtained on the infectivity of vesicle fluid materials or on decay of infectivity after freezing. Only from two cases were accurately measurable quantities of fluid collected. *Rob.*, a 31-year-old female with varicella was seen 48 hours after the appearance of the initial lesion, at a time when many relatively large young vesicles were present. Aspiration of 28 vesicles yielded 0.04 ml. of fluid which was diluted to 10^{-1} in buffered milk and quick frozen in the dry ice cabinet. Seventeen months later tenfold dilutions to 10^{-8} were made in buffered milk, 0.1 ml. amounts of each dilution were inoculated into three cultures of SM tissue, and the cultures were observed for 28 days. The $TCID_{50}$ of the stored varicella material was found to be $10^{-3.5}$. A 70-year-old lady with thoracic herpes zoster, unusual in that numerous young vesicles were present on the 7th day of the eruption, provided material for an experiment with freshly collected fluid. This was diluted and inoculated within an hour of collection; the $TCID_{50}$ was $10^{-1.2}$. Virus was also demonstrable in the supernatant of a portion of this specimen following centrifugation in a capillary tube of a 1:2 milk dilution for 5 minutes at 2000 r.p.m.

As recorded in Tables I and II, cytopathogenic changes commonly were first apparent in cultures of SM or FS tissue between the 6th and 8th day after inoculation. However, these changes have appeared as early as the 3d day, and as late as the 22nd day after the introduction of vesicle fluid. The time of appearance and development of focal lesions were adversely affected in cultures in which tissue growth was suboptimal.

Routine Serial Passage of Varicella and Zoster Strains in Vitro.—

Although, on occasion, as described below, virus could be recovered from frozen infected tissue culture materials, to assure the availability of selected strains, it was found desirable to maintain the agents in serial passage. The characteristic, slowly progressive cytopathic process facilitated this maneuver inasmuch as subculture could be accomplished at 15- to 35-day intervals, depending on the rapidity of extension of the cytopathic process and on the availability of suitable uninoculated cultures. Maintenance of strains for relatively long periods in this manner increased the hazard of introduction of viral and bacterial contaminants. It therefore was found desirable to continue some of the parent cultures and to discard them only after an additional passage had been accomplished. To minimize contamination and resultant loss of a strain, the unused cultures of the parent material were usually maintained with lots of media distinct from those employed for the new passage. In this manner, 198 routine subcultures of selected varicella strains were accomplished, as were 99 passages of zoster strains. Of the varicella strains, the *Wel.*

virus, maintained for 38 passages during an elapsed period in cultivation of 799 days, and the *McEl.* agent, passed 34 times over a period of 789 days, were propagated the longest. Three zoster strains, *Blu.*, subcultured 28 times over a 678 day period, *Bag.*, maintained for 23 passages during an elapsed time of 560 days, and the *Sto.* strain, carried for 18 passages over a 444 day period of cultivation, were extensively studied. Throughout these prolonged periods of cultivation no alteration was observed in the cytopathic behavior of the viruses.

In subcultures inoculated with crudely ground infected tissues, cytopathic changes appeared more rapidly than in primary cultures. Frequently, early changes could be associated with the implantation of tissue fragments derived

TABLE III

Representative Data on Serial Passage in Vitro of V-Z Agents

Wel. V strain					*McEl.* V strain					*Blu.* Z strain				
Passage	Tissue	No. cult. + / No. cult. inoc.	Day foci first noted	Day subculture	Passage	Tissue	No. cult. + / No. cult. inoc.	Day foci first noted	Day subculture	Passage	Tissue	No. cult. + / No. cult. inoc.	Day foci first noted	Day subculture
10	SM	6/6	4	10	24	FS	3/3	<6	15	20	SM	3/3	3	23
11	SM	3/3	5	34	25	SM	4/4	4	19	21	SM	4/4	4	22
12	FS	2/3	<12*	33	26	SM	3/3	4	10	22	FS	3/4	6	32
13	FS	4/4	2	21	27	SM	6/6	<6	17	23	FS	6/6	3	18
14	SM	4/4	<5	37	28	FS	1/3	9	33	24	FS	4/4	<7	34
15	SM	3/3	<5	24	29	FS	4/4	2	20	25	FS	4/4	5	37
16	SM	4/4	<6	24	30	FS	4/4	<7	53	26	FS	4/4	<6	16
17	SM	10/10	5	22	31	FS	4/4	2	30	27	FS	4/4	3	19

* Focal lesions well developed when observed on day indicated; last previous observation 2 or more days earlier.

from the inoculum, and foci developing in the outgrowth therefrom could occasionally be seen within 48 hours. In Table III are summarized data indicating the usual sequence of events in the course of serial passage. Delay of cytopathogenic effect or failure to infect subcultures usually was related to poor tissue growth. For example, in the single instance cited in Table III in which subculture was unsatisfactory, two of the three cultures inoculated with material from the 27th passage of the *McEl.* strain were noted as showing "fair" growth at the time of inoculation; although 2 weeks later the appearance of the outgrowth was termed "good," these cultures failed to develop focal lesions.

Characteristically, the focal lesions in an infected culture slowly increased in size and in number with progression determined by the physical disposition of the cell outgrowth as well as the rapidity thereof, and by the size of the inoculum. Representative data showing the rate of appearance of foci after

introduction of inocula of moderate size are given in Table IV. With inocula of a greater order of infectivity, the development and resulting fusion of adjacent foci occurred more rapidly. The involvement of an ever-increasing proportion of the outgrowth was characteristic and was recorded by estimating the proportion (expressed in per cent) of the cell sheet affected. Under the

TABLE IV

Average No. of Focal Lesions Observed per Culture during First 11-Day Period; Fourth in Vitro Passage of McEl. and Wel. Varicella Strains in FS Tissue

Strain	Day after inoculation						
	4	5	6	7	8	10	11
McEl.							
Average No. per culture*	0	1.3	1.5	1.8	3.1	4.1	5.0
Range	0	0–4	0–4	0–4	1–5	1–8	1–10
Wel.							
Average No. per culture*	0	0.4	0.5	0.7	1.5	2.3	3.1
Range	0	0–1	0–1	0–1	1–3	1–5	1–8

* Based on nine cultures in each group.

TABLE V

Representative Readings to Illustrate the Slow Progressive Nature of the Cytopathic Process Induced by V-Z Strains

Strain	No. of focal lesions or *per cent* of cell sheet involved on day:						
	5	12	16	23	30	40	55
Blu. (Z) 9th passage in FS tissue (Average 3 cultures)	4	*18%*	*25%*	*65%*	*85%*	*90%*	*98%*
McEl. (V) 20th passage in FS tissue (Average 4 cultures)		4		*10%*	*45%*	*85%*	
Wel. (V) 29th passage in SM tissue (Average 3 cultures)	1.2	5	*15%*	*40%*	*75%*		

conditions of routine passage, 50 per cent of the available cell sheet usually became involved between the 15th and 30th day after inoculation; subculture was often timed to coincide with this degree of cytopathic change. The end point of the process of peripheral extension and fusion of the lesions was destruction of all cells. Occasionally, however, islands of cells would escape involvement; in one instance, cellular repopulation of a long term culture occurred after specific destruction was almost complete, and the new growth was

then in turn slowly destroyed by a second progressive cytopathogenic wave. Representative data indicating the pattern of spread of the cytopathic process are given in Table V. As long as new areas continued to be involved in a culture, the agent could be subcultured without difficulty. Thus strains maintained *in vitro* at 35° C. were passed in 11 instances after intervals ranging from 40 to 70 days, with a maximum period of 82 days of cultivation elapsing before successful passage. Failure to subculture virus from infected cultures kept at 35°C. occurred in a single instance after 71 days, and frequently in attempts made after intervals exceeding 80 days.

TABLE VI

*Increase in Size of Three Cytopathic Foci as Followed by Serial Camera Lucida Drawings**

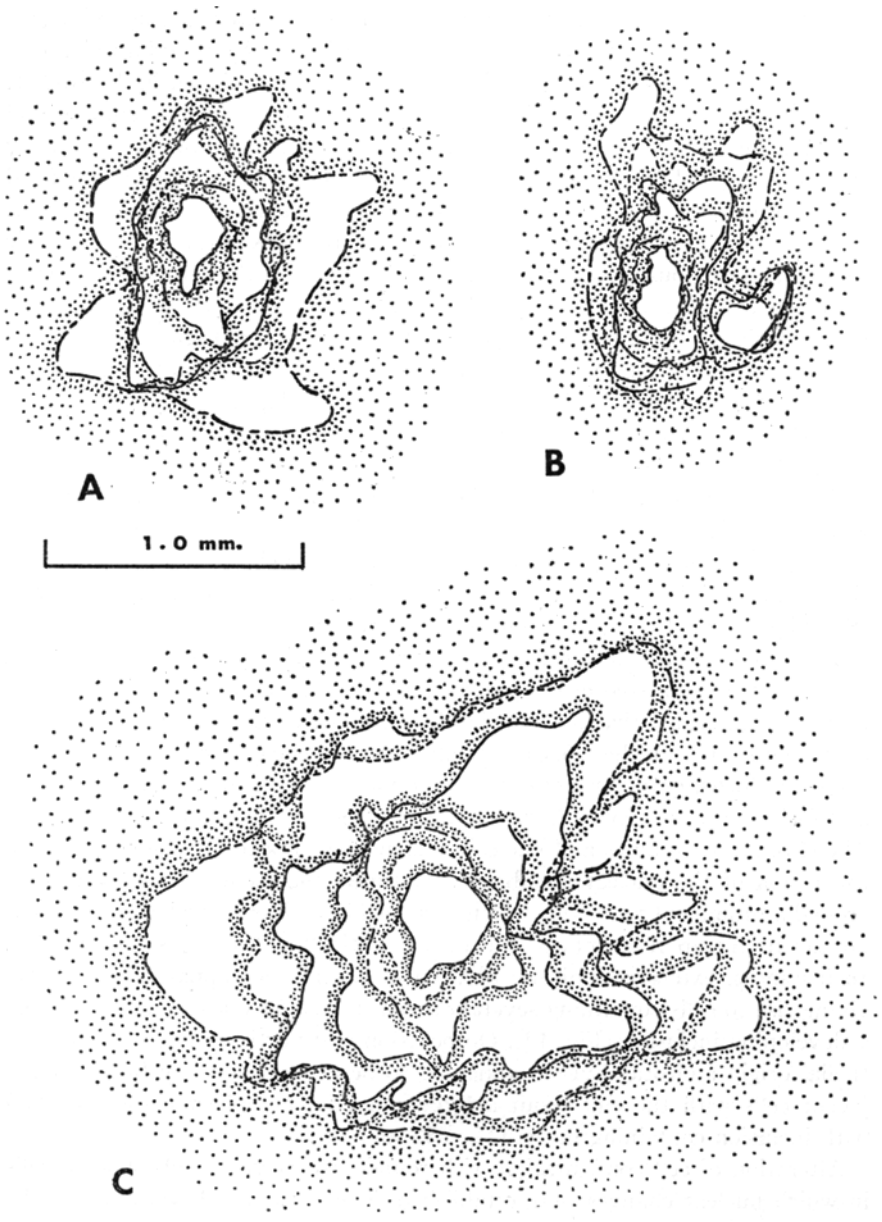
Day after inoculation	Circumferential measurements		
	Focus A (Culture No. 4)	Focus B (Culture No. 8)	Focus C (Culture No. 9)
	mm.	*mm.*	*mm.*
5	0.7	0.7	1.4
7	1.1	1.2	2.2
9	1.6	1.3	2.7
12	2.5	3.3	4.3
15	3.0	3.6	6.1
19	5.4	3.7	9.8

* Varicella *Cic.* strain; 9th *in vitro* passage; human embryonic SM tissue; foci selected in different cultures.

The Focal Cytopathic Process.—

The development of individual focal lesions as observed *in vitro* was a matter of interest. Small groups of rounded, refractile cells appeared, with the initial changes most noticeable in areas where the outgrowth consisted of a sheet of clear fibroblast-like cells. Similar focal changes, however, occurred in the variety of cell types present in FS or SM cultures. Considerable variation in response was observed. In some foci small round cells predominated and giant cells were infrequent; in others, giant cell formation was a prominent feature. Extension of the cytopathic process appeared to be by direct involvement of contiguous cells; relatively rapid development occurred in regions where cells were in close approximation, and extension was slow if the outgrowth pattern was a loose cellular network. The changes occurring over a 3 day period are illustrated in Figs. 1 to 6 which picture three varicella foci in FS tissue cultures as observed on the 7th day after inoculation and again on the 10th day.

The progress of selected cytopathic lesions as followed by serial camera lucida drawings was one of continuing slow increase in size (See Table VI). A



TEXT-FIG. 1. Serial outline drawings of single focal lesion in each of three cultures of V virus (strain *Cic.* 9th passage) in human embryonic SM tissue; superimposed camera lucida drawings done on days 5, 6, 8, 12, 14, 16, and 19.

better concept of the somewhat irregular manner of extension, as influenced by shifting and retraction of the cell sheet, is afforded by superimposing certain of the serial outline tracings in this experiment, as shown in Text-fig. 1. Not apparent from these particular tracings is the common tendency of foci to extend most rapidly along the long axis of cellular outgrowth. Whenever there was a radial orientation of the outgrowth from a tissue implant, the focal lesions often were aligned in a radial fashion and resembled petals of a flower. With the progressive replacement of the central portions of the foci by necrotic cells and amorphous granular debris, there was a loss of the usual transparency manifested by the unaltered cell sheet. Older foci could then be visualized macroscopically by transmitted light and appeared as radiating bright areas if illuminated obliquely while viewed against a dark background; the "petal" effect as demonstrated in this manner is shown in Fig. 7.

Study of stained preparations added to an understanding of the cytopathic process. Usually the focal lesion was sharply delineated from unaffected tissue, as illustrated in Fig. 8. In areas where the cells were in close proximity, the zone of activity was broad, as evidenced by the presence of a number of cells showing approximately the same stage of cytopathic change; in regions where the cell network had an open pattern, the active zone was irregular and narrow (See Figs. 9 and 10). Just beyond the apparent margin of the focus, nuclear changes were observable in cells that were morphologically otherwise unaltered. These changes consisted of the presence of one to several minute intranuclear granular aggregates that stained lightly eosinophilic or amphophilic, and occupied clear areas in the nuclear reticulum. In the nuclei of cells at the edge of the overt focus, granular aggregates were more prominent, increasing in size, and fusing until usually a single large intranuclear eosinophilic mass resulted. The chromatin granules and nucleoli, meanwhile, appeared to be displaced to the region of the nuclear membrane. At times, the inclusion would develop around a prominent nucleolus, which remained in a clear central area, or would appear to entrap remnants of the nuclear reticulum (See Figs. 11 and 12). In instances, extensive amitotic division of the nucleus apparently occurred giving rise to cells containing several or even masses of nuclei each containing a developing inclusion (Fig. 12). On occasion and particularly in cells of epithelial origin amitotic division appeared to be accompanied by fusion of adjacent cells with the end result a large syncytial cytoplasmic sheet packed with inclusion containing nuclei (Figs. 13 and 14).

Alteration of cell configuration with rounding occurred in fibroblastic cells in which nuclear changes were established. There was much variation in the size of infected cells, some appearing little enlarged and others transforming into large giant cells. A collection of poorly defined, finely granular material that stained with eosin, or more rarely was slightly basophilic, could be seen in the cytoplasm of some of the infected cells. This material usually was paranu-

clear in location and at high magnifications (see Figs. 15 and 16) appeared as a fine network or honeycomb containing poorly differentiated bodies.

Cells at the inner edge of the zone of cytopathic activity showed changes leading to complete degeneration. The intranuclear inclusions in this region were less prominent, tended to be amphophilic, and the nucleus was progressively more pyknotic; the end result appeared as a small irregular rounded clump of basophilic granules representing the nuclear chromatin material, in which the remnants of the inclusion could not be identified. The cytoplasm often appeared irregularly vacuolated and finally underwent dissolution releasing amorphous eosinophilic granular debris and the basophilic clumps of nuclear remnants.

This summary of changes observed in stained preparations indicates in a general way the nature of the progressive cytopathic process induced by the V-Z viruses. To be emphasized are the focal nature and at the same time the great variability of the host-cell response, depending on cell type and other undefined factors.

The in Vitro Susceptibility of Other Tissues of Human and Non-Human Origin.—

Various tissues were tested as to their capacity to support growth of the varicella-zoster agents. It was noted that cells derived from the inoculum, *i.e.* crudely ground infected tissue, would on occasion proliferate, and if not morphologically of a type distinct from the strain under examination could give equivocal results. For this reason, some experiments were performed using vesicle fluid materials as inocula. Serial passage of the agents in the tissues under study was also utilized in an effort to minimize possible misinterpretation of observed changes.

1. Human Tissues.—

(*a*) *Uterus:* Subculture of the *Pat.* strain was accomplished on two occasions to myometrial cultures, and this observation indicating the susceptibility *in vitro* of this tissue was confirmed by the primary isolation in uterus cultures of the *Dru.* strain. The cellular changes induced in the myometrial outgrowth were less sharply focal and were more pronounced than in the fibroblastic growth from SM. The involved cells lost the typical spindle appearance, became rounded, large, and at times showed an irregular flattened ameboid configuration. In stained preparations, multinucleate cells were common. Masses of paranuclear, cytoplasmic, finely granular material were usually more prominent than in comparable FS or SM preparations (see Figs. 17 and 18).

(*b*) *Testis:* Specific cytopathic changes developed in roller cultures of adult human testis following inoculation with passage materials of two V strains and one Z strain and also with *Dru.* (V) vesicle fluid. The resulting alterations in the characteristically delicate fibroblast-like cell outgrowth were less marked than

in SM or FS cultures. Individual infected cells became round but usually remained small. The areas of focal activity were at times poorly demarcated, irregular, and uninvolved cells often remained scattered throughout regions of viral activity (see Figs. 19 and 20).

(*c*) *Brain:* Roller cultures of human embryonic brain tissue when inoculated with the *Wel.* or *McEl.* varicella strains and with the *Sto.* zoster strain developed specific cytopathic changes. The response in infected glial cells was striking with development of large, flattened, ameboid multinucleate cells (see Figs. 21 and 22).

(*d*) *Kidney:* In trypsinized human postnatal kidney cell cultures, serial passage of three varicella strains (*McEl.*, *Orc.*, and *Cam.*) and of one zoster strain (*Blu.*) was accomplished without difficulty; the *Cam.* line was maintained for 9 successive transfers. The lesions developing in the epithelial cell sheets were plaque-like, and extended centrifugally in an irregular manner. Foci in the early stages appeared as sharply demarcated, irregular hyaline or glassy areas that were distinct from the transparent uninvolved cell sheet. The rounded, infected cells not infrequently contained prominent cytoplasmic vacuoles. Progressive involvement of the area of the cell sheet was again characteristic; usually 80 per cent of the tissue was involved by the 15th to 20th day after inoculation.

(*e*) *Amnion:* Trypsinized human amnion cultures were satisfactory for the isolation of two strains from vesicle fluid materials (*Orc.* (V) and *Szu.* (Z)) and for the serial propagation of the *Wel.* agent. In general, the plaque-like foci were comparable to those observed in cultures of renal epithelium. The infected cells frequently showed delicate radiating cytoplasmic processes and multinucleate giant cells were a common feature (Figs. 23 and 24).

(*f*) *HeLa cells:* In an experiment performed by one of us at the Rocky Mountain Laboratory in Hamilton, Montana, it was shown that HeLa cell cultures were satisfactory for the maintenance of varicella virus in serial passage and that the natural history of the cytopathic process was similar to that observed in other epithelial cell cultures of human origin (Figs. 25 and 26).

2. Rhesus Monkey Tissues.—

(*a*) *Monkey testis:* The introduction of vesicle fluid inocula into roller cultures of monkey testicular tissue resulted in the irregular development of focal cytopathic lesions. In one experiment, foci of rounded fibroblasts were observed on the 15th day after inoculation of *And.* vesicle fluid and on the 20th day after *Blu.* material; it is to be noted that the foci took longer to become apparent and progressed more slowly than in human tissues. The two agents so isolated were passed to additional monkey testis cultures; when stained, these showed sharply circumscribed necrotic foci, and the cells at the periphery of the areas of destruction were rounded and contained intranuclear inclusions. Two additional experiments indicated that monkey testis cultures are inferior to cul-

tures of human foreskin for the isolation of varicella virus; inoculation of *Cic.* and *Poo.* vesicle fluids respectively, in parallel, into three cultures of monkey testis and into three of human foreskin, resulted in no isolations in the cultures of testis, while 5 of the 6 foreskin cultures were positive.

The inoculation of monkey testis cultures with 6th passage *Orc.* virus, previously maintained in human SM tissue, resulted in an actively progressive cytopathic process that involved 90 per cent of the monkey cells by the 21st day (See Fig. 27); in these cultures the macroscopic "petal" effect was striking. This strain continued to manifest relatively rapid cytopathic activity during two additional monkey testis passages.

(*b*) *Monkey kidney:* Virus in the form of infected tissue culture materials, representing in different experiments three varicella strains (*Orc.*, *McEl.*, and *Cam.*) and one zoster strain (*Blu.*), produced cytopathic changes in cultures of trypsinized monkey kidney cells that closely resembled those seen in human kidney cell cultures (Figs. 29 and 30). Plaque-like lesions developed 5 to 7 days following inoculation of infected human SM tissue or kidney cells. By the 20th day usually 60 to 95 per cent of the monkey cells were involved. One line (*Orc.*) was passed five times in monkey kidney cultures, and another (*Cam.*) was alternately maintained for five passages in human and in monkey kidney cells; in neither instance was any modification of *in vitro* behavior noted as the result of these manipulations. Extraneous simian agents, morphologically similar to Rustigian's foamy virus (19) appeared in certain of the monkey kidney cultures, and also in uninoculated and inoculated cultures of monkey testis. The strain of foamy virus of monkey testis cell origin also produced granular swelling, syncytial changes, and subsequent degeneration in cultures of human FS tissue. Although the simian virus problem made serial propagation of varicella virus in monkey cells difficult, it was observed that, with suitable controls, bottle cultures of monkey kidney cells could be used for the preparation of a satisfactory varicella complement-fixing antigen (11).

3. Tissues Derived from Other Species.—

(*a*) *Rabbit tissues:* Two types of tissue from the domestic rabbit were investigated as to ability to support growth *in vitro* of V-Z viruses, with suggestive results. Cultures of rabbit testis inoculated with an infected tissue suspension of 7th FS passage *Orc.* virus developed characteristic focal lesions. Subculture to rabbit testis cultures on the 12th day resulted in slowly progressive focal lesions in 2 of 4 tubes (see Fig. 28); material from one of these stained at the 30th day contained typical lesions with intranuclear inclusions in the involved cells. A second attempt at subculture failed. Negative results with cultures of rabbit testis were obtained using 28th human tissue passage *McEl.* virus as inoculum, and in limited attempts with vesicle fluid specimens of known infectivity derived from two varicella cases and one zoster case.

Cultures of rabbit kidney tissue were inoculated on different occasions with

two strains of *Orc.* virus. Of four cultures receiving 4th passage human tissue material, one developed progressive focal cytopathic changes; passage of this material on the 26th day of cultivation to a culture of human uterus tissue gave characteristic lesions. Two of three rabbit kidney cultures inoculated with *Orc.* 2nd monkey kidney passage material developed foci; subculture attempts at the 7th day and at the 21st day to additional cultures of rabbit kidney failed but in each instance parallel attempts were positive in human SM tissue.

(*b*) *Other tissues:* Roller cultures of mixed chick tissues derived from 5-day-old embryos, and also of amniotic membrane tissue from 9-day-old embryos were inoculated with 14th passage *McEl.* virus and with 17th passage *Wel.* virus. No lesions were observed in the latter group, but in those inoculated with *McEl.* virus, three of the mixed chick tissue cultures and two of the amnion cultures developed focal lesions between the 12th and 19th day that appeared to involve chick cells as well as the outgrowth of cells derived from the inoculum; however, passage attempts were negative. The same virus suspensions produced no lesions in cultures of mixed mouse embryonic tissues in a single experiment. Cultures of bovine embryonic SM tissue inoculated with first passage *Fit.* strain material of known infectivity produced small focal lesions in one of four cultures. In one experiment, no foci developed in cultures of trypsinized porcine kidney tissue inoculated with culture material in the form of a human kidney strain of *Cam.* virus of known infectivity.

Characteristics of the V-Z Viruses under in Vitro Conditions.—

1. Infectivity of the Fluid and Tissue Phases of Infected Cultures.—Only two experiments were done with infected tissue suspensions. In one, aliquots of crude infected tissue suspension obtained on the 15th day of cultivation of the *McEl.* strain (26th passage) were ground either by hand or with a motor-driven Teflon tissue grinder for 3 minutes; the $TCID_{50}$ of each suspension (not centrifuged) was of the order of $10^{-3.0}$. A suspension of infected human kidney cells (*Cam.* strain, 5th passage, 16th day) dispersed mechanically by scraping and pipetting was found to have a titer of $10^{-1.5}$.

More attention was paid to attempts to demonstrate infectious material in the fluid phase of infected cultures after centrifugation of the fluid at slow speeds to sediment cells. Fluids from infected cultures between the 13th and 45th day after inoculation were centrifuged for 10 to 15 minutes at speeds of 1500 to 2000 R.P.M. and then tested *in vitro.* Eight such experiments were carried out in which the materials tested were derived from the 1st to the 20th passage of four V strains and of one Z strain; only in a single experiment and in that instance in a single culture was a focal cytopathic agent recovered; this occurred in a test of original passage *McEl.* strain fluid collected on the 13th day, and may have indicated survival of the inoculum.

2. Storage of Infected Tissue Suspensions in the Frozen State.—The ease with which virus could be subcultured when the inoculum consisted of infected tissue focused attention on the stability of the agent stored in this form in the dry ice cabinet. Numerous attempts were made to isolate V-Z agents from tissue

TABLE VII

Summary of Results of 33 Experiments in Which Attempts Were Made to Recover Virus from Infected Ground Tissue Suspensions, after Varying Periods of Storage in the Dry Ice Cabinet

Period tissue suspension frozen	Tissue cultures inoculated	
	No. positive	No. negative
0–7 days	4	2
8–14 "	14	1
15–28 "	0	11
29–59 "	7	5
2–3 mos.	4	35
4–5 "	3	41
6–11 "	3	45

TABLE VIII

Attempts to Recover Two Strains of Virus from Frozen Tissue Suspensions Derived from Varying in Vitro Passages

Strain	Tissue from *in vitro* passage	Months frozen	Cultures + / Cultures inoc.	Day cytopathic changes noted
Sto. (Z) virus	1	24	0/3	
	3	15	1/3	15th
	6	12	0/3	
	8	10	1/3	14th
	12	6	0/3	
	14	3	2/3	11th
Wel. (V) virus	23	26	0/2	
	25	24	0/2	
	28	22	0/2	
	30	20	1/2	21st
	32	9	0/2	

suspensions prepared in culture medium that had been quick frozen in a dry ice–alcohol bath and kept at −40 to −50°C. in sealed glass ampoules. These experiments are summarized in Table VII. Infectivity could be demonstrated with fair regularity after periods of storage under 2 months; survival of the agent was irregular and unpredictable after longer periods. In two experiments, summarized in Table VIII, the question of possible adaptation to *in vitro* conditions and the better survival in the frozen state of high passage V-Z virus was

explored. Large inocula, ranging from 0.3 to 0.6 ml. of tissue suspension per culture, were employed in the *Wel.* experiment. Here again, recovery of virus was sporadic and suggested that the interval of storage was more important than the previous *in vitro* experience of the strain. Focal cytopathic changes in cultures inoculated with frozen suspensions occurred relatively late, but the mode of progression thereafter was not unusual.

In two experiments ampouled suspensions of infected tissue, frozen for 6 and 13 days respectively, were rapidly subjected to four additional freeze-thaw cycles, with the thawing done in cold water. Whereas the suspensions frozen once infected 5 of 8 cultures, those frozen five times failed to infect 13 cultures inoculated with 0.1 ml. amounts.

The foregoing observations suggested that maintenance of infectivity in stored tissue suspensions might be related to the presence of infected cells that survived the freeze-thaw process. Two experiments designed to explore the effect of substances that might promote cell survival were carried out with inconclusive results. Suspensions of infected tissue were prepared in media with and without the incorporation of 5 per cent dextrose. When tested after periods of storage of 10 and 38 days, there was no loss of infectivity of the suspensions—at 67 days, each infected one of 3 cultures. In another experiment, infected tissue suspensions were prepared with 10 per cent glycerin in medium and aliquots thereof frozen rapidly or slowly at −60°C.; when tested after storage for 4 months in the frozen state, neither retained significant levels of infectivity.

3. The Effect of Modifying Conditions of Cultivation.—

(*a*) *Incubation at lower temperatures:* It was noted that the cytopathic process was slower at room temperature than at 35°C. For purposes of preserving strains, infected cultures containing well developed foci were therefore transferred to roller drums maintained at 25 or 28°C. and the interval between media changes was lengthened to 7 to 10 days. Subculture could then be accomplished after periods of maintenance at 28°C. as long as 4½ months.

The behavior of the cytopathic process at the lower temperatures was studied in several experiments. When cultures containing discrete well developed foci were transferred to a roller machine at 25°C., the individual lesions either ceased to show peripheral extension or did so at a much slower rate; degeneration of infected cells continued, however, and thus the lesions appeared as an area of amorphous and granular debris separated from the normal appearing cellular outgrowth by relatively few rounded, swollen fibroblasts. Reincubation of such infected cultures at 35°C., after periods of 30 to 60 days at the lower temperature, resulted in reactivation of focal activity in the usual manner. The incubation of cultures at 25°C. immediately following inoculation with infected tissue suspensions gave variable results. In one

instance no foci appeared in cultures incubated for 15 days at 25°C. and then held for 14 days at 35°C. In another group of cultures thus handled, two small focal lesions were seen after 21 days at 25°C.; these developed very slowly through the 50th day at which time transfer to an incubator at 35°C. was followed by the usual sequence of progression. Again, in the experiment summarized in Table IX, foci were not observed during a 21 day period at 25°C. but developed on transfer to 35°C. This particular experiment also documents the observed stabilization of pre-existing foci on transfer from 35 to 25°C. and reactivation on incubation at the higher temperature.

(*b*) *Modifications in the medium:* The tissue culture medium was altered in various respects in unsuccessful attempts to demonstrate a hypothetical viral inhibitor, to accelerate cytopathic activity, or to effect simplification of the

TABLE IX

*Relationship between the Development of Cytopathic Changes and Temperature of Incubation**

Cultures of SM tissue maintained after inoculation:	Average No. foci or *per cent* tissue involved on day:									
	3	6	10	13	19	21	25	35	42	56
At 35°C. throughout (3 cultures)	4	26	52	*35%*	*65%*	*80%*	*85%*	*98%*	*98%*	
At 25°C. to day 21; then 35°C. (2 cultures)	0	0	0	0	0	0‡	1.3	2	*30%*	*85%*
At 35°C. to day 3; at 25°C. day 3 to 21; at 35°C. day 21 to 56 (6 cultures)	6.5	11.1	10.9	6.8	8.6	7.2	*30.5%*	*85%*	*95%*	

* Inoculum: Infected tissue suspension from 20th *in vitro* passage *Blu.* strain.

‡ Transferred to higher temperature on 21st day.

procedures for strain maintenance. In tests with representative V and Z agents, no adverse effects on the cytopathic process were observed when the bovine amniotic fluid was reduced from a concentration of 90 to 45 per cent by substitution of Hanks's salt solution. The observations of Amos (20) on the inactivation of herpes simplex virus by phosphatase enzymes suggested comparative trial of the amniotic fluid–horse serum–embryo extract medium heated to 65°C. for 30 minutes; groups of 6 SM cultures were inoculated and maintained on regular medium, on heated medium, and on ox serum ultrafiltrate (20 per cent)–Hanks's salt solution (80 per cent). No differences were observed in the focal changes developing in the cultures receiving regular and heated medium; those on serum ultrafiltrate averaged two small foci per culture by the 7th day, and these failed to extend in the usual manner.

(*c*) *Attempts to dissociate virus from suspensions of infected tissue:* Tissue suspensions of known infectivity were ground in a mortar using various diluents,

centrifuged at 0°C. for 15 minutes at 2000 R.P.M., and the supernatants then tested for infectivity. Negative results were obtained when the diluent was regular medium containing 0.01 M cysteine, 20 per cent glycerin in phosphate buffer (pH-7.1), or buffered skimmed milk (pH-7.1). In three experiments no virus was demonstrable in the supernatant following trypsinization of infected tissue suspensions. Infected tissue suspensions in regular media were held at 35 and at 5°C. for 24 hours and then centrifuged; on inoculation the sediments were shown to have retained infectivity and the supernatants were negative.

(*d*) *Behavior in the presence of other viruses:* Cultures of FS and SM tissue infected with three strains of V virus and containing discrete focal lesions were inoculated with herpes simplex virus. No overt evidence of interference was observed. By the 9th day, the varicella lesions appeared as dense masses of finely granular debris and all surrounding cells had been destroyed by the cytopathic activity of the simplex virus. Superinfection of monkey kidney cell cultures containing typical V virus foci with Type I polio virus produced a similar end result. In cultures of monkey testicular tissue infected with V virus and with a virus similar to Rustigian's simian agent (19), the distinctive cytopathic changes again appeared to develop in an independent fashion.

Control Studies and Other Observations Made in the Course of Serial Propagation of the V and Z Agents.—

1. In Vitro Observations.—In the course of serial propagation tissue suspensions were prepared as routine from the "control" cultures as well as from "infected" cultures and used as inocula for passage. During the study, with one exception, focal cytopathogenic changes of the varicella type were not observed in uninoculated cultures of FS or SM tissues or in inoculated "control" cultures; the exception occurred in the course of a single passage wherein control cultures developed focal lesions,—this was probably the result of an error in labelling, and the passage was discarded.

Three additional observations, two dealing with extraneous agents, and another with the appearance of a "refractory" strain of cells, are briefly described as problems encountered with the use of primary explant cultures in the course of the prolonged propagation of V-Z viruses. Apart from the occasional loss of a strain due to bacterial or mold contamination, or to technical factors of a non-specific nature, these three episodes comprise the complications recognized over a 5 year period of the routine use of primary cultures of human embryonic or of foreskin tissues maintained on the bovine amniotic fluid medium.

A cytopathogenic agent, termed Wild Virus No. 1, producing changes similar to those described for the adenoviruses, appeared in concurrent passages in FS tissue of three different V-Z strains. The cultures in these experiments were prepared from a single prepuce, set up on July 6, 1955, and inoculated on August

8, 1955; 4 of 28 cultures developed bizarre rounding of scattered fibroblasts between the 23rd and 41st day after inoculation. The responsible agent was studied by Dr. F. A. Neva, and subsequently kindly identified as an adenovirus Type 2 by Dr. W. P. Rowe of the National Institutes of Health. Circumstantial evidence suggested that the agent had derived from the foreskin tissue but other sources could not be excluded; the virus had not previously been studied in this laboratory.

The nature and origin of a second extraneous agent were less clear. In the 15th passage of the *Sto.* Z strain in FS, distinctive cytopathic changes occurred in a single culture on the 7th day after inoculation, at a time when the zoster foci were starting to appear; these changes were reproduced on subculture to SM and FS cultures after a 20 day incubation period and the term "FS agent" was arbitrarily applied. With subsequent passage, the incubation period shortened to 4 to 8 days. The cytopathic changes associated with the "FS agent" were unique in that initially there was focal involvement wherein the fibroblastic cells first became slightly enlarged and flattened or rounded and then appeared to undergo cytoplasmic dissolution leaving masses of shrunken nuclei; the nuclei simulated closely the appearance of leucocytes in a counting chamber after treatment with dilute acetic acid (see Figs. 31 and 32). In single experiments, similar cytopathic changes developed in monkey testicular and kidney cell cultures following inoculation. In hematoxylin-stained coverslip preparations, masses of minute intra- and extracellular granules and filaments were demonstable in the areas of degeneration suggesting a relationship to the pleuropneumonia group. The nature of the agent was not further established. It appeared to derive from one of the components of the culture system.

A different type of phenomenon resulted in the loss of a strain of V virus due to the overgrowth of the cultures by a type of cell that was refractory to infection. Three strains of V virus (*Pat.*, *Wel.*, *McEl.*) were subcultured on the same day to groups of SM cultures that came from the tissues of a single embryo and had been prepared 29 days previously. Typical focal lesions appeared; however, spread was slow and in all three experiments the presence of a rapidly proliferating small, transparent, stellate type of cell was noted. These appeared not to be involved in the cytopathic process. Using SM cultures from a different source, two of the V strains were successfully subcultured on the 13th and 23rd day, respectively. Bottle cultures were successfully inoculated with 11th day 8th passage *Pat.* material, but on routine passage in cultures inoculated with 29th day *Pat.* 8th passage material, there was rapid proliferation within 48 hours of the flat, stellate cell derived from the inoculum, and no specific focal changes developed over a 28 day observation period. Two of the "refractory" cultures were reinoculated with a suspension of *Sto.* virus; again no cytopathic changes appeared, although the same *Sto.* inoculum induced characteristic changes in a group of FS cultures.

The strain of refractory cells was readily transferred and repeatedly subcultured over a 26 month period (Fig. 33) before it was inadvertently lost owing to contamination. In a single experiment roller cultures were prepared by planting refractory cells and fresh human embryonic SM tissue in the same culture tube; inoculation of these cultures with Type III poliomyelitis virus promptly produced cytopathic changes in the SM tissue and no changes in the clear, stellate cells. Although the "refractory" cells appeared in cultures prepared from a single human embryo, their origin from bovine or chicken constituents of the medium could not definitely be excluded.

DISCUSSION

It is apparent that with the use of the procedures of tissue culture, isolation of the etiologic agent of varicella and of herpes zoster from the cutaneous lesions of man may be accomplished without difficulty. To obtain material of optimal infectivity for this purpose it is desirable that the inoculum be prepared from the contents of immature vesicular lesions prior to the appearance of pustulation. A wide variety of cell types of human origin will suffice for the demonstration of the specific and unusual focal cytopathic process induced by the V-Z viruses. However, for this purpose, it is presently essential that the conditions of cultivation permit active and sustained cellular growth and that the spatial distribution thereof is such that the cells are in close physical approximation.

The focal nature of the cytopathic process is of interest. The agent manifests a similar tendency *in vivo*. Tyzzer (6) emphasized the focal distribution of inclusion-containing cells in his description of the histopathology of the cutaneous lesion in varicella, and localized lesions are also characteristic of the disseminated process in fatal cases of varicella (18). In experimental studies, Rivers (1) described a similar circumscribed distribution in the inoculated testis of the monkey.

The evolution of the focal varicella lesion *in vitro* strongly suggests that infectious material passes from cell to contiguous cell. This concept is further supported by the fact that development of a focus continues in the presence of specific antibody (11). The slow increase in number of focal lesions in an infected roller culture may indicate the intermittent presence of minimal amounts of infectious material in the medium, or may represent the transfer of virus via the occasional dislodged infected cell. The observed behavior *in vitro* is difficult to reconcile with the apparent ease of spread of the agent from infected individual to susceptible subject *in vivo*. It is possible that the behavior *in vitro* may reflect a quantitative difference in the amount of virus produced or in the rate of its inactivation. It would seem necessary in this instance to postulate almost instantaneous inactivation of virus on release from the infected cell in order to account for the observed phenomena. A second

explanatory hypothesis might evoke the concept of qualitative differences between particles produced *in vitro* and *in vivo* in terms of infective potential. The ease with which virus may be recovered from frozen varicella vesicle fluid and the difficulties of similarly preserving the agent as propagated *in vitro* may here be relevant. The electron microscopic studies of Tournier, Cathala, and Bernhard (9) have demonstrated the presence of masses of viral particles in the nucleus and cytoplasm of cells and also in extracellular spaces in cultures infected with varicella virus. We have prepared specific complement-fixing antigens from the fluids obtained from infected cultures (11). Viral material does therefore appear extracellularly under *in vitro* conditions, although the biological properties thereof are not, as of now, defined.

Under conditions of *in vitro* cultivation, other viruses show cytopathic patterns somewhat analogous to the V-Z viruses. Herpes simplex virus cytopathogenicity develops initially as a focal process which then rapidly becomes generalized as infectious material appears in the culture fluid (21–23). Working with herpes B virus, Black and Melnick (24) observed, as had we with varicella, that spread of virus from cell to contiguous cell was not blocked by the presence of specific immune serum. Even more analogous is the behavior of the cytomegalic disease–salivary gland virus group of agents that likewise induce formation of intranuclear inclusions. Viruses of this group produce focal lesions on primary isolation and initially are subcultured most easily with the use of inocula containing infected tissue; subsequently, however, adaptation to *in vitro* conditions usually occurs and infectious material appears in the culture fluid (25–29). The tendency for late generalization of the cytopathic process and the character of the focal lesion is of use in differentiation of these viruses from varicella (28). In the course of propagation of another intranuclear inclusion producing agent, the virus of infectious laryngotracheitis of fowls, Atherton and Anderson (30) failed to demonstrate infectious material in the fluid phase of the cultures, although the cytopathic process appeared to be generalized.

In the present study, primary explant cultures were employed by preference for the serial propagation of the V-Z viruses. The extensive experience thus accumulated with cultures of SM and FS tissue has been summarized to emphasize the infrequency with which extraneous agents were encountered. Only in the instance of the appearance of the "refractory cell" strain did the question of an extraneous origin or of alteration in character of the cellular substrate arise. All of the human and monkey tissues tested in culture were susceptible to infection with the V-Z viruses, although there was some evidence that those of monkey origin were comparatively less sensitive. The morphological changes observed following inoculation of cultures of rabbit, bovine, and chick origin with the V-Z viruses can not be interpreted with equal confidence. The findings suggested a degree of susceptibility but the area requires further study.

The question of the relationship of varicella and herpes zoster will be considered in a subsequent paper dealing with immunologic studies on the viruses. Here, it is pertinent to reiterate that, on morphological grounds, no points of differentiation were noted between the cytopathogenic behavior of strains of virus obtained from typical cases of varicella in children and that of strains from typical cases of herpes zoster in adults.

SUMMARY

Fourteen strains of virus derived from the cutaneous lesions of cases of varicella and eight from patients with herpes zoster were propagated serially in primary explant cultures of human preputial or embryonic skin-muscle tissue. Infectious material could not be demonstrated in the fluid phase of infected cultures and inocula for passage therefore consisted of suspensions of infected tissue. Such tissue suspensions when stored in the frozen state did not regularly retain infectivity.

The cytopathic process was focal and appeared to develop as the result of transfer of infectious material from cell to contiguous cell. Optimum development of the focal lesions *in vitro* related directly to conditions favoring optimum tissue growth and was further influenced by the spatial relationship of the tissue outgrowth.

A variety of types of cells of human origin and several of monkey origin were susceptible to infection and responded with the formation of intranuclear inclusion bodies. The cellular response otherwise was variable, ranging from simple rounding with little change in size to the formation of large multinucleated cytoplasmic syncytia.

Strains of virus recovered from patients with varicella and from patients with herpes zoster could not be distinguished on the basis of their cultural attributes.

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EXPLANATION OF PLATES

PLATE 55

FIGS. 1 to 6. Focal changes produced by V virus in roller cultures of FS tissues, showing extension of cytopathic process occurring between 7th and 10th day after inoculation. All 4th tissue culture passage. × 89.

FIGS. 1 and 2. 7 and 10 days, *McEl.* strain.

FIGS. 3 and 4. 7 and 10 days, *McEl.* strain.

FIGS. 5 and 6. 7 and 10 days, *Wel.* strain.

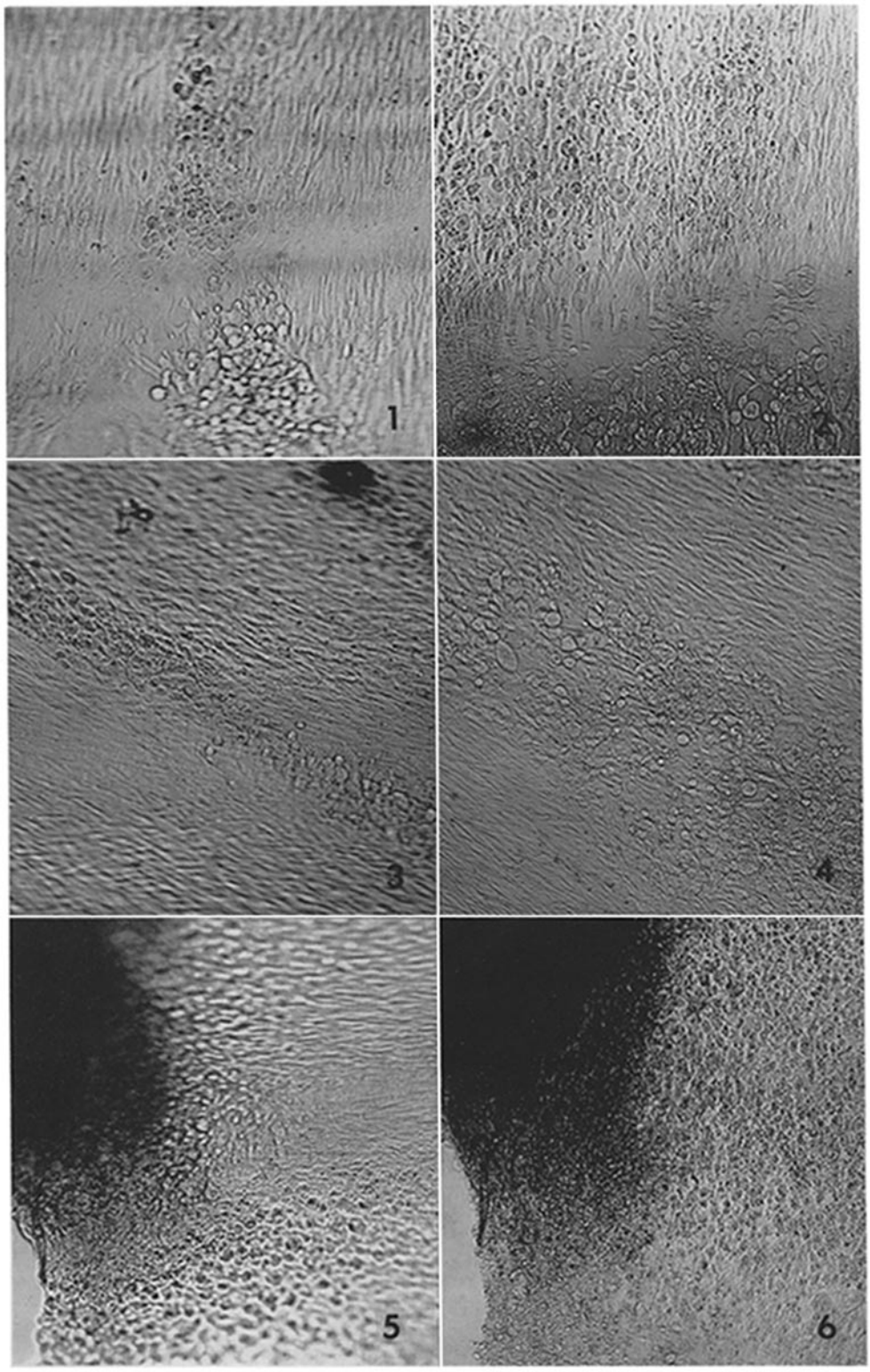

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PLATE 56

FIG. 7. Roller tube tissue culture showing macroscopic "petal" degeneration; transilluminated against black background. Z strain (*Blu.*, 20th passage, 21st day of cultivation) in human embryonic SM tissue. × 2.3.

FIG. 8. Small focal lesion, human embryonic SM tissue, on 6th day after inoculation with *Wel.* vesicle fluid. Hematoxylin and eosin. × 71.

FIG. 9. Edge of focus in dense tissue showing wide zone of involvement with many cells at same phase of inclusion formation. *Sto.* agent, 2nd passage, 12th day, human SM tissue. Hematoxylin and eosin. × 583.

FIG. 10. Edge of focus in loose tissue network; narrow zone of activity. *McEl.* agent, original passage, 11th day, human embryonic SM tissue. Hematoxylin and eosin. × 388.

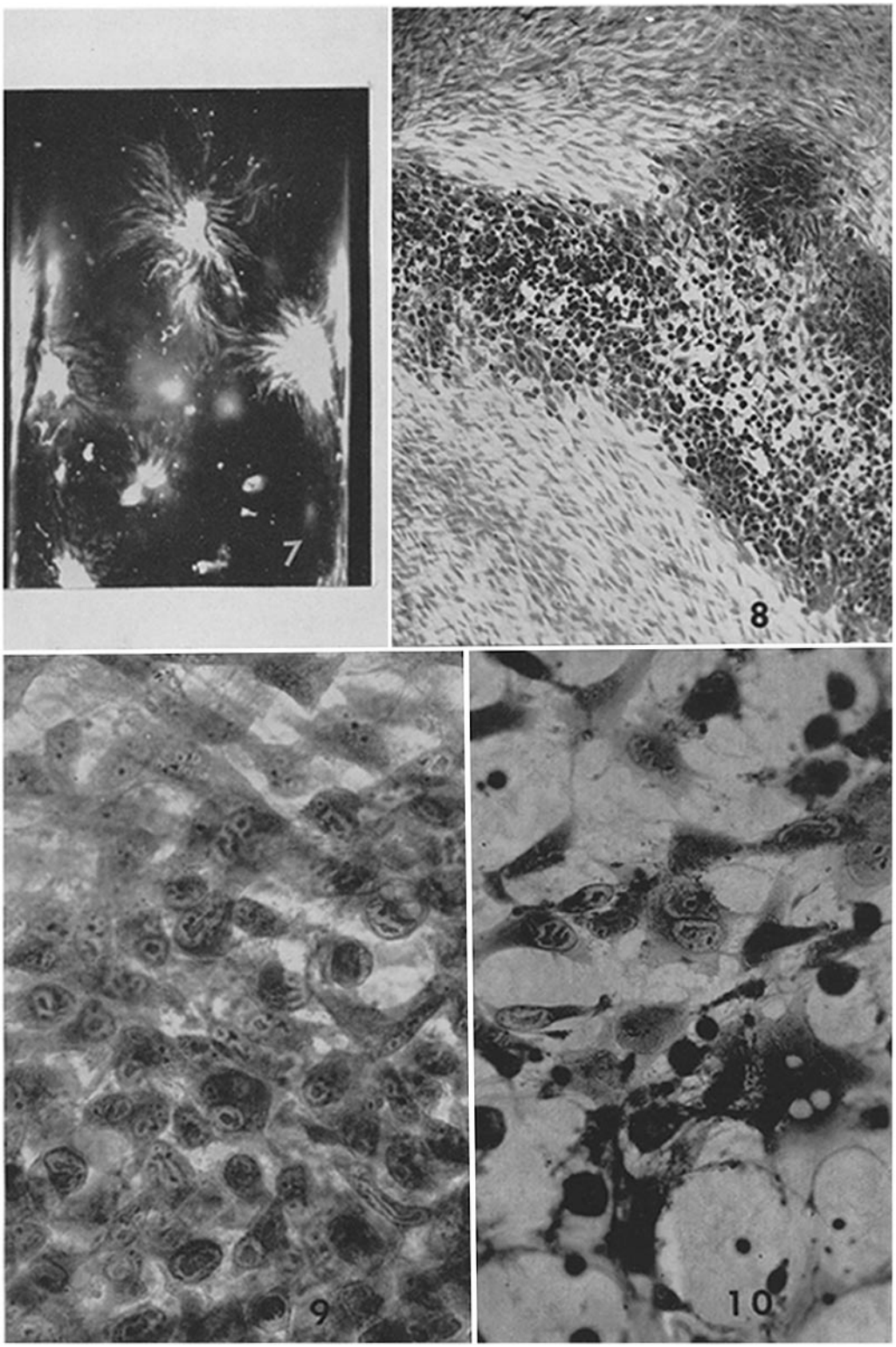

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PLATE 57

FIG. 11. Developing inclusions with apparent entrapment of nuclear elements. *Sto.* agent, 5th passage, 8th day, human embryonic SM tissue. Iron hematoxylin and phloxine. × 493.

FIG. 12. Multinucleate giant cell with intranuclear inclusions. *Wel.* strain, 34th passage, 11th day, FS tissue. Hematoxylin and eosin. × 493.

FIG. 13. Syncytial cytoplasmic mass apparently of epithelial cell origin in culture of FS tissue infected with *Blu.* virus, 26th passage, 6th day. Hematoxylin and eosin. × 146.

FIG. 14. Edge of syncytial mass, shown in Fig. 13, packed with nuclei containing developing inclusions. × 540.

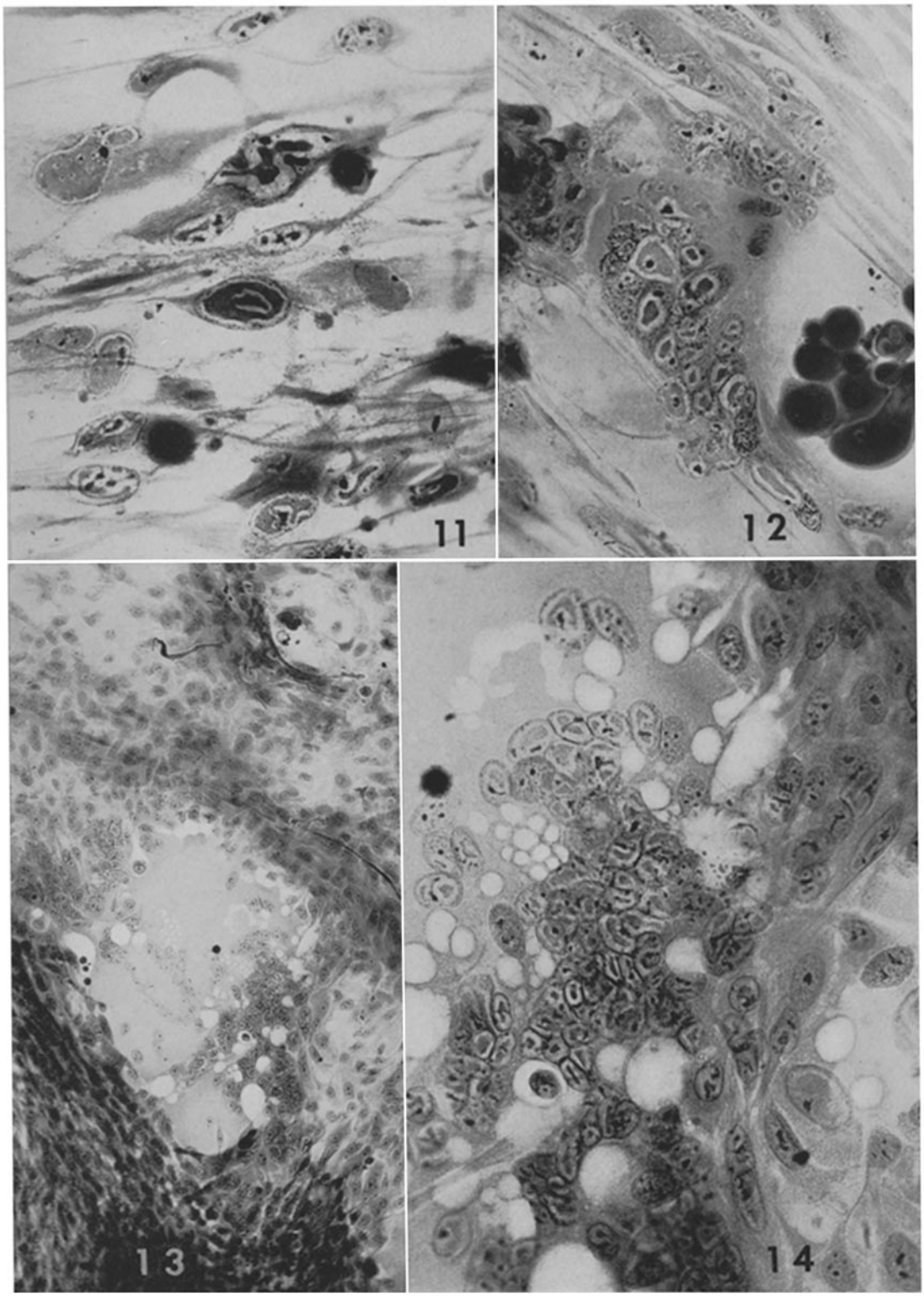

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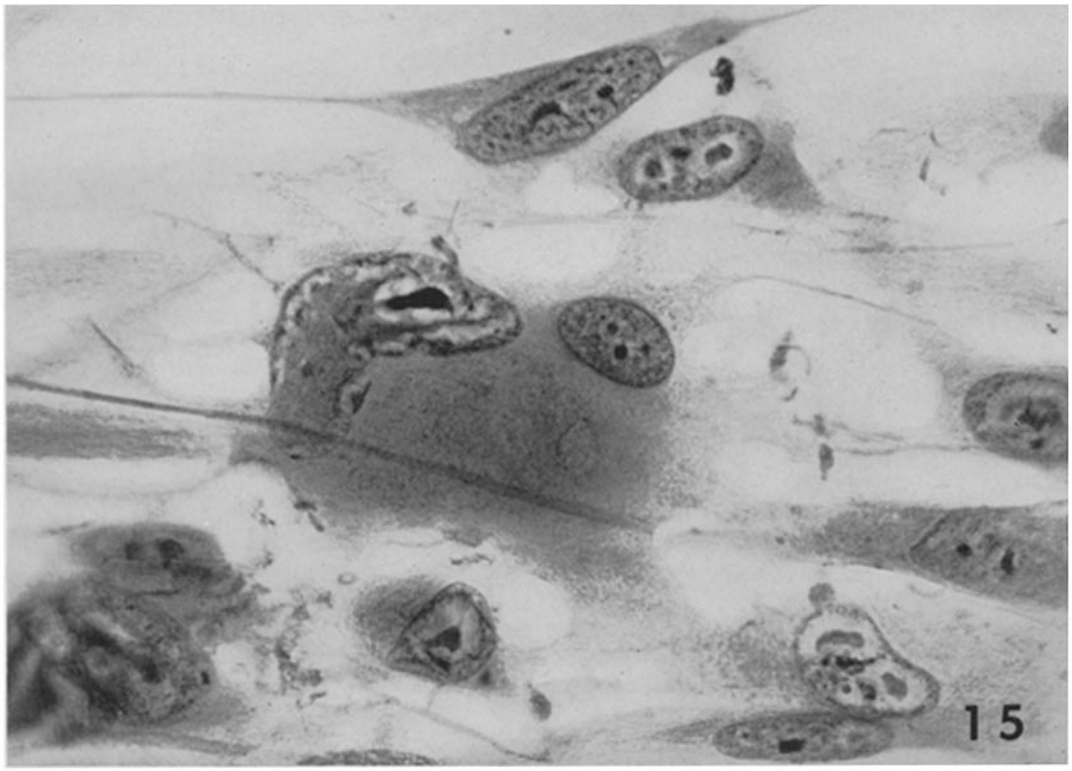
PLATE 58

FIGS. 15 and 16. Human embryonic SM cells infected with *Blu.* virus, 3rd passage, 6th day. Differentiation of the cytoplasm in the paranuclear area of several of the cells is apparent with the presence of poorly defined cytoplasmic bodies. Hematoxylin and eosin. × 1077.



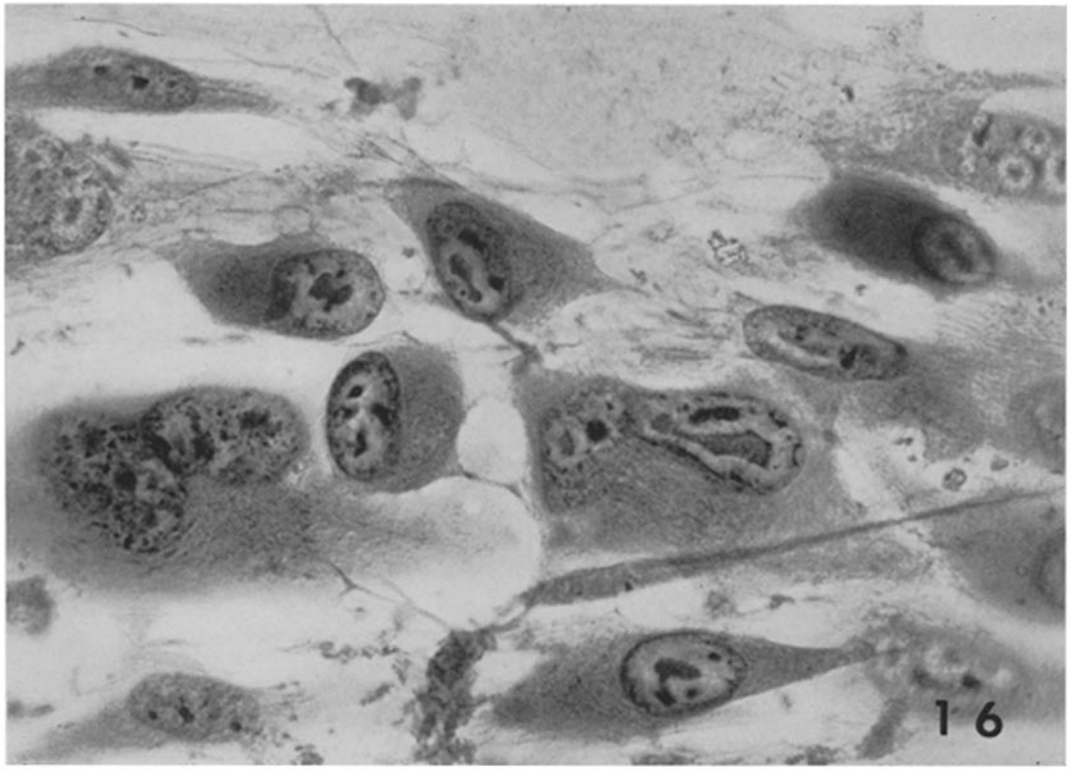


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PLATE 59

FIG. 17. Primary explant culture of human uterine tissue showing the rounding and enlargement of the spindle-shaped myometrial cells on the 10th day following inoculation with *Dru.* vesicle fluid material. × 93.

FIG. 18. Cytopathic changes produced by 17th passage *Pat.* virus in primary explant cultures of human uterus; 17th day following inoculation. Hematoxylin and eosin. × 179.

FIG. 19. Irregular focus in culture of human testis on 10th day following inoculation with *Dru.* vesicle fluid material. × 93.

FIG. 20. Cytopathic changes produced by 7th passage *Sto.* virus in culture of human testis on 17th day following inoculation. Hematoxylin and eosin. × 440.

FIG. 21. Edge of focal process showing zone of large amoeboid cell reaction in culture of human embryonic brain tissue 12 days after inoculation with 11th passage *McEl.* virus. × 96.

FIG. 22. Preparation from culture depicted in Fig. 21. Hematoxylin and eosin. × 447.

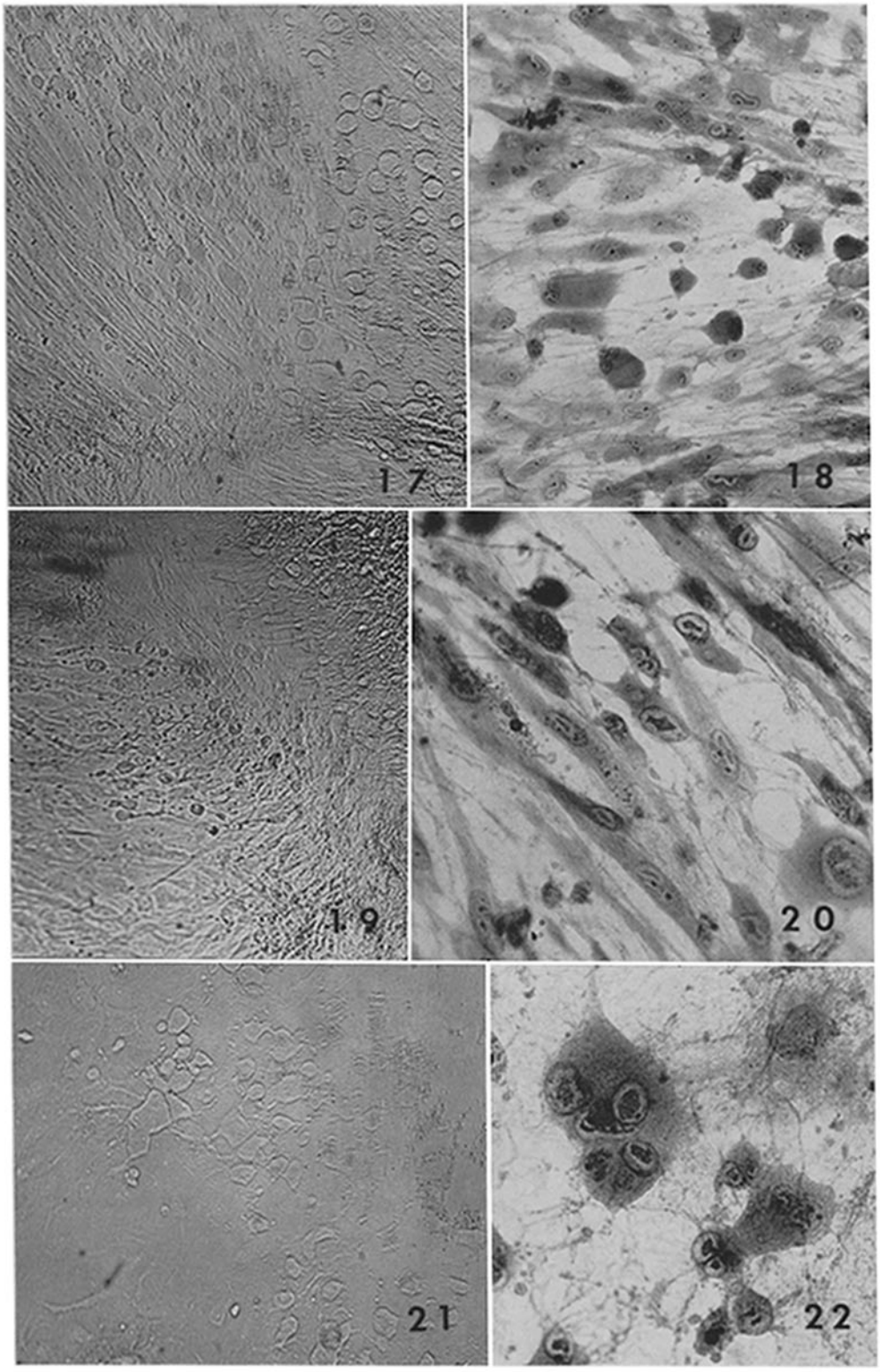

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PLATE 60

FIG. 23. Small focal lesion in culture of human amnion on 13th day after inoculation with 33rd passage *Wel.* strain virus. Hematoxylin and eosin. × 179.

FIG. 24. Detail of cytopathic changes in human amnion cells 6 days after inoculation with *Szu.* strain, 2nd passage material. Hematoxylin and eosin. × 358.

FIG. 25. Early focal lesion in HeLa cell culture on 4th day after inoculation with 7th HeLa cell passage of Collins strain varicella virus. × 440.

FIG. 26. HeLa cell culture infected with 7th HeLa cell passage Collins strain showing degenerative changes toward center of focal lesion on 6th day after inoculation. × 440.

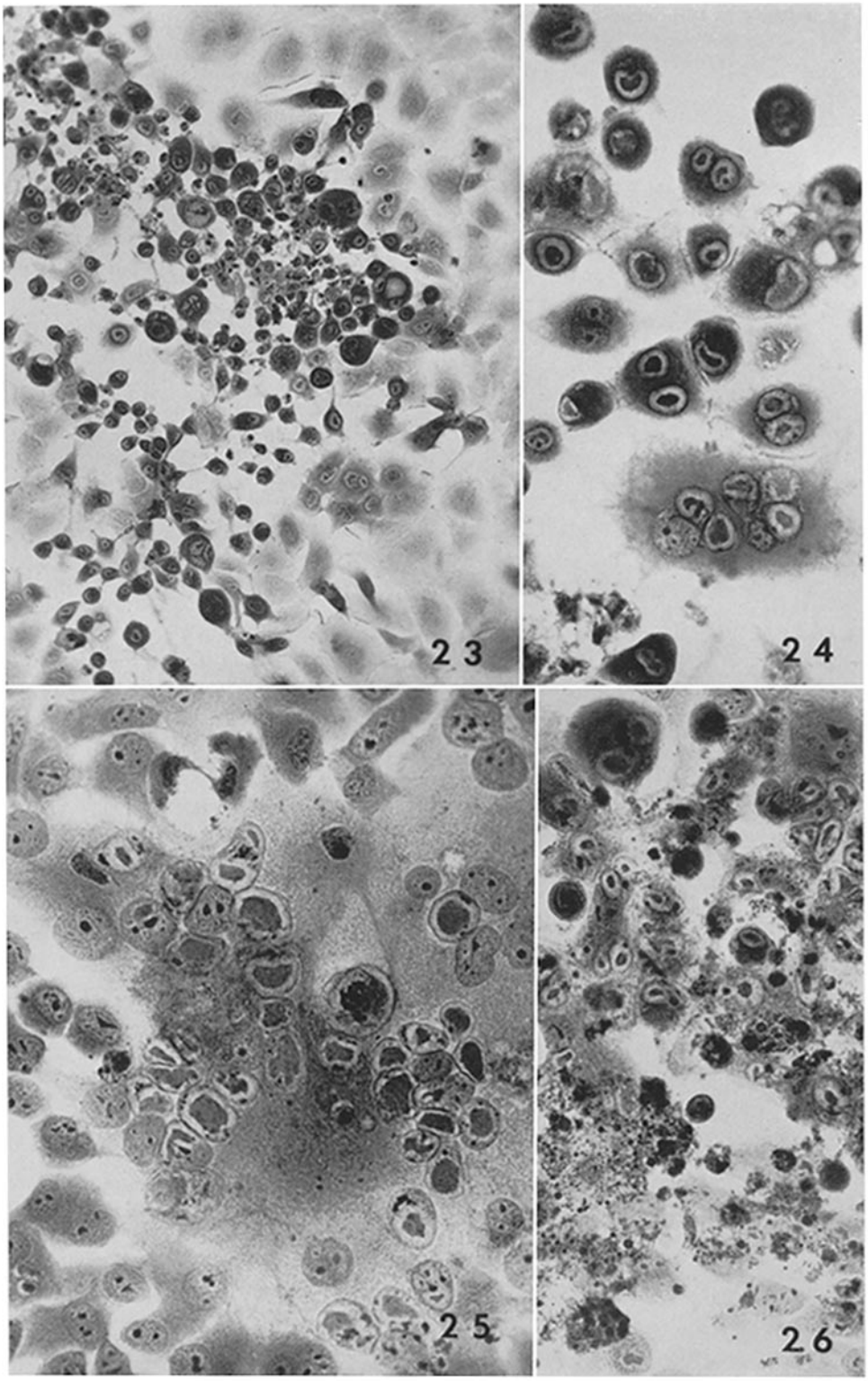

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PLATE 61

FIG. 27. Edge of progressive focal area of degeneration in culture of monkey testicular tissue on 41st day after inoculation with first monkey tissue culture passage *Orc.* strain material. × 137.

FIG. 28. Edge of focal cytopathic process in culture of rabbit testicular tissue inoculated with first rabbit testis passage *Orc.* virus 13 days earlier. × 137.

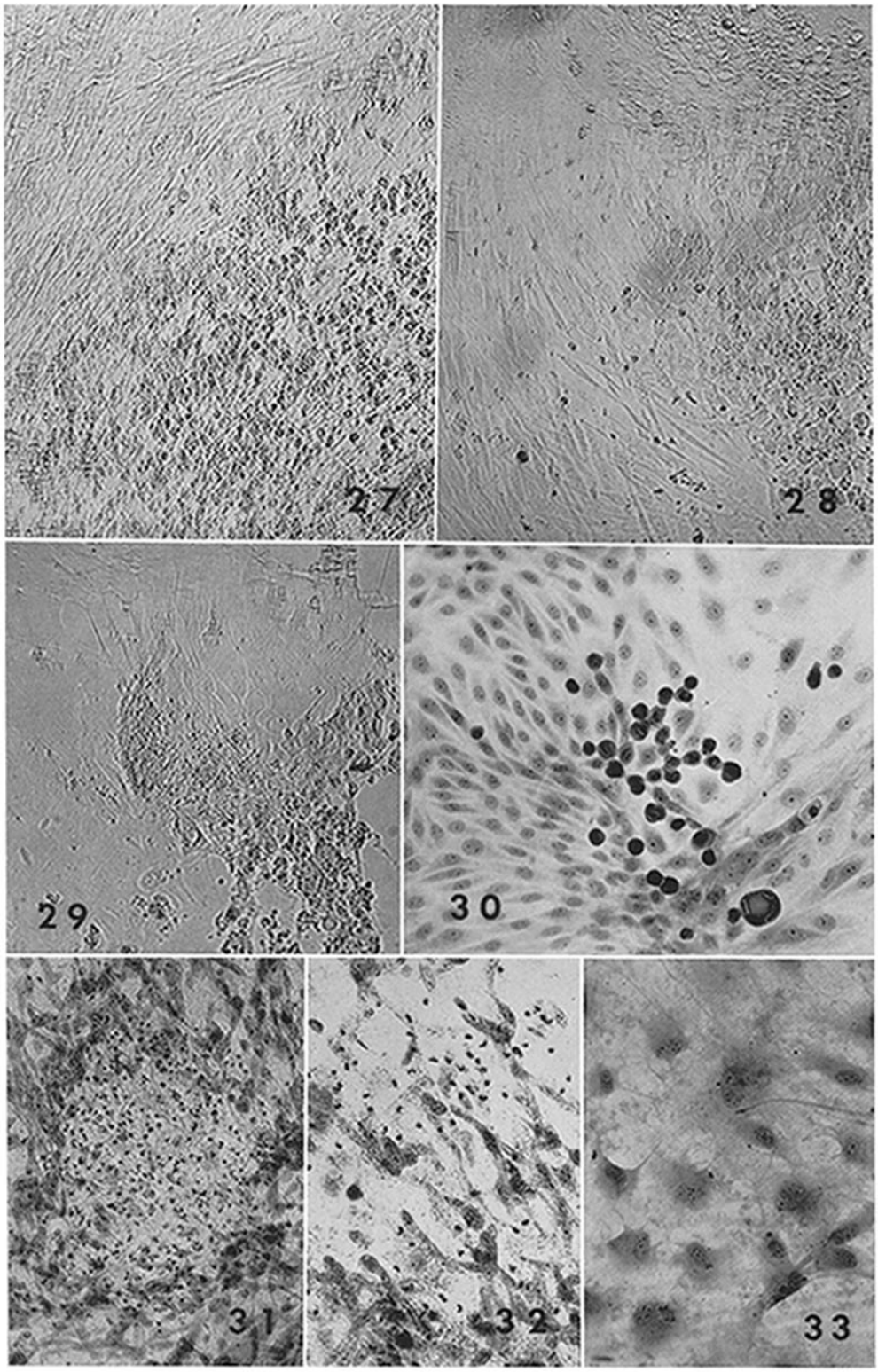
FIG. 29. Margin of area of specific degeneration in monkey kidney culture, 22 days after inoculation with 4th monkey kidney passage *Orc.* virus. × 105.

FIG. 30. Early focus of rounded intranuclear inclusion containing cells in preparation from culture of monkey kidney tissue, 14 days after inoculation with suspension of monkey tissue infected with *Blu.* virus. Hematoxylin and eosin. × 177.

FIG. 31. Focal degeneration in culture of FS tissue on 14th day after inoculation with "FS agent." Hematoxylin and eosin. × 123.

FIG. 32. Higher magnification of preparation pictured in Fig. 31, showing residual pyknotic nuclei and dissolution of cytoplasm of infected cells. × 204.

FIG. 33. Culture of "refractory cells" apparently originating from cultures of SM tissue. Hematoxylin and eosin. × 177.



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