CHANGING VIRAL SUSCEPTIBILITY OF A HUMAN CELL LINE IN CONTINUOUS CULTIVATION*

I. Production of Infective Virus in a Variant of the Chang Conjunctival Cell Following Infection with Swine or N-WS Influenza Viruses

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Plates 21 and 22

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Influenza viruses may be propagated in a variety of primarily explanted tissue (1, 2) and cell cultures (3–6) including cells from human embryos (7), and these viruses may also induce cytopathic effects (CPE) associated with an incomplete multiplication cycle in certain cell lines of human origin (8, 9). However, serial propagation of influenza virus with formation of infective virus has not been reported for any cell type in continuous cultivation. This paper will present evidence that swine influenza virus and the neurotropic variant of the WS strain of influenza A virus (N-WS) will multiply in variant cells of the Chang conjunctival cell line with the induction of CPE and the formation of infective virus.

Materials and Methods

Viruses.—Most of the influenza viruses used in these studies are well known laboratory strains. These include (*a*) influenza A viruses: PR8, N-WS (Francis), Japan 305, Cornell/A1/56 (a 1956 A1 strain isolated in this laboratory), swine influenza virus (S 15 strain), and (*b*) influenza B viruses: Lee, B/G1/54, and 1760 (a monkey kidney culture-adapted strain obtained from Dr. William Mogabgab). All of them were grown in the allantoic sac of the chick embryo and infected allantoic fluid virus seed was stored at −65°C.

Cell Lines.—Lines of Chang's conjunctival and liver cells and HeLa cells were obtained from Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, through the kindness of Dr. Seymour Levine. The conjunctival and liver cell lines were adapted in this laboratory to grow in a calf serum-199 medium. HeLa cells were obtained as the horse serum–adapted cell line and were so carried. Other cell lines used will be mentioned where appropriate.

Propagation and Maintenance of Cell Lines.—All cell lines used in the present study were

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grown in a basic 199 medium enriched with 10 per cent animal serum. With the exception of the HeLa cell, all cells used for virus propagation were maintained in a medium consisting of 2 per cent horse serum inactivated at 56°C. for 30 minutes in 199 adjusted to pH 7.2. For HeLa cells, Ginsberg's maintenance medium (10) was used. With few exceptions, procedures for the propagation and maintenance of other cell lines were the same as those used for the conjunctival cell. Therefore, a detailed description of the propagation of this line suffices.

The Chang line of human conjunctival cell was cultivated and maintained in serum dilution bottles of 250 ml. capacity. Each bottle was usually seeded with 0.5×10^6 cells contained in 10 ml. of growth medium consisting of 10 per cent calf serum in 199, pH 7.1 to 7.2. The medium was changed every 4 or 5 days. Between changes of growth medium, the pH of the culture fluid was adjusted with 5 per cent autoclaved sodium bicarbonate. A solid sheet of cells was usually formed in about a week. In general, cells were divided every 7 to 10 days employing the following trypsinizing procedure: The old medium was discarded and the cell sheet washed twice with approximately 30 ml. of phosphate-buffered saline (PBS). Then 0.5 ml. of 0.25 per cent trypsin solution was added to the bottle. The bottle was rocked back and forth for about 10 seconds by hand to insure proper contact between cells and trypsin. The excess trypsin was discarded and the bottle incubated at 36°C. for 3 to 5 minutes. Trypsinization was judged complete when clumps of cells slid off the glass surface with gentle rocking of the bottle. The action of trypsin was arrested by the addition of 6 to 10 ml. of growth medium. The cell clumps were dispersed by pipetting the cell suspension back and forth 6 to 8 times with a 5 ml. pipette. An aliquot was then taken for direct cell count in a hemocytometer under 70 magnification. The cells were finally diluted in growth medium to contain the desired number of cells.

Stationary Tube Culture.—Screw cap tubes measuring 16 x 125 mm. were used. Each tube was seeded with 10,000 cells contained in 0.5 ml. of growth medium placed in stationary racks, shaken to insure even distribution of cells and incubated at 36°C. At the end of 48 hours, cells were fed 0.5 ml. of growth medium. On the 4th or 5th day, the old medium was replaced with 1 ml. of fresh medium. The cells were usually ready for use on the 6th or 7th day. Before virus propagation the tubes were washed twice with 2 ml. of PBS before the addition of 1 ml. of maintenance medium. Virus titers were determined by the inoculation of 0.1 ml. of serial 10-fold dilutions of the virus in PBS into tubes employing 3 to 4 tubes per dilution. Control tubes received comparable volumes of PBS. After 2 hours of incubation at 36°C., the unabsorbed virus was discarded and fresh medium added. This procedure was necessary to remove residual virus particles which may give rise to false positives in performance of the hemagglutination (HA) and the microscopic hemadsorption (MHA) tests. CPE (cytopathic effect) was scored as 1+ to 4+ according to the degree of cellular destruction. Thus 1+ represents 25 per cent cellular involvement and 4+ complete destruction. A 1+ reading was considered positive in calculation of the TCD_{50}.

When 10- to 11-day-old embryonated hens' eggs were used for ID_{50} determinations, comparable numbers of eggs and volumes of inocula were used as for tube cultures. The virus was inoculated in the allantoic sac and eggs were incubated at 36°C. for 40 to 48 hours. Allantoic fluid was removed for virus assay after overnight refrigeration of eggs.

Hemagglutination (HA) titrations were carried out by adding equal volumes of 0.5 per cent human "O" red blood cells to serial 2-fold dilutions of virus. Readings were made at the end of 60 minutes at 22°–26°C. by the pattern technique. Culture fluids infected with the same dilution were usually pooled for HA determinations.

The term *microscopic hemagglutination* (MHA) is employed here as an inclusive term to describe both the attachment of human "O" cells to the infected conjunctival cells (hemadsorption) (11) and to the glass surface. In the early stage of viral infection in which minimal CPE was observed, hemadsorption was seen. As infection of the cells progressed, red blood cells were also seen attaching to the glass surface. Finally, with advanced infection as shown

by detachment of the cultured cells from the glass surface, massive attachment of erythrocytes to the glass wall was observed. The test was performed on cell cultures by adding 1 ml. of 0.05 per cent human "O" cells to the tubes which had been previously washed with 2 ml. of PBS. After thorough mixing, the tubes were slanted and held at 22°C. for 10 minutes or longer. Readings were made under 70 magnification.

Specific *viral antiserum* was obtained from rabbits following one intravenous and two intraperitoneal injections spaced 1 week apart. A total of 15 ml. of allantoic fluid virus was used per animal. Animals were bled 8 days after the last injection.

Preparation of Reagents and Media.—Adjustment of the proper pH of tube cultures between changes of media was made with 0.5 per cent sodium bicarbonate solution. Phosphate-buffered saline (PBS), pH 7.3, was made of CP chemicals as follows: NaCl, 8 gm., KCl, 0.2 gm., Na_2HPO_4 (anhydrous), 2.10 gm., KH_2PO_4, 0.2 gm., and distilled water, 1,000 ml. After autoclaving, 5 ml. of a sterile 20 per cent dextrose solution was added. The trypsin (Difco 1:250) solution was made up in double strength by adding 0.5 gm. to 100 ml. of PBS. To dissolve the enzyme, the mixture was left at 4°C. overnight. Bacterial sterility was achieved by Seitz filtration. An equivalent volume of PBS was added to the trypsin before storing at −20°C. Sterility tests for possible bacterial contamination of growth and maintenance media, virus inocula, and other solutions were made on blood agar plates and thioglycollate broth. All media for tissue culture contained 100 units of penicillin and 100 μg of streptomycin per ml. Calf serum and medium 199 were purchased from Microbiological Associates, Bethesda. The horse serum was generously supplied by the Bureau of Laboratories of the New York City Department of Health.

RESULTS

The Comparative Susceptibility of Conjunctival and HeLa Cells to the Non-Transmissible Cytopathic Effect of Influenza Virus.—During the course of *in vitro* studies of viral interference, it appeared that the Chang conjunctival cell (as cultivated in this laboratory) was more susceptible to the non-transmissible CPE described by Henle *et al.* (8) than our HeLa cell line. This impression was verified by a systematic comparison of the susceptibility of the two cell lines to the effects of 8 influenza virus strains (Table I). It was found that 6 of the 8 viruses in the concentrations indicated induced marked CPE within 48 hours in the conjunctival cell, while only 3 of the 8 strains did so in HeLa cell cultures. Furthermore, passage of culture fluids in 10^0 or 10^{-1} dilution from cells showing CPE resulted in the induction of CPE in fresh cultures of conjunctival cells in the case of swine influenza and N-WS viruses, but not in HeLa cells with these or other viral strains. The formation of hemagglutinating virus and the demonstration of positive hemadsorption in this second passage suggested that transmissible infectious virus was being formed and that adaptation of these viruses to the conjunctival cell was occurring. That this was indeed the case is illustrated in Text-fig. 1, in which the steps required for the adaptation of swine influenza virus are outlined. That only a minority of viral particles were initially capable of inducing formation of infective virus in conjunctival cell culture is suggested by the critical adjustments of dose which were necessary for serial propagation of virus. Thus, the higher multiplicity provided by the 10^0 inoculum precluded (perhaps through autointerference) CPE on 2nd pass-

age. However, on 2nd passage from the 10^{-1} inoculum only the 10^{0} rather than the 10^{-1} dilution provided sufficient virus to induce CPE. A repetition of this "adaptation" procedure resulted in almost identical results. In each case, on and after the 4th passage, virus could be passed with induction of CPE, HA, and MHA at dilutions of 10^{-4} to 10^{-6}. After 11 conjunctival cell passages of the swine virus and 8 passages of the N-WS strain, cumulative dilutions of the original inocula of 10^{-23} and 10^{-18}, respectively, have been attained. Thus, evidence for multiplication of these viruses in the conjunctival cell is unequivocal.

The Properties of Swine Influenza Virus Adapted to Growth in Conjunctival Cells (Swine-cc Influenza Virus).—The necessity for several passages for the

TABLE I

Comparative Susceptibility to Non-Transmissible Viral Effect of Conjunctival Cell-D and HeLa Cell*

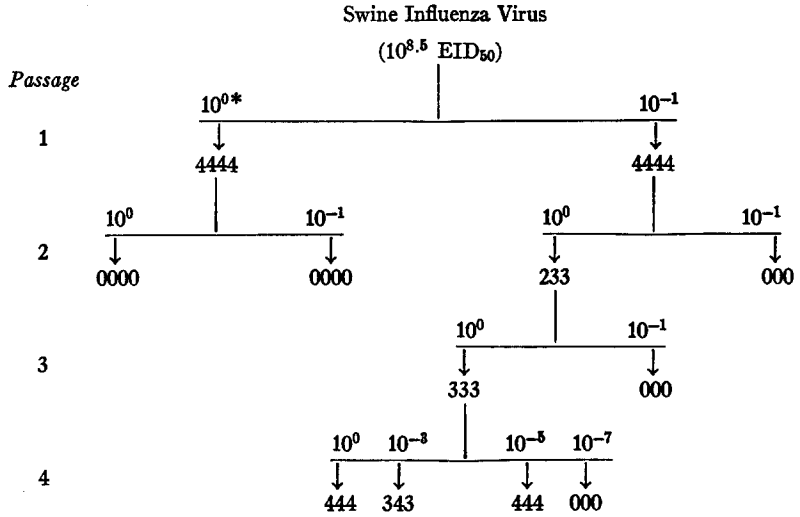
Influenza virus	Strain	Inoculum egg inf. doses	Cytopathic effect involving more than 50% of cells (2+)	
			Conj. cell-D	HeLa cell
		log		
Swine	S-15	7.5	+	+
A	N-WS	8.3	+	+
A	PR8	8.5	0	0
A1	Cornell/A1/56	7.5	+	0
A2	Jap. 305	8.3	0	0
B	Lee	7.7	+	0
B	B/G1/54	8.3	+	+
B	1760‡	—	+	0

* Derived in this laboratory from Chang (original) conjunctival cell line.

‡ Monkey kidney cell—adapted (Mogabgab).

evolution of "adapted" swine influenza virus suggested that either selection of a pre-existing minority constituent or concomitant mutation and selection of virus had occurred and that the adapted virus (swine-cc) might differ demonstrably in other properties from the original strain. Virus of the 8th passage in conjunctival cell culture was inoculated into 10-day-old chick embryos as 10^{3} TCD_{50}. Allantoic fluid virus was harvested in the usual manner after 48 hours of incubation. Virus so produced was compared with the original strain with respect to mouse pathogenicity, hemagglutinating characteristics and viral morphology. Groups of 4 CFW male mice were inoculated intranasally under ether anesthesia with 10^{-1} to 10^{-4} dilutions of either adapted or unadapted swine influenza virus comprising equivalent EID_{50}. 7 days after inoculation, mice were killed and their lungs examined for lesions. On the basis that these lesion scores did not differ significantly, it was concluded that the mouse lung virulence of

the original and derived strains was the same. On the other hand, comparative studies of hemagglutination of human, sheep, and chicken RBC demonstrated the acquisition by the derived virus (swine-cc) of the capacity to agglutinate sheep RBC to a titer of 1:256 at 30°C., whereas the same human RBC agglutinating dose of original virus did not agglutinate sheep RBC at 1:4 dilution. Moreover, the adapted swine-cc virus was 8-fold *less* effective in agglutinating chicken RBC then the original strain. Although the capacity of certain influenza viruses to agglutinate sheep RBC is of equivocal validity as a genetic



* Dilution of virus suspension.

TEXT-FIG. 1. The adaptation of swine influenza virus to conjunctival cell culture. (Numbers below passage arrows designate degree of CPE in individual tubes.)

marker (12), these results indicate a change in the activity of the adapted virus which has thus far proved stable. Electron microscopy of original and derived virus by methods previously described (12) did not disclose any change from a predominantly spherical morphology.

Adaptation of N-WS virus to conjunctival cell culture proved unnecessary. Repeated experiments with this virus demonstrated comparable TCD_{50} in conjunctival cells of parent and 8th TC passage virus (titers of $10^{6.7}$ and $10^{6.3}$ respectively). It is of interest that the non-neurotropic parent WS strain did not possess this intrinsic virulence for conjunctival cells, nor could it be adapted to serial propagation.

Changes in N-WS passed in conjunctival cells were not demonstrated.

The Comparative Susceptibility to Swine-cc Virus of Allantoic Sac and Con-

junctival Cells.—Comparative infectivity end points were determined for the tissue culture-adapted swine-cc virus by simultaneous titration of serial 10-fold dilutions of virus in chick embryos and freshly seeded conjunctival cell cultures. 10-day-old chick embryos (4/dilution) were inoculated with viral dilutions and infectivity end points determined in the usual manner after 40 hours of incubation. Conjunctival cell cultures (4 tubes/dilution) were inoculated and observed for CPE and positive hemagglutination over an 8 day period. The EID_{50} titer of swine-cc virus of allantoic fluid origin was $10^{-8.5}$ and the TCD_{50}, $10^{-6.2}$. A viral preparation of conjunctival cell culture origin titered $10^{-4.5}$ in conjunctival cell culture and $10^{-6.5}$ in the allantoic sac. Thus, regardless of the source of virus, about 100 times more virus was required to initiate infection of conjunctival cell cultures than cells of the allantoic sac. This was also true of N-WS virus.

Comparative Sensitivity of CPE, MHA, and HA in Estimating Cellular Infection.—Although CPE and MHA were usually in close agreement in conjunctival cells infected with either swine-cc or N-WS viruses, hemagglutinin (HA) in tissue culture fluids was not always detectable. This was particularly true when CPE and MHA were minimal. Of the 3 properties, the most sensitive and reproducible was the MHA test.

Susceptibility of Other Cell Lines to Swine-cc and N-WS Viruses.—The effect of the 2 strains "pathogenic" for the conjunctival cell on other human cell lines was studied.

The following cells as monolayer tube cultures were purchased from Microbiological Associates, Bethesda: HeLa (horse serum-adapted), human skin and muscle, human heart, human intestine, and KB cells. In addition, other specimens of Chang's conjunctival cell line were obtained from two other laboratories. We are indebted to Dr. Robert Chang and Dr. Seymour Levine for their cooperation in this regard. Both of these conjunctival cell lines had been grown in a 10 per cent human serum–199 medium. Chang's human liver cell and the Lederle HeLa cell line have been maintained in this laboratory for 2 years. All cell lines were not tested simultaneously, but the susceptible conjunctival D cells were always included in each test. In addition, the same seed viruses (stored at −65°C.) were used in all tests.

Cells were infected with serial 10-fold dilutions of swine-cc or N-WS virus. Cultures were observed for 10 days for CPE and for the formation of HA or the development of a positive hemadsorption (MHA) reaction. As shown in Table II, the conjunctival cell-adapted swine-cc and N-WS viruses induced CPE, Ha, or MHA in other cell lines only if inoculated in concentrations 4.5 to 6.5 logs greater than were required to induce such effects in the conjunctival cell D line. The resistance of the original (conjunctival O) line to all but large amounts of both viruses is notable. To ascertain whether production of infective virus had occurred in these or in other cell lines. serial decimal dilutions of

pooled culture fluids were subinoculated into 10-day-old chick embryos. By this method it was demonstrated that only in conjunctival cell D cultures had increases in infective virus occurred. It was concluded that the adaptation of swine influenza virus to the conjunctival cell D line thus did not represent a

TABLE II

Comparative Susceptibility of Human Cell Lines to Infection with Swine-cc and N-WS Influenza Viruses

	Minimal viral dose (log EID_{50}) inducing CPE, HA, or MHA		Increase in infective virus (egg sub-inoc.)	
	Swine-cc	N-WS	Swine-cc	N-WS
Conj. D	2.0	2.8	+	+
Conj. O (Lederle)	8.5	6.5	0	0
Conj. O (Chang)	7.5		0	0
HeLa (Lederle)	8.5	7.5	0	0
HeLa (Gey)	8.5	ND*	0	0
Liver (Chang)	8.5	ND	0	0
KB (Eagle)	6.5	ND	0	0
MAF (Gray)	8.5	ND	0	0
Heart (Girardi)	8.5	ND	0	0
Intestine (Henle)	8.5	ND	0	0
Amnion	ND	6.5	0	0
Monkey kidney‡	4.0	3.3	+	+

* ND, not tested.

‡ Primary cultures of monkey kidney cells.

general enhancement of its virulence, but was a specific adaptation of virus to this host cell. Similarly, the primary ability of N-WS to multiply in the D cell was sharply specific.

Confirmation of the Identity of Swine-cc Virus.—Following the 8th passage of swine influenza virus in conjunctival cell culture, CPE, HA formation, and MHA were inhibited with rabbit antiserum specific for swine influenza virus, but not by normal rabbit serum. Hemagglutination by the swine-cc virus was also inhibited with specific antiserum in HI tests. Thus, the serially transmissi-

ble virus was definitively identified as swine influenza virus and did not represent a contaminating virus from the environment or one endogenous in culture.

Susceptibility of the Variant Conjunctival Cell Line (D) to Other Viruses.—Apart from its susceptibility to influenza virus infection, the derived (D) line of conjunctival cells did not differ from the original (O) lines with respect to susceptibility to infection with polioviruses types 1, 2, and 3; adenovirus, type 4; herpes simplex and Coxsackie B3 viruses inoculated as 1000 TCD_{50}.

Evidence of Variation or Mutation of the Conjunctival Cell Line.—Our Chang conjunctival cell line was originally obtained in the summer of 1958 from Dr. Seymour Levine of Lederle Laboratories, who, in turn, had obtained it from Dr. Theodore Puck. It had been cloned at the latter's laboratory so that the cell population would be relatively homogeneous. The cell had been grown in a 20 per cent human serum, 10 per cent horse serum-Weymouth medium. Upon arrival in our laboratory a second line was initiated employing a 10 per cent calf serum–199 medium. The cells grew readily in the new medium. After 5 passages, the susceptibility of the two cell lines was tested against 6 viruses known to infect conjunctival cells. When little or no difference was demonstrated in the viral susceptibility of the two lines, maintenance of the parent cell line was discontinued. Cells were subdivided approximately once a week. As transfers of cells continued, it was noticed that the original tendency of the fibroblast-like cells to "round up" in maintenance medium was no longer evident so that the characteristic morphology had become predominantly cuboidal. By the 15th to the 20th transfer, the cells appeared distinctly different from the parent culture in morphology. Fig. 1 illustrates the pleomorphism of the parent conjunctival cell and in Fig. 2 the relatively homogeneous, tightly packed cells of the mutant line are shown in a characteristic contiguous monolayer.

When our cell line was found to support the growth of swine and N-WS influenza viruses, a new culture of the original conjunctival cell was obtained from Dr. Levine in the summer of 1959. The differing viral susceptibility of the "D" cells maintained in this laboratory and that of the original (O) cells maintained elsewhere has been shown in Table II. It has also been determined that the original conjunctival cells are resistant to the non-transmissible effect induced by swine-cc, N-WS, and other strains of influenza viruses, an observation consistent with the "partial cytopathogenic effect" noted by Deinhardt and Henle with PR8 in their strain of Chang conjunctival cell (9).

Evidence That the Changed Viral Susceptibility of the Conjunctival Cell D Line Is Stable and Not Environmentally Conditioned.—The possibility existed that the changed morphology and viral susceptibility of the conjunctival cell maintained in calf serum–199 in this laboratory was nutritionally conditioned. That this was not true was demonstrated in experiments in which the O and D cell lines were cultivated for a total of 4 transfers, each in the medium usually used

for the other. Under these reversed nutritional conditions, the relative susceptibility of the two lines to influenza viral multiplication remained unaltered.

Evidence that the Virus-Susceptible D Cell Line Is of Human Origin and Not a Contaminating Cell Type.—It was recognized that the apparently changed viral susceptibility of the so called conjunctival D line might be explained by postulating exogenous contamination of the original line with a different cell. This postulation seemed most unlikely in view of the following facts: (*a*) the change in viral susceptibility was minor in that it was limited to only 2 virus strains; otherwise, the spectrum of viral susceptibility remained unchanged, (*b*) the continuing susceptibility of the D line cells to polio- and adeno-virus infections is strong evidence of their primate, if not human, origin (13), (*c*) most tellingly, because no existing cell line of any type is known to support full cycle influenza virus multiplication, one must ask, "What is the hypothetical contaminating

TABLE III

Production of Agglutinins for Human RBC in Rabbits Injected with O or D Conjunctival Cells

Rabbit injected with conjunctival cell	Serum hemagglutination titer for human O RBC	
	Initial	After injection
O	64*	8192
D	64	2048

* Reciprocal of serum dilution at titration end point.

cell?" Additional evidence against the possible non-human origin of the D cell has been provided by analysis of its chromosome number and morphology by Dr. Mathilde Krim of this laboratory.

Cytologic Analysis of the Chromosome Complement.—Although both original and derived conjunctival cell lines were found to be composed of cells showing a range of chromosome numbers, thirty counts of preparations of cells from each line have disclosed a shift from a modal number of 62 in the original cell line to 73 in the derived cell line. Such shifts in chromosome numbers of stem-line cells have been observed in other established cell lines (14, 15). Whether the higher chromosome number in the conjunctival D cell is necessarily related to its new property of supporting the growth of infective influenza virus is now being tested through cloning of the cells and submitting clones–with nearly diploid and a range of hyperdiploid numbers of chromosomes–to virus infection (16).

The chromosome morphology of a number of animal species from which most cells established in tissue culture have been derived is now well known. Definite features characterize the normal chromosome complement of each of the fol-

lowing species: chicken (17), mouse (18–20), rat (21), and monkey (22–23). These features include location of the centromeres and relative size and frequency of chromosomes of specific morphology.

Study of chromosome morphology of the O and D conjunctival cell lines has produced evidence against the contamination of the former by a cell of animal origin, since neither line exhibits any of the characteristics of the chromosome complements of the above mentioned species. Moreover, both O and D cells exhibit in the morphology of their individual chromosomes and in the size and frequency of the different types of chromosomes, characteristics which are displayed by normal human diploid cells (24–27). These are the presence of at least one pair of large metacentric chromosomes, at least 2 pairs of short telo-

TABLE IV

Species Specificity of Response to Injection of Conjunctival Cell D

RBC from	Hemagglutination titer*	
	Before	After
Human	64‡	2048
Mouse	64	64
Rat	128	64
Monkey (*rhesus*)	<4	<4
Cow	<4	<4
Guinea pig	<4	<4
Sheep	<4	<4
Chicken	<4	<4

* Before and after injection of rabbit with conjunctival cell D.

‡ Reciprocal of serum dilution at titration end point.

centric chromosomes, at least 2 pairs of short metacentric chromosomes and a number of chromosomes of intermediate sizes, with submedian or subterminal centromeres.

Antigenic Evidence of the Human Origin of O and D Conjunctival Cells.—Direct evidence that the susceptible D cell is of human origin was obtained through the following experiments:

Rabbits were bled from the heart to obtain base line serum specimens. Each animal then received 5 weekly injections of 5×10^6 of O or D conjunctival cells contained in 5.0 ml. of buffered saline (pH 7.3). To obtain these cell suspensions, cells grown in milk dilution bottles were washed 3 times with 30.0 ml. of PBS prior to trypsinization, then 3 more times after enzyme treatment. Animals were bled 1 week after the last injection. Serum aliquots were heated at 56°C. for 30 minutes prior to use. The antisera thus produced were tested for their capacity to agglutinate human type O erythrocytes. Equal volumes of 0.5 per cent washed RBC and serial 2-fold dilutions of serum were mixed and held at 4°C. for 16 hours before reading the results as macroscopic hemagglutination. The results of these hemagglutination tests are presented in Table III.

It will be noted that despite the initial presence in pre-injection rabbit serums of hemagglutinins for human RBC, that significant increases in titer occurred after injection of either O or D conjunctival cells. The specificity of this response to the injection of D cells is attested by the data in Table IV which indicate that the increase in hemagglutination titer is confined to human erythrocytes. It is emphasized that no human serum was used in the cultivation of the conjunctival cells used for immunization.

Further evidence that the agglutinins for human RBC resulted from the injection of conjunctival cells was provided by absorption of such agglutinins from serum by either D or O conjunctival cells. The data in Table V demonstrate the results of absorption of anti-D serum with D cells and with human RBC and those of other species. To remove specific hemagglutinins, serums were

TABLE V

Homologous Specificity of Absorption of Hemagglutinins for RBC of Several Species. Absorption of Agglutinins for Human RBC by Conjunctival Cell D

Initial hemagglutinin titer*	Tested with RBC from	Hemagglutinin titer after absorption with			
		Conj. cell D	RBC		
			human	mouse	rat
2048‡	Human	16	<10	1024	2048
64	Mouse	128	64	<10	32
64	Rat	64	64	64	<10

* Of serum from rabbit injected with conjunctival cell D.

‡ Reciprocal of serum diluted at titration end point.

mixed with either 1.0 ml. of packed RBC or 6×10^6 trypsinized conjunctival cells. Following overnight refrigeration, the serum-cell mixtures were centrifuged at 1500 R.P.M. for 10 minutes and the clear supernates used in the hemagglutination tests. It is evident that only absorption with human RBC or D cells reduced the agglutinin titer for human RBC. Similarly, absorption by mouse and rat RBC did not reduce the serum agglutinins for the human RBC but only the agglutinins naturally present for either mouse or rat RBC. In other experiments the relatedness of the O and D conjunctival lines is suggested by reciprocal cross-absorption of hemagglutinins engendered by each cell. The use of hemagglutination for the determination of the species origin of cultivated cells has been reported by other investigators (28–30).

The foregoing cytologic and antigenic evidence thus strongly suggests the human identity of both the original (O) and derived (D) conjunctival cells.

DISCUSSION

That cells in continuous culture may change in their susceptibility to the effects of viral infection is not a new observation (31–35). However, previous

studies have delineated either: (*a*) the acquisition of resistance by cells surviving exposure to virus (31), or (*b*) a varying degree of resistance to viral cytopathic effect of derived cell clones (32–35), or (*c*) variation in production of virus by clonal variants (35). The evidence in the present paper appears to be unprecedented in demonstrating a qualitative change in cellular viral susceptibility from a complete incapacity to produce infective virus to acquisition of this ability. Of special interest is the sharp restriction of this new capacity to synthesize virus to certain influenza virus strains. It is easy to see how change of this subtlety might be overlooked in studies of cellular biologic differences. Thus, with respect to the criterion of change usually employed by the virologist, namely susceptibility to a wide range of viruses, the derived cell line is unchanged. Furthermore, in the case of swine influenza virus, adaptation or selection of *virus* as well as change in the cell is also necessary for the establishment of a host-parasite relationship productive of infective virus. This specificity of cell-virus relationship is reminiscent of similar specificity manifested with a type 2 poliovirus variant and a HeLa clone with respect not to multiplication but to the inducibility of CPE (34).

Although the derived conjunctival cell differs in morphology and chromosome number from the original cell, its susceptibility to viruses which infect only primate cells (adeno- and polioviruses) and its possession of antigens and chromosome morphology characteristic of human cells offers strong presumptive evidence of its identity as a human cell and its derivation from the original conjunctival cell. This evidence, plus the lack of evidence that a similarly susceptible cell exists elsewhere, makes it only reasonable to assume that the derived cell is an altered variant of the original rather than a contaminating cell exogenously acquired. A summary comparison of the O and D conjunctival cells is presented in Table VI.

The reasons for the altered reaction of the derived cell to N-WS and swine influenza viruses are not apparent. These reasons will be particularly important to find in this situation in which neither viral replication nor CPE is inducible in the parent cell, but *complete* viral synthesis occurs in the variant progeny cells. Comparative biochemical studies of the 2 cell types may prove productive in view of recently published evidence that conjunctival cell variants may be isolated which have differing capacities to utilize carbohydrate (36). The capacity of the derived cell type to produce infective virus (or complete viral RNA) might well be related to a hypothetically greater capacity in ribose synthesis, for example.

Studies of 9 established clones of the derived conjunctival cell will be presented in another paper in which the cell variation responsible for the change in viral susceptibility is discussed further (16).

It is pertinent to consider the nature of the 2 influenza virus strains which are capable of full cycle multiplication in the variant cell. Both the swine and

N-WS viruses possess virulence for a mammalian species as manifested by their capacity to induce pneumonia in the mouse. N-WS is a neurotropic variant of the WS strain which is also virulent for the mouse by the intracerebral route of inoculation. In the mouse brain it has a capacity unique among influenza strains to multiply with the production of infective virus. The conjunctival cell therefore represents a second system in which this virus can uniquely undergo full cycle propagation *de novo*. Other evidence of the increased "destructive effect" of N-WS has been cited (37). Is such a strain less demanding in its need for nutrilites and therefore more "pan-virulent"? Similarly, the swine virus may well represent a variant from a virus of multitropic potential if, as postulated, it emanated from man but passed to swine (38).

TABLE VI

Comparative Properties of Original (O) and Derived (D) Conjunctival Cells

	O cell	D cell
Human antigenicity	+	+
Susceptibility to viruses specific for primate cells	+	+
Morphology	Rounded	Polygonal
Chromosome number	62	73
Chromosome morphology	Human	Human
Susceptibility to CPE and non-infective virus formation with influenza viruses	0	+
Capacity to produce infective virus after swine or N-WS virus infection	0	+

Incidentally, the capacity of this virus to multiply in human cells deserves more than casual notice.

In conclusion, the derivation of the original conjunctival cell may be considered in relation to the manifest susceptibility of its variant to influenza viruses. Was the "normal conjunctival cell" (39) which was originally cultivated susceptible to influenza virus *in vitro* then later overgrown by resistant variants? The *in vivo* susceptibility of conjunctival cells to other respiratory viruses (measles and adenoviruses) is well known, and the frequency of conjunctivitis in influenza (40) may indicate a susceptibility of conjunctival cells to influenza viral infection.

SUMMARY

During its serial transfer and cultivation in this laboratory, a human conjunctival cell line (Chang) was observed to change in morphology. Concurrently no change was noted in the susceptibility of the cells to viruses capable of infecting the original cell line.

However, it was noted that the derived variant cell line had acquired suscepti-

bility to the induction of cytopathic effects and incomplete virus formation by several strains of influenza viruses.

It was then discovered that swine influenza virus and the N-WS strain of influenza A virus could be serially propagated in the derived cell line with production of infective virus. The swine virus required adaptation, but the N-WS strain did not.

N-WS and swine influenza viruses multiply with infective virus formation only in the variant conjunctival cell and in no other cell line.

Antigenic, cytologic, and virologic evidence is presented that the influenza virus-susceptible variant cell is of human origin and is not a contaminating cell exogenously introduced.

Transition of a cell line from complete insusceptibility to susceptibility to virus infection and multiplication has not been described previously.

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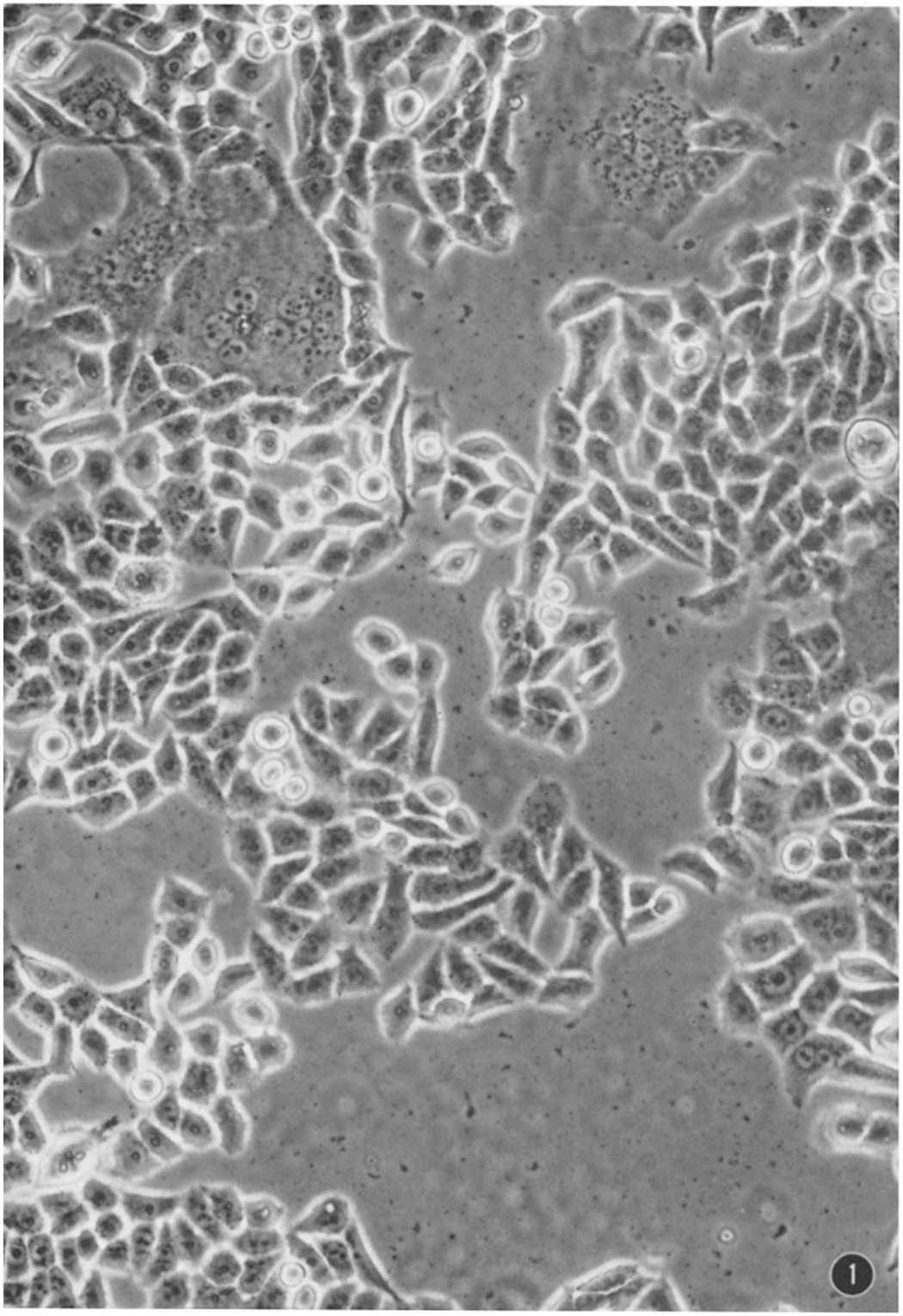
EXPLANATION OF PLATES

PLATE 21

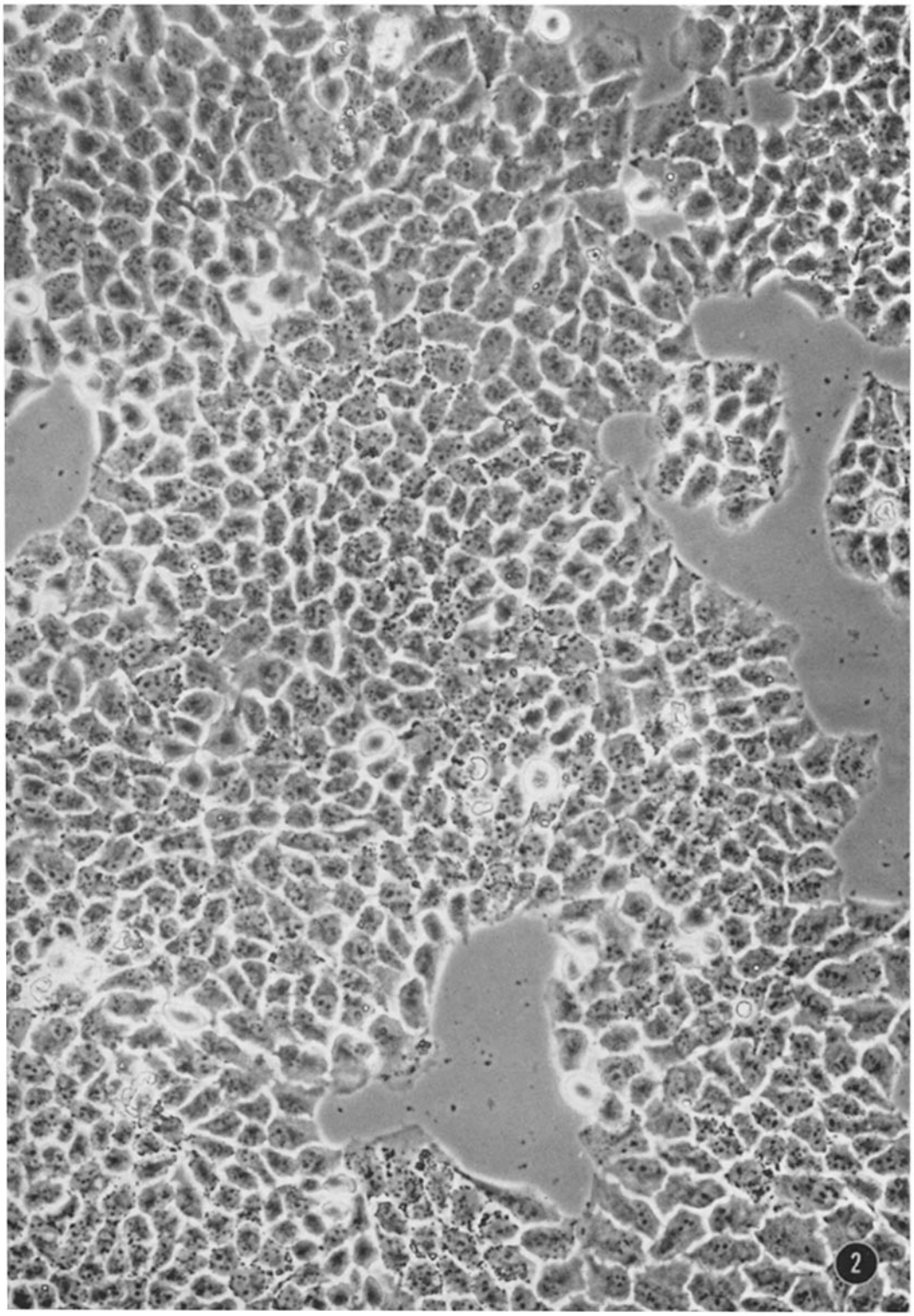
FIG. 1. The morphology by phase contrast of the original (O) conjunctival cell line (young culture in growth medium). Note polygonal shape and presence of giant cells. (microscopic magnification, 100; photographic enlargement, 2.1).



(Wong and Kilbourne: Viral susceptibility of human cell line)

PLATE 22

FIG. 2. The morphology by phase contrast of the derived (D) conjunctival cell line (young culture in growth medium). The cells are homogeneous in size and shape. (microscopic magnification, 100; photographic enlargement, 2.1).



(Wong and Kilbourne: Viral susceptibility of human cell line)