Cytocidal Assay and Propagation of Human Influenza Viruses in Cultured Primary Hamster Kidney Cells.* (28422)

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Human influenza viruses have been grown in cultured cells from various species and tissues(1-11). Total or easily visible cellular degeneration and death have not been regularly demonstrable features of all such infections *in vitro*, except for type B and relatively few other strains of influenza virus. Assay of virus and neutralizing antibody has largely depended upon the use of *in vivo* technics. In the studies reported here, commonly employed strains of influenza virus subtypes A, A_1, and A_2, as well as type B, propagated in and overtly destroyed hamster kidney cells maintained in fluid medium free of serum. Hamster kidney cell culture therefore appeared useful for assay of influenza viruses and their corresponding neutralizing antibody as well as for virus production.

Materials and methods. Viruses. The following influenza viruses were used: WS strain, and its neurotropic variant, NWS(12), FM-1, 3 lines of A_2/Japan/305/57, and Lee. (See Tables I-III for passage histories). Strains FM-1 and Lee had been passed twice allantoically after receipt from the American Type Culture Collection. Allantoic fluid virus preparations were pooled harvests from 12-day-old chicken embryos 48 hours following inoculation with 0.1 ml of a 10^{-3} dilution of allantoic fluid and incubation at 35°C. Virus-infected allantoic and cell culture fluids were stored in sealed ampoules in a dry ice chest for virus titration and/or passage.

Cell culture. Decapsulated kidneys aseptically removed from young, weaned Syrian hamsters *(Mesocricetus auratus)*‡ were finely minced with scissors and washed 4 times with Hanks' balanced salt solution (BSS) (13) without bicarbonate or antibiotic. Tissue fragments were treated with 20-40 volumes of 0.1% Difco trypsin (1:250) in BSS (pH 7.4-7.0) at room temperature for 2-3 hours on a magnetic stirrer. Dispersed, unfiltered cells were centrifuged and washed twice with 50 volumes of BSS. After centrifugation at 190 × *g* for 2 minutes, 1 ml aliquots of a 1/200 to 1/300 dilution (wet weight/volume) of cells in medium were distributed into 16 × 125 mm Pyrex screwcap tubes. Cells were established on glass in medium consisting of calf serum§ (heat-inactivated at 56°C for 30 minutes), 100 parts, lactalbumin hydrolysate, 2.5 parts, BSS, 897.5 parts with penicillin, 100 units/ml, and streptomycin, 100 μg/ml.‖ Cultures kept slanted in stationary racks formed confluent, usable monolayers after 3 to 5 days incubation at 37°C.

Infection of cells and virus assay. Prior to virus adsorption cells were rinsed 3 times with GLB medium which contained gelatin, 1%, and lactalbumin hydrolysate, 0.25%, in BSS with penicillin and streptomycin. Virus diluted in GLM medium (consisting of gelatin, 1%, and lactalbumin hydrolysate, 0.5%, in maintenance solution (MS)(14) with penicillin, 100 units/ml, and streptomycin, 100 μg/ml) was adsorbed on monolayers in 0.1 ml volume at 37°C for 1 hour, with frequent rocking to distribute inoculum and to keep cells moist; 0.9 ml of GLM medium was then added to each tube. Cell mono-

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‖ During initial experiments it was learned that when calf serum in 4% final concentration was used in growth medium, monolayers subsequently did not maintain well upon change to serum-free medium; also, yeast extract (Difco yeastolate), 0.1%, could not be substituted for lactalbumin hydrolysate.

layers not inoculated with virus but treated in all other respects like virus-exposed cells were kept as controls. Cultures were incubated at 37°C and examined daily at 50-100 × magnification.

Infectivity titers in hamster kidney cell cultures ($TCID_{50}$) were determined by inoculation of tube cultures in triplicate with 0.1 ml of each decimal dilution of virus; a monolayer was considered infected if ≧ 50% destruction became evident 7-10 days after incubation at 37°C. Egg infectivity (EID_{50}) titers were obtained by allantoic sub-inoculation of 0.1 ml into five 10- or 11-day-old chicken embryos per decimal dilution of virus made in BSS containing bovine albumin, 0.1%, penicillin, 100 units/ml, and streptomycin, 100 μg/ml. The method of Reed and Muench(15) was used to calculate 50% infectivity end points. Hemagglutinin (HA) titers were measured at room temperature utilizing erythrocytes of white Embden geese in 0.5% final concentration in physiological saline.¶ Hemadsorption tests were performed as recommended(16) at 4°C with 0.4% washed guinea pig erythrocytes in saline.

Immune sera. WS viral antiserum prepared in rabbits had been previously obtained from Dr. R. R. Wagner. Rabbit antisera against NWS virus and FM-1 virus were kindly provided by Drs. E. W. Hook and I. Tamm, respectively. All sera were stored frozen at −20°C and after thawing were heated to 56°C for 30 minutes prior to testing in neutralization or hemagglutination-inhibition (HI) tests. Serum samples used in HI tests were pre-treated with trypsin (17) to remove non-specific inhibitors.

Results. Destruction of cricetine cells by influenza viruses. Primary hamster kidney (HK) cell monolayers under fluid (GLM) medium free of serum were rapidly destroyed by influenza viruses belonging to subtypes A, A_1, A_2, and type B. Fig. 1-5 show the characteristic pattern of cellular destruction by NWS virus. With large inocula of NWS virus, rounding of scattered cells was seen about 24 hours after infection. Over the subsequent 24 hours progressive cell lysis began, and changes in cellular membranes led to detachment of cells from glass while those remaining attached tended to aggregate; long cellular processes extended from such small groups of cells, which exhibited marked hyperchromicity upon staining with hematoxylin and eosin. Only cellular debris and scattered fibroblastic-type cells remained attached for an additional 24-96 hours. Varying pathologic stages could, of course, be observed in different cultures at the same time interval after infection depending on virus dose (Fig. 4 and 5). Cytoplasmic vacuolation and nuclear pyknosis were pronounced in HK cells (Fig. 4 and 7), as had been noted previously in influenza viral infection of calf kidney cells in culture(18) and alveolar cells in mouse lung(19). Nuclear fragmentation of HK cells was not notable. Eosinophilic paranuclear inclusions were seen in infected HK cells but could also be found in rare uninfected cells (Fig. 6-7). Whether these eosinophilic aggregates are viral or cellular in nature has yet to be determined, but they occurred with greater frequency and were larger and more deeply staining in infected cells than those in uninfected cultures.

The cytodestructive effects of other influenza viruses followed a similar sequence. Marked cytoplasmic elongation and cellular aggregation did not appear to be constant features of infection, however, with Japan/305 virus; infected cells tended to round up and drop off the glass. The time after infection when initial cytopathic effects could be discerned varied with the virus inoculated. Cytopathic changes began earlier after infection with NWS (24-48 hours) than with WS, FM-1, and Lee strains (about 48 hours) or with Japan/305 (72-96 hours).

Epithelial-like cells and cellular debris in cultures infected with NWS virus adsorbed guinea pig erythrocytes, but fibroblastic-type cells which temporarily persisted on glass after destruction of the monolayer did not adsorb red cells, an observation suggesting differential susceptibility of cell types in these primary cultures. When a cytonecrotic effect was absent 7 days after infection with NWS virus, no hemadsorption occurred.

¶ Titers have been comparable to those obtained with rooster erythrocytes done in parallel simultaneous titrations.

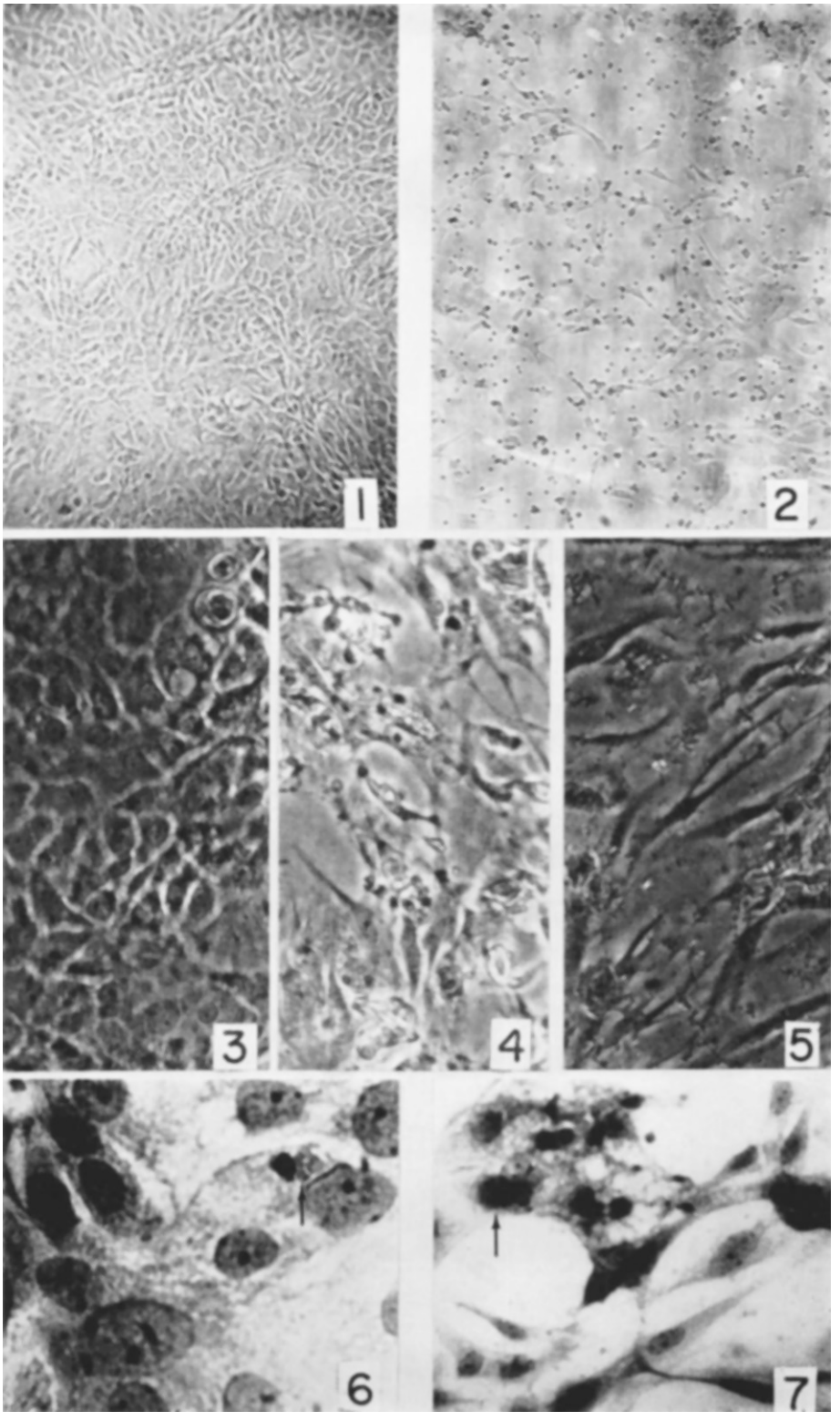
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FIG. 1. Normal uninoculated hamster kidney cells 44 hr after change to serumless medium. Alcohol-fixed, unstained, coverslip preparation, phase contrast, 75×.

FIG. 2. Hamster kidney cells 44 hr after adsorption of $10^{2.5}$ $TCID_{50}$/0.1 ml of NWS virus. Alcohol-fixed, unstained coverslip preparation, phase contrast, 75×.

FIG. 3. Normal uninoculated hamster kidney cells 44 hr after change to serumless medium. Alcohol-fixed, unstained, coverslip preparation, phase contrast, 234×.

FIG. 4. Hamster kidney cells 44 hr after adsorption of $10^{0.5}$ $TCID_{50}$/0.1 ml of NWS virus. Note membrane retraction and cytoplasmic vacuolation. Alcohol-fixed, unstained coverslip preparation, phase contrast, 234×.

FIG. 5. Hamster kidney cells 44 hr after adsorption of $10^{2.5}$ $TCID_{50}$/0.1 ml of NWS virus. Note debris from cell lysis. Alcohol-fixed, unstained coverslip preparation, phase contrast, 234×.

FIG. 6. Normal hamster kidney cells with lightly staining eosinophilic inclusion. Hematoxylin and eosin, 351×.

FIG. 7. NWS virus-infected hamster kidney cells with deeply staining eosinophilic inclusion. Note cytoplasmic vacuolation. Hematoxylin and eosin, 225×.

Thus, cytopathic end points could be correlated with viral multiplication as manifest by hemadsorption.

Serial propogation of viruses in hamster cells. To pass virus in series, virus was titrated (usually 10^{-1} to 10^{-7} in decimal dilutions) in HK cell cultures in triplicate. When cytonecrosis became evident, fluids were pooled from cultures inoculated with the highest dilution that showed approximately 50% destruction in all 3 cultures, titrated for hemagglutinin, and frozen for passage and EID_{50} determination. As shown in Table I, influenza A viruses, NWS and WS, and an A_1 virus, FM-1, were propagated serially in HK cell cultures. Propagation of Japan/305 virus was not attempted. Titers of virus-infected cultural supernatant fluids measured *in ovo* were consistently higher ($10^{6.8}$ to $10^{8.5}$ EID_{50}/0.1 ml) than titers measured in HK cells ($10^{4.5}$ to $10^{7.0}$ $TCID_{50}$/0.1 ml). Hemagglutinin was produced in infected HK cell cultures, although titers were somewhat variable. NWS or WS hemagglutinin titers of cultural harvests were generally comparable to the original allantoic fluid virus preparations, whereas FM-1 hemagglutinin was produced in lesser amounts (Table I). The highest titers of HK-infectious virus could be obtained by early harvest of infected HK cell culture supernatant fluids when no more than about 30% destruction of cells was seen. No evidence of autointerference as manifest by a sparing effect or delay in cellular destruction at the lowest dilutions (10^{0}-10^{-2}) of virus inocula was observed on passage in HK cells.

Limited passage of influenza virus strains

TABLE I. Serial Propagation of Influenza A Viruses in Primary Hamster Kidney (HK) Cell Cultures.

Passage No.	Titer of inoculum *in ovo* (EID_{50}/0.1 ml)	Titer of inoculum in HK cells ($TCID_{50}$/0.1 ml)	Duration of of virus passage (days)	Total days in culture	Dilution of original inoc (log_{10})	HA* in HK cultural harvest†
		NWS strain (allantoic passage 48)				
1	$10^{7.5}$	$10^{5.5}$	3	3	6	256
2	$10^{7.1}$	$10^{6.5}$	3	6	12	32
6	$10^{7.6}$	$10^{5.7}$	2	17	22	1024
7	$10^{7.8}$	$10^{6.3}$	2	19	27	512
		WS strain (allantoic passage 156)				
1	$10^{7.0}$	$10^{3.5}$	4	4	3	128
2	$10^{7.5}$	$10^{3.3}$	3	7	6	64
3	$10^{6.7}$	$10^{5.8}$	2	9	10	256
4	$10^{7.8}$	$10^{6.5}$	1	10	13	256
		FM-1 strain (allantoic passage 48)				
1	$10^{7.8}$	$10^{6.3}$	3	3	5	256
2	$10^{7.7}$	$10^{4.5}$	3	6	9	128
3	$10^{7.0}$	$10^{4.0}$	4	10	12	32
4	n.t.‡	$10^{4.8}$	2	12	15	64

* Hemagglutinin (HA) as reciprocal of dilution of cultural supernatant fluid/0.5 ml.
† HA titers of original allantoic fluid virus inocula: NWS (256); WS (512); and FM-1 (1024).
‡ Not tested.

NWS (more than 7 times) and FM-1 (4 times) in HK cell cultures produced no observable increase in their pathogenicity for these cells. WS virus appeared to become somewhat more pathogenic upon cell culture passage, as evidenced by an increase in titer measured in HK cell culture and a shortening of the interval between inoculation and appearance of cytopathic effect (Table I). The characteristic ability of NWS virus to produce severe cerebral hemorrhages in chicken embryos upon intravenous inoculation(20,21) was preserved after 2 passages in HK cell culture but was lost after 6 passages, even when injected undiluted.

Viral neutralization and hemagglutination-inhibition. To determine whether influenza virus infectivity could be neutralized by specific antibody in HK cell culture, influenza A hyperimmune serum was tested against A and A_1 viruses. WS rabbit serum had a homologous $\log_{10}$ neutralization index, calculated for undiluted serum, of 4.6 as measured *in ovo*. This serum (inactivated at 56°C for 30 minutes) was diluted 1/10 and 1/100 and serially 2-fold thereafter in MS; serum dilutions were mixed with equal volumes of NWS or FM-1 virus dilutions (made in GLM medium), incubated 30 minutes at 22-24°C, and 0.1 ml of the mixtures adsorbed in triplicate onto washed HK cell cultures for 60 minutes at 37°C, following which 0.9 ml of GLM medium was added. NWS and FM-1 virus dilutions similarly incubated and adsorbed were titrated simultaneously in HK cell cultures. This WS antiserum, diluted 1/10 (but not 1/100), neutralized $10^{3.2}$ $TCID_{50}$ of NWS virus, but at 1/10 dilution, it did not neutralize $10^{2.2}$ $TCID_{50}$ of FM-1 virus. Tests performed *in ovo* simultaneously with those in cell culture likewise showed neutralization, at 1/10 serum dilution, of NWS but not FM-1 virus. Homologous *in vitro* neutralization of WS virus was demonstrated by a plaque-inhibition technique (Grossberg, S. E., unpublished studies).

Virus passaged in HK cell culture produced hemagglutinin (Table I) in sufficient quantities to allow for identification of viruses serially propagated in these cells. Serum titers obtained with cultural fluid as source of hemagglutinin were comparable to those measured against allantoic fluid virus which had not undergone passage in HK cell culture (Table II).

TABLE II. Homologous Hemagglutination-Inhibition (HI) Titers of Immune Rabbit Antisera Tested with Influenza Viruses Grown in Allantois or Hamster Kidney (HK) Cell Culture.

Antiserum versus	Source* of influenza virus hemagglutinin	HI titer† (as serum dilution)
NWS	E_{43} (allantoic fluid)	1:1024
"	$E_{43}HK_8$ (cultural fluid)	1:1024
WS	E_{155} (allantoic fluid)	1:1024
"	$E_{156}HK_4$ (cultural fluid)	1:2048
FM-1	E_{49} (allantoic fluid)	1:2048
"	$E_{48}HK_4$ (cultural fluid)	1:2048

* E_zHK_a = passaged in embryonated egg z times, hamster kidney cell culture a times; for prior passage history, see Table III.

† Tested against 2-4 units of hemagglutinin in simultaneous titrations for a given serum.

Titers in ovo and in cricetine cells. Titration of the infectivity for HK cells of common laboratory strains of influenza viruses could be readily accomplished and was compared with egg infectivity (Table III). End points of virus titrations in HK cell culture manifest as $\geqq$ 50% destruction (usually 90-100%) could frequently be determined within 5 to 7 days after inoculation. Uninfected cell monolayers were regularly well maintained for 8 to 10 days and occasionally for 12 to 14 days without requiring supplementation of GLM medium.

Passage histories (since isolation from man) of the viruses employed appeared to influence titers attained in HK cell cultures. The A, A_1, and B strains which had undergone a number of passages in rodents produced higher titers than 2 Japan/305 substrains which had no rodent and limited egg passages (Table III). However, after 160 egg passages, another substrain had a titer similar to other influenza viruses tested. Again, titers measured in allantois were higher than in HK cell culture.

Five per cent calf serum in GLM medium put on cells following virus adsorption significantly reduced NWS virus titer (from 10^5 to 10^3 $TCID_{50}$), delayed NWS cytopathic effects by several days, and prevented complete destruction of cells which occurred in simultaneously infected cells maintained

TABLE III. Comparative Titrations of Allantoic Fluid Preparations of Influenza Viruses Measured *in ovo* and in Hamster Kidney (HK) Cell Culture.

Immunologic type	Strain designation	Passage history	Infectivity titer (log_{10}) *in ovo* (EID_{50}/0.1 ml)	Infectivity titer (log_{10}) in HK cell culture ($TCID_{50}$/0.1 ml)
A	NWS	$F_{166}M_{196}E_{34}M_{35}E_{48}$*†‡§	7.5	5.5
"	WS	F?M?E_{156}†	7.0	4.7
A_1	FM-1	$E_1M_{87}E_{14}M_3E_{48}$§	7.3	6.3
A_2	Japan/305	E_3‖	5.5	3.0
"	"	E_7¶	6.1	1.5
"	"	E_{160}**	7.5	5.3
B	Lee	$F_7M_{310}E_5$§	6.3	5.5

* $F_xM_yE_z$ = passaged in ferret x times, mouse y times, and in embryonated egg z times, since isolated from man.

† Dr. R. R. Wagner, personal communication.

‡ Reference citation No. 12.

§ American Type Culture Collection Registry, 2nd ed., 1959 and Supplement, 1961.

‖ Forwarded in 1961 from U. S. Army 406th Med. General Laboratory, Japan, as first passage material.

¶ Obtained from Walter Reed Army Inst. of Research.

** Obtained from Dr. J. Y. Sugg.

under serum-free medium. Indeed, by day 10 after infection, monolayers nourished by medium containing calf serum appeared to recover from viral effects they previously had demonstrated.

Discussion. The rapid, overt destruction of cultured cricetine kidney cells by influenza viruses provides a simple means for detection and measurement of viral infectivity and neutralizing antibody. Production of cytopathic effects was not dependent on viral adaptation to cricetine cells but could be observed with allantoic fluid virus. Maintenance of primary hamster kidney cell cultures in a satisfactory nutritional state for 7 to 10 days without serum allowed the cytopathic effects of influenza viruses to become evident. When calf serum was added to cultural fluid, cytodestruction was suppressed, presumably due to inhibitors for influenza virus known to be present in mammalian serum; stimulation of cellular growth by serum may also have played an important role in the reparative process following the minimal destructive effects observed with NWS virus in medium containing serum.

Propagation *in vitro* of certain influenza viruses has been shown to occur in primary cultures of the chicken embryo and its viscera(1,2), human embryo lung and kidney (3,4,5), simian kidney(6,7,8), and porcine lung(4), as well as in continuous cell lines of human(9) and canine(10) origin. Influenza B virus(6,10) the neurotropic NWS variant (subtype A)(2,9), and occasionally other strains have been shown to induce cytopathic changes in some of these cell types. Usually influenza A viruses of human origin have been propagated in such cells either not at all, to only low titer, or with variable or incomplete overt destruction(2,4,5). When virus was either isolated in or intentionally adapted to a given host cell system, extensive cytopathic changes often became evident(6,7,8). Plaque formation under solid medium has been most readily observed with such adapted strains in simian kidney cells (7,8) and with egg-passaged strains in chicken embryo fibroblasts(11). Using some of the methods described here, a rapid plaque assay has been developed for human influenza A viruses without the necessity for adapting them to cricetine cells (Grossberg, S. E., unpublished studies).

Several influenza viruses used in these studies had been passaged in mice since isolation from man (Table III). Inasmuch as hamsters and mice belong to the same rodent suborder (Myomorpha), it was first thought that the propensity of these viruses to destroy cricetine cells in culture might be related to prior murine passage. However, strain Japan/305 was never passaged in mice but nonetheless destroyed hamster kidney cells, although lower titers were achieved with two A_2 substrains in low egg passage than the other

viruses tested. Moreover, it should be noted that all viruses tested, other than two A_2 substrains (which also had somewhat lower egg infectivity titers than other type A viruses), had undergone numerous passages in the chicken embryo allantois since last exposure to murine tissues.

Allantoic fluid virus destroyed HK cells no less well than cell culture passaged virus. However, the basis for the consistent disparity between titers achieved *in ovo* and those in cricetine cell culture, even for virus passaged in HK cells, is unclear. All strains of virus used were either isolated in chicken embryos or propagated serially over a long period of time in them; it is likely that under these circumstances selection pressures were exerted on virus populations that could multiply more readily in chicken embryos than in other hosts. Further adaptation of influenza viruses to HK cells may obliterate such disparity. The difference in temperature of incubation of virus-infected embryos (35°C) and cell cultures (37°C) may be another determining factor.

The cytopathology of HK cells infected with influenza virus generally appeared similar to that observed previously *in vitro*(18) and *in vivo*(19), *i.e.*, cytoplasmic vacuolation, nuclear pyknosis, cellular granularity and degeneration. The finding of eosinophilic aggregates in cytoplasm is at variance with previous observations of Harford and Hamlin(22) who described cytoplasmic basophilic inclusions in mouse bronchial epithelium, and others(18,19) who noted no inclusions. This finding deserves further investigation.

Influenza virus subtype A infection in the intact hamster has been shown to be an inapparent process with production of specific antibody(23), an observation which provided a host as sensitive as the ferret and more reliable than the chicken embryo(24) for detection, *but not recovery*, of strains of influenza subtype A virus present in throat washings. Serial passage of influenza virus increased its virulence for the hamster with subsequent production of pneumonia and death(25); although in the present *in vitro* studies, no adaptation was required to produce hamster kidney cell destruction.

Cricetine kidney tissue in primary culture has been used in detection and propagation of certain arthropod-borne viruses(26) and selected enteroviruses(27). The demonstrated wide range of cellular susceptibility to a variety of viruses including influenza may also prove these cells to be of value diagnostically. However, their usefulness for isolation of influenza viruses from man needs to be evaluated. Because of their ready availability, ease of cultivation, and ability to grow influenza viruses to relatively high titer in serum-free medium, hamster kidney cell cultures may be a useful (non-avian and non-primate) source of virus for production of vaccines for the *Myxovirus influenzae* group.

Conclusion. Influenza viruses belonging to immunologic subtypes A, A_1, A_2, and type B, rapidly and completely destroyed hamster kidney cells cultured in serum-free medium. Intracellular pathologic changes resembled those described for influenza virus infection in other cells. Strains NWS, WS, and FM-1 were serially propagated in cricetine cells beyond theoretical extinction of original inoculum with the production of virus which was infectious for hamster kidney cells as well as chicken embryos and which could be neutralized by immune serum. Hemagglutinin was also produced in cell cultures in sufficient quantities to allow for identification of virus by means of hemagglutination-inhibition tests.

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1. Francis, T., Magill, T. P., *Science,* 1935, v82, 353.
2. Tyrrell, D. A. J., *J. Immunol.,* 1955, v74, 293.
3. Mogabgab, W. J., Green, I. J., Dierkhising, O. C., *Science,* 1954, v120, 22.
4. Hinz, R. W., Syverton, J. T., PROC. SOC. EXP. BIOL. AND MED., 1959, v101, 22.
5. Mogabgab, W. J., Simpson, G. I., Green, I. J., *J. Immunol.,* 1956, v76, 314.
6. Mogabgab, W. J., Green, I. J., Dierkhising, O. C., Phillip, I. A., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 654.
7. Henry, C., Youngner, J. S., *J. Immunol.,* 1957, v78, 273.

8. Choppin, P. W., *Virology,* 1962, v18, 332.

9. Wong, S. C., Kilbourne, E. D., *J. Exp. Med.,* 1961, v113, 95.

10. Green, I. J., *Science,* 1962, v138, 42.

11. Simpson, R. W., Hirst, G. K., *Virology,* 1961, v15, 436.

12. Stuart-Harris, C. H., *Lancet,* 1939, v29, 113.

13. Hanks, J. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 196.

14. Scherer, W. F., *Am. J. Path.,* 1953, v29, 113.

15. Reed, L. J., Muench, H., *Am. J. Hyg.,* 1938, v27, 493.

16. Vogel, J., Shelokov, A., *Science,* 1947, v126, 358.

17. Sampaic, A. A., Isaacs, A., *Brit. J. Exp. Path.,* 1953, v34, 152.

18. Negroni, G., Tyrrell, D. A. J., *J. Path. and Bact.,* 1959, v77, 497.

19. Mulder, J., in *Ciba Foundation Study Group No. 4-Virus Virulence and Pathogenicity,* Little, Brown and Co., Boston, 1960, p43.

20. Burnet, F. M., Fraser, K. B., *Austr. J. Exp. Biol. and Med. Sci.,* 1952, v30, 447.

21. Grossberg, S. E., Hook, E. W., Wagner, R. R., *J. Immunol.,* 1962, v88, 1.

22. Harford, C. G., Hamlin, A., *J. Exp. Med.,* 1952, v95, 173.

23. Taylor, R. M., Parodi, A. S., PROC. SOC. EXP. BIOL. AND MED., 1942, v49, 105.

24. Eaton, M. D., Corey, M., Van Herick, W., Meiklejohn, G., *ibid.,* 1945, v58, 6.

25. Friedewald, W. F., Hook, E. W., *J. Exp. Med.,* 1948, v88, 343.

26. Diercks, F. H., Hammon, W. McD., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 627.

27. Barron, A. L., Karzon, D. T., *ibid.,* 1959, v100, 316.

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Antigenicity of Polypeptides (Poly Alpha Amino Acids). X. Studies with Polymers of D Amino Acids.* (28423)

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Several groups have studied the antigenicity polymers of L-α-amino acids. The polymers have been either of the "straight chain" linear random copolymer(1,2) or the multichain type(3,4). Investigations have indicated that the *copolymers* of L-α-amino acids in contrast to the homopolymers of a single amino acid(5) may be antigenic in rabbits, (5-9), guinea pigs(10,11) and man(12). The use of synthetic polymers also afforded insight into some of the structural factors controlling immunogenicity of proteins. To learn more about these parameters, the antigenicity of copolymers consisting of 2 and 3 D amino acids has been studied in both rabbits and guinea pigs. This report presents data which indicate that polymers made solely of D amino acids have little or no immunogenicity.

Materials and methods. The copolymers studied for antigenicity are listed in Table I. They were prepared by the random polymerization of mixtures of the N-carboxy anhydrides of the amino acids which were present in the mol percent as indicated in the final product. Before use, the solution of the polymer was dialized to remove any low molecular weight materials. Polymerization was allowed to go to completion. The weight average molecular weight was estimated from viscosity measurements(13). Nomenclature for the polymers is as follows: G, A and T refer to glutamic acid, alanine and tyrosine respectively and subscripts refer to the mol percent composition of the amino acid in the random copolymers. The optical configuration of the polymers is indicated by (D) or (L) preceding the polymer. The arsanilic acid derivative of D(GAT), (As(D)(GAT)), was prepared by Dr. B. Benacerraf. Five mg of the polymer were treated with an excess of diazotized arsanilic acid. The mixture was stirred for 1 hour, then dialyzed for several days to remove unreacted arsanilic acid. Arsanyl groups were determined spectrophotometrically at 333 mμ using the mono arsanyl derivative

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