taining 30% corn oil produce marked acceleration in excretion of C^{14} as non-digitonin precipitable neutral sterols in rat feces, accounting in these animals for 17-25% of the administered C^{14}. This non-digitonin precipitable neutral sterol does not contain a ketonic group and is presumably a 3-alpha-hydroxy sterol.

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Characteristics of the Asian Strain of Influenza A.* (24266)

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Besides the marked antigenic variation found in the Asian strain of influenza A, several other characteristics differentiate it from influenza A viruses isolated in recent years (1,2). Included were growth behavior in the embryonated egg, and reaction with various erythrocytes, with antibody and with non-specific inhibitors. Although the virus propagated in monkey kidney cultures tended to minimize such differences, even in this case hemagglutination behavior set it apart from earlier strains. Pertinent features of Asian virus isolated and propagated in monkey kidney cultures and in the chick embryo are here described.

Materials and methods. Cultures (TC) were prepared with 0.25% trypsin (Difco, 1-250) digested Rhesus monkey kidneys, grown at 35°C in stationary tubes with 0.5% lactalbumin hydrolysate (Nutritional Biochemicals) and 2% calf serum (Microbiological Associates) in Earle's solution. After 7-12 days they were washed and maintained at 35°C with Mixture 199 (Microbiological Associates), and pH held between 7.0 and 7.6 during experiments by adding $NaHCO_3$ solution or allowing escape of CO_2. Ten day White Leghorn embryonated eggs were used for virus isolation in the amniotic sac or propagation in the allantoic sac (E). Throat garglings were obtained with Mixture 199, 0.5% lactalbumin hydrolysate in Hanks' solution or boiled skimmed milk and were collected from July through Dec. 1957 in Louisiana. Except where references are made to observations on several strains, experiments were done with Asian virus isolated in New Orleans from a Boy Scout on board a train from the 1957 Jamboree, Valley Forge, Pa. Separate passage lines were derived from the original specimen and maintained in the egg and in TC. The egg-ferret-mouse-egg line ($E_4F_1M_3E_{12}$) of A/Japan/305/57 (FE) was obtained from the Communicable Disease Center, U.S. Pub. Health Serv. Erythrocyte (rbc) suspensions for hemagglutination (Ha) were based on packed cell volume in a 15 ml conical tube after centrifugation in an International No. 1 at 1500 rpm for 10 minutes. A final concen-

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TABLE I. Virus Isolations in Egg and in Tissue Culture (TC) from Specimens Obtained from Serologically Diagnosed Cases.*

Isolation system	No. cases	No. isolations	% isolations
Egg	100	62	62.0
TC (7 day)	64	10	15.6

* Four-fold or greater increments in hemagglutination-inhibition were obtained in paired sera using the monkey kidney line of Asian virus and RDE treated sera.

tration of 0.25% rbc was used in all experiments. Receptor destroying enzyme (rde) was prepared from filtrates of *Vibrio cholerae* cultured at room temperature on a shaking apparatus. For hemagglutination-inhibition (HaI) titrations sera were treated overnight with rde or M/90 potassium periodate, or heated at 56°C for 30 minutes. Titers were recorded as initial dilution of antiserum inhibiting agglutination of 0.25% human type O rbc by 4 Ha units of virus. Neutralization titers were determined with sera inactivated at 56°C for 30 minutes and read as the initial dilution of serum inhibiting growth of 10^2TCID_{50} at 7 days or 10^2EID_{50} at 3 days.

Primary isolation. A larger percentage of virus isolations was made in the amniotic sac even on first passage than in TC (Tables I, II). Second passage was not used in TC since previous results with influenza B isolations had suggested that a medium change on day 3-4 accomplished as much(3). In contrast to that virus, 10 to 100 times as much Asian strain was required to infect TC as the amniotic sac. Although the percentage of isolations of Asian virus was slightly less than might have been expected with influenza A strains in the chick embryo, the results in TC were not unlike those with previous strains of this type of virus(4). Serologic identification of hemagglutinins in amniotic fluid was sometimes difficult because of low titers or lack of combination with antibody (Q phase) until additional passages had been made(5). The latter characteristic was not encountered in strains isolated in TC. Perhaps the poor results shown in Table II with the hemadsorption technic might have been improved by use of an intermediate medium change to remove inhibitory substances(6). Although hemadsorption was satisfactory for detection of virus growth with a TC line of Asian virus, a conventional hemagglutination pattern could also be observed in positive tubes.

TABLE II. Results of Inoculation of Egg Positive Specimens in Egg and in Monkey Kidney Cultures.

Isolation system	No. specimens	No. positive	% positive
Egg*—E_1		33	43.4
E_2		37	48.7
Q (E_3–E_7)		6	7.9
Total	76	76	100.0
TC (7 day)†	51	10	19.6
TC (HaAds)‡	27§	3	11.1

* More than 2 amniotic passages (E_1 and E_2) were done if hemagglutinins were not inhibited by specific antibody (Q phase).

† Fluids were replaced at 3 and 7 days and hemagglutinating titers determined.

‡ Human type O RBC were added at 1-2 days for hemagglutination-adsorption.

§ 9 were also positive in TC (7 day).

Hemagglutination. Titers of agglutination of various rbc by Asian virus as shown in Table III resembled influenza B strains(7,8).

TABLE III. Titers of Agglutination of RBC from Various Species by TC and E Lines of Asian Virus.

RBC, .25%	Temp., °C	TC_{2-4} (MK) Active	TC_{2-4} (MK) Inactive*	E_{3-5} Active	E_{3-5} Inactive*
Human	4	64	32	256	64
	24	64	32	256	64
Fowl	4	64	32	512	256
	24	64	32	512	128
Sheep	4	16	8	64	32
	24	8	4	64	32
Monkey	4	32	16	512	64
	24	32	8	256	64

* Viruses were inactivated by heating at 56°C for 30 min.

Absent were the lower titers with fowl rbc and lack of agglutination of sheep rbc at 24°C characteristic of early egg passages of previous influenza A strains. Differences between the TC and E lines of Asian virus were manifest only by lesser affinity of the TC line with sheep rbc and disagglutination was more rapid and extensive especially with the latter as well as fowl rbc. This resembled the 1953 strain of influenza A in which passages in the egg increased reactivity with fowl and sheep rbc receptors to a greater degree than pas-

sages in human kidney cultures. Adaptation of the 1953 TC line to monkey kidney cultures also resulted in equivalent titers with human, fowl or monkey rbc and lesser affinity for sheep rbc(4,8).

Growth of virus. Although Asian virus titers in the amniotic or allantoic sacs were considerably lower than those observed with the 1953 strain of influenza A, growth in monkey kidney cultures was similar to the latter virus(4). Differences in isolated strains were reflected by variability in virus titers obtained on early amniotic passages, some requiring several to equal titers obtained with others upon initial inoculation. After adaptation to the allantoic sac an incubation period of 2 days was adequate for determination of infectivity (EID_{50}). Asian virus often killed chick embryos inoculated in the amniotic sac and produced relatively marked cytopathogenic effects in monkey kidney cultures. Strains isolated from human lung autopsy specimens demonstrated no distinctive features in the egg or TC.

Serum inhibitors. Agglutination of human type O rbc by the egg line of Asian virus, either active or heated at 56°C for 30 minutes, was not inhibited by normal human, rabbit or fowl sera until more than 4 passages had been made. The TC line, in contrast, was quite sensitive to non-specific substances, and heating human or fowl sera at 56°C for 30 minutes increased degree of inhibition by fourfold or greater. Additional passages in the allantoic sac resulted in similar inhibition of the E line with human and rabbit, but not fowl, sera. The EFME line of Asian virus behaved much like the TC line, but periodate was required for inactivation of inhibitor rather than rde, which was adequate for the latter. Treatment of serum at 35°C overnight with equal quantities of 1.6% trypsin (9) removed inhibitors of hemagglutination in all instances except with rabbit serum and the TC and EFME lines. Temperatures of 65°C for a half hour did not reduce inhibitor titers.

Reaction with antibody. Early egg passage Asian virus reacted weakly with HaI antibody in rde treated sera. In fact, diagnostic, 4-fold or greater, increments in titer were found in only half the acute-convalescent serum pairs that were positive with TC virus. With the TC line diagnostic HaI antibody increments were found in all cases in which virus had been isolated or antibody increase demonstrated by complement-fixation with egg propagated virus. Increased reactivity of the E_4 virus resulted when sera treated with heat alone were used. The same was accomplished by additional egg or intermediate animal passages (EFME), but it became necessary to remove non-specific inhibitors in sera with both the latter. Antibody titers in convalescent sera from influenza cases were higher when determined with the TC than any of the E lines (Table IV), either because of a closer antigenic relation to the infecting virus or due to greater reactivity of the TC line with antibody. On the other hand antibody titers in post-vaccinal sera were similar with the TC or EFME lines(10,11). It was unreasonable to assume that the lower HaI antibody titers with E_4 and rde treated sera were due to destruction of antibody since this did not occur with the E_7 and TC lines. Although it seemed likely that an additional serum component was participating in the inhibition of E_4 by untreated convalescent serum, titers were not increased by addition of fresh normal serum or calcium to rde treated serum. Lower titer antibody increments were often not demonstrable in paired sera from influenza cases regardless of treatment of sera when tested with the E lines. Even with the TC virus and a measurable 4-fold antibody increase the convalescent serum titer was sometimes 1-4. This indicated little previous antigenic experience with influenza A, and also suggested limits to

TABLE IV. Mean Hemagglutination-Inhibition Antibody Titers Determined with TC and E Lines of Asian Virus.*

	Virus				
	TC_{2-4}	E_4	E_4	E_7	EFME
Treatment of serum	RDE	△56°C	RDE	RDE	Periodate
Acute serum titer	<4	<4	<4	<4	<4
Convalescent serum titer	120	37	16	32	46

* Geometric mean titers determined from 10 paired sera.

TABLE V. Mean Neutralization Titers Determined with TC and E Lines of Asian Virus.*

	Virus			
	TC_{2-4}	E_4	E_8	EFME
Host system	TC	Egg	Egg	Egg
Acute serum titer	<4	<4	<4	<4
Convalescent serum titer	91	4	4	10

* 10^2 ID_{50} of virus was added to dilutions of heat inactivated sera. Results are geometric means from 6 pairs of sera from influenza cases.

sensitivity and a threshold of the technic of determining HaI antibody.

Avidity of the E line for neutralizing antibody determined in the allantoic sac appeared slight as shown in Table V. Increasing the temperature from 4° to 37°C and the time from 30 to 60 minutes did not change the combining capacity of the E line with neutralizing antibody. Reduction in amount of virus by 10-fold also did not greatly increase titers as compared to those obtained in TC. Quantitatively, the results of antibody determinations in the latter system were similar to those of HaI with TC virus and rde treated sera. It appeared that the E line had more affinity for neutralizing antibody in post-vaccinal rather than post-influenzal sera, again suggesting its lack of close relationship to the natural virus(11).

Although complement-fixation was necessary to establish relationship of Asian virus to other influenza A strains, some evidence could be obtained by HaI. Antibody increments in the order of 2-fold were not uncommon in paired sera from Asian influenza cases when tested with the 1953 Great Lakes or 1957 Denver influenza A viruses. Also, the TC line of Asian virus was inhibited at low titers with Denver rooster antiserum, although not with Great Lakes rabbit antiserum.

Discussion. Features of Asian virus characteristic of influenza A as a group in addition to antigenic relationship were behavior upon primary isolation in TC and the egg and rate of growth of a minimal infecting dose in the allantoic sac. Thus, the proportion of cases from which virus could be isolated in the amniotic sac was at least 60% and was as much as 80-90% when specimens were carefully collected early in the illness. In addition, primary isolation in TC required 10-100 times as much virus as the chick embryo in contrast to results with influenza B(3). Also, an incubation period of 2 days was adequate for determination of infectivity with adapted virus in the allantoic sac, while influenza B required 3 days(12).

Among the differences between the Asian and other influenza A strains reactivity with receptors was of particular interest. The lack of normal serum inhibition of early egg passages of the strain of Asian virus described herein was reminiscent of similar behavior of the human TC line of a 1953 influenza A with non-specific inhibitors(8). Although the monkey kidney culture line of Asian virus was quite sensitive to normal serum inhibition, additional passage of the 1953 human TC line in monkey kidney cultures also produced a similar change(4). Accordingly, it is possible that lack of sensitivity to inhibitors was a feature of the natural virus. Support for the contention that such a characteristic might facilitate spread was obtained by demonstration of an inhibitor of infectivity of the monkey kidney line of the 1953 virus in normal heat inactivated rabbit sera, and this inhibitor was removable by rde(4). Affinity for receptors of fowl and sheep erythrocytes was another unexpected attribute of the Asian strain. Since this was found even on initial passages in the egg or TC it is unlikely that this was caused by the laboratory host system. Nevertheless, studies with Asian virus in human tissue culture might serve to answer some of these questions. Surprisingly in this regard it has not yet been possible to isolate or propagate virus in human embryo kidney cultures from 6 original specimens that had previously been positive both in the egg and monkey kidney cultures. This occurred despite two passages as well as several fluid changes in cultures that supported growth of the human tissue culture line of the 1953 influenza A line (4).

The occurrence of strains that were initially weak reactors with antibody (Q phase) was of especial interest in a pandemic virus even though this has been previously described with epidemic strains of influenza A (5). Al-

though this might have been caused by growth in the chick embryo, since similar characteristics were not observed in the TC isolates, it is also reasonable to assume that preferential growth of Asian virus in the egg allowed retention of certain natural characteristics longer than TC. Probably the same mechanism accounted for lack of reactivity with inhibitors and antibody and might be of consequence in explaining spread of the virus if it occurred during infection of man. One might speculate that virus propagated in TC was antigenically closer to the natural agent, but that grown in the egg was reactively more similar.

Summary. The amniotic sac of the chick embryo was much more effective than monkey kidney cultures for isolation of the Asian strain of influenza A, and virus was obtained from at least 60% of serologically diagnosed illnesses. Both monkey kidney culture and chick embryo propagated virus agglutinated human, fowl, sheep and monkey erythrocytes at 4° or 24°C, but titers were lower with sheep red blood cells. Asian virus multiplied readily in monkey kidney cultures, but several passages were required for adaptation to the allantoic sac. Some strains during early egg passages were not inhibited by non-specific substances in normal sera, and some were initially non-reactive with antibody, especially in rde treated sera. Increased sensitivity to inhibitors and antibody occurred with additional chick embryo passages, but remained lesser than the tissue culture line. Asian virus propagated in monkey kidney cultures was preferable to the egg line for determinations of hemagglutination-inhibition and neutralizing antibody by virtue of the higher titers, comparability of response and reproducibility.

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Antibody Response to Asian Influenza Vaccination in Man.* (24267)

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Although much information has been available on the immune response and protective effects resulting from influenza vaccines, the novelty of the Asian strain of influenza A provided an opportunity for pre-epidemic investigations in populations that were essentially

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