as a source of infant antibody. If the mechanism involved this fluid the discrepancies between the 2 circulations would be expected to be much greater than the data indicate. Furthermore, many of the amniotic fluids do not have detectable antibody to some of the poliovirus types and the cord serums have appreciable levels of antibody.

Summary. Amniotic fluid of humans at birth has been demonstrated to contain poliocidal substance, active against all 3 serotypes of virus. Evidence has been presented which indicates that the activity is associated with the gamma globulin present in the amniotic fluid. The poliocidal activity is believed to be specific antibody. The presence of these antibodies has no correlation as far as can be determined with the age, color, polio immunization status of the mother nor with her previous obstetrical history. The level of antibody in the amniotic fluid for the 3 polioviruses was in proportion to the levels in the blood serum and in general was demonstrable when the serum level was high. There is also in amniotic fluid an inhibitor to the 3 polioviruses which is heat labile. Its character has not been determined. Enterobacterial hemagglutinins have been demonstrated in 1 of 6 AF tested.

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Porcine Kidney Cell Cultures for Propagation and Assay of Japanese Encephalitis Virus.* (24426)

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Observations in 1956 at U.S. Army 406th Medical General Laboratory near Tokyo, Japan showed that swine serve as important source of Japanese encephalitis (JE) virus for *Culex tritaeniorhynchus,* Japanese vector mosquito, (unpublished). This finding together with the knowledge that JE virus causes stillbirth in swine(1), stimulated inquiry into possible effects of JE virus upon swine tissues *in vitro.* The initial study employed cultures of swine kidney because the basic principles were known for preparation of primary cultures from renal tissues(2). This article reports that cells in primary cultures from porcine kidney (PK) are rapidly destroyed by each of 5 strains of JE virus and concurrently support virus multiplication. Cultures of swine kidney cells thus furnish a tissue culture system for assay and propagation of JE virus.

Materials and methods. Viruses. Four strains of JE virus were isolated in Japan during 1955 and 1956 from swine blood, Black-crowned Night Heron blood, human brain, and triturated mosquitoes (*Culex tritaeniorhynchus*) (Table I). The fifth virus was Nakayama strain from Dr. C. M. Eklund (USPHS Rocky Mountain Laboratory). Mouse brain suspensions (10% in 10-40% rabbit serum in Hanks' solution) and cultural fluids were stored in sealed glass ampules at −70°C for as long as 2 months before being assayed. Cultural fluids were kept above pH 7.2 by addition of 1.4% aqueous sodium bicarbonate solution to minimize inactivation of JE virus that occurs at lower pH's(3). Kid-

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neys were from fetuses to 18 inches in length (head to foot) from 59 swine of various breeds slaughtered at a local abattoir and from 16 specially bred miniature pigs(4) less than 8 months old.† Fetal kidneys were most satisfactory because kidneys from mature pigs yielded few viable cells with the methods employed and kidneys from young swine were difficult to obtain regularly. Renal cortex was minced to 1 mm diameter fragments and rinsed free of erythrocytes with Hanks' balanced salt solution (BSS) without sodium bicarbonate (RBSS). The fragments, in a 100 ml screw capped bottle, were covered by Difco 1:250 trypsin, 0.25% in BSS at pH 7.6, and stirred on a magnetic mixer at 5°C for 15-20 hours or until they disappeared. Cells were sedimented at 38 g for 5 minutes, rinsed 2 or 3 times with 10-20 volumes of RBSS and suspended in measured volume of swine serum, 20%, and BSS, 80% (SwS-20, BSS-80) with or without Difco yeastolate at 0.1% final concentration. Penicillin and streptomycin were incorporated at 50-100 μg/ml. Cells were counted under 200x magnification in a hemocytometer, the cell concentration adjusted with SwS-20, BSS-80 to 10^6 cells/ml, and 0.5 or 1 ml ($5x10^5$, or 10^6 cells) inoculated into each 16 x 125 mm screw capped test tube. Cultures were incubated in stationary slanted position at 36-37°C for 2-4 days before medium was changed or supplemented by addition of 1 ml of SwS-20, BSS-80. After another 3-4 days of incubation either the sheets of epithelial cells were satisfactory for virus experiments or the medium was again changed and incubation continued for several additional days. *Cultivation of virus in PK cultures.* The medium was changed to 0.9 ml of a mixture of swine serum, 5% (or 20% in one experiment), yeast extract, 0.1% (optional), penicillin and streptomycin, 50-100 μg/ml, and maintenance solution(5). To initiate experiments, mouse brain suspensions of virus were inoculated, 0.1 ml/tube culture, while for subsequent passages, harvests of cultural fluid and cells were made from 2-6 cultures when greater than 80% of cells were destroyed. For growth curve experiments, fluid and cells were pooled from 3 cultures at selected time intervals regardless of CPE. Incubation was at 36-37°C in a stationary position. Virus was assayed usually simultaneously in mice and PK cultures by inoculating decimal dilutions, in 0.03 ml volume, intracranially into 5 or 6 3-4 week old weanling Swiss albino mice or in 0.1 ml volume into 2 or 3 PK cultures containing 0.9 ml of SwS-5, MS-95/culture. For the Nakayama strain 1 ml of diluted virus (in SwS-5, MS-95) was placed in tube cultures void of fluid medium. Mice were observed for 10 or 14 days and cells for 7 days before LD_{50} and TCD_{50} were calculated(6). Virus from cultures was identified by virus dilution neutralization tests in PK cultures and/or in mice using specific rabbit antiserum made either at 406th Medical General Laboratory against the Nakayama strain, or at the USPHS Rocky Mountain Laboratory (furnished by Dr. C. M. Eklund).

Results. Evidence that JE virus propagates in porcine kidney cells was obtained by 2 types of experiments: 1) passage of virus through a series of cultures, and 2) measurement of virus growth in a single set of cultures. The cytopathogenic effects (CPE) of virus became evident within 2-5 days after inoculation of PK cultures with each of 4 virus strains, either as mouse brain suspensions or harvests from PK cultures. With Nakayama strain, total cell destruction did not occur until 6-10 days after inoculation.

Serial passage of JE virus in porcine kidney cultures. Five strains of JE virus derived from different natural sources were inoculated as mouse brain suspensions into PK cultures, and subsequently passed serially 5-28 times (Table I). Each passage was made when 80% or more of cells were destroyed, *i.e.,* at 2-4 day intervals for recently isolated strains, and at 6-10 day intervals for high mouse-and egg-passaged Nakayama strain. Mouse infectivity of each virus was maintained for 20-132 days, and although original mouse brain suspensions were cumulatively diluted 10^{6-35} times, virus titers of cultural harvests remained $10^{-5.0}$ to $10^{-8.0}$ LD_{50}/ml for PK cultures and for weanling mice (Table I). Of

† We are indebted to Swift Co. for weekly supply of porcine kidneys and to Hormel Fn. for kidneys from their specially bred line of miniature pigs.

TABLE I. Serial Passage of JE Virus in Porcine Kidney Cell Cultures (5 Experiments).

JE virus strains						Virus harvested from serial passages as determined by simultaneous titrations of	
Original source and designation	Mouse brain passage used as original inoculum	No. of virus passage in PK cultures	Duration of virus passage (days)	Total days in culture	Cumulative log dilution of mouse brain inoculum	CPE in PK cultures, $-\log TCD_{50}$/ml	Viral lethality in weanling mice, $-\log LD_{50}$/ml
Blood from pig in mosquito trap, Sagiyama, 21 Aug., '56 Pig-6	2	[mouse brain inoculum]				(6.3)	(8.9)
		1	4	4	4	4.5	7.3
		6	4	19	11	6.6	6.0
		11	3	35	16	6.3	5.1
		18	4	61	25	7.0	5.2
		21	3	69	28	5.5	4.7
		24	4	83	31	5.2	6.3
		27	4	93	34	4.8*	n.t.†
		28	4	97	35	5.8	5.0
Blood from wild Black-crowned Night Heron, Shinhama, 29 July, '55 281801	4					(7.5)	(9.3)
		6	4	23	7	6.7	5.0
		7	4	27	8	5.0*	n.t.
Mosquito pool (14 *C. tritaeniorhynchus*), Sagiyama, 8 Aug., '55 M5/596	3					(6.5)	(10.2)
		4	4	18	5	5.0*	n.t.
		5	2	20	6	6.5	5.4
Human brain, Yokosuka, 5 Sept., '55 55-1735	3					(5.5)	(9.2)
		6	2	20	7	6.6	5.8
		8	4	27	9	n.t.	5.7*
Human spinal fluid, Japan, 1935 Nakayama	>40 (+41 egg passages)	1	10	10	1	5.2	8.2
		5	7	42	5	5.2	8.0*
		10	6	72	10	6.2	8.3
		15	6	102	15	6.5	7.5
		20	6	132	20	6.7	7.1

* CPE or mouse infectivity of virus was neutralized with specific JE virus rabbit antiserum.
† n.t. = not tested.

interest were changes in ratio, mouse LD_{50}/PK culture TCD_{50}, that occurred with serial passage of virus. Whereas each strain of virus as mouse brain suspension or first PK-passage harvest titrated significantly higher in weanling mice than in PK cultures, virus derived from fifth or higher passages in porcine kidney cells assayed approximately as high in PK cultures as in mice (Table I). This finding has significance since it indicates that PK-passaged JE virus can be detected as well in PK cultures as in weanling mice, and that PK-adapted virus could be employed for assay of serum neutralizing antibodies in PK cultures.

Cytopathogenic effect of JE virus for porcine kidney cells and its neutralization by specific antibody. Each of 4 strains of JE virus in low mouse passage (Table I) regularly destroyed 80-100% of PK cells in 2-5 days, even at terminal dilutions in virus titrations; Nakayama strain did not totally destroy cells until 6-10 days after inoculation. Cells first rounded, then aggregated in clusters, and finally detached from the glass. Usually peripheral cells of epithelial sheets were first affected, but central foci of degeneration were also seen. Uninoculated cells and cells undergoing destruction by JE virus are presented for comparison in Fig. 1 and 2.

The cytopathogenic effect as well as mouse infectivity of JE virus could be neutralized by specific rabbit antiserum. Each strain of virus employed for serial passage experiments

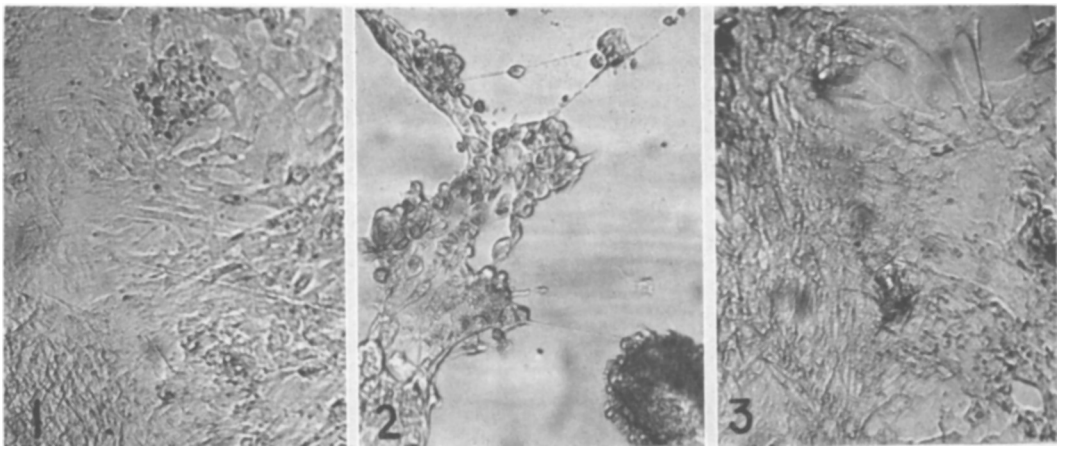

FIG. 1-3. Cytopathogenic effect of Japanese encephalitis virus (pig-6 strain of 27th passage, Table I) for porcine kidney cells in test tube cultures; neutralization of CPE by specific rabbit antiserum. Final magnification, × 100.

FIG. 1. Normal cells, uninoculated with virus.
FIG. 2. Cells 3 days after virus inoculation.
FIG. 3. Cells 3 days after inoculation of virus admixed with antiserum.

was neutralized either in PK cultures or in mice at passage levels indicated in Table I. Mouse infectivity of the human strain virus (55-1735) as 8th PK cultural harvest was reduced from $10^{4.2}$/0.03 ml without antiserum to $<10^{0.9}$/0.03 ml in the presence of antibody. A photograph of cells protected from destructive effects of pig-6 virus by antiserum is shown in Fig. 3.

Growth curve of JE virus in porcine kidney cultures. Growth curves were determined for 2 strains of JE virus in PK cultures to learn the optimal time for harvest of virus. Pig-6 and mosquito 5/596 strains of JE virus were inoculated as mouse brain suspensions into PK cultures in quantities calculated to yield 6.9 and 4.3 mouse LD_{50} of virus/ml of cultural fluid immediately after inoculation. Quantities of mouse infectious virus in pooled cells and fluid were then determined 1, 3, 4, 5 and 7 days after inoculation. Virus grew in cultures of porcine kidney cells and reached maximal titers during 3-5 days after inoculation (Fig. 4). Thereafter essentially 100% of cells were destroyed and virus merely deteriorated.

Discussion. Until recently JE virus was in the group of arthropod-borne viruses which fail to exhibit a consistent cytopathogenic effect in tissue culture. Although some arbor viruses, such as Western equine encephalomyelitis and West Nile fever regularly destroy HeLa(7,8), Henle's human intestinal cells(8), monkey kidney(9), and chick embryonic tissue cultures(9), JE virus has with 2 exceptions failed to produce total and/or consistent CPE(7,8,10). The exceptions are the report of McCollum and Foley that 2 strains in 4th and 8th mouse passages incompletely destroy Detroit-6 human epithelial cells(10), and the recent discoveries of Kissling(11), and Diercks and Hammon(12) that high mouse passage Nakayama strain produces CPE and can be titrated in primary cultures of hamster kidney. The findings reported herein with porcine kidney cells thus provide a second cell culture system in which JE virus can be assayed. However, because porcine kidneys are available in almost unlimited supply and at little expense from abattoirs, they may prove to be under certain circumstances, potentially superior to hamster kidneys for mass production of virus and widespread, inexpensive utilization of tissue cultures in study of JE virus. Moreover, it is conceivable that PK cultures might isolate virus strains biologically different from the mouse pathogenic strains currently known, and that their inexpense and ease of mass production may make them equal or superior to mice in tests for neutralizing antibody. The possibility that a vaccine for Japanese encephalitis could be made either

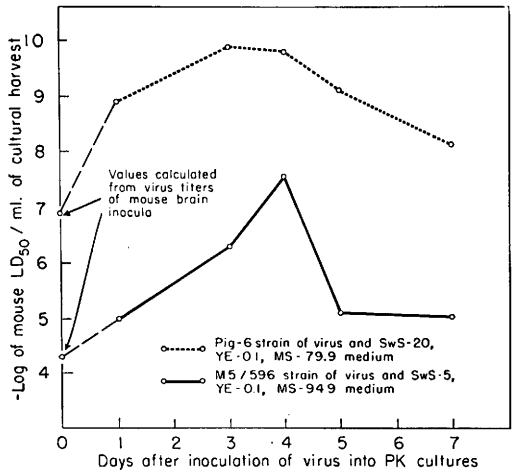

FIG. 4. Growth curves of Japanese encephalitis virus in porcine kidney cultures.

from inactivated or attenuated virus grown in porcine or hamster kidney cultures also deserves exploration because the mouse brain- and chick embryo-derived vaccines now available have been unsatisfactory for large scale use in humans. Since swine are plentiful in Asia, porcine kidneys would be readily available for vaccine production in countries where Japanese encephalitis occurs.

The observation that JE virus grows in porcine kidney cells *in vitro* cannot currently be related to the pathogenesis of swine infection. Although Japanese pigs are frequently infected in nature and develop viremia and antibodies (unpublished observations), they rarely manifest disease (encephalitis or stillbirth). Whether the kidney *in vivo* is a site of virus proliferation following natural infection by mosquito bite remains to be determined, although if virus actually grows in swine kidneys, it certainly does not destroy cells as extensively as *in vitro* since no obvious renal pathology occurs. Similar, incompletely understood, situations also exist with the viruses of Newcastle disease, fowl plague, hemagglutinating virus of Japan and vesicular exanthema which have recently been reported to grow in porcine kidney cultures(13-15).

Summary. Cells cultured *in vitro* from porcine kidneys support growth of JE virus and are concomitantly destroyed. Each of 5 virus strains (from pig, bird, mosquito and man) was propagated serially through 5-28 passages. Virus harvests from porcine kidney cultures titrated 10^{5-8} ID_{50}/ml in mice and in PK cultures. Total destruction of cells by recently isolated viruses in low mouse passage occurred usually within 2-5 days after inoculation of cultures, and could be prevented by specific antiserum.

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