treponemes and numerous artifacts are factors which can make interpretation difficult. The indirect FA procedure is time-consuming and employs a non-specific antibody. Neither of these two procedures can detect *T. pallidum* present as a minority in a mixture of treponemes. The RIS technique specifically stains *T. pallidum* in smears from syphilitic lesions in less time than would be required for making a gram stain preparation of other microorganisms.

These results indicate that a further evaluation of this process should be made. Confirmation of our conclusions may allow smears to be collected at distant points and shipped to central laboratories for rapid and specific diagnosis.

Summary. An immunofluorescent staining technique has been devoloped which specifically stains *T. pallidum* or *N. gonorrhoeae* in less than one minute. Application of the technique to direct smears of either organism has demonstrated its practicability as a diagnostic tool.

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Characteristics of Five Rhesus Monkey Kidney Cell Lines. (29091)

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The monkey kidney tissue cultures commonly used in studies of viruses are primary: Monkeys are killed and cells from their kidneys are cultured usually on a glass surface for 3 to 12 days. The cultures are then inoculated with viruses, or control materials. Such a procedure has several drawbacks, including the following: (a) The monkey colony is often seriously depleted, especially when the purposes of the virus research require thousands of such cultures. (b) The cultures themselves often carry viruses.

From September 1961 to January 1962, we tried to establish monkey kidney cell lines with 24 batches of cells from rhesus monkeys *(Macaca mulatta)* and 3 batches from cynomolgus monkeys *(M. irus)*. These batches were among those routinely supplied in our laboratories for investigations of the enteroviruses and certain other viruses. Five of these 27 attempts succeeded. In this report we describe the distinctive morphological features of these 5 lines, compare them with primary cultures in susceptibility to intact "wild-type" polioviruses and to ribonucleic acid (RNA) therefrom, and characterize 8 poliovirus mutants selected through passages in these lines.

A note on the RNA susceptibility of these lines appeared earlier(1).

Materials and methods. Primary cultures. The primary cultures were prepared in our laboratory by a standard procedure: Diced kidneys from rhesus or cynomolgus monkeys were digested with trypsin to obtain cell suspensions, which were finally suspended either in lactalbumin growth (LG) medium(2) or in medium S, which is high-cystine altered Eagle's maintenance ($hcAE_m$) medium(3) containing 4% calf serum. Usually 2-5 monkeys were used in the preparation of each batch of cells. The calf serum used in the

media LG and S had been Seitz-filtered. The cells in medium LG or S were delivered to bottles, tubes or Petri plates, which were then kept for 5 to 11 days at about 37°C in a humidified incubator continuously flushed with 95 to 97% air and 3 to 5% CO_2; the bottle caps were left slightly loose and the tubes were cotton-plugged.

Passages of cultures. Other kinds of tissue culture cells were not handled in the laboratory room during these passages. Cells were scraped from glass with cylindrical wooden applicator sticks 2 mm in diameter and commonly about 200 mm long. Part of the cell suspension was transferred to one or more fresh containers and fresh medium S was added to each. The containers were commonly 4.5 × 4.5 × 15.5 cm screw-cap Pyrex bottles. Incubation for growth was the same as for the primary cultures. Medium S was used since it supported more rapid growth of primary cultures than did medium LG.

Wild-type polioviruses. Three wild-type polioviruses were used: those of the Brunhilde (antigenically type 1), Brooks (type 2), and Mabie (type 3) strains.

Poliovirus stocks and passages. Supernatant stocks were obtained by passaging virus on cultures under medium $hcAE_m$ or S without agar; the supernatant suspension was harvested when viral cytopathic action was complete, or nearly complete. Plaque stocks were prepared in the same way except that the medium contained agar; plaques were revealed with neutral red, and the harvest was made by suspending a cylinder of agar from a plaque in 1 ml phosphate-buffered saline (PBS)(4).

Production of plaques. Essentially the method of Dulbecco and Vogt(4) was used: Plaques developed in 60-mm Petri plates on cultures under medium $hcAE_m$ or S containing 0.9% washed agar. The volume of agar-$hcAE_m$ or agar-S added per plate varied from 3 to 6 ml, depending on the length of this plaque development period, the number of ml roughly corresponding directly to the number of days. During plaque development, plates were kept at about 36°C in a continuous-flow incubator, as above, for 3 to 6 days. Neutral red was then added and plaques scored. Relative efficiency of plating (eop) is expressed herein as the titer of a viral stock on the system being tested divided by its titer on a standard system; the standard system used was plaque development for 3 days on LG-grown primary cultures maintained under $hcAE_m$.

Poliovirus RNA preparations. Wild-type Brunhilde poliovirus preparations having ribonuclease-labile infectivity were obtained by the monophasic phenol method(3,5): To 7 volumes of water-saturated phenol (0°C) were added 93 volumes of a supernatant stock of Brunhilde at 0°C. After mixing till all the phenol was dissolved, the mixture was kept at 0°C for 5 minutes. Phenol was then removed by 3 serial extractions with diethyl ether; residual ether was evaporated.

Facilitation of infection with RNA. The system employing undissolved facilitator(6) was used: Cultures were depleted of calcium and the RNA inocula contained 0.5% talc.

Test of progeny from poliovirus variants. The genetic nature of the 9 variant polioviruses obtained in these studies through passages on cultures, primary or nonprimary, under medium S was tested as follows: Stocks were prepared from plaques produced by the variants on primary cultures under $hcAE_m$. The viruses in these stocks were characterized for the property distinguishing their progenitor variant from wild-type. Variants yielding such progeny like themselves in the property concerned are termed genetic variants, or mutants.

Results. Establishment of the five lines. All 27 of the primary cultures used in attempts to establish lines had grown rapidly and appeared to be "healthy" at the time of starting the secondary cultures. Growth continued to be rapid, or moderately rapid, for awhile. In all 27 attempts, however, growth became very slow between the second (secondary cultures) and sixth passage levels; this was 1 to 4 months after starting the primary cultures. Twenty-two of the attempted lines died at this time or were aban-

TABLE I. Starting Dates, Passage Levels, and Some Characteristics of the 5 Rhesus Kidney Lines.

Line designation	Date (1961) primary started	Current* passage levels	Relative rates of growth†	Some morphological characteristics
α	Sept. 29	19-22	++++	Cells in whorls.
ϵ	Oct. 12	16-20	+	" often granular.
η	" 19	29-44	++++	Very compact colonies.
κ	" 31	35-37	++++	Cells often with large vacuoles.
π	Nov. 21	24-29	++	Very clear cells.

* Dec., 1963.

† A rough guide to relative growth rate under medium S; recently, however, rates of growth of α and π have decreased considerably.

doned because of their nearly complete failure to multiply. One of these 22 exhibited a cytopathology similar to that associated with several of the enteroviruses; type 1 poliovirus was isolated from the supernatant over one of the dying cultures of this attempted line.

Five of the attempted lines "spontaneously" began growing rapidly again. This rapid growth occurred "focally"; that is, at 1 to about 6 loci in the culture, colonies of rapidly growing cells appeared. Cells scraped from these colonies grew rapidly, either at new places on the glass of the same container or of a fresh container. The designations, starting dates, current passage levels, relative growth rates, and some morphological characteristics of these 5 lines are given in Table I.

Tests for cytopathic agents in the lines. Supernatants from cultures of all 5 lines were inoculated onto primary cultures, which were incubated for 7-10 days and then stained with neutral red. None of the supernatants exhibited any cytopathic effect. The supernatants tested were from: α culture, 7th passage level; ϵ, 9th; η, 7th; κ, 10th, 17th and 18th; and π, 7th.

Maintenance of the cultures under agar media. The standard agar-hcAE$_m$ medium was adequate for maintenance of the ϵ and π cultures during the plaque development period. With α, η, and κ cultures usually a fairly large fraction of the cells died when the cultures were kept under this medium. Cultures of these 3 lines, however, were adequately maintained under agar-S medium. Primary cultures were of course adequately maintained under either of these 2 media.

Comparative susceptibility of the 5 lines to intact wild-type polioviruses. The 5 lines were compared with primary cultures as hosts for plaque production by 3 wild-type polioviruses. Each of the 5 was found to be susceptible to all 3 viruses; in most cases, however, fewer and smaller plaques were produced on the line than on the primary culture. Average values derived from many experiments are shown in Table II; longer plaque development periods were used for the line cultures because of the usually slower plaque growth on them than on primary cultures. The π line decreased in susceptibility during the course of the experiments; when relative eop is used as the criterion, π is the least susceptible line. The κ line, on the other hand, has maintained its rather high susceptibility through many passages over many months.

Comparative susceptibility of the 5 lines to RNA from wild-type Brunhilde virus. Using undissolved facilitator, we tested several batches of the 5 lines and of primary cultures for susceptibility to Brunhilde RNA. All 5 lines were susceptible but apparently not equally so (Table III); the ϵ line was the most and α the least susceptible. The variation in susceptibility among batches of a line is probably related to the age of the batch at time of inoculation with the RNA; such variation in age is a major source of variation in susceptibility among primary batches (1). When facilitator was not used, few or no plaques were produced by RNA on any of the lines(1).

Capacity of the 5 lines to select poliovirus

TABLE II. Comparisons of Primary and Line Cultures in Susceptibility to Wild-Type Polioviruses.

Plaque development system				Avg relative eop			Avg plaque diameter (mm)		
Cell	Growth medium	Maintenance medium	Days	Bh*	Bk*	M*	Bh	Bk	M
Primary	LG	$hcAE_m$	3	1	1	1	6.0	4.0	3.8
"	S	"	3	1.4	2.3	1.1	6.1	5.3	4.5
"	S	S	3	1.2	.96	.80	5.0	3.1	2.1
α	S	S	6	.21	.38	.36	2.7	2.3	2.1
ϵ	S	$hcAE_m$	4; 5†	.35	.21	.43	4.0	2.0	2.4
η	S	" S‡	4	.19	.37	.086	4.1	6.5	1.8
κ	S	" S‡	4	1.0	.46	1.3	3.6	8.0	2.2
π (e)§	S	"	4	1.6	1.0	.81	5.2	3.0	3.5
π (l)§	S	"	5; 6‖	.053	¶	.0047	1.8	¶	3.0

* Bh = Brunhilde, Bk = Brooks, and M = Mabie.
† 4 days for Brooks; 5 for the other two.
‡ $hcAE_m$ for Brooks; S for others.
§ e = early π passage (7th); l = late (23rd for Brunhilde and 24th for Mabie).
‖ 5 days for Brunhilde; 6 for Mabie.
¶ Not done.

mutants. Passages were initiated with wild-type Brunhilde and Mabie viruses on each line and, for comparison, on medium S-grown primary cultures, thus starting 12 series for possible selection of mutant polioviruses. Ten serial passages under medium S were made for each of these 12; for each passage the inoculum was about 10^3 to 10^6 plaque-forming units. Using specific antisera, the antigenic type of each of the 12 tenth-passage levels was confirmed. The virus in each of these 12 was then compared with the corresponding wild-type virus. For the 10 where the passages had been on line cells these comparisons were in sizes of plaques produced on primary and corresponding line cultures and relative eop on such line cultures; for the 2 primary-passaged tenth levels the comparisons were made using all 6 kinds of cultures. In 8 of the tenth-passage levels the virus was found to be different from the corresponding wild-type virus; in 1 of these 8 (π-passaged Brunhilde) 2 different variants were detected. Cloned stocks of each of these 9 variant viruses were prepared as follows: From the second of 2 serial plaque purifications a supernatant stock was prepared, all 3 passages being in the corresponding system. All 9 variant viruses were shown to differ from wild-type genetically (see *Materials and methods*). The differentiating characteristics of these 9 mutants are shown in Table IV. The mutants are designated *m (minute-plaque)* or *r (rapid)*, with a subscript indicating the culture used for the serial passages. The 3 *m* mutants exhibited relative eop's on their corresponding line systems markedly greater than those of wild-type Brunhilde: 19 times greater for the $m\alpha$ mutant, 5 times for $m\epsilon$, and 64 for $m\pi$ (compare data of Tables II and IV). Also the 3 *m* mutants produced smaller plaques than wild type. The differences in size were particularly large on primary cultures; this difference between wild type and $m\pi$ is shown in Fig. 1. The r_{pr} mutant produced somewhat larger plaques on primary cultures

TABLE III. Comparison of the 5 Lines with Primary Cultures in Susceptibility to Poliovirus RNA.

Culture*	Plaques/plate† Individual cell batches	Mean of means
Primary	40 ± 3; 28 ± 3; 14 ± 2; 11 ± 1.3; 7 ± 1.2	20
α	$1.5 \pm .5$; $<.25$; $<.5$; $<.2$; 6 ± 2; $<.12$	1.2
ϵ	95 ± 15; 50 ± 10; 60 ± 38; 86 ± 4	73
η	5.5 ± 1.5; 5.0 ± 1.2; 18 ± 1.5; 28 ± 2	14
κ	4.5 ± 1.5; $.38 \pm .26$	2.4
π	40 ± 11; $3.4 \pm .6$	22

* All were grown under medium S and then calcium-depleted.
† Each plate received 0.3 ml PBS without $CaCl_2$ containing 0.5% talc and a Brunhilde RNA preparation at concentration 0.05; infection period was at 37°C for 1 hour.

TABLE IV. Characters of Viral Mutants Selected.

Strain	Mutant	Plaque development system*	Avg relative eop†	Avg plaque diameter (mm) †
Brunhilde	m_α	pr. (Lh)	1	1.4
		α	3.9	2.0
	m_ϵ	pr. (Lh)	1	1.4
		ϵ	1.7	2.0
	m_π	pr. (Lh)	1	.62
		π (l)	3.4	.73
	r_π	pr. (Lh)	1	6.3
		π (l)	.25	3.1
Mabie	r_{pr}	pr. (Lh)	1	4.2
		pr. (SS)	.49	3.1
	r_α	pr. (Lh)	1	3.6
		α	.48	3.2
	r_η	pr. (Lh)	1	4.6
		η	.24	2.3
	r_κ	pr. (Lh)	1	2.9
		κ	3.1	2.8
	r_π	pr. (Lh)	1	4.1
		π (l)	.034	5.8

* See Table II. pr. = primary cultures; Lh = LG-grown and $hcAE_m$-maintained, and SS = S-grown and S-maintained. Development on ϵ cells was for 5 days and on π cells was for 5 days for Brunhilde and 6 for Mabie. Tne η and κ cells were S-maintained.

† For corresponding data for the 2 wild-type viruses see Table II.

than did wild type (Tables II and IV); limited tests suggested that also on ϵ and η cultures its plaques were somewhat larger. The other 5 *r* mutants exhibited higher relative eop and produced larger plaques than wild type on their corresponding line cultures. On primary cultures, plaques of Mabie r_η and r_π were slightly larger whereas those of Mabie r_κ were smaller, more turbid, and more irregularly shaped than the plaques of wild type.

Discussion. Hull, Cherry and Tritch(7) established 2 rhesus kidney lines from primary cultures initiated in 1955, and Takaoka *et al*(8) established a cynomolgus kidney line from a primary culture initiated in 1961. The results of both of these groups of investigators suggest that their monkey kidney lines should be useful for viral studies. Our 5 rhesus kidney lines, particularly the rapidly growing η and κ lines, should also be suitable, convenient, and relatively economical for several kinds of research with the enteroviruses, and perhaps with some other viruses.

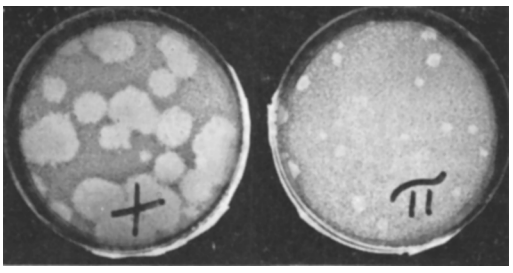

FIG. 1. Comparison of Brunhilde wild-type (+) and m_π mutant (π) viruses. Plaques developed for 3 days on medium S-grown primary cultures maintained under medium $hcAE_m$.

Though an excellent medium for growing primary cynomolgus or rhesus kidney cultures, S is inadequate to support continued rapid growth of these kidney cells. Apparently, however, in these cultures there do sometimes occur a very few cells which have the capacity to continue to grow rapidly under this medium.

We cannot conclude that our 5 lines carry no cytopathic agent: Though our tests of supernatants from the lines detected no such agent, other tests in other systems might. Furthermore, tests of the lines at passage levels later (or earlier) than those tested might reveal such agents; this would of course depend on the time of "contamina-

tion" with and on the state (latent?) of such agent.

Four of our 5 lines became more resistant than primary cultures to wild-type polioviruses; the relative resistance of the later passage levels of the π line was particularly marked. The fifth line (κ) remained highly susceptible; using eop as criterion, it was consistently even slightly more susceptible than primary cultures to the type 3 poliovirus used. These results are rather similar to those of Hull, Cherry, and Tritch(7), who found their 2 lines, when tested uncloned, to be slightly more resistant than primary cultures to type 1 poliovirus, and to the results of Takaoka *et al*(8), who found their cynomolgus line to be clearly less sensitive than primary cultures to type 1 polioviruses; both of these groups of workers used end point titrations in tube cultures under liquid medium.

Some of the 9 mutants described herein may be identical with each other: Insofar as tested, the Brunhilde m_α and m_ϵ mutants did not differ significantly from each other; nor did the Mabie r_{pr}, r_η, and r_π mutants. Because of the relative ease of distinguishing them from wild type, the 3 *m* mutants and the Mabie r_κ mutant should be appropriate for certain studies requiring genetically marked polioviruses.

Summary. Five lines of rhesus monkey kidney cells were established. Four of the 5 became more resistant than primary rhesus kidney cultures to wild-type polioviruses; the sensitivity of the remaining line (κ) to wild-type polioviruses was roughly equal to that of primary cultures. All 5 lines were susceptible to poliovirus RNA, though some were more susceptible than others. Passages of polioviruses on the lines selected 3 *minute-plaque* and 5 *rapid* poliovirus mutants.

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Multiplication of Canine Hepatitis Virus in the Presence of 5-Fluorodeoxyuridine.* (29092)

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In a recent study(1) canine hepatitis (CH) virus was observed to increase in cultures of dog kidney (DK) cells when the cultures had been treated with 5-fluorodeoxyuridine (FUDR) at concentrations that inhibit other DNA viruses. This lack of inhibitory effect of FUDR upon CH virus was observed in cultures incubated 30 hours or longer but not in cultures incubated 18 to 24 hours. A possible explanation for this lack of inhibition is presented in this report. Further studies have been made of cultures with and without FUDR; changes in cell morphology, DNA, and virus were determined.

Materials and methods. The procedure

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