able container were found.

The rapid adsorption of myxoma virus to cell monolayers which is achieved by using centrifugal force will facilitate studies on the growth cycle of this virus and on virus-cell interactions during the early stages of viral replication. Also, it is now feasible to investigate such matters as rate of heat inactivation of myxoma virus at 37°C; a study which would have been meaningless with a plaque procedure requiring a 4-hour adsorption period.

Summary. Centrifugation of myxoma virus onto preformed monolayers of RK and RHF-1 cells substantially increased the number of PFU compared to other methods of virus adsorption. The maximum number of PFU was obtained after only 15 minutes of centrifugation at 1900 *g* at room temperature. Virtually simultaneous infection of all RK cells growing as a monolayer on the bottom of shell vials can be accomplished by centrifuging a low multiplicity of myxoma virus onto the cells at 1270 *g* for 10-20 minutes.

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Induced Latent Infection of Monkeys with Vacuolating SV-40 Papova Virus. Virus in Kidneys and Urine.* (27794)

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In nature, vacuolating or SV-40 virus(1), the simian member of the papova virus group (2), commonly infects rhesus (*Macaca mulatta*), less commonly cynomolgus (*M. irus* or *M. cynomolgus*), and apparently infects African primates (*Cercopithecus* spp., *Papio* spp.) only if these are placed in contact with infected Asian animals(1,3). When inadvertently given to human beings, low grade subclinical infection has occurred(4,5). Although no illness has been observed in primates (monkeys or man) infected with SV-40 virus, the agent is tumorigenic in newborn hamsters, producing fibrosarcomas(6,7).

The present report forms part of a study with the latent infection in African primates (green monkeys and baboons) infected experimentally with a purified line of the virus. Two features of the study reported here have been (a) to follow the animals for excretion of virus and subsequently of antibody in the urine, and (b) to follow the persistence of latent virus in the kidneys. The latter was done by carrying out repeated biopsies on the same monkey and testing the biopsy tissue for virus by direct and indirect means.

Materials and methods. The study included 23 primates: 7 baboons (*Papio doguera*) about 6 months old, and 16 green monkeys (*Cercopithecus aethiops*). The green monkeys included 8 young animals about 1 year old, 4 newborns about 7 days old and their respective mothers. All animals were flown from Africa directly to Houston or to New York, where they were kept separate and sent on within a day or two.

The routes and amounts of virus inocu-

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TABLE I. Recovery of SV-40 Virus and Antibody in Urine of Inoculated Monkeys.

Monkey	Route	Wk after infection initiated									
		0	1/3	1	2	3	4	6	8	10	12
G - 2	i.k.		0	+	+	+	+	(>4)*			
G - 3	"		0	+	+	+	0	0		(>8)	(8)
G -10	i.c.						(>16)				(4)
G -11	"			+	+						
G -12	"			0	+		(0)				
G -13	s.q.			+	+	+	(>16)				
G -14	"					+	(4)				(4)
G -15	"				+						
BG-17	"						+		+		
BG-22	i.pul. + s.q.				+		+	+	D		
B - 1	i.k.		0		+	0	(2)				
B - 4	i.q.	(0)		0	+			(8)			(>16)
B - 5	"	(0)			+		+				(2)
B - 6	"		0	+	0		0	(8)			(2)
B - 7	i.n.			0	0			S			
B - 8	"	(0)	0	0			0		(0)		(4)
B - 9	oral	(0)	0				0	(2)			

G, green monkey; BG, baby green monkey; B, baboon.

i.k., into the kidney; i.c., intracerebral; s.q., subcutaneous; i.q., intracutaneous; i.n., intranasal; i.pul., intrapulmonary.

D, died; S, sacrificed.

* Figures in parentheses indicate antibody titers. All specimens tested for antibody had previously been examined for virus but none was found.

lated are shown in Table I. One baboon was inoculated directly into the kidney with 0.4 ml, three baboons were inoculated intracutaneously in 20 piqures of 0.1 ml each in both hind legs, 2 baboons had 1 ml instilled into each nostril, and one baboon was given 5 ml of virus orally.

Two green monkeys were inoculated into the kidneys with 0.4 ml virus suspension, 3 were inoculated intracerebrally with 0.4 ml in each hemisphere, 3 were inoculated with 2 ml subcutaneously. Of the babies, 3 were inoculated subcutaneously with 2 ml, one baby was inoculated with 1 ml in the right lung and 0.6 ml subcutaneously. The mothers were left uninoculated to follow contact infection. Two babies are not included in the table because of early death, 5 and 7 days after inoculation. The mothers are omitted from this report because too little time has elapsed for a meaningful follow-up.

Virus. The virus used was isolated(8) from Sabin's seed stock of type 3 oral poliovaccine and purified by passing 3 times at terminal dilution in continuous line green monkey cells (BSC-1). This cell line was developed and kindly supplied by Hopps and Smadel(9) who showed that it was highly susceptible to SV-40(9,3). Stock virus was prepared by inoculation of 16-ounce bottle cultures, 12 days after seeding with BSC cells. The nutrient fluid was drained, 0.5 ml of seed virus was inoculated, adsorbed for 4 hours, and 20 ml of medium 199 with 2% fetal bovine serum were added. Four days later 75% of the cells showed cytopathic changes (CPE). They were frozen and thawed 3 times before use. The fluid was centrifuged at 1500 rpm for 10 minutes, dispensed and stored at –20°C. The titer of the stock virus was $10^{7.5}$ TCD_{50}/ml.

Collection of urine. Monkeys were immobilized and allowed to urinate on sterile aluminum foil. Some samples were collected by bladder puncture.

Kidney biopsies were performed percutaneously on monkeys under Sernyl† anesthesia with the Franklin modification of the Vim Silverman needle. The biopsies were performed on alternate kidneys, *i.e.*, first time on the left kidney, the second time on the right, etc. Part of the biopsy specimen was fixed in Zenker's fluid, embedded in paraffin and the sections stained with hematoxylin and

† Kindly supplied by Parke Davis & Co.

eosin were examined for histologic lesions.‡ The remaining part was washed free of body fluids (antibody) and part was grown in tissue culture by a method previously described for kidney fragments(11). Because of the small amount of tissue, we modified the method by planting 4 to 5 tiny pieces with a bent capillary pipette. Excellent results were obtained using new disposable culture tubes made of lime glass(12). The tubes are clean when received from the manufacturer; they are simply dry-sterilized before use. After the tissue was added, the tube was incubated at 37°C for 30 minutes. Then 0.4 ml of M-H medium containing 5% calf serum was added to bathe the fragments. After wetting the tissue, the tube was turned so that the fragments were on the top surface as in a hanging drop preparation. Once each day the tubes were briefly rotated, then reincubated as described above. On the 4th-5th day of culture, outgrowths of cells were seen. An additional 0.4 ml of M-H medium was added and the cells were now incubated on the bottom of the culture vessel so that they were constantly submerged in the medium. The next day, the medium was changed to M-E maintenance medium containing 2% calf serum. The tubes were observed every 2 days for CPE and were changed every 4-5 days. The cultures were kept for 30 days.

The fluid in which the washed pieces of kidney taken by biopsy were minced and the left-over fragments were frozen and thawed 3 times and inoculated in green monkey (GM) tube cultures which were then observed for CPE for 30 days.

Tissue cultures and virus isolations. BSC-1 cells(9) grown in tubes suspended in medium 199 + 20% fetal calf serum (FCS) were changed to medium 199 + 2% calf serum and inoculated. Green monkey (GM) kidney monolayer cultures were grown and maintained in lactalbumin (M-H) medium as described(10). In all experiments a number of uninoculated cultures was observed to insure that SV-40 was not a spontaneous contaminant of the test cultures.

‡ We are grateful to Dr. Harvey S. Rosenberg, Texas Children's Hospital, for examining these specimens.

The specimens to be tested for virus were treated with 1 mg of penicillin and 1 mg of streptomycin per ml. Three tube cultures were inoculated with 0.1 ml of test suspension. The inoculated tube cultures were observed for CPE every 2-3 days for 30 days. The nutrient medium was changed every 5 days.

Typing of isolates from kidney biopsies and urine. The CPE produced was typical of SV-40 but nevertheless isolates were typed against rabbit and monkey SV-40 antiserum. This was done by mixing 100 TCD_{50} of virus per 0.1 ml with 20 units of antiserum per 0.1 ml, incubating for 2 hours at 37°C, inoculating 0.2 ml of the serum-virus mixture into each of 3 GM tube cultures. The cultures were changed once at 6 days and observed for 14 days. A virus of known titer was run as a control in each test.

Neutralization tests with urine as a source of antibody. Two-fold dilutions of urine were mixed with equal amounts of virus dilution containing 100 TCD_{50}/0.1 ml and incubated for 2 hours at 37°C and overnight at 5°C. Parallel controls of urine collected before SV-40 inoculation were mixed with virus and incubated as above. (Such controls had no inhibitory effect on the virus.) The virus-urine mixture was inoculated into 3 tubes of GM cells, each tube receiving 0.2 ml. The cells were observed for 14 days and the level of antibody was considered as the reciprocal of the maximal dilutions preventing CPE. At the same time the control virus titration showed 3-4+ CPE with the 100 TCD_{50} concentration and 1-2+ CPE with 10 TCD_{50}.

Results. Viruria. Nine of 10 green monkeys tested yielded virus in the urine (Tables I and II). Of 26 specimens collected in the first 4 weeks after infection, 18 were positive. In the 2 babies that survived more than 4 weeks, virus was recovered from the urine 5

TABLE II. SV-40 Virus and Antibody in Urine.

Monkey	Material	Weeks after inoculation 1–3	4–6	8–12
Green	Virus	16/18	4/11	1/1
"	Antibody	—	4/11	4/4
Baboon	Virus	4/4	1/5	—
"	Antibody	—	4/10	4/5

TABLE III. Recovery of Virus from Kidney Biopsies.

	Months after inoculation					
	3	6	8	3	6	8
	Green monkeys			Baboons		
Spontaneous appearance of virus in outgrowth from biopsy	5/8	3/6	1/2	1/6	0/6	0/1
Virus in suspension of ruptured kidney cells	0/8	0/6	0/2	0/6		

and 8 weeks after exposure to the virus. In some animals the titer of virus in the urine reached as high as $10^{6.5}$ TCD_{50}/ml.

Virus was recovered from all 4 baboons that were inoculated parenterally; 5 of 12 samples were positive. Three baboons given the virus intranasally or orally failed to yield the virus in 7 urine specimens examined.

Neutralizing substance in the urine. Starting in the 4th week after infection, when viruria was waning, neutralizing substance appeared in the urine. Of 18 urine specimens which had been tested and found negative for virus, 16 were positive in the neutralization tests (Table I). The titer of urine for neutralizing substance ranged from 1:2 to more than 1:16. The positive samples when retitrated yielded essentially the same endpoints. Attempts to dissociate a hypothetical virus-antibody complex in the urine by Mandel's low pH method(13) were unsuccessful, which is not surprising in view of the fact that the SV-40 virus control was destroyed by pH 2.5. Experiments on the physiochemical identification of the neutralizing substance as antibody, as shown by Lerner, Remington and Finland(14) for urine in poliomyelitis infection, are in progress. No neutralizing substance was found in urine specimens collected before infection from 4 monkeys which subsequently yielded antibody in the urine.

Recovery of virus from kidney biopsies. When fragments of tissue were prepared for testing by rupturing the cells by grinding, freezing, and thawing, not a single positive virus isolation was made from any of the 22 biopsies examined by inoculation of GM cultures (Table III). However, by the outgrowth method in which spontaneous appearance of virus was sought in the new cells, virus was found in 5 of 8 green monkeys tested 3 months after infection. The 5 monkeys with positive biopsies at 3 months were retested (other kidney) at 6 or 8 months, and the virus was recovered again in 4 animals. The 3 greens negative at 3 months were also negative in the later tests. Only 1 of 6 baboons yielded virus. This was also by the outgrowth method. The positive baboon response was obtained 3 months after direct inoculation into the kidney. At this time, the 2 babies are too early in their experimental period to be reported.

The CPE appeared in the explanted biopsies between the 18th and 29th day after explantation. Even in the positive tests, not all the tubes with growing explants showed CPE. Usually 6 to 10 explant cultures were made from one biopsy and in the positive tests only 50% of cultures yielded virus. This means that virus was not present in every kidney cell but was scattered in cells throughout the kidney tissue.

In every monkey with a positive kidney biopsy, virus had been recovered earlier in the urine. However, some animals excreted the virus in the urine, but did not yield virus in the biopsy. Perhaps this was due to the small amount of tissue sampled. In one case abundant material was available. A baboon was sacrificed 5 weeks after inoculation, but no virus was recovered from its kidney by direct inoculation of kidney cell suspension or from cells grown from the trypsinized kidney.

Typing of recovered virus. In every case in which explant cultures yielded spontaneous CPE, virus could be successfully passed to GM cultures. The virus showed the same antigenic characteristics as our stock SV-40 virus, for antiserum (which had an endpoint of 1/20,000) when diluted 1000 times neutralized 100 TCD_{50} of the recovered virus. Virus strains recovered from urine were likewise uniformly neutralized by the specific SV-40 antiserum.

Histopathology. In 1 of 8 biopsies examined, changes compatible with pyelonephritis

were found by Dr. Harvey S. Rosenberg. The other 7 examined were normal. One month later from the monkey with pyelonephritis a second biopsy was taken and found negative.

Discussion. That green monkeys in contact with rhesus may become infected with SV-40 virus had already been known to us and to others from the occasional spontaneous contamination of their kidney cultures. Meyer *et al.*(3) studied certain aspects of the experimental infection in 3 green monkeys, particularly the presence of virus in the throat, feces, and blood, and the development of serum antibody. We have confirmed and extended their results. It is these new findings relative to the infection of some 17 African monkeys that we wish to emphasize here.

Following exposure to a genetically purified line of the virus, no apparent disease was produced, but a latent, chronic infection was established. Regardless of route of entry, the virus was excreted in the urine for about a month. When the urine became negative for virus, neutralizing substance appeared, most probably antibody as shown for poliomyelitis(14). In this late stage, virus could be demonstrated in the kidneys by performing and growing kidney biopsies. The virus persisted in this latent stage in the kidneys of 4 of 8 green monkeys for at least 6 to 8 months after infection. Later biopsies are contemplated.

The growing of the kidney biopsies in culture as a means for isolation of virus was essential for success. The attempt to grow the virus from the mincing fluid and ground fragments of the kidney biopsy, as well as from early fluid changes of the cultures, were regularly negative. As with the isolation of adenovirus from adenoids(15), the recovery of virus from the biopsy may be dependent upon gradual elimination of the antibody present in the extracellular fluid, or presence of the virus in latent state in the kidney cells which can be detected only after replication outside the influence of the host.

From our inability to recover virus from every tube in the positive biopsies, the virus does not seem to be present in every kidney cell or even in a large percentage of them. Some of the monkeys with negative biopsies might well have had virus elsewhere in the kidney, especially those green monkeys with viruria. The situation with the baboons was different. The inability of the virus to colonize the kidney, with one exception, may be due to a species difference. That the baboons were less responsive to SV-40 infection is also shown by lesser amounts of virus in the throat, stools, urine, and blood, and also by a lower level of serum antibody than was found in the green monkeys.§

Like other papova viruses, SV-40 can establish latent infections, as has been documented here. The virus has produced low grade infections in man(4,5), and has infected and transformed human cells, even producing chromosome changes in them(16, 17). In hamsters, enormous fibrosarcomas are produced which may be larger than the host animal itself(6,7,16).

Summary. A latent infection was induced by exposure of African primates by a variety of routes to the vacuolating SV-40 virus, the simian member of the papova virus group. Virus multiplied in the green monkey *Cercopithecus aethiops* more abundantly than in the baboon *Papio doguera,* without inducing an apparent illness in either species. Early in the infection SV-40 was found in the urine, with 18 isolations obtained among the 26 specimens collected from 10 green monkeys in the first 4 weeks after infection. The titer of the urine was as high as $10^{6.5}$ TCD_{50}/ml. Virus was recovered from the urine of all 4 baboons inoculated parenterally, but not from 3 baboons infected by intranasal or oral administration of virus. Later in the course of the infection when viruria was waning, neutralizing antibodies appeared in the urine (as well as in the blood). Of 18 late urine specimens which were negative for virus, 16 were positive in neutralization tests. Three to 8 months after infection, when virus was no longer recoverable from the urine, kidney biopsies were obtained. Part of each biopsy was tested for virus by the conventional method of inoculating ground tissue fragments into susceptible green monkey cultures,

§ Ashkenazi, A., Melnick, J. L., unpublished data.

but not a single positive was obtained. However a number of biopsies yielded virus when they were grown as explants in tissue culture. Positive biopsy cultures spontaneously developed vacuolating type of degeneration and virus was then readily detected by passage. Kidneys from 5 of 8 green monkeys tested yielded virus by this method 3 months after infection was initiated. This latent infection persisted and a second biopsy 6 to 8 months after infection was positive in 4 of the 5 previously positive monkeys. Only 1 of 6 baboon kidneys yielded virus, again by the biopsy explant procedure.

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Failure of Transient Progesterone Deprivation to Induce Ova Implantation in Ovariectomized Rats.* (27795)

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When nidation is delayed in the ovariectomized rat treated daily with progesterone, the administration of small quantities of estrogen along with progesterone causes prompt implantation of the blastocysts(1,2,3). On the other hand, implantation of ova has also been induced by increasing the amount of progesterone(4,5). The apparent discrepancy existing between these two methods has been explained partially on the basis of time of ovariectomy(6). More recently implantation has been reported to occur when progesterone is temporarily discontinued for one day during the 12th to 15th day of pregnancy(7). Data to be presented here do not support the conclusion that transient progesterone deprivation initiates nidation.

Methods. Mature virgin female rats weighing 190-230 g were caged with adult males in the evening and insemination confirmed the next morning by the presence of spermatozoa in vaginal smears. The day spermatozoa were found was designated as day 1 of pregnancy. The inseminated animals were ovariectomized on the third day of pregnancy according to the method of Cochrane and Meyer(2) and placed in 3 experi-

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