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Comparative Transmission of Multiple Herpesviruses and Simian Virus 40 in a Baboon Breeding Colony

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Little is known about the natural history of herpesviruses indigenous in baboons. Here, we describe the development of ELISAs for five herpesviruses. These assays were used to test more than 950 serum samples collected from approximately 210 infant/juvenile and 130 adult baboons in a captive breeding colony over a period of seven years. Results indicated that baboon cytomegalovirus, lymphocryptovirus, and rhadinovirus are transmitted efficiently within the colony and are acquired at an early age. Baboon α-herpesvirus HVP2 and polyomavirus simian virus 40 (SV40) were acquired later and by fewer juveniles than were the other three herpesviruses. More than 60% of baboons acquired HVP2 before reaching sexual maturity, indicating that oral infection of infants and juveniles, rather than sexual transmission between adults, is the predominant mode of transmission for this virus. Antibody to simian varicella virus (SVV) was found in about 40% of baboons. SVV was acquired principally by infants and juveniles; few adults seroconverted despite seronegative adults being in constant contact with infants and juveniles undergoing primary infection. Time of seroconversion was not statistically correlated to specific individual herpesviruses, suggesting that each virus is acquired as an independent infection event rather than multiple viruses being acquired at the same time. Several baboons that were delivered by cesarean section and were housed separate from, but in close proximity to, other baboons remained free of many or all viruses for several years, suggesting that, similar to human herpesviruses, baboon herpesviruses and SV40 are transmitted principally by direct contact.

Baboons have long been used in many capacities in biomedical research. With the current national shortage of rhesus monkeys, use of baboons as an alternative species has been increasing. It has long been realized that indigenous viruses can be a confounding factor in the use of macaques for various research purposes. Retroviruses and herpesviruses are particular concerns due to their ability to influence the immune system and to persist in the host long after the initial infection event. Since rhesus and other macaques have been the most widely used primate species, more is known about the viruses of macaques than about those of other primate species. By comparison, relatively little is known about the viruses that naturally infect baboons.

Aside from their potential effect on research results, indigenous baboon viruses are a concern, given the proposed use of baboons as organ donors for xenotransplant procedures. There is fear that transplanting a baboon organ into a human recipient may allow indigenous baboon viruses contaminating the organ to infect and adapt to the human host, raising the specter of generating another situation like that of human immunodeficiency virus/acquired immune deficiency syndrome (1, 31). Biomedical experimentation using similar immune suppression procedures can lead to reactivation of latent viruses, which in turn can affect experimental results. Development of neurologic lesions due to simian virus 40 (SV40) in simian immunodeficiency virus-infected macaques and lethal generalized cytomegalovirus infections following baboon/rhesus xenotransplant experiments are just two such examples (7, 27, 45). Furthermore, testing of human viral vaccines in primates can yield erroneous results if the experimental subject animals carry an endogenous virus that is antigenically related to the test immunogen. Thus, there are many reasons for developing baboons that are free of specific viral agents. Knowing which viruses baboons actually carry and something about their biology in their natural host species is a requisite first step in producing baboons free of such viruses.

Herpesviruses and SV40 have proven to be complicating factors in biomedical research using macaques. Although serologic evidence suggests that baboons may harbor a virus related to SV40, nothing is known about the actual virus or its prevalence in baboons. Herpesviruses are widespread throughout the animal kingdom, and as more isolates have been found and analyzed, it has become clear that nonhuman primates likely harbor a full complement of herpesviruses homologous to those that infect humans (36). Humans harbor herpesviruses of all three subtypes: α-herpesviruses (herpes simplex virus types 1 and 2 [HSV1, HSV2] and varicella zoster virus [VZV]), β-herpesviruses (cytomegalovirus [CMV] and human herpesviruses 6 and 7 [HHV6, HHV7]), and γ-herpesviruses (lymphocryptovirus Epstein-Barr virus [EBV] and rhadinovirus Kaposi's sarcoma-associated herpesvirus [KSHV or HHV8]). Monkeys appear to have a single virus homologous to HSV1 plus HSV2 that infects the oral and genital tracts (*Cercopithecine herpesvirus* 1 [monkey B virus; BV in macaques] and *C. herpesvirus* 16 [*Herpesvirus*

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Table 1. Polymerase chain reaction (PCR) primers used in this study

Virus	Primer	Gene	Sequence*
HVP1	372	p18 (F)	GCAGGCTCAG*GGATCC*GGCGCCGGTGTTTCGGCATC
	373	p18 (R)	GTGCCCCCC*AAGCTT*TCTACTGTTTCTTGCGTGCC
	366	p23 (F)	GATA*GAATTC*CAGCCCCACGAAAGACCAGGTTGC
	371	p23 (R)	TCAGAA*ACTAGT*ACTTTCTTGGATTCGCGTCCTC
RV	312	gB (F1)	AAGTTCGTRGGMGACGCCATYTCCGTGAC
	313	gB (F2)	TGCAAAGASACSTGCGAACACTACTTC
	314	gB (R1)	TGYGTGTAGTAGTTGTAYTCCCTGAAC
	315	gB (R2)	TTTCTCYGCRCKGCTGTACAGCTCGATG

*Nucleotides in bold italics indicate the restriction sites used for cloning and constructing the recombinant protein antigens. Degenerate positions are defined as follows: M = A+C, S = G+C, Y = C+T, and R = A+G.
HVP = *Herpesvirus papio*; RV = rhadinovirus.

papio 2; HVP2] in baboons). Macaques also carry homologues of VZV (*C. herpesvirus* 9 [simian varicella virus; SVV]), CMV (*C. herpesvirus* 8), EBV (*C. herpesvirus* 15), and HHV8 (*C. herpesvirus* 17, rhesus rhadinovirus; RRV) (30, 35, 36, 50). To date, baboon viruses corresponding only to HSV (HVP2), CMV (BaCMV), and EBV (HVP1) have been isolated (5, 14, 15, 37).

Although a number of studies on the presence of antibodies to various viruses in baboon sera have been reported, few studies have examined in more depth epidemiologic aspects of multiple viruses in baboons. The study reported here was undertaken to try to better understand basic temporal aspects of the transmission of herpesviruses and SV40 in baboons.

Materials and Methods

Baboons and sera. Olive baboons (*Papio cynocephalus anubis*) were housed at the AAALAC-accredited Oklahoma University Health Science Center (OUHSC) breeding facility. Since 1996, the breeding colony has undergone considerable expansion. The colony originally comprised five harems consisting of 1 adult male and 7 to 10 females housed in indoor gang cages. Infants were routinely removed from the harems at approximately 6 to 9 months of age and were group housed with other juveniles. These animals were not included in the serologic analyses as long as they were housed in these juvenile gangs. In May 2001, a new breeding facility was completed and the breeding colony was transferred into it. The original five harem groups were moved into four quarter-acre outdoor corrals with attached indoor housing, and additional juvenile and adult females were periodically added. From this time, female infants and juveniles were usually left in the breeding troops, while male juveniles were routinely removed at 1 to 2 years of age. As the number of breeding-age females increased, additional males were added to each troop. As of May 2003, each of the four troops consisted of 3 to 4 adult males, approximately 30 adult females, and their associated infants and juveniles.

As part of the routine colony health monitoring program, blood samples were collected from the animals during bi-annual physical examinations and TB testing. Some serum samples were obtained from blood samples collected as part of experimental studies, and all such studies were reviewed and approved by the institutional IACUC. Baboons used as negative controls for all serologic assays were animals that, for medical reasons, were delivered by cesarean section. These baboons were hand reared as infants and were housed in cages physically separate from other baboons. Although space limitations sometimes necessitated keeping them in the same room as other baboons on experimental study, an effort was made to keep their cages sufficiently separated such that they did not have direct contact with other baboons in the facility. True seronegativity of these animals for each virus was evidenced by the decay of maternal antibodies and the persistent absence thereafter of any reappearance of anti-viral titers.

Viruses and cells. *Herpesvirus papio* 2 strain OU1-76 and baboon cytomegalovirus (BaCMV) strain OCOM4-37 were originally isolated from olive baboons at the OUHSC facility (5, 12). Simian varicella virus strain Delta was generously provided by Dr. W. Gray (University of Arkansas, Little Rock, Ark.), RRV strain H29-95T was provided by Dr. R. Desrosiers (New England National Primate Research Center, Southborough, Mass.), and *H. papio* 1 (HVP1)-infected S594 cells were provided by Dr. F. Wang (Harvard University, Boston, Mass.). HVP2 and SVV were propagated in Vero cells, BaCMV was propagated in HEL 299 cells (ATCC CCL-137, Rockville, Md.), and RRV was propagated in telomerase-immortalized rhesus fibroblast cells (11) kindly provided by Dr. D. Dittmer (OUHSC, Oklahoma City, Okla.). SV40 was obtained from the ATCC (VR-239) and was propagated in BSC-1 cells. S594 cells were maintained in RPMI medium supplemented with 10% fetal bovine serum, 2 m*M* L-glutamine, and 100 µg of streptomycin and 100 U of penicillin/ml; all other cells were propagated in minimal essential medium (BSC-1 cells) or Dulbecco's minimal essential medium containing the same supplements.

HVP1 expression constructs. Initial primer sequences for polymerase chain reaction (PCR) amplification of HVP1 homologues of the EBV p18 and p23 genes were designed by alignment of homologous EBV and rhesus lymphocryptovirus sequences. S594 cell DNA was prepared, using DNAzol according to the manufacturer's instructions (Molecular Research Center Inc., Cincinnati, Ohio), and was used as template in PCR analysis, which was performed using a hot-start protocol with Ampli-Taq (Perkin-Elmer, Foster City, Calif.). The PCR products were purified using Wizard PCR Preps (Promega, Madison, Wis.), and were sequenced at the Oklahoma Medical Research Foundation (Oklahoma City, Okla.). Based on the sequence data, new primers (Table 1) were designed to amplify specific regions of the HVP1 p18 and p23 genes for cloning into the prokaryotic expression vector pPROEX-1 (Life Technologies, Gaithersburg, Md.). Polymerase chain reaction analysis contained 15 m*M* $MgCl_2$, 0.2 m*M* dNTPs, 10% dimethyl sulfoxide (DMSO), 1*M* betaine (Sigma Chemicals, St Louis, Mo.), and 25 p*M* each primer. The PCR analysis involved an initial denaturation step of 96°C for 3 min; 35 cycles of 96°C for 1 min, 65°C for 30 sec, and 72°C for 1 min; followed by a final extension at 72°C for 7 min. The PCR products and vector DNA were digested using the appropriate restriction enzymes, gel purified, ligated into pPROEX-1 either separately or together, and transfected into *Escherichia coli*

DH5α. The final construct consisting of p18 and p23 sequences (p18/23) had an N-terminal 6xHis tag followed by the N-terminal 158 codons of the HVP1 p23 ORF (excluding the Met initiation codon), an eight-codon spacer from pPROEX-1, and the C-terminal 66 codons and termination codon of the HVP1 p18 ORF.

Polymerase chain reaction detection of rhadinovirus sequences. A similar approach was used to design a set of primers for nested PCR amplification of RV sequences from baboon peripheral blood lymphocytes (PBL). Sequences of HHV8 and RRV glycoprotein B genes were aligned. Primers with minimal degeneracy were then sited in areas of maximal homology. Baboon PBL DNA was prepared using a QIAamp DNA Blood Kit (Qiagen, Valencia, Calif.) and was used as template for PCR analysis. Reaction mixtures contained 5% DMSO, 0.2 m*M* each dNTP, 2.5 m*M* $MgCl_2$, 25 p*M* each primer (312 and 314; Table 1), and 250 U of *Taq* polymerase. Initial PCR analysis to amplify a 465-bp product was done with an initial denaturation step at 94°C for 4 min; 40 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min; followed by a final extension at 72°C for 7 min. The second PCR analysis used 0.25 µl from the first PCR analysis as template and primers 313 and 315, and was done using the same amplification conditions to amplify a 189-bp product. The PCR products were visualized on ethidium bromide-stained agarose gels. Products were purified for sequencing as described.

Viral immunoassays. Established ELISA protocols for detection of HVP2 and BaCMV IgG in serum have been described (5, 39). The same protocol was used for preparation of infected cell extracts for antigens and assay of serum IgG to rhadinovirus (using RRV), SVV, and SV40. A commercial ELISA kit was used to assay for HHV6 (Advanced Biotechnologies Inc., Columbia, Md.). Initial testing for HVP1 involved use of commercial EBV viral capsid antigen (VCA)- and EBV nuclear antigen (EBNA)-based ELISA kits (Sigma-Aldrich, St Louis, Mo.) for detection of anti-HVP1 IgG in baboon sera before tests based on recombinant HVP1 proteins were developed.

Recombinant p18, p23, and p18/23 proteins for use as ELISA substrate antigens were prepared by inoculating 10 ml of Luria broth (LB) containing 1 µg of ampicillin per milliliter (LB/amp) with a single colony, followed by overnight incubation. Cultures were then diluted 1:33 with fresh LB/amp and were grown for 6 h, then isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 0.2 m*M*. After 2 h, bacteria were pelleted by centrifugation and lysed by re-suspension in 0.5% TX-100/0.01% sodium dodecyl sulfate, followed by sonication. Lysates were then clarified, and the recombinant proteins were purified from the supernatant via their 6xHis tag on NiNTA resin using the manufacturer's protocol (Novagen, Madison, Wis.).

Statistical analysis. Data were analyzed using PC SAS Version 8.2 (SAS Institute, Cary, N.C.), and $P \leq 0.05$ was used to judge significance. PROBIT regression models were fit using PROC PROBIT to predict the age that a baboon becomes seropositive for each of the viruses in question. The age, in years, at which 50, 75, and 90% of the baboons were seropositive was calculated and these predicted values for the viruses were compared using the fiducial limit multiple comparison approach of Payton and co-workers (40). To ascertain differences in ELISA OD values for baboons that seroconverted at various ages, each baboon was classified in one of three age groups: younger than 2 years, between 2 and 4 years old, and older than 4 years. The intensity of the IgG response was analyzed by use of one-way analysis of variance (ANOVA) with PROC MIXED. Least significant difference procedures were performed if the ANOVA results were deemed significant. Using PROC FREQ, the observed (nonmodel based) seroconversion proportions at various ages were calculated and compared using a χ^2-test for equal proportions, along with pairwise tests for proportions to compare the rate of conversion for the viruses. Finally, the rate of acquisition of the viruses at or before sexual maturity was compared using PROC FREQ.

Table 2. Comparison of antigens for the HVP1 ELISA

Baboon	Assay antigen			
	p18	p23	p18/23	EBV VCA
1702	0.058* (-)	0.378 (-)	0.132 (-)	0.076 (-)
2802	0.036 (-)	0.330 (-)	0.072 (-)	0.081 (-)
3302	0.046 (-)	0.336 (-)	0.101 (-)	0.055 (-)
PC14	0.330 (+)	0.403 (±)	0.363 (+)	0.258 (+)
PC9605	0.401 (+)	0.968 (+)	0.993 (+)	0.409 (+)
PC9502	0.152 (±)	1.027 (+)	0.556 (+)	0.401 (+)
PC9604	0.180 (±)	0.929 (+)	0.803 (+)	0.318 (+)
Wyatt	0.330 (+)	1.325 (+)	1.348 (+)	0.665 (+)
ORP 003	0.132 (±)	0.804 (+)	0.674 (+)	0.292 (+)
O24	0.100 (±)	0.868 (+)	0.943 (+)	0.345 (+)
57-308	0.129 (±)	0.462 (±)	0.508 (+)	0.066 (-)
9511	0.195 (±)	0.857 (+)	0.562 (+)	0.247 (+)
57-288	0.066 (-)	0.548 (±)	0.298 (±)	0.247 (+)
Kramer	0.118 (±)	0.738 (±)	0.556 (+)	0.346 (+)
57-295	0.205 (+)	1.220 (+)	1.188 (+)	0.275 (+)

*Values are ELISA OD values, with the positive/negative result in parentheses. The positive/negative cut-off value for each test was: p18, 0.100; p23, 0.400; p18/23, 0.150; and EBV VCA, 0.100. Results are expressed as negative (-), weak positive (less than twice the cut-off value, ±), and positive (+).

Results

Development of an ELISA for HVP1. Initial testing using commercial kits indicated that, although an EBNA-based EBV kit did not detect any HVP1-infected baboons, a VCA-based EBV kit did detect infected baboons. However, OD values were lower than those for EBV-positive human sera. To develop an HVP1-specific assay, several recombinant HVP1 polypeptides were tested. Based on the results of Hinderer and co-workers (16, 23), who tested a number of EBV gene products for detection of EBV-positive humans and found the p18 and p23 capsid protein antigens to be the best for diagnostic purposes, we focused on HVP1 homologues of the EBV p18 and p23 genes. The N-terminal 158 amino acids of p23 and the C-terminal 66 amino acids of p18 were expressed individually or together as a fusion protein, all having an N-terminal 6xHis tag.

In comparative testing of sera from adult baboons which tested positive for HVP1 in the EBV VCA test, all had positive results when tested for the p18/23 recombinant HVP1 antigen and all had higher OD values with the p18/23 antigen than with the EBV VCA test (Table 2). Also, one serum which tested negative for HVP1 in the EBV VCA test was identified as HVP1 positive using all three HVP1 recombinant antigens (animal 57-308). All sera that were positive with the p18/23 antigen also were positive when either p18 or p23 was used. By contrast, use of the p18 antigen alone yielded marginal positive reactions for several sera; however, one serum that was positive with the p23, p18/23, and VCA assays was missed by use of the p18 antigen. The recombinant p23 antigen gave a comparatively high background level of reactivity for all sera tested, making this antigen less desirable for use as a diagnostic test. Comparative testing of additional sera from adult baboons yielded similar results (not shown). Based on these results, the recombinant p18/23 antigen

Table 3. Prevalence of herpesviruses in adult baboons

Corral	No. of baboons	Virus*					
		HVP2	SVV	BaCMV	HVP1	RV	SV40
SW	31	31 (100)	15 (48.4)	31 (100)	30 (96.8)	31 (100)	26 (83.8)
NW	35	31 (88.6)	24 (68.6)	35 (100)	27 (77.1)	35 (100)	28 (80.0)
NE	30	30 (100)	14 (46.7)	30 (100)	20 (66.7)	30 (100)	28 (93.3)
SE	33	33 (100)	10 (30.3)	33 (100)	28 (84.8)	33 (100)	28 (84.8)
Total	129	125 (96.4)	53 (41.1)	129 (100)	105 (81.4)	129 (100)	110 (85.3)

*Values are the number of animals positive, with the percentage of positives in parentheses.
SVV = simian varicella virus; BaCMV = baboon cytomegalovirus; and SV40 = simian virus 40.
See Table 1 for key.

was adopted for use for all further testing of baboons for anti-HVP1 serum IgG.

Prevalence of viruses in adult baboons. To determine the prevalence of each virus in the breeding colony, all adult baboons were tested by ELISA for HVP1, HVP2, BaCMV, SVV, RV, HHV6, and SV40. Although sexual maturity takes slightly longer in males than females, and females are typically considered mature at 3 to 3.5 years of age, for simplicity we herein consider all baboons older than three years as adults. Results for the colony as of May 2003 (137 adult animals) are summarized in Table 3. No baboons tested positive based on results from the HHV6 kit (not shown). All adult baboons in the colony were seropositive for BaCMV and RV, and almost all were positive for HVP2 as reported (12, 13). HVP1 was somewhat less prevalent in the colony, although nearly 80% of adults were seropositive for this virus. In contrast to these four herpesviruses, only about 50% of adult baboons were seropositive for SVV. Also, seroprevalence for SVV was lower in animals of one of the four corrals than in those of the other three. Simian virus 40 was prevalent in all four corrals, with approximately 80 to 90% of adults being seropositive.

Although a rhadinovirus has not been isolated from baboons, there is every reason to expect that, similar to other monkey species, they harbor a virus analogous to the rhesus virus. Since the rhesus rhadinovirus was used as antigen in RV assays and baboon serum had positive reactions associated with its use, it was of interest to determine whether the virus detected in baboons was the same or different from the rhesus virus. A nested PCR analysis was performed on PBL DNA samples from baboons in one corral; this test was designed to amplify sequences from the conserved gB glycoprotein gene. Of the five seronegative animals tested, none had positive results. However, PCR products were generated from 13 of 70 seropositive animals (18.6%) that ranged from old adults to infants younger than one year. Sequencing of these products indicated that, although the predicted amino acid sequence was almost identical to that of the rhesus virus, there were consistent differences at the nucleotide level between RRV and PCR products amplified from baboon PBL (Fig. 1). Among the eleven 186-bp baboon PCR products that were sequenced and aligned, variation among baboon sequences was noted at only five positions. All of these substitutions were in the third codon position, but one resulted in a conservative amino acid change (Met versus Ile). By contrast, the BaRV sequences in 19 positions varied from the two RRV sequences (which only differed from each other by a single silent nucleotide substitution), and 18 of these were in the third codon position. Consistent with the highly conserved nature of the gB gene, only 1 of the 19 differences between the rhesus and baboon sequences resulted in an amino acid change, and this also was a conservative change (Ala in BaRV, Ser in RRV). Thus, although they were closely related to the rhesus RV sequence, RV sequences amplified from baboons appear to be distinct from those found in macaques. We, therefore, denote this putative virus as baboon rhadinovirus (BaRV).

Acquisition of viruses by young baboons. The age at which each of the herpesviruses is typically acquired by baboons was assessed by testing 961 serum samples collected over 7 years from 213 infant and juvenile baboons ranging in age from newborns to older than 8 years. For PROBIT regression analysis of ELISA results, samples collected from animals younger than 6 months were excluded to avoid bias resulting from the possible presence of maternal IgG. The results are summarized in Table 4. It was readily evident that BaCMV is acquired more rapidly by young animals than are any of the other herpesviruses. BaCMV also appeared to be readily transmitted, since almost all animals were infected by 3 years of age. Results for BaRV were similar in that this virus was also acquired principally at a young age, with ≥ 75% of animals being infected before 2.5 years of age. The acquisition of HVP1 lagged somewhat behind BaRV, and HVP2 and SV40 were acquired even later, consistently lagging BaCMV acquisition by almost 2 years. Acquisition estimates for SVV were somewhat surprising, given that varicella in humans is typically a childhood infection. Since only about 50% of adult baboons displayed evidence of SVV infection, this model-based approach is not accurate for estimating ages for high (> 0.5) infection proportions.

Although this statistical modeling approach indicated that different herpesviruses are acquired with variable efficiency, this approach did not consider infections acquired before 6 months of age. The results for SVV also raised concerns about relying on this approach. Temporal acquisition of the various viruses was, therefore, also examined by identifying all animals from which paired sera had been collected that allowed a determination to be made of the age (within a 1-year period) at which they seroconverted. A total of 612 seroconversion events were thus identified. Based on the results of the aforementioned samples, the time of seroconversion to each virus was determined (Table 5). Consistent with the regression analyses, the overwhelming majority (82.2%) of baboons that seroconverted to BaCMV did so by the age of 1 year, with 97.7% of seroconversions occurring by the time animals were sexually mature (3 years minimum). Temporal acquisition of HVP1 and BaRV was similar, both viruses being acquired principally during the first 2 years of life. Although acquisition of HVP2 has been assumed to occur principally by sexual transmission, > 70% of the seroconversion detected in young baboons occurred before the age of 2 years. Seroconversion to SVV had a similar temporal pattern, and SV40 also was acquired principally during the first two years of life. Thus, all of these viruses appear to be usually acquired early in life.

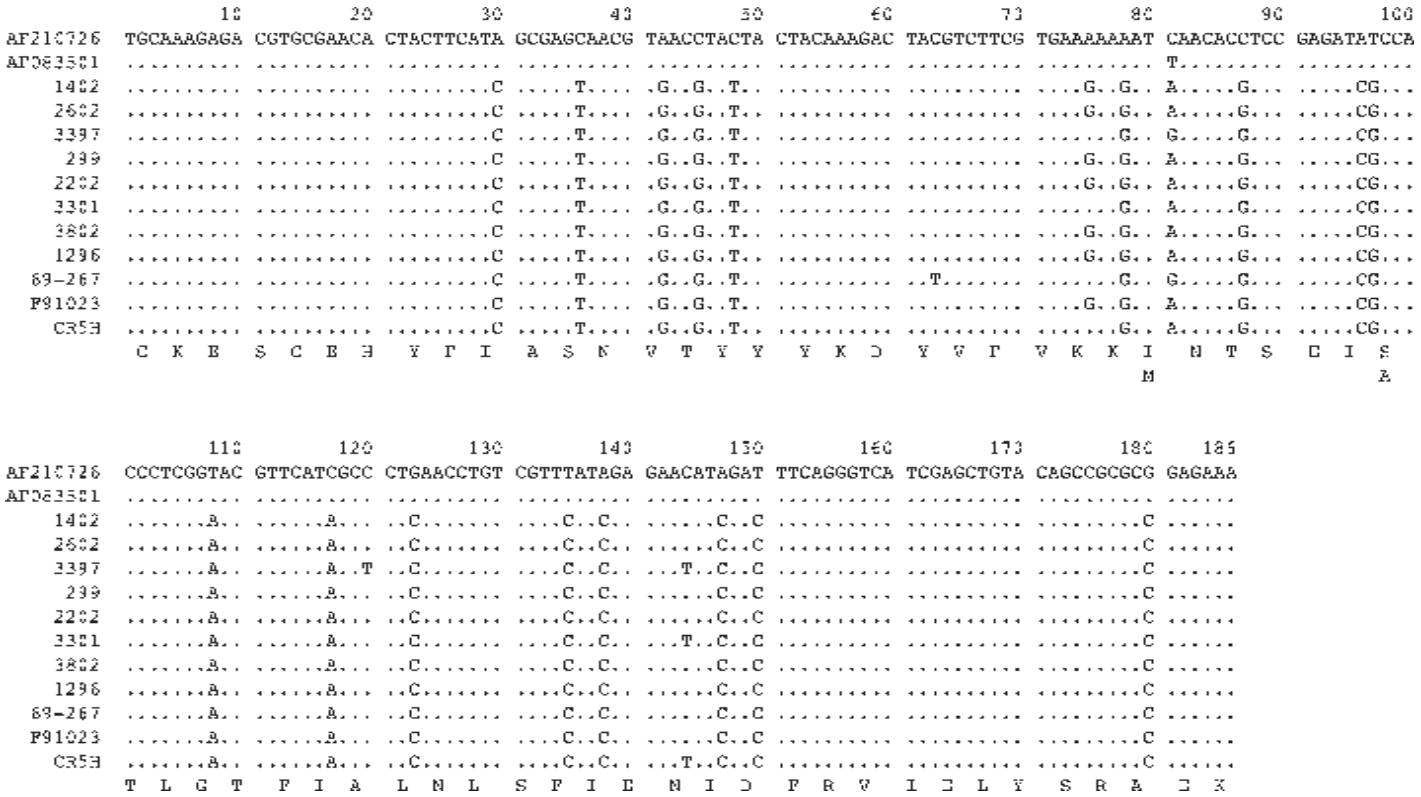

Figure 1. Comparison of rhadinovirus sequences derived from rhesus macaques and baboons. Polymerase chain reaction (PCR) products amplified from baboon peripheral blood lymphocytes (PBL) were purified and sequenced. The two RRV sequences are identified by their GenBank accession numbers (AF210726 and AF083501). All sequences were aligned to the rhesus sequence AF210726. Only nucleotides that differed from that of the reference sequence are indicated. The encoded amino acids are indicated below the nucleotide sequence. Codons affected by nucleotide substitutions are identified by the two amino acids are shown below the sequence.

Table 4. Probit model-based age of acquisition of herpesviruses by baboons

	Population infection rates[a,b]		
Virus	50%	75%	90%
HVP2	2.0[§]	3.9[‡]	5.7[†,§]
SVV	5.3[#]	8.7[#]	11.8[‡]
BaCMV	0.1[*]	1.7[*]	3.1[*]
HVP1	1.4[†]	3.2[§]	4.8[†]
BaRV	1.1[†]	2.4[†]	3.6[*]
SV40	2.5[‡]	4.5[‡]	6.3[§]

[a]Age in years at which the given percentage of baboons are predicted to be seropositive for the virus.
[b]Two age estimates within the same column having the same symbol are not significantly different at a 0.05 level of significance.
BaRV = baboon rhadinovirus.
See Tables 1 and 3 for key.

The aforementioned analyses indicated the age of young baboons when they seroconverted, but these results were based only on data from animals that seroconverted. To obtain a better estimate of the percentage of young baboons that actually acquire each of these viruses before reaching sexual maturity, sera collected from baboons at close to 3 years of age were tested. As shown in Table 6, these results were consistent with the apparent efficient transmission of BaCMV and BaRV, in that > 90% of baboons were infected by 3 years of age. Fewer baboons were infected with HVP1 before reaching maturity, but still, > 70% had acquired this virus. SVV infected only slightly > 50% of baboons before the age of three years. For these four viruses, the percentages are close to the prevalence of these viruses in adults (Table 3), suggesting that these viruses are acquired almost exclusively in childhood. By contrast, approximately 60% of baboons acquire HVP2 by 3 years of age, and the prevalence in adult baboons is > 95%. This implies that, although HVP2 is often acquired early, a substantial percentage of baboons acquire the virus after reaching sexual maturity. Results for SV40 were similar to those for HVP2: about 60 to 65% of baboons acquire the virus before reaching sexual maturity, and another 20% appear to acquire the virus later. However, unlike HVP2, it appears that not all baboons eventually acquire SV40 since the prevalence of this virus in adult baboons is only 80 to 90%.

It was of interest to determine whether animals that experienced primary infection at an older age responded differently than those that acquired the virus at a younger age. To examine the level of the IgG response to primary herpesvirus infections as related to the age of the animal at the time of seroconversion, samples were separated into three groups consisting of those where the first positive sample had been obtained when the animal was younger than 2 years, 2 to 4 years old, or older than four years. For each virus, the ELISA OD values for each group were then compared, using the ANOVA techniques with SAS PROC MIXED. Although there was a visible trend toward stronger responses by animals that experienced initial infection with BaCMV, HVP1, and HVP2 at an older age, the differences observed were statistically significant at the 0.05 level only for BaCMV; for SVV, BaRV, and SV40, the intensity of the IgG response was equivalent at all ages (data not shown). Blood samples collected serially from animals that had been bled on at least five occasions were also examined to see whether IgG con-

Table 5. Age distribution of young baboons at initial seroconversion

Virus	Age at seroconversion (years)[a,b]				
	0-1	1-2	2-3	3-4[c]	> 4
HVP2	30.0[§,‡]	42.0 (72.0)[†]	11.0 (83.0)[§,‡]	4.0 (87.0)[§]	13.0 (100)
SVV	20.8[‡]	49.1 (69.9)[†]	11.3 (81.2)[‡]	11.3 (92.5)[†,§]	7.5 (100)
BaCMV	82.2[†]	10.1 (92.3)[*]	5.4 (97.7)[*]	0.8 (98.5)[*]	1.5 (100)
HVP1	65.6[†]	26.7 (92.3)[*]	3.3 (95.6)[*,†]	1.1 (96.7)[*,†]	3.3 (100)
BaRV	62.1[†]	26.3 (87.9)[*]	7.5 (95.5)[*,†]	3.0 (98.5)[*]	1.5 (100)
SV40	34.4[§]	43.0 (77.4)[†]	12.9 (90.3)[†,§]	0 (90.3)[§]	9.7 (100)

[a]Based on analysis of a total of 612 seroconversion events, with n values for each virus as follows: BaCMV, 139; HVP1, 83; HVP2, 99; BaRV, 136; SVV, 60; and SV40, 99. Values represent the percentage of the total number of seroconversion events that occurred in each age group of baboons, with cumulative percentages in parentheses.
[b]Percentages in the same column having the same symbol are not significantly different at a 0.05 significance level.
[c]Reflects the approximate age of sexual maturity.
See Tables 1, 3, and 4 for key.

Table 6. Percentage of baboons acquiring herpesviruses before reaching maturity

	Virus[a]					
	HVP2	SVV	BaCMV	HVP1	BaRV	SV40
No. positive (%)	41 (62.1)[a,*,†]	35 (53.0)[*]	63 (95.5)[§]	48 (72.7)[†]	60 (90.9)[§]	42 (63.3)[*,†]
No. negative (%)	25 (37.9)	31 (47.0)	3 (4.5)	18 (27.3)	6 (9.1)	24 (36.4)

[a]Percentages with the same symbol are not significantly different at a 0.05 significance level.
See Tables 1, 3, and 4 for key.

centration continued to increase with age. All animals had fluctuations (increases and decreases) in serum anti-viral IgG concentration over time. Fluctuations in serum IgG values to different viruses in individual animals were not coincident, nor did different animals have increases or decreases at the same time as did other animals (not shown).

Since stress appears to be a common factor in reactivation of latent herpesviruses, it is possible that an adult baboon which has reactivated and is shedding one herpesvirus could also shed many others simultaneously. If so, it is conceivable that young baboons could acquire many viruses at once from a single source (a shedding adult). Thus, it was of interest to determine whether all of the various herpesviruses and SV40 are commonly acquired at the same time by individual animals. For all baboons that had sera available which allowed detection of seroconversion to any virus within a time interval of one year or less, their serologic status to the other five viruses was assessed. A total of 301 serum samples were analyzed by constructing 2×2 contingency tables for each virus pair and the significance of animals acquiring both viruses in the same timeframe was assessed, using a right-tailed Fisher's exact test with PROC FREQ in PC SAS. There was not a significant relationship between the acquisition of any one virus with acquisition any of the other viruses. This was true even for BaCMV and BaRV, both of which are acquired early in life by infant baboons. Of the 131 animals that seroconverted to at least one of these two viruses, only 53 (40.5%) seroconverted to both viruses in the same time period (in the same paired serum set). It also should be noted that, for these 53 animals that also that had dual seroconversion, the span of time between the paired sera defining this seroconversion varied from one month to almost a year, allowing a considerable period in which non-simultaneous infection with these two viruses could have occurred. Thus, although it may sometimes happen, it does not appear to be normal or common for baboons to acquire multiple viruses simultaneously.

Another question that arises is whether there is seasonal or otherwise temporal grouping of infection events within the colony. For example, in humans, VZV infection can occur in small epidemics, as the virus is transmitted among non-immune children in schools. When the times of seroconversion to each virus were mapped over the 6.5 years of surveillance, there was no indication of any temporal clustering of seroconversion events for any of the viruses examined (Fig. 2). The one exception was that in the last 6-month period when testing was performed (between October 2002 and May 2003); many animals seroconverted to all of the viruses. This was probably due to the presence of more infants and juveniles in the colony and, thus, sampling of more paired sera from juveniles and infants collected at these particular times.

Cesarean-derived baboons. From 1996 to 1997, four infant baboons were delivered by cesarean section for medical reasons. These female infants were hand reared by laboratory animal care personnel and, as juveniles, were kept in cages separate from other baboons. However, due to space limitations in the facility, these baboons were kept in the same room as other baboons, although direct contact with other baboons was avoided. These baboons were housed in this manner for 3 to 5 years. One of these baboons remained seronegative for all of the herpesviruses and SV40 for 3 years, at which point it was group-housed with "dirty" baboons. Within 3 months of introduction to the other baboons, this animal seroconverted to BaCMV, BaRV, HVP1, and HVP2. Two of the cesarean-sectioned baboons seroconverted to HVP1 (one at 1.5 and the other at 2 years of age), and one of these two also seroconverted to BaRV 3 months later. One of these two baboons died at the age of 3.5 years, at which time it was positive only for HVP1. The fourth baboon did not seroconvert to any of the viruses. When the two surviving baboons were almost 5 years old, they were introduced to a "dirty" male baboon. Within one month, both females were seropositive for BaCMV, HVP1, and BaRV. One of these females also seroconverted to HVP2 within 1 month, and the second was positive for HVP2 at the next testing one year later. Neither these two nor the one introduced to "dirty" baboons at 3 years of age had evidence of SVV or SV40 infection.

Discussion

In this study, we determined the serologic status of baboons in the OUHSC breeding colony for various herpesviruses and SV40 over time. Also, infant and juvenile animals were followed over time where possible. This allowed us to not only look at the sta-

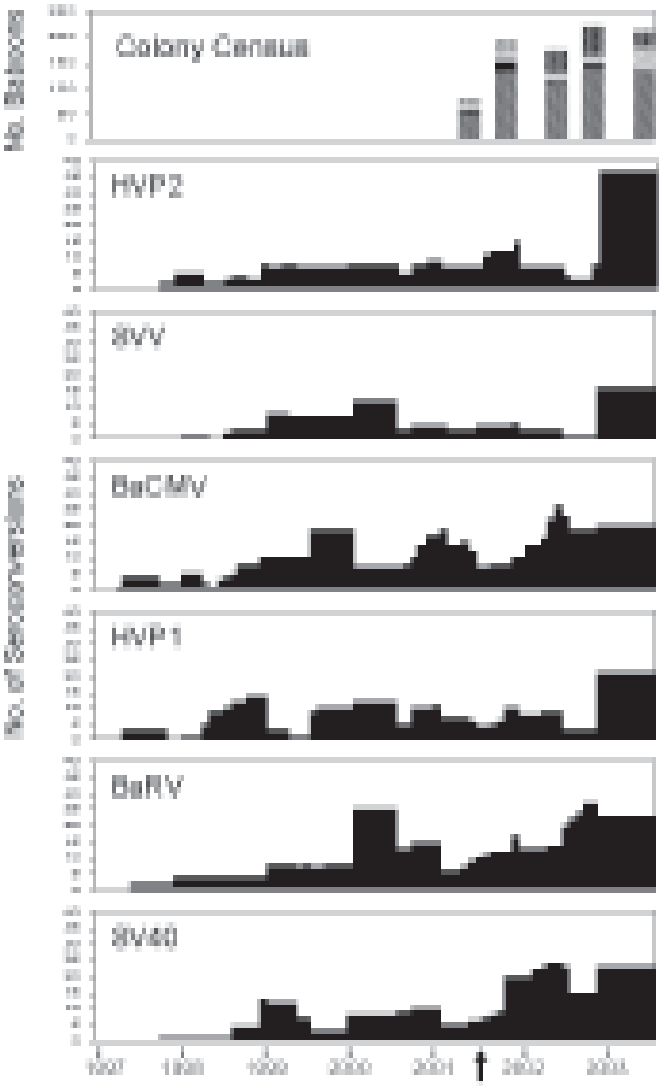

Figure 2. Temporal distribution of seroconversion events for six viruses in the Oklahoma University Health Science Center colony between 1997 and 2003. For each virus examined, paired sera that were temporally separated by one year or less and that defined seroconversion to the virus were plotted. The arrow between 2001 and 2002 indicates the movement of the colony from the indoor gang cages to the new indoor/outdoor corrals. The top panel represents the colony census at the semi-annual testing dates. Numbers of animals of various ages are indicated as follows: 0 to 1 year (▦), 1 to 2 years (▧), 2 to 3 years (□), 3 to 4 years (■), and adults > 4 years (▨).

tus of individual animals and the colony as a whole for each virus, but also to compare transmission and acquisition of the various viruses within the colony. However, the results need to be considered in light of the problems inherent in the sample sets analyzed. Although there are no significant concerns regarding the sample set representing adult baboons in the colony, there are shortcomings associated with the infant/juvenile sera. First, management of the colony unavoidably affected the animals available for sequential testing. Until May 2001, infant baboons were routinely pulled from the breeding harems at 6 to 9 months of age and were housed in peer groups. Also, juvenile baboons were often assigned to experimental studies, some of which were at other institutions, thereby preventing repeated sampling of these animals. A second confounding element was the rapid growth of the colony during the study period. In May 2001, the breeding colony was moved from a small indoor facility to a new indoor/outdoor facility that provided more room for each harem, allowing re-introduction of many juvenile animals back into the breeding harems and incorporation of additional adults into each troop. These events translated into major changes in the social dynamics of the troops and to a significant increase in breeding productivity (6). Thus, although we can draw many inferences regarding the transmission of herpesviruses in the colony, which may well reflect "normal" events, it must be borne in mind that these temporal, constitutional, and management changes in the colony may have had an influence.

Several years ago, cytomegalovirus was independently isolated from baboons at two laboratories (5, 37). Molecular characterization of BaCMV indicates that it is closely related to the rhesus and vervet CMVs (5, 34). Several serosurveys of rhesus colonies have found that > 95% of adult animals are positive for rhesus CMV (RhCMV), and studies in which infants were tested indicated that most had seroconverted by 1 year of age (2, 29, 49). Thus, BaCMV and RhCMV appear to have similar epidemiologic behaviors. Furthermore, these findings closely reflect the epidemiology of CMV in human populations: high prevalence in adults with primary exposure to the virus mainly in early childhood (9, 32, 42, 47). In humans, population density and mother-baby contact appear to be significant risk factors for CMV transmission (32). BaCMV was acquired by more baboons at a younger age than were any of the other viruses, most being infected by 1 year of age. This implies that BaCMV is transmitted efficiently and possibly from the dam, which in turn implies that it is frequently shed long term by infected baboons and/or shed in high concentrations. This again is consistent with results reported for RhCMV (24).

Given that many infants appear to acquire BaCMV infection within the first 6 months of age, shedding of the virus by their dam in the saliva or milk seems likely. Since some infants never experienced any decrease of antibody titer to BaCMV after birth, BaCMV must be able to infect infants even when maternal antibodies are present. Our initial efforts at establishing specific-pathogen-free baboons have suffered from this phenomenon. Infant baboons taken from their dam within 12 h of birth experience a normal decay in maternal antibodies, only to have reached seronegative status, only to have BaCMV break before maternal antibody titers have decayed to background values and rapidly spread to the rest of the cohort (unpublished data). Despite the rapid spread of BaCMV among animals in direct contact, lack of direct contact between animals appears effective at preventing transmission, as evidenced by lack of seroconversion of cesarean section-derived animals housed near to, but not in direct contact with BaCMV-positive baboons for over 4 years. It is, however, apparent that CMV is spread efficiently in captive primate breeding colonies. Given the close parallels between human and baboon CMVs, baboons offer an ideal model system in which to apply molecular epidemiologic techniques to further elucidate details of the shedding and transmission of this virus in a natural host species.

Rhadinoviruses are a recently discovered group of herpesviruses (8,10). Using degenerate PCR primers, rhadinovirus sequences have been detected in many macaque as well as other monkey species (21, 30, 46, 54). Using RRV as test antigen, it

was apparent that the OUHSC baboon colony is widely infected with an antigenically related virus. Using minimally degenerate primers, we were able to use PCR analysis to amplify products from PBL of seropositive, but not seronegative baboons. Sequencing of these PCR products confirmed that baboons carry a virus (or at least virus-like sequences) that are closely related to, but distinct from the rhesus rhadinovirus. These results confirm those recently published by Whitby and co-workers (54). Given the seroconversion of infant baboons, there seems little question but that these sequences are derived from an intact, infective virus, rather than representing a non-infective virus-like genetic element. Our data document that this baboon rhadinovirus (BaRV) infects baboons at an early age. Since > 95% of baboons become seropositive by the time they reach sexual maturity, this virus is probably transmitted as an oral infection. Indeed, results of recent studies on HHV8 in humans also have suggested that this virus may be acquired in early childhood and can be transmitted horizontally, possibly from the mother (3, 17, 41). The high prevalence of anti-BaRV antibodies in adult baboons also is consistent with what has been observed in macaques (8, 10, 20, 55).

The recombinant protein antigen ELISA described here for HVP1 testing was based on the results of Hinderer and co-workers (16, 23), who tested a number of recombinant EBV antigens for use as in diagnosis of EBV infections. Although use of the p18 and p23 homologues of HVP1 detected HVP1 infected baboons, assays using either antigen alone missed some animals that tested positive by use of the EBV VCA and p18/23 assays. Use of a p18/23 fusion peptide was found to detect all EBV VCA-positive baboons. Using this recombinant antigen-based ELISA to test sera of adults, it was evident that almost all baboons are seropositive for HVP1. Consistent with the results of others, we found high prevalence of anti-HVP1 antibody in adult baboons (26, 48). From testing of sera from infants and juveniles, it was clear that HVP1 is commonly acquired at an early age, with approximately 70% of animals being seropositive by three years of age. This seroconversion rate is somewhat lower than that reported by Jenson and co-workers (26), who estimated > 95% seropositivity by two years of age using a different serologic assay (immunofluorescence) and a smaller sample size. Despite this slight difference, the epidemiology of HVP1 in baboons appears to parallel that of EBV in humans.

It has been reported that SVV is transmitted among adult patas monkeys, but that not all adults that seroconvert develop clinical signs of infection (43). Of all the sera from adult baboons we tested (which included all adults every 6 months for 3 years), we detected seroconversion in only a few adult baboons. There were, however, many infant and juvenile baboons that seroconverted to SVV during this same period, indicating that the virus was circulating and being transmitted within the colony. However, these seroconversion events did not occur in clustered outbreaks, but rather in a continuous, long-term pattern (Fig. 2). Particularly interesting was that, although 30 to 60% of adult baboons were seronegative for SVV (Table 3), seroconversion of adult animals to SVV was infrequent. This suggests that, in baboons, SVV is transmitted principally among infant and juvenile animals. This, in fact, is consistent with primary VZV infections in humans where about 90% of cases are children under 10 years of age and < 3% of cases are persons over 15 years of age (18). During semi-annual TB testing roundups, we have occasionally observed papular rashes located principally on the lower portion of the abdomen of young baboons. Although not vesicular in nature, these rashes were morphologically consistent with those observed in other monkey species (20), and so may represent SVV infections. If these rashes are due to SVV, isolation of a varicella virus from baboons should be possible. The reference strains of SVV were originally isolated from vervets and macaques. Although there is some variation in restriction endonuclease cleavage patterns of macaque and vervet SVV isolates, SVV isolates from vervets and patas monkeys do not appear to vary significantly from each other (19). It will, therefore, be of interest to determine whether a baboon varicella isolate is identical to either vervet or macaque SVV isolates.

HVP2 is genetically similar to BV of macaques and HSV1 and HSV2 of humans (14). BV has long been known to infect infant macaques as oral infections, but the principal means of spread has always been presumed to be via sexual contact. It has been noted by many investigators that, in macaques, the prevalence of antibodies to BV increases with age, and that a substantial increase in seroconversion occurs when animals reach sexual maturity (28, 44, 52, 56). However, it also has been reported that about 60% of BV seroconversions occur before monkeys reach sexual maturity, leading those investigators to conclude that, although sexual transmission is relevant, it is not the predominant mode of BV transmission among macaques (51, 53). We previously reported that almost all adult baboons are infected with HVP2 (12-14). Levin and co-workers (33) described an outbreak in baboons due to HVP2 and, on the basis of lesion type, frequency, and location, concluded that the virus was transmitted sexually. In this study, we found that nearly 60% of baboons were seropositive for HVP2 by 3 years of age, and that > 70% of all these seroconversions occurred by the age of 2 years. Consistent with this, we previously reported a mini-outbreak of HVP2 in a small breeding harem, in which three infants were infected (13). Restriction analysis of isolates from these three infants as well as an isolate recovered from an oral swab specimen from one dam indicated that all were probably derived from a single source, presumably from a dam shedding HVP2 in saliva. These results are entirely consistent with the conclusion reached by Weigler and co-workers (53) for BV in macaques, that HVP2 is predominantly transmitted at a young age as an oral infection.

Using a commercial HHV6 ELISA, we did not detect any positive baboons. Higashi and co-workers (22) tested sera from 10 monkey species (although not baboons) for antibody to HHV6 using an immunofluorescence assay, and detected reactivity in eight species. Thus, there is reason to expect that baboons may well harbor an HHV6 homologue. Although it is possible that the HHV6 assay we used was not able to detect antibodies to a baboon HHV6 homologue, this seems unlikely since the assay utilized an HHV6-infected cell lysate as antigen. Such an antigen would contain all structural and non-structural proteins of the virus, and so, should detect antibody to a related virus. It is also possible that the OUHSC colony is free of HHV6, or even that baboons do not harbor an HHV6-like virus. Further investigation will be needed to clarify this.

Although SV40 is not a herpesvirus, it was included in these assays since reactivity of baboon sera with SV40 has been reported (25, 54). SV40 has been reported to be prevalent in adult macaques (4, 38). Similarly, we found that almost all adults have serologic evidence of SV40 infection. Minor and co-workers (38) followed several newly formed cohorts of macaques and found that

transmission of SV40 appeared to be derived principally from an environmental source (as opposed to vertical transmission from the dam) and occurred rapidly, although not all susceptible monkeys seroconverted. Our results were consistent with these findings, in that more than half of the seroconversions to SV40 occurred in animals after the age of 1 year, with approximately a third of baboons remaining seronegative at the age of 3 years. Thus, SV40 does not appear to be as readily transmitted among young baboons as are BaCMV, BaRV, and HVP1. As for HVP2, a significant proportion of SV40 seroconversion events were noted in baboons older than 4 years. Whether this indicates a role for sexual transmission of this virus remains to be determined.

Overall, the epidemiology of herpesviruses in baboons appears to reflect that of homologous herpesviruses in human and macaque populations. The early seroconversion to BaRV, HVP1, and especially, BaCMV by a substantial number of infants prior to total decay of maternal antibodies raises questions about the degree of protection offered by these antibodies. Certainly the ability of these viruses to infect infants in the face of maternal antibody constitutes a situation where an infant could become latently infected without mounting a substantial IgG response of their own. The specter of such antibody-negative, latently infected animals represents a serious potential problem that needs to be considered in the establishment of virus-free colonies. Also, this infection at an early age when infants remain closely associated with their mothers suggests that these viruses may be primarily transmitted to infants from their mothers via saliva or possibly through breast milk. Perhaps somewhat surprisingly, we did not find evidence that simultaneous infection with one or more viruses is common, even in infants that seem to acquire a number of viruses before 1 year of age. The fact that several cesarean section-derived baboons that were hand reared and housed largely without any direct contact with "dirty" baboons still acquired a few herpesviruses over several years indicates that, although direct contact with the mother may be the most effective or principal means of transmission of these viruses, transmission from other animals or possibly even fomites also can occur. Thus, care should be taken in the husbandry practices used in derivation of specific-pathogen-free colonies.

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