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In the fall of 1987, the establishment of a Herpesvirus simiae (B-virus) negative rhesus monkey (Macaca mulatta) breeding colony was initiated at the Armstrong Laboratory. A serological testing program was used to categorize all monkeys into groups of either positive or negative to B-virus. Segregation of the groups allowed the creation of breeding harems that were exclusively serum positive or negative to B-virus while allowing maintenance of a similar level of infant production. Decreasing numbers of animals converted to a positive status during the first three followup serum tests for B-virus in the program. During 1990 an increase in the number of monkeys converting to positive status and the discovery of an indeterminate status demonstrated that the latency of B-virus in the rhesus may have the potential to defeat an eradication attempt not conscientiously pursued.

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An Attempt to Eradicate *Herpesvirus simiae* from a Rhesus Monkey Breeding Colony

Jerome J. Sauber, John W. Fanton, Roger C. Harvey, and John G. Golden

Abstract | In the fall of 1987 an attempt to establish a *Herpesvirus simiae* (B-virus)-negative rhesus monkey *(Macaca mulatta)* breeding colony was initiated at the Armstrong Laboratory. A serologic testing program was used to identify all monkeys into groups that were either positive or negative to B-virus based on serologic tests. Segregation of the groups allowed the creation of breeding harems that were exclusively seropositive or -negative to B-virus. Animals that were serologically positive were kept in breeding to maintain infant production levels not unlike those previous to segregation. Decreasing numbers of animals converted to a positive status during the first three serum tests for B-virus in the program. During 1990, an increase in the number of monkeys converting to positive status and the discovery of an indeterminate status demonstrated that latency of B-virus in the rhesus may have the potential to defeat an eradication attempt not conscientiously pursued.

In 1934 Sabin and Wright described the first case of human B-virus encephalomyelitis in the *Journal of Experimental Medicine* (1). Since that first case in 1934, the Centers for Disease Control has acknowledged an additional 31 published cases of human B-virus infection (2). It is often indeterminable if there was actually a B-virus infection in some of these early published cases. This could reduce the number of known human B-virus infections (3, 4). The severity of human B-virus infection has been described in detail (5, 6) and warrants detailed preventive measures for animal care staff.

The entry site for human B-virus infection is usually attributed to either scratches or bite wounds contaminated with virus-infected saliva. Improper handling of infected monkey tissues or specimens has also been incriminated as a source of human B-virus infection. During the 1987 outbreak at Pensacola, Florida, the first known case involving human-to-human transmission of B-virus (7, 8) was documented.

When the human B-virus deaths in Pensacola occurred, the Armstrong Laboratory (AL), Brooks Air Force Base, placed a moratorium on the research use of macaques that were positive for B-virus. Serologic testing (Julia K. Hilliard, PhD, Southwest Foundation for Biomedical Research) was selected as the method to identify B-virus carriers in our colony. A small breeding colony had been operated at our laboratory for several years, prompting a decision to attempt to derive a B-virus-negative colony from the rhesus monkeys already in the AL breeding colony. Zwartouw had reported in 1984 that B-virus-positive dams give birth to offspring with only transient positive titer (from transplacental passive immunity) to B-virus (9, 10). Breeding colony records revealed that almost all of the test-negative native-born animals were the offspring of B-virus-positive parents. It had been initially thought that B-virus-positive breeders would have to be totally culled from production. The success of Charles River Laboratories' free-ranging (and B-virus SPF) breeding colony on Key Lois, Florida (11), was considered to be sufficient evidence to attempt to make our colony B-virus-negative.

Materials and Methods

Until 1987, rhesus had been assigned to the primate breeding colony without regard to their B-virus status. The housing (breeding) format was group harems of 7 to 10 females with 1 male monkey. At the time of the moratorium, the breeding colony had a total assigned population of 176 male and female rhesus monkeys. Slightly over one-half (95/176, 54%) of these rhesus were wild-born. They were estimated to have been born from 12 to 20 years earlier in either India or present-day Bangladesh.

The decision was made to segregate B-virus-seropositive dams and their newborn infants to individual runs in a maternity building from birth. Segregated sections in the building were designated as B-virus-positive or -negative. Seronegative dams and their infants would not be housed near seropositive dams or their infants. All animals in this study were used and cared for in accordance with Air Force Regulation 169-2 and Public Health Service guidelines (12).

An initial 100% serum titer sampling of the entire breeding colony population was conducted during November and December 1987 before harems were established for the 1988 breeding season. Serologic testing was performed again at the end of March 1988 and subsequently every year just before breeding harem assembly to assure segregation of any seropositive converter rhesus from the negatives. Sera were tested by enzyme-linked immunosorbent assay (ELISA). Suspicious ELISA tests were rechecked by Western blot test-

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ing to detect reactivity with viral antigens. Retesting was occasionally performed for monkeys with borderline positive or suspicious test results.

Results

The initial 100% sampling of the breeding colony population in November and December 1987 disclosed that almost two of every three (117/176, 66.5%) breeding rhesus were seropositive for B-virus. This percentage of positives closely paralleled the percentage of wild-born adolescent rhesus reported by Orcutt *et al.* after their original test sample (13). The first and second serologic rechecks also indicated decreased B-virus-positivity in previously seronegative rhesus as reported by Orcutt (13).

In March 1988, when the breeding harems were disassembled, all seronegative animals (no apparent or detectable antibodies to B-virus) on the first test were again sampled. Serologic testing revealed that another 16.9% (10/59) of previously seronegative monkeys were seropositive for B-virus.

Again in October 1988, before the formation of breeding harems, all previously negative rhesus, and additions from the vivarium, were retested. One monkey, then the last negative wild-born male breeder, was found to have converted to a positive B-virus status.

No testing was done at harem disassembly in the spring of 1989. In the fall of 1989 testing, before harem assembly, two additional females were discovered to have converted to positive B-virus status. These females were of diverse breeding backgrounds with no relationship to each other in positioning in the breeding harem building.

The fifth testing of all previously negative monkeys was accomplished in August and September 1990 as harems were to be assembled. This testing disclosed 11 monkeys with either positive or suspicious ELISA test results.

A follow-up retesting of the same sera by Western blot disclosed that five rhesus were negative (no apparent or detectable antibodies to B-virus). Three each of the remaining six animals' sera were judged to have either indeterminate (not negative) or positive test results. Another test sample of all 11 monkeys by ELISA and Western blot analysis yielded identical results. The six rhesus found to be positive or indeterminate were all classified as being B-virus-positive. This policy of placing all positive or indeterminate animals in the B-virus-positive groupings was adopted by the AL for all rhesus with suspicious test results.

The sixth ELISA testing (fall 1991) disclosed that three more rhesus, one male and two females, were judged suspicious. Western blot testing confirmed that the wild-born male and a native-born female were reclassified as indeterminate. These animals had previously tested negative since the initiation of the B-virus serum testing in 1987. The second female was an older wild-born female whose ELISA results were suspicious in 1990 and 1991 but had been ruled negative on Western blot both years.

Additionally, the three rhesus that had been classified indeterminate (i.e., positive) in 1990 were retested. The ELISA results were deemed negative for both the male and the female that did not conceive. The other female, which still had her infant at breast, again had indeterminate ELISA and Western blot results (Table 1).

Table 1. A chronological order of serum testing to determine *Herpesvirus simiae* positive (not negative) animals in the Brooks AFB primate breeding colony

Samples (date taken)	Wild born origin	Native born origin	Total* number
Baseline 100% sample (Nov-Dec 1987)	89.5% (84/95)	40.7% (33/81)	66.5% (117/176)
First recheck of negative animals (March 1988)	40.0% (4/11)	12.2% (6/48)	16.9% (10/59)
Second recheck of negative animals (Aug-Oct 1988)	16.7% (1/6)	0.0% (0/63)	1.4% (1/69)
Third recheck of negative animals (Sep-Oct 1989)	0.0% (0/5)	3.1% (2/65)	2.9% (2/70)
Fourth recheck of negative animals (Aug-Oct 1990)	0.0% (0/11)	7.8% (6/77)	6.8% (6/88)**
Fifth recheck of negative animals (Dec 1991-Jan 1992)	14.3% (1/7)	1.5% (1/66)	2.7% (2/73)**

*The number of B-virus-negative rhesus tested yearly varies due to culling from and additions to the breeding colony from vivarium pool rhesus. Added rhesus had been individually housed and B-virus titer tested a varying number of times prior to entry into the breeding colony. The B-virus titer checks prior to entry into the breeding colony are not included in these totals.

**Three (50%, 3/6) animals were classified as indeterminate in 1990 and two (100%, 2/2) in 1991.

Discussion

The original 100% sampling disclosed that 66.5% (117/176) of the breeding rhesus were B-virus-seropositive. B-virus-positive animals represented 88.4% (84/95) of wild-born breeders. Fourteen of the 16 wild-born males (87.5%) and 70 of the 79 wild-born females (88.6%) in the breeding colony were positive for B-virus. Of special interest, however, were native-born rhesus that had been in the breeding colony for three or fewer breeding seasons. These animals were assigned to the breeding colony in the summers and falls of 1984, 1985, and 1986. These animals should have had smaller percentages of positive blood tests, provided they were negative at entry to the breeding colony. No animals were tested before 1987. In the fall of 1987, there were 81 sexually mature, native-born, male and female rhesus in the primate breeding colony. These native-born animals represented slightly less than one-half (81/176, 46.0%) of the total breeding colony in 1987. In the initial testing, 33 (40.7%) of these rhesus had positive titers to B-virus. The rhesus females had from 1 to 6 active years in the breeding colony. The 54 females added in 1984 (13 monkeys) and 1985 (41 monkeys) were generally older rhesus that had been in the vivarium for several years. Thirty-nine (72.2%) of them were 4 or more years old at entry and considered sexually mature at that time. These rhesus had been individually caged for various numbers of years in the vivarium and were research naive. These 54 females ranged in age from as young as 2 years (15 monkeys) to as much as 8 years old (3 monkeys) on assignment to the breeding colony. In contrast, the 30 females that were added in

Vol 42, No 5
Laboratory Animal Science
October 1992

Table 2. Prevalence of *Herpesvirus simiae* in virgin rhesus females shortly after exposure to rhesus males following entry into breeding colony

Number Entry year	% Positive (after 1 year)	% Positive (after 2 years)	% Positive (after 3 years)	Total % positive to date	% of Total exposed to "B" positive males
13 (1984)	Not tested (1985)	Not tested (1986)	61.54% 8/13	69.23% 9/13	10.0% 13/13
41 (1985)	Not tested (1986)	29.27% 12/41	41.46% 5/29	51.28% 20/39	58.54% 24/41*
30 (1986)	3.33% 1/30	3.45% 1/29	3.57% 1/28	10.00% 3/30	46.67% 14/30*
0 (1987)	0.00% N/A	0.00% N/A	0.00% N/A	0.00% N/A	0.00% N/A
4 (1988)	0.00% 0/4	0.00% 0/3	0.00% 0/2	0.00% 0/2	0.00% 0/4**
9 (1989)	22.22% 2/9	0.00% 0/7	N/A (1992)	22.22% 2/9	44.44% 4/9***
0 (1990)	0.00% N/A	0.00% N/A	0.00% N/A	0.00% N/A	0.00% N/A

*In 1986 and 1987, 14 immature females were exposed in a group housing situation to an immature male that was positive on the initial 100% screening in 1987.
**Never exposed to a male rhesus with positive *Herpesvirus simiae* titer in a breeding situation.
***One of two exposed to an indeterminate status male prior to detection.

1986 were primarily (27/30, 90.0%) prepubescent (1 to 2 years old) females. This was reflected in only three pregnancies that first year after their entry into the breeding colony. Only the three oldest females were placed with mature males in that first breeding season.

The 13 females that entered the breeding colony in 1984 were exposed to seropositive males for three full breeding seasons (1985, 1986, 1987). All were 4 or more years old on entry to the breeding colony. Eight (61.5%) conceived and seven delivered live infants in the spring of 1985. All 13 had conceived and delivered at least one infant by various males that were determined to be positive in the fall of 1987. Correspondingly, these 1984 additions had the highest percentage (9/13, 69.2%) of native-born female rhesus infected with B-virus. All nine of these positive titers were detected in the first two B-virus tests. The 41 females that entered breeding in 1985, with two seasons of exposure (1986 and 1987) to B-virus-positive rhesus, have had considerably less positive titers (20/41, 48.8%) to date. Eighteen (90%) of these 20 positive titers were detected in the first two blood tests in the fall of 1987 and spring of 1988. For reasons due primarily to their youth, only some of the 30 females that entered the breeding colony in 1986 were ever exposed to mature positive males. If exposed, it was only for one breeding season. However, 14 immature females were in the company of an immature male the year after their entry (1987). This male was positive on the first blood test in the fall of 1987. Thus far, four of these females have converted to a positive titer to B-virus. Only one of the four females was detected as positive on the first two tests. She was a sexually mature female that was bred and delivered a live-born infant. Of interest is the sire of this infant. He was a negative male that could be considered suspect, as he appeared in the breeding history of several females that have later converted to B-virus-positive. Because he was a strong breeder and had been bred to several females, he was in the breeding histories of several females. The majority (29/33, 87.9%) of native-born females having positive titers were those that 1) had been in the breeding colony the longest (i.e., entered the colony before the segregation of monkeys that were seropositive from negative for B-virus in December 1987); or 2) were sexually mature at entry (Table 2).

Nine of the 10 breeder serum conversions discovered in March 1988 were females. Three of the females and the single male were wild-born. The other six were born in our colony. However, five of the six native-born animals had only been in the breeding colony one breeding season before the segregation of B-virus-positive and negative monkeys. All five had been with rhesus males that were positive in the fall of 1987. Only one of the five had conceived and borne an infant from her single year in the breeding colony with a positive male and female harem mates. All five were old enough to be considered sexually mature, but two were later removed as breeding colony culls due to infertility.

The wild-born rhesus male which converted in the fall of 1988 had been used for four breeding seasons and twice had tested seronegative (fall 1987 and spring 1988) before converting to antibody-positive. This seroconversion to B-virus-positive was more than 15 months since his last exposure to positive females. Fourteen of the 15 (93.3%) females he had been in the company of between 1985 through 1987 were found positive to B-virus on the initial serum check in the fall of 1987. Nine (64.3%) of these 14 females had conceived and borne infants by this male. The 15th female was bred by this male in 1986 and delivered a live infant but remains B-virus seronegative to date. In the 1988 breeding season, this male had been placed in a harem with 12 virgin females. None of these females had attained 4 years of age by the end of the breeding season. Seven (58.3%) of the 12 became pregnant, and all 7 had live births. One of the seven females that conceived by this male converted to a positive B-virus titer, but not until the fall of 1990.

In 1989, only 2 native-born female rhesus of the 77 breeders tested (2.6%) converted to positive status. Both had been

in the breeding colony for the entire time of the B-virus antibody testing and had tested negative in three previous tests. Their breeding histories were totally dissimilar despite their entry into the breeding colony only a year apart. One had entered the breeding colony as an adult and had produced four infants, the first three of which were sired by males that were seropositive on the initial 100% serum sampling in 1987. The other female had been assigned to the breeding colony shortly after weaning and had only been placed with a mature (and negative) male in 1989. The only factors these two females had in common were that both were U.S.-born of wild-born monkeys, and their parents had tested B-virus-positive on the initial 1987 serum sampling.

The results of an ELISA performed in the fall of 1990 initially indicated that samples from 11 (11/93, 11.8%) animals had equivocal B-virus antibody titers. A search of the breeding history of the 2 males and 9 females in this group disclosed occasional or chance meetings of most of the 11 in breeding situations during the years since the initiation of testing for B-virus. All but one of these animals were native-born. The three oldest females had been bred in harems containing B-virus-positive male and female breeders for 1 or more years before this B-virus eradication program was initiated. All of these older animals had tested negative for B-virus antibodies on at least four previous occasions since the initial testing. Two of the females had only been in the breeding colony for 10 months before converting to a positive serology. They had been tested and were negative before their assignment to the breeding colony. A review of all these animals' breeding records for their placements in breeding harems indicated a commingling in the 1990 breeding season. Zwartouw (9, 10) contends that B-virus in rhesus and other *Macaca* species is a venereal disease. If Zwartouw's belief is correct, a theory for the possible source of this spike in B-virus is that there had been an acute outbreak of B-virus in the 1990 breeding season. One or more females or the male could have had an acute case of B-virus from a latent infection which caused passage of B-virus infection at breeding. The male then may have sexually transmitted the B-virus to other females in the harem. It is also possible that B-virus was passed between females by fight trauma (bites and scratches) with transmission to the male. In accordance with the AL policy, these indeterminate animals were considered to be positive (not negative) and were segregated from both positive and negative animals. For research purposes, these monkeys were kept separate from known positive monkeys to assist in the possible determination of either a true intermediate stage or a possible transitional stage enroute from a negative to a true positive test. The indeterminate male was used in 1:1 cage breeding with the indeterminate females during the 1991 breeding season. This breeding resulted in the production of one infant. An ELISA in the fall of 1991 disclosed that the male and the female that did not conceive were negative by both ELISA and Western blot. The female that conceived was bled at the weaning of the infant. This female's serum was again indeterminate on ELISA and Western blot.

Also of note are four wild-born females that had been introduced into the breeding colony in the fall of 1981. These females had produced 12 live-born infants before the initial blood tests in 1987. The majority of the infants were sired by males determined to be B-virus positive on the initial 1987 testing. These females remained seronegative for all six testing cycles reported here. One female's ELISA test results were equivocal twice but were considered negative on all Western blot follow-ups. Eradication of B-virus from this breeding colony was not fully successful during the first 4 years of monitoring. Those monkeys that had been exposed to or were carrying B-virus were not always identified in a timely manner by positive virus antibody titers. Failure to detect the virus in these animals is crucial to segregating B-virus-positive animals from a breeding, or any type of group housing, situation. The reasons for the slow or delayed arrival of a strong and easily detectable serum titer could possibly be attributed to one or more of the following: 1) a latency of B-virus that failed to present sufficient viral antigen to the monkey's immune system to induce a strong serum antibody response; 2) B-virus does not always quickly induce strong positive titer in all animals after exposure; or 3) the seropositive status is possibly either indistinct, weak, or transient in some rhesus. A weak (indeterminate) titer could revert to a false-negative or possibly a viral carrier state later. Two of three rhesus thought to be indeterminate in 1990 were classified as negative in 1991. B-virus can possibly remain quiescent (or at least not produce a detectable antibody titer) in many rhesus after exposure to known positive rhesus. Some examples of these animals were the five native-born females not detected as being positive until March 1988. These animals did not have a positive ELISA titer for 9 or more months after the known end of a single breeding season and exposure to positive males and females. They were placed with negative breeders in 1988. Also possibly in this extended transition to positive are: 1) two females discovered to be positive in 1989, 2) six animals considered positive or indeterminate in 1990, 3) the four wild-born but still negative females and probably other rhesus with no serum antibody titers at this time. Another consideration in our eradication attempt was that all sexually mature breeding rhesus, at the time of the initial testing in 1987, had been exposed to rhesus that were seropositive for B-virus antibodies for one or more breeding seasons. It has been determined that B-virus can be isolated from rhesus that are seronegative and that the virus is, at times, impossible to isolate from seropositive rhesus (4). Culling of all positive and exposed rhesus breeders, consistently shortening the serology submission interval to 6 months, and diverting young female production rhesus back into the breeding colony may have hastened eradication, but it was not completely accomplished.

In our situation, semiannual testing may be the procedural modification to improve the likelihood to detect B-virus titers in a transitional state, speed the identification and isolation of positive animals, and lessen the probability of viral transfer. With our breeding methods, any additional shortening of the interval between tests to less than 6 months would not decrease the possibility of B-virus transfer between animals. This is because, in our system, we divide the year into two almost identical intervals of approximately 6 months each.

Vol 42, No 5
Laboratory Animal Science
October 1992

The first interval is after the birth of the infant when females are singly housed during nursing and raising their infants to a weaning age and weight. A second approximately 6-month interval is devoted to group-housing in breeding harems. This is initiated immediately after serologic test results are available. The progress that we have made in our program hopefully will mean that the elimination of positive and indeterminate rhesus will continue to reduce the numbers of converters until they will be a rarity.

We have presented the quandary of B-virus eradication in our colony. We encountered great early success with the identification of almost two of every three of the colony as serum antibody-positive to B-virus. The numbers of seropositive animals in the breeding colony showed substantial reductions through the first three serum samples and were possibly instrumental in the decision to change to annual serum testing. Replacement of aged positive breeders with young negative females was delayed in part because of high birth rates in the 15- to 17-year-old, B-virus-positive (serum) females. Always possible is the passage of B-virus between monkeys by any of a multitude of proven methods of viral disease transmission. These possibilities become even more likely as the investigation of one of the latest human cases of B-virus has failed to provide any unequivocal evidence of commonly accepted means of transmission from animal to human.

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