The replicative capacity of rhesus macaque peripheral blood mononuclear cells for simian immunodeficiency virus in vitro is predictive of the rate of progression to AIDS in vivo

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Survival of rhesus macaques (Macaca mulatta) experimentally infected with simian immunodeficiency virus (SIV) varies significantly from animal to animal. Some animals die within 2 months while others survive for more than 5 years, even when identical inocula are used. This diversity in survival creates a significant problem in the design of therapeutic and vaccine trials using the SIV–macaque model because the use of small numbers of animals may provide results that are misleading. Identifying an in vitro assay that could determine the survival of monkeys prior to infection would prove extremely useful for stratifying experimental groups. Analysis of the survival of a cohort of 59 control animals obtained from over a decade of vaccine and therapeutic trials has demonstrated that the ability of peripheral blood mononuclear cells (PBMC) from a naïve animal to produce virus in vitro was highly predictive of disease progression in vivo following experimental inoculation. Animals classified in vitro as high producers of virus progressed to disease significantly more rapidly than animals classified as either low (P = 0.002) or intermediate (P = 0.013) producers of virus. The hierarchy of high and low virus production was maintained in purified CD4+ T cell cultures, indicating that this phenotype is an intrinsic property of the CD4+ T cell itself. These findings should significantly aid in the design of vaccine and therapeutic trials using the SIV–macaque model. Furthermore, since these studies suggest that the rate of virus replication is controlled by innate characteristics of the individual, they provide new insight into the pathogenesis of AIDS.

Introduction

Simian immunodeficiency virus (SIV) causes a fatal AIDS-like disease in rhesus macaques (Kindt et al., 1992; Lackner, 1994; Murphey-Corb et al., 1986). For this reason, the nonhuman primate serves as a useful animal model for the study of the pathogenesis of human immunodeficiency virus (HIV) and AIDS in humans. The average time until death of an SIV-infected macaque is 1–2 years depending upon the strain of SIV used (Dittmer et al., 1994; Kindt et al., 1992). However, a wide range of survival is observed (Buchbinder et al., 1994; Cao et al., 1995; Letvin & King, 1990; Lifson et al., 1988, 1997; Michael et al., 1997; O’Brien et al., 1996; Operskalski et al., 1997; Zhang et al., 1988), with some animals dying within months while others live for many years. This pleiotropy is particularly striking because, in most SIV studies, the same route and dose of a cryopreserved inoculum are used to infect the animals. A considerable variation in the length of survival has also been noted in HIV-infected humans (Lifson et al., 1988; Munoz et al., 1989). It is estimated that 20% of HIV-infected people will progress to AIDS within 5 years, while 12% will remain free of disease for nearly 20 years (Munoz et al., 1995). Although the dose and strain responsible for human HIV infections are highly variable, some studies have shown that a difference in survival persisted even though an identical route and strain could be documented (Liu et al., 1997; Operskalski et al., 1997).

As for HIV-infected humans (Haynes et al., 1996), SIV-infected macaques have been classified into three groups: fast progressors, progressors and slow/nonprogressors (Dykhuizen et al., 1998; Kindt et al., 1992; Letvin & King, 1990; Zhang et al., 1988). SIV-infected rapid progressors have a persistent viraemia with little or no virus-specific antibody...
response. Survival of these animals is approximately 2–3 months. Progressors have detectable viraemia, but also have a strong antibody response and survive for 1–3 years. Slow/ nonprogressors remain persistently infected because viral sequences can be detected by PCR, but virus is difficult to recover from peripheral blood. These animals can survive for 5 or more years.

We have determined that cultured peripheral blood mononuclear cells (PBMC) from different naive animals vary widely in their ability to support virus replication in vitro. To determine whether a relationship exists between this phenomenon and the variable survival noted in vivo following experimental infection, data from a cohort of 59 control animals used over the last decade were evaluated. These data demonstrate that there is a statistical relationship between the ability of an animal's PBMC to produce virus in vitro and the rate of progression to disease and death following experimental infection. Furthermore, comparative evaluation of virus production in purified T cell cultures produced similar results, a finding that demonstrates that this phenotype is a property of the CD4+ T cell itself.

Methods

- **Cells and virus.** Blood (20–40 ml) was collected via the femoral vein from individual rhesus macaques using acid citrate dextrose (ACD) as the anticoagulant. PBMC were isolated from whole blood by Ficoll–Paque (Pharmacia Biotech) density-gradient centrifugation. Cells were grown in modified RPMI 1640 supplemented with 15% foetal bovine serum, penicillin-streptomycin (100 U/ml; 100 U/ml), t-glutamine (2 mM), HEPES buffer solution (10 mM) (GibcoBRL), and recombinant human IL-2 (40 U/ml) (GibcoBRL), and SIV/DeltaBe70 stocks; in vitro infections employed stocks propagated on rhesus PHA blasts; in vivo infections employed stocks propagated on either rhesus or human PHA blasts as described (Murphey-Corb et al., 1989). All virus stocks were cryopreserved in 1 ml aliquots in liquid nitrogen until needed.

- **Animal manipulations.** Rhesus macaques (Macaca mulatta) of either sex were obtained from the Tulane Regional Primate Center breeding colony. Animals were infected by inoculation of 1 ml of 10–100 monkey infectious doses into the saphenous vein using a 23 gauge butterfly needle. Each injection was chased with 1–2 ml sterile saline to assure accurate delivery of the inoculum. Physical examinations were performed at biweekly intervals, and animals were provided full supportive care until they were deemed moribund by the attending veterinarian. Blood was drawn at these times for measurements of antigenaemia and for quantification of T lymphocytes. Antigenaemia was determined by an SIV core antigen ELISA (Coulter) according to the manufacturer's instructions. Flow cytometric determination of lymphocyte subsets was performed as described (Martin et al., 1993). Complete necropsies were performed following humane sacrifice.

- **Analysis of virus replication in vitro.** PBMC were obtained as described above. Cell pellets containing 1 x 10⁷ PBMC were infected by incubation with 1 ml of a cryopreserved stock of SIV/DeltaBe70 containing 1000–100000 tissue culture infectious doses for 1–2 h at 37°C. No difference in the hierarchy with respect to virus production was observed when inocula within this range of infectivity were used (data not shown). After infection, cells were washed twice in complete medium (RPMI 1640 supplemented with 15% FBS, t-glutamine, and penicillin-streptomycin at concentrations indicated above). Twenty-four hours after infection, 10 µg/ml of phytohaemagglutinin (PHA) (GibcoBRL) was added. On day 4, the PHA was removed by washing the cells three times in complete medium. Cells were cultured in complete medium supplemented with human IL-2 (40 U/ml) for the entire experiment (21 days) and maintained at a concentration of 1 x 10⁸ cells/ml. On days 7, 10, 14, 18 and 21, 1 ml of supernatant was removed and stored at −70°C until analysis. The amount of virus in culture supernatants was determined by measuring reverse transcriptase (RT) activity in cell culture supernatants as described below. Alternatively, virus production was measured using an SIV Core antigen ELISA (Coulter), as per the manufacturer's instructions. Measurement of RT activity or p27 antigen yielded similar results.

- **Measurement of RT activity.** Cell-free culture fluid was collected in 1 ml aliquots and subjected to centrifugation at 12,000 g for 45 min in a refrigerated microfuge. The supernatant was decanted, and the virus pellet was stored at −70°C until needed. The pellet was thawed on ice and 50 µl of solubilization buffer (0.5% Triton X-100, 0.8 M NaCl, 0.5 mM PMSF, 20% glycerol and 50 mM Tris–HCl, pH 7.8) was added to lyse the pelleted virus particles. The pellet was resuspended by vortexing, and the sample was retained on ice until incorporation into the assay. In each well of a 96-well flat-bottomed microtitre plate, 10 µl of the solubilized pellet was added to 90 µl of a solution containing 10 mM MgCl₂, 5 mM dithiothreitol, 83 µg/ml dATP, 5 µg/ml polyr-A(p-dT)₁₅₋₁₈ (Pharmacia), 52 µCi/ml [³²P]dTPP (New England Nuclear) and 52 mM Tris–HCl, pH 7.8. The plates were then incubated at 37°C for 2 h, and the reaction was stopped by the addition of 10 µl 25 mg/ml tRNA (GibcoBRL). To each well, 90 µl of cold 10% TCA containing 5 mM PMSF, 20% glycerol and 50 mM Tris–HCl, pH 7.8 was added and the reaction was allowed to sit for 30 min. Acid-precipitable radiolabelled nucleotides were harvested using a Skatron plate washer and the resulting radioactivity counted using a scintillation counter.

- **Purification of CD4+ T cells.** CD4+ T cells were purified from PBMC using MACS, a magnetic cell selection system (Miltenyi Biotec). After isolation from peripheral blood as described above, PBMC were washed twice in PBS and once in MACS buffer, which consisted of PBS containing 5 mM EDTA (Sigma), 0.5% BSA (Sigma) and 10 mM HEPES (GibcoBRL). Cells were incubated in 200 µl of buffer and 50 µl of CD4+ microbeads per 4 x 10⁵ cells for 20 min at 4°C. For all experiments, cells were run over two MS selection columns. Purity of selected cells was determined using flow cytometry and was >95% for all assays. Rhesus-reactive antibodies used for flow cytometry were: phycoerythrin-conjugated anti-CD4 clone MT477 (Pharmingen), fluorescein-conjugated anti-CD8 clone SK1 (Becton-Dickinson) and either phycoerythrin- or fluorescein-conjugated anti-CD3 clone SP34 (Pharmingen).

- **Statistics.** Data were analysed with Microsoft Excel 9.0 for data entry and coding. Statistical analysis was performed using SPSS Version 9.0 (SPSS Inc.). A P value of <0.05 was considered significant. Kaplan–Meier analysis was used to evaluate survival among groups. Statistical relationship of maximum RT and survival was analysed by one-way ANOVA.

Results

**Classification of animals based on in vitro SIV production from PBMC.**

To determine the capacity of rhesus macaque PBMC to replicate SIV in vitro, Ficoll–Paque-purified PBMC were...
Macaque susceptibility to SIV-induced disease

Fig. 1. In vitro replicative capacity of SIV in PBMC from randomly chosen rhesus monkeys. Ficoll–Paque-banded PBMC from each animal were infected with 1000 TCID_{50} per 10^6 cells of a cryopreserved stock of SIV/DeltaB670, and virus production was assessed by quantifying RT activity in culture supernatants as described. Animals were classified as high, intermediate or low producers of virus based on overall rank in the group. Animals classified as low producers have RT values below 10 000 c.p.m./ml, which is considered negative for this assay. Animals: H779 (+); H727 (○); H695 (▲); H780 (●); H696 (▼); H644 (◇); H737 (▽); H746 (●); H783 (□); H822 (△).

Exposed to a cryopreserved stock of SIV/DeltaB670, and virus production was measured at biweekly intervals by analysis of RT activity in culture supernatants. Isolated PBMC were infected with SIV immediately following purification and prior to the addition of PHA to ensure that the state of these cells would most closely approximate their condition in vivo. The outcome of infection of PBMC from 10 monkeys selected randomly from the Tulane Regional Primate Center breeding colony is shown in Fig. 1. A wide range of virus production was observed, even though the input virus and the cell number were held constant. Cells from three monkeys (animals H779, H727 and H695) had maximum (peak) RT levels in culture supernatants > 400 000 c.p.m./ml. Four monkeys had peak RT values ranging between 80 000–105 000 c.p.m./ml and three animals had RT values of < 25 000 c.p.m./ml. The difference observed in these cultures was not due to a failure of the cells to become infected, because all cultures contained SIV sequences readily detectable by PCR of PBMC DNA (data not shown). Based on these results, these animals were arbitrarily assigned to high, intermediate and low producer groups, respectively.

To determine whether the virus replication phenotypes observed among these 10 animals were consistent over time, in vitro infections of PBMC were repeated twice more over the course of a year. The results for representative animals from each of the subgroups (high, intermediate and low producers) are shown in Fig. 2. In each case, the amount of virus produced was consistent over time for cells from a given animal. These data suggest that the virus replicative capacity of PBMC is a property of the animal itself, and that the differences in replication between animals was not due to variability in the assays.

Fig. 2. Reproducibility of the in vitro SIV producer phenotype. All 10 animals shown in Fig. 1 were assayed three times over a period of a year. Data from a representative animal from each group are depicted. RT activity (in c.p.m./ml) was measured for all three trials. (A) Animal H779, a high producer; (B) H780, an intermediate producer; (C) H822, a low producer.
Fig. 3. Relationship of post-infection SIV p27 antigenaemia among different in vitro replicative phenotypes. All animals were infected intravenously with 10–100 monkey infectious doses of SIV/DeltaB670 as described. Antigenaemia (ng/ml) in the blood was monitored serially by ELISA until sacrifice due to the onset of AIDS. (A) High producers: H695 (●), H727 (○) and H779 (▲). (B) Intermediate producers: H644 (●), H780 (○) and H737 (▲). (C) Low producers: H746 (●), H783 (○), H822 (▲) and H696 (▼).

Fig. 4. Relationship of changes in CD4⁺CD29⁺ T helper lymphocytes in monkeys with different in vitro replicative phenotypes. At regular intervals after infection, the percentage of memory (% CD4⁺CD29⁺/total % CD4⁺) T cells in peripheral blood was evaluated by flow cytometry as described. (A) High producers: H695 (●), H727 (○) and H779 (▲). (B) Intermediate producers: H644 (●), H780 (○) and H737 (▲). (C) Low producers: H746 (●), H783 (○), H822 (▲) and H696 (▼).
**In vitro classification is predictive of disease progression in vivo**

To determine whether our *in vitro* classification of animals as high, intermediate or low producers correlated with the rate of disease progression *in vivo*, the 10 animals described in the previous section were inoculated intravenously with 10–100 monkey infectious doses of the same virus stock of SIV/DeltaB670 that had been used for the phenotyping *in vitro*. The rate of disease progression was determined by (1) quantifying SIV p27 antigenaemia, (2) analysing changes in T cell subsets in the peripheral blood and (3) survival. SIV p27 antigenaemia was used to determine the virus burden in these animals because adequately preserved plasma samples for quantification of plasma RNA were not available. The relationship between the amount and persistence of antigenaemia to disease progression and survival, however, has been well documented (Farzadegan *et al*., 1996; Martin *et al*., 1993, 1994, 1997; Zhang *et al*., 1988). In our analysis of peripheral blood T cell subsets, we were particularly interested in determining whether a selective loss of the CD4+CD29+ memory subset of T lymphocytes in the peripheral blood had occurred, since we had previously shown that these changes were the most indicative of rapid disease progression (Martin *et al*., 1993, 1994, 1997). Persistent antigenaemia was observed in all three high producers (Fig. 3A), a finding indicative of rapid disease progression and early death. The remaining animals showed declines in antigenaemia by 3 weeks post-infection (p.i.). Two of three intermediate producers (monkeys H737 and H644), however, had recurrences of antigenaemia that were detectable by 18 and 24 weeks p.i., respectively. In contrast, low producers had the lowest peak antigenaemia, with recurrences detectable only after 28 weeks p.i.

The changes in the CD4+CD29+ memory T cell subset identified by flow cytometry in each animal (Fig. 4) were consistent with the antigenaemia observed. All three high producers had rapid selective declines in CD4+CD29+ T lymphocytes that coincided with persistent antigenaemia. This decline was evident by the first month p.i., whereas a decline in this population was much more variable in intermediate and low producers and generally progressed at a much slower rate. The intermediate producer that had the earliest recurrence of antigenaemia and earliest death in the group (monkey H737) also had a very rapid selective decrease in CD4+CD29+ cells. An increase in the CD4+CD29+ T cell population was also observed in some animals late in the infection. This finding is consistent with the observation of others (Nicholson *et al*.,...
and likely reflects activation of these cells associated with the increases in virus burden that occur near death.

Physical examinations were performed at biweekly intervals, and animals were provided with full supportive care until they were deemed moribund by the attending veterinarian. Moribund animals were humanely sacrificed and given complete pathological examinations. As expected from the differences observed in virus replication, a wide range of survival was observed among the ten animals. Deaths occurred from 50 to 557 days p.i. with a median time of 381 days. The three high producers survived less than the median, while the four low producers survived beyond the median survival time. Survival of the intermediate producers was more variable. The patterns of clinical and pathological findings were also associated with the RT rank and rate of disease progression, with high producers developing a generalized rash soon after infection, showing little evidence of lymphadenopathy, and dying early with severe opportunistic infections. In contrast, low producers had more chronic disease progression characterized by lymphadenopathy, splenomegaly, chronic diarrhoea and wasting. Lymphoma occurred in one low producer, a finding associated with lengthened survival in macaques (Habis et al., 1999). Histopathological examination of tissues taken from these animals at necropsy confirmed the clinical profile of simian (S)AIDS in all cases.

Although the data from these 10 animals suggested a striking relationship between SIV production in vitro and rate of SIV-induced disease progression and time to death in vivo, larger numbers of animals were required to definitively establish this phenomenon. Since adequate cage space was available at the Center, the majority of animals enrolled in SIV studies spanning a decade of research were provided with supportive clinical care until they were moribund so that crucial data on survival in a large cohort of infected animals could be obtained. Furthermore, because we believed that the results of virus production in vitro were highly useful in the selection of animals for experimental groups, this analysis was performed on most of the animals used during this time period. Fifty-nine animals were chosen for further analysis. This cohort comprised all animals infected with SIV/DeltaB670 that were (1) inoculated intravenously, (2) received no other effective therapeutic or vaccine and (3) died of an AIDS-defining illness. However, different cryopreserved stocks (both human and monkey propagated) and different doses (10–10000 animal infectious doses) were used over this time period. Maximum RT determined for each animal is presented graphically in Fig. 5(A). The overall mean peak RT was 101120 ± 118764 c.p.m./ml. Animals were divided into three cohorts using natural breakpoints detected by direct visualization of the data. Each breakpoint is indicated by the vertical line in Fig. 5(A). These cohorts consisted of: (1) the upper 20th percentile, peak RT > 160000 c.p.m./ml (mean = 298119 ± 128355 c.p.m./ml), 12 animals; (2) the lower 20th percentile, peak RT < 15000 c.p.m./ml (mean = 7838 ± 4004 c.p.m./ml), 12 animals; (3) the remaining animals falling into the median range, 160000 > peak RT > 15000 c.p.m./ml (mean = 66548 ± 32793 c.p.m./ml), 35 animals. The cause of death in these cohorts was consistent with their survival, with SIV-giant cell disease, pneumonia and colitis most prevalent in rapid progressors, and lymphoma, lymphoproliferative disease and amyloidosis being the predominant findings in slow progressors.

The overall mean survival for the 59 animals was 289 ± 151 days. Kaplan–Meier analysis of survival for the individual cohorts is shown in Fig. 5(B). Striking differences in survival were apparent in the three groups, with early mortality almost exclusively within animals in the highest peak RT group and long-term survivors associated with the lowest peak RT group.
Some of the animals in the lowest peak RT group lived over 400 days p.i., which was over 100 days beyond the mean survival for the entire study group.

The statistical relationship between peak RT and survival of the three cohorts is shown in Fig. 5(C). Mean survival differed markedly among the groups, with survival in high producers less than half that of low producers \((168 \pm 123 \text{ vs } 367 \pm 106)\). This difference was highly significant by one-way ANOVA \((P = 0.002)\). High producers also experienced reduced survival compared to that of the intermediate group \((304 \pm 152, P = 0.013)\). However, no significant difference in overall survival between intermediate and low producers was observed.

The phenotypic differences observed between high and low producers were not due to infection with other viruses commonly encountered in macaques \((e.g.\) simian retrovirus, simian T-lymphotropic virus-I, herpesvirus B) because differences in virus replicative capacity were observed in cells from monkeys that were PCR- and/or antibody-negative for these viruses \(\text{data not shown}\).

**High/low producer phenotype is maintained in purified CD4\(^+\) T cell populations**

To determine the relative contribution of other cell types in PBMC to virus production during the kinetics assay, cultures of purified CD4\(^+\) T cells and whole PBMC were run in parallel. CD4\(^+\) T cells were purified using anti-CD4 antibodies coupled to magnetic beads according to the Miltenyi Biotec system. Flow cytometry was used to monitor the purity of the CD4\(^+\) T cell cultures, and in all cases, purity was equal to or greater than 95% \(\text{data not shown}\). The results from these assays are shown in Fig. 6. A comparison of virus production from whole PBMC cultures from high and low producer animals shows at least a log difference in the amount of virus produced, as measured by p27 antigen in the cell culture supernatants \(\text{Fig. 6A, B}\). When comparing virus production from purified CD4\(^+\) T cell cultures \(\text{Fig. 6C, D}\), there is still a difference of at least a log in virus production \(\text{notice difference in y-axes}\). This confirms that the CD4\(^+\) T cell itself is responsible for the differences in virus production \(\text{in vitro}\).

**Discussion**

In a survey of rhesus monkeys infected intravenously with the primary virulent isolate SIV/DeltaB670, we have shown that the virus replicative capacity of the naıve animal’s PBMC following \(\text{in vitro}\) infection is highly predictive of disease progression and survival \(\text{in vivo}\) following experimental inoculation. Monkeys whose cells produce high amounts of SIV following an \(\text{in vitro}\) infection \(\text{high producers}\) are more likely to develop SAIDS sooner and die more quickly than those animals whose cells support little virus replication \(\text{low producers}\). As expected, these animals displayed a continuum of virus production and survival spanning high virus production/early death to low virus production/long-term survival. The relationship between the maximum RT activity produced in cell culture supernatants during a 21 day culture period and survival was highly significant when high producers were compared to intermediate and low producers \((P = 0.013 \text{ and } 0.002, \text{respectively}; \text{ANOVA})\). The direct correlation between high \(\text{in vitro}\) replicative capacity and shortened survival that has persisted throughout these experiments is even more striking given that results obtained over a long time interval were compared, and multiple virus stocks propagated in both human and monkey PBMC were used during this time period. Furthermore, this property determines the fate of the animal once infected, with low producers progressing to SAIDS more slowly and surviving longer than high producers. This relationship is particularly intriguing in view of the likelihood that \(\text{in vitro}\) control of virus does not involve virus-specific immunity.

These data support observations reported by Lifson \textit{et al.} \(1997\) in which RNA plasma loads and survival in eight macaques correlated with the amount of virus required to infect macaque PBMC \(\text{in vitro}\). These two studies thus clearly identify an innate property of individual macaques that controls virus replication \(\text{both in vitro and in vivo}\). Further insight into this phenomenon was gained by determining that the hierarchy associated with high and low producer phenotypes is retained in cultures of infected purified CD4\(^+\) T lymphocytes. These findings indicate that this phenotype is an intrinsic property of the CD4\(^+\) T cell itself. Control of virus production could be at the level of virus entry \(\text{e.g. virus receptor[s]}, \text{or post-entry, with the differential production of either suppressor (in low producer) or enhancer (in high producer) proteins}\).

The intrinsic property of macaque PBMC to support the replication of SIV \(\text{in vitro}\) may be analogous to the differences in replicative capacity observed for human PBMC infected \(\text{in vitro}\) with HIV-1 \(\text{Williams \\& Cloyd, 1991}\). Together with the variable length of time from infection to death also observed in HIV humans \(\text{Buchbinder et al., 1994; Cao et al., 1995; Lifson et al., 1988; Michael et al., 1997; Munoz et al., 1995; Oberskalski et al., 1997}\), the data reported here may be highly relevant to the pathogenesis of HIV as well. The experimental system described in this report should enable the identification of the specific host gene(s) responsible for this phenomenon as well as provide crucial information for the development of effective strategies for disease intervention.

The advantage of accurately predicting survival prior to selection of monkeys for SIV studies is obvious, particularly in experiments where both control and test groups are infected and more subtle parameters of efficacy are required. Since limited numbers of animals are often used in macaque trials due to the expense, optimal stratification of groups is crucial so that differences in delays in disease progression be appropriately identified. In this regard, it is important to note that \(\text{in vitro}\)
determination of virus replicative capacity not only predicted overall survival in the animals reported here, but also correlated with other parameters such as antigenaemia and the rate of T cell decline (reported here) and plasma virus loads (Lifson et al., 1997), which are all used to determine the rate of disease progression before death.

We have successfully used this simple, in vitro assay in the design of many therapeutic and vaccine trials performed on SIV/DeltaB670-infected macaques at the Tulane Primate Center. Stratification of groups prior to the initiation of the study using this assay has enhanced the interpretation of these studies. For example, in a therapeutic trial evaluating the efficacy of treatment with cyclosporin during the acute viremic episode (Martin et al., 1997), the parameters of survival, T cell changes and antigenaemia were used as indicators of efficacy. Stratification of the treatment and control groups with respect to maximum RT obtained from the in vitro assay permitted pairwise analysis of efficacy. This analysis identified differences in persistence of antigenaemia among the two groups that were statistically significant, even though the variability among the group as a whole precluded a significant outcome. The opposite effect can also occur. Differences in survival may be suggested when statistical analysis of small groups (n ≤ 4 animals) of randomly chosen animals is performed, but when the group size is doubled, the difference may no longer be significant. The inclusion of analysis of the in vitro replicative capacity of monkeys used in experimental trials should permit an appropriate interpretation of the outcome of these studies.

This work was supported by Public Health Service contracts NOI AI 2560 and 15119 and grant PO1 AI28243 from the National Institutes of Health, National Institute of Allergy and Infectious Diseases, and P51-RR-0164 support of Regional Primate Centers from the National Center for Research Resources.

We gratefully acknowledge the technical support of Calvin Lanelo, Eileen Deharo, Maury Duplantis, Gail Plache and Robin Rodriguez and the professional staff in the Departments of Veterinary Sciences and Pathology at the Tulane Regional Primate Center.

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Received 3 May 2000; Accepted 16 June 2000