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Protection of Macaques against AIDS with a Live Attenuated SHIV Vaccine is of Finite Duration

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Abstract

Using background data that live vaccines against several viral pathogens are effective in inducing life-long protection against disease, we undertook studies in macaques to determine the duration of protection that two live SHIV vaccines could induce against AIDS. Earlier studies had established that macaques immunized once with a live vaccine and challenged 6 months later were protected, and that other macaques given two sequential inoculations of live vaccines were protected for at least one year. Protection was associated with persistence of the vaccine viruses. In this study, we sought to determine whether the duration of protection in macaques given a single inoculation of replication competent live vaccines would extend beyond three years. Two groups of four rhesus macaques were inoculated with two live SHIV vaccines, respectively. The viruses replicated transiently in all animals but at the 3 year time point, PCR analysis of PBMC did not detect DNA of either virus in any of the animals, and all were negative for CMI responses in the blood. All 8 animals succumbed to disease when challenged with pathogenic viruses.

INTRODUCTION

The well known biological properties of HIV that include its preferential infection in HIV-specific CD4 T cells (Douek *et al*., 2003), its propensity for undergoing continuous mutation (Nickle *et al*., 2003) and the long incubation period of disease (Lassen *et al*., 2004) have created major road blocks for development and evaluation of safety, immunogenicity, and efficacy of a vaccine that could prevent AIDS. The macaque models of HIV pathogenesis using SIV and SHIV strains of virus in different species of macaques have provided important insights into the feasibility of developing an efficacious vaccine against disease (Igarashi *et al*., 1999; Joag *et al*., 1996; Luciw *et al*., 1995; Reimann *et al*., 1996a; Reimann *et al*., 1996b). Use of this animal model system has made conditions for development and assessment of efficacy relatively simple and has clearly established the principle that it is possible to develop an

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efficacious vaccine for use in humans, even though it has not been possible as yet to develop an efficacious vaccine that could predictably prevent infection in macaques with pathogenic challenge viruses (Johnston and Fauci, 2007).

Efficacy of long term protection against well known human viral pathogens such as measles, polio, mumps, small pox viruses provided the initial major thrust for efforts to develop a live vaccine against HIV. However, the data from macaques showing that attenuated SIVmac239 which proved to be highly efficacious in preventing AIDS in adult macaques was still pathogenic in infant animals (Baba *et al*., 1995) greatly dampened enthusiasm for pursuing development of such vaccines for use in humans. Despite this, live vaccine studies in macaques are still being pursued using SIV and SHIV vaccines (Amara *et al*., 2005; Desrosiers, 1998; Johnson and Desrosiers, 1998; Johnson *et al*., 1997; Koff et al., 2006) because such vaccines have proven to be efficacious in adult animals and still safe in young animals even after considerable effort to bring out the pathogenic potential of such viruses . Such efforts have included IV inoculation of animals at birth and observation for more than six years (Smith, unpublished observation), IC inoculation (Smith *et al*., 2002) and serial passage of the virus in young animals (Mackay *et al*., 2002) all without pathological effects.

Deletion of accessory genes from pathogenic SIV provided the first method for development of live vaccines against macaque lentiviral pathogens (Daniel *et al*., 1992; Johnson and Desrosiers, 1998; Wyand *et al*., 1999; Wyand *et al*., 1996). Use of this concept has been exploited for development of SIV and later, safe SHIV vaccines (Abel *et al*., 2003; Amara *et al*., 2005; Desrosiers, 1998; Hu, 2005; Joag *et al*., 1998b; Johnson and Desrosiers, 1998; Johnson *et al*., 1997; Koff *et al*., 2006; Silverstein *et al*., 2000). The process resulted in development of viruses that have attenuated replication potential and pathogenicity, thereby making them candidates for live virus vaccines against AIDS. These SHIV agents have been used to test proof of concept hypotheses about mechanisms of vaccine-induced protection, identification of immunological correlates or protection, and duration of the protection following immunization (Joag *et al*., 1998b; Johnson *et al*., 1997; Kumar *et al*., 2002; Wyand *et al*., 1999). Studies on the SHIV vaccines showed that the viruses replicated productively for a few weeks, after which they persisted in a minimally productive state for an indefinite period (Joag *et al*., 1998b; Kumar *et al*., 2002; Mackay *et al*., 2004; Silverstein *et al*., 2000). Control of productive replication was associated with development of CMI responses (Johnson *et al*., 1997; Kumar *et al*., 2001; Kumar *et al*., 2002; Silverstein *et al*., 2000) similar to the responses associated with control of HIV in humans (Pantaleo and Koup, 2004).

In one of our previously reported studies (Joag *et al*., 1998b), we had immunized two cohorts of six macaques with Δ*vpu*Δ*nef* SHIV-4 (Vacc-I) and Δ*vpu*SHIVppc (Vacc-II), respectively. The parental viruses from which the vaccines were derived varied greatly in replication competence in macaques, SHIV-4, being poor (Joag *et al*., 1996; Mackay *et al*., 2002), and SHIVppc, being highly competent. Vacc-I replicated poorly, similar to the parental virus, SHIV-4, and viral DNA was found in only 4 of the 6 animals six months later. Similar to its parent, SHIVppc, Vacc-II replicated vigorously and established latent infection in all six animals. The 12 animals were challenged vaginally 6 months after immunization with pathogenic $SHIV_{KU1}$, a virus that underwent massive replication in non-immunized animals, and within two weeks, caused near complete elimination of $CD4^+$T cells. Nearly all such animals developed fatal AIDS within one year (Joag *et al*, 1998b). The 2 animals in cohort 1 (Vacc-I group) that lacked vaccine viral DNA in PBMC developed a disease pattern similar to that seen in the control animals (Joag *et al*., 1998b). The remaining 10 immunized animals all became infected with the challenge virus as indicated by infectivity in PBMC and plasma, but this was only transient. Animals that lacked infectivity in PBMC still had cell-associated viral DNA (Silverstein *et al*., 2000) indicating that both vaccine and challenge viruses had established persistent low grade infections. The vaccine viruses had thus induced protection

against acute disease but not against infection by the challenge virus. Control of pathogenic virus was associated with strong CMI responses. However, these responses waned with time, in concert with minimal replication of either virus. Studies on these animals for the next six years showed that the animals eliminated one or the other of their "latent" viral genomes at different time points varying from 1 year to 5 years. Elimination of pathogenic virus and retention of the vaccine virus left animals in a permanently healthy state (Silverstein *et al*., 2000; Mackay *et al*., 2004). However, elimination of the vaccine virus but retention of the pathogenic virus was invariably associated with activation of the pathogenic virus and onset of AIDS (Mackay *et al*., 2004).

In a second study (Kumar *et al*., 2002), we sought to determine whether macaques that were immunized first with Vacc-I, followed by immunization with Vacc-II, would develop a more limited period of productive replication of Vacc-II, but yet still benefit from the protection induced by Vacc-II, as had been seen in the study reported above (Joag *et al*., 1998b). This report (Kumar *et al*., 2002) showed that the immunized macaques did develop an extremely limited period of replication of Vacc-II, but when challenged 58 weeks later with a mixture of three viruses, SHIV89.6P, $SHIV_{KU2}$ (homologous with the vaccine viruses), and SIVmac17E, all of the animals were protected against disease (Kumar *et al*., 2002). Thus, persistence of Vacc-II virus must have been responsible for protection against disease.

In the present study, we focused the question on duration of protection following a single inoculation of replication-competent vaccine viruses, and asked whether the umbrella of protection induced by Vacc-II, and a derivative of Vacc-II, Vacc-III, would extend beyond three years. Vacc-II that had been used in the previous studies was used to inoculate four rhesus macaques, and Vacc-III, a replica of Vacc-II except that *nef* was deleted from the genome, was used to inoculate four other rhesus macaques. Replication-competence and safety of the Vacc-III virus was confirmed in earlier studies (Mackay et al., 2002; Smith et al., 2002). None of the animals were protected when challenged at three years with the three viruses described above. This established a new principle with expectations from live vaccine viruses. Classically, these types of vaccines have been known to confer life-long protection against disease. In the case of lentiviruses such as HIV however, protection from disease even by a safe and efficacious live vaccine may be of considerably shorter duration than expected.

RESULTS AND DISCUSSION

Following the observation that Vacc-II could induce protection for at least six months prior to challenge, we undertook another study in which we used a double immunization protocol of Vacc-I followed 4 months later by Vacc-II to ask three questions: (i) Would the productive replication of Vacc-II be modulated in macaques previously immunized with Vacc-I? (ii) Would Vacc-II induce the same level of protection in these animals as it had in the earlier study reported by Joag? (Joag *et al*., 1998b) and (iii) Would the induced protection extend to heterologous viruses such as SHIV89.6P and SIVmac17E? Studies on the animals prior to challenge showed that replication of Vacc-II was greatly restricted, but the animals nevertheless developed potent CMI responses that were still present at the time of challenge 58 weeks later. The pathogenic viruses had replicated transiently followed by control that lasted 74 weeks (Kumar *et al*., 2002). Having shown that the Vacc-I/Vacc-II protocol could induce protection for as long as one year, we now sought to determine whether a single inoculation with replication competent vaccine virus, Vacc-II and Vacc-III, (Vacc-III being Vacc-II that had been attenuated further by deletion of nef) would induce protection that would last at least three years.

In this new study, we inoculated four rhesus macaques orally with Vacc-II and another four with Vacc-III. These animals, along with two non-immunized macaques, were then challenged

rectally three years after immunization with the same three viruses that had been used in the previously reported study (Kumar *et al*., 2002). Plasma viral RNA loads and CD4 T cell counts in these animals are shown in Fig-1 and 2, respectively. Both vaccine viruses replicated productively in all eight macaques. Peak viral RNA concentrations, ranging between 2×10^3 and 1.2×10^5 RNA copies/ml plasma, were established by four weeks post inoculation. The virus replication then became undetectable in plasma within 3 months after administration of Vacc-II or III. However, sporadic spurts of virus replication evidenced by presence of viral RNA in plasma were seen occasionally in some of the animals during the first year following immunization. At the time of challenge at three years, none of the animals had vaccine virus RNA in plasma (Fig-1 and 2), and PBMCs lacked antiviral T cells measured by ELISPOT (Table-I). The animals were challenged rectally with the three-virus combination at Year 3. After pathogenic challenge, the virus-specific T cells became detectable in peripheral circulation within a week suggesting development of virus-specific cellular immune response. However, we can not rule out the possibility of these cells being present in secondary lymphoid organs at the time of challenge which could have migrated to peripheral circulation after challenge.

Following rectal infusion of the three challenge viruses into the eight vaccinated and two unvaccinated animals, systemic infections became established in all of the animals as evidenced by detection of viral RNA in plasma (Fig-1 and 2). Unlike our previous study where all vaccinated animals eventually controlled virus replication at one point or other (Kumar et al., 2002), the pathogenic viruses in this study continued to replicate in animals in both vaccine groups until the animals progressed to clinical disease, at which time they were euthanized (Fig-1 and 2).

The animals were monitored for the rapidity of development of SIV/SHIV-induced AIDS. The control animals progressed to clinical AIDS and lost >25% body weight within 6 weeks after challenge. Both animals developed neurological signs (hyper-reactivity, movement-coordination, hand tremor and balance). These animals showed high virus replication and massive CD4 loss in the blood (Fig-3). They were euthanized at weeks 6 and 7 and showed meningitis, mild encephalitis, and severe depletion of $CD4^+$ T cells from lymphoid tissues (data not shown). On the other hand the animals in both vaccine groups survived for more than one year (Fig-1 and 2), suggesting that the vaccines had induced a measure of protection against clinical disease. Compared to the virus control animals, the challenged vaccinates had an increased survival time by almost 9-fold.

In addition to developing high viral RNA concentrations in plasma, the animals developed progressive loss of $CD4^+$ T cells. Examination of plasma by *env*-specific RT-PCR prior to necropsy of the animals showed that SIV was present in all animals, and that $SHIV_{KU2}$ and SHIV89.6 were present in only some of the animals (Table-III). Examination of tissues from the animals showed that there was no trace of vaccine viral DNA [truncated vpu (Silverstein *et al*., 2000)], except for one macaque, RKq5, that had vaccine viral DNA in the brain (Table-II). Analysis of tissues exemplified in studies on RKq5 and RMt5 showed a wide distribution of the three viruses which were identified by *env*-specific sequences of the three viruses (Table-II). There was no particular pattern of distribution of the three viruses. However, in general, lymph node and spleen were specifically targeted by the three viruses, compared to the CNS, where there was minimal infection.

In summary, our findings indicated that the transient productive infection caused by a single inoculation of the live attenuated vaccine viruses failed to induce protection against disease at three years. In earlier studies, protection correlated with continuous sporadic bursts of virus replication that presumably contributed to maintenance of protective CMI responses (Daniel *et al*., 1992; Daniel *et al*., 1985; Desrosiers, 1998; Evans *et al*., 2005; Gauduin *et al*., 2006;

Johnson and Desrosiers, 1998; Johnson *et al*., 1997). In the animals reported in this study, there was no evidence of such bursts of virus replication between Years 2 and 3. In an earlier report (Mackay et al., 2004), we have previously shown that elimination of the vaccine virus was associated with activation of pathogenic virus. In the present study, lack of persistence of the vaccine virus in the plasma and cell-mediate immune response in the blood could have been a possible reason for the susceptibility of the animals to disease.

MATERIALS AND METHODS

Viruses

The construction of Vacc-II, Vacc-III and derivation of $SHIV_{KU-2}$ have been previously described (Joag *et al*., 1998a; Kumar *et al*., 2002; Mackay *et al*., 2002). Vaccine-II was derived from a non-pathogenic virus $SHIV_{PPC}$ by deleting 60 bp from *vpu* (Joag *et al*., 1996) whereas Vacc-III was derived by deleting *vpu* and *nef* from $SHIV_{PPC}$ (Mackay et al., 2002). $SHIV_{KU-2}$ was derived from non-pathogenic SHIV-4 first by passaging into pigtailed macaques (Joag *et al*., 1996) followed by passage in rhesus macaques (Joag *et al*., 1998a). $SHIV_{89.6}P$ was kindly provided by Dr. N. Letvin (Reimann *et al*., 1996a). $SIV_{mac}R71/17E$ is a M tropic and neurovirulent virus (Sharma *et al*., 1992). Stock preparations of these viruses were propagated in macaque mitogen-stimulated PBMC and viral titers were determined in C8166 cells.

Vaccination of macaques and challenge with $SHIV_{KU-2}$, $SHIV_{89.6}P$, and $SIV_{mac}R71/17E$

Ten 3-5 year old rhesus macaques (*Macaca mulatta*) were obtained and maintained in the AAALAC-approved Animal Facility of the first in Yerkes Primate Center, Atlanta, GA followed by transportation to University of Kansas Medical Center where they were housed in AAALAC-approved facility for post-challenge observation. These animals were divided into 3 groups of 4, 4 and 2 macaques. First 2 groups were orally inoculated with 1 ml of Vacc-II and III, respectively. Third group of 2 animals were administered equal volume Hank's buffered salt solution (HBSS). The vaccinated and control animals were challenged twice, one day apart, by intrarectal route with a mixture of undiluted $SHIV_{KU-2}$, $SHIV_{89.6}P$, and $SIV_{mac}R71/17E$ stocks. The Vacc-II and Vacc-III animals were challenged 148 and 140 weeks post-immunization, respectively. The animals were bled regularly to monitor $CD4^+$ T cell profiles and plasma viral loads.

Flow cytometry

Lymphocyte subset cell profiles were determined by staining for CD3, CD4 and CD8 cell surface markers using the whole blood lysis technique (Wyand *et al*., 1996). Briefly, 10 μl of the antibody mix against CD3, CD4, and CD8 (Becton Dickinson) was added to 100 μl of whole blood and incubated for one hour in the dark. Lysing solution (Becton Dickinson) was then added and the samples were incubated for another 10 min at room temperature. Stained cells were fixed with 0.5% paraformaldehyde and analyzed in a flow cytometer (Becton Dickinson FACS Calibur).

Total viral load measurements

Plasma viral RNA levels were determined by real-time reverse transcriptase PCR (ABI Prism 7700-sequence detection system, Perkin-Elmer) essentially as described previously (Mackay *et al*., 2002). Briefly RNA samples were subjected to real time RT-PCR using *gag* primers and a 5'-FAM- and 3'-TAMARA- labeled Taqman probe homologous to SIVmac239 *gag*, which is identical in both vaccine and 3 challenge viruses. Viral RNA copy numbers were calculated per ml plasma.

Differential detection of challenge virus in plasma of vaccinated macaques

Total RNA was extracted from plasma samples collected at the time of necropsy and used as template in a RT-PCR reaction using primers and conditions described previously (Kumar *et al*., 2002). Following the second round of amplification, a 5-μl aliquot was electrophoresed on a 1.5% agarose gel, and the bands visualized by staining with ethidium bromide. Different proviral DNA were identified using a set of primers specific for vaccine and different challenge virus. The primer sequence and PCR condition has been reported in our earlier publication (Kumar *et al*., 2002).

ELISPOT Assay

We measured viral antigen-specific cells in PBMC in an ELISPOT assay using their ability to secrete IFN-γ. The assay was performed on weeks 0, 1, 3 8, 12, 16 and 21 after pathogenic challenge. This assay used overlapping peptides encompassing the full length HIV Env (Cat no. 6451), Tat (Cat no. 5138) and Rev (Cat no. 6445) as well as SIVmac239 Gag (Cat no. 6204) and Nef (Cat no. 6206) proteins, provided by NIH AIDS Research and Reference Reagent Program. Env and Gag peptides were dissolved into 10 and 5 pools respectively whereas other 3 set of peptides were dissolved into individual pools with final concentration of 1 mg/ml. The peptide stocks were stored at -80°C. High binding Immobilon-P plates (Billerica, MA) were coated overnight at 4°C with 50 μl/well of 5 μg/ml of anti-monkey IFN-γ Abs (mAb G2-4, Mabtech Stockholm, Sweden). The unbound antibodies were removed next by washing 4 times with PBS. The wells were blocked with 10% fetal bovine serum (FBS). Fifty μl mixture of 5 peptide pools were added to each well except positive and negative control wells that received Con-A and FBS, respectively. 10^5 PBMC were added to each wells in triplicate for 6 hrs incubation at 37°C, followed by washing with PBS-Tween. The wells were incubated again with another anti-monkey biotinylated IFN-γ Abs (7-B6-1, Mabtech Stockholm, Sweden). The wells were then washed and 50 μl of Vectastain (Vector Laboratories, Buringame, CA) was added. The reaction was developed using 100 μl/well Nova-Red for 4 minutes. The plate was washed with tap water and spots were counted using a stereomicroscope. The negative controls (un-stimulated PBMC in the assay) showed 1-3 positive spots every time. These numbers were subtracted from total number of spots obtained in the wells containing peptide-stimulated PBMC.

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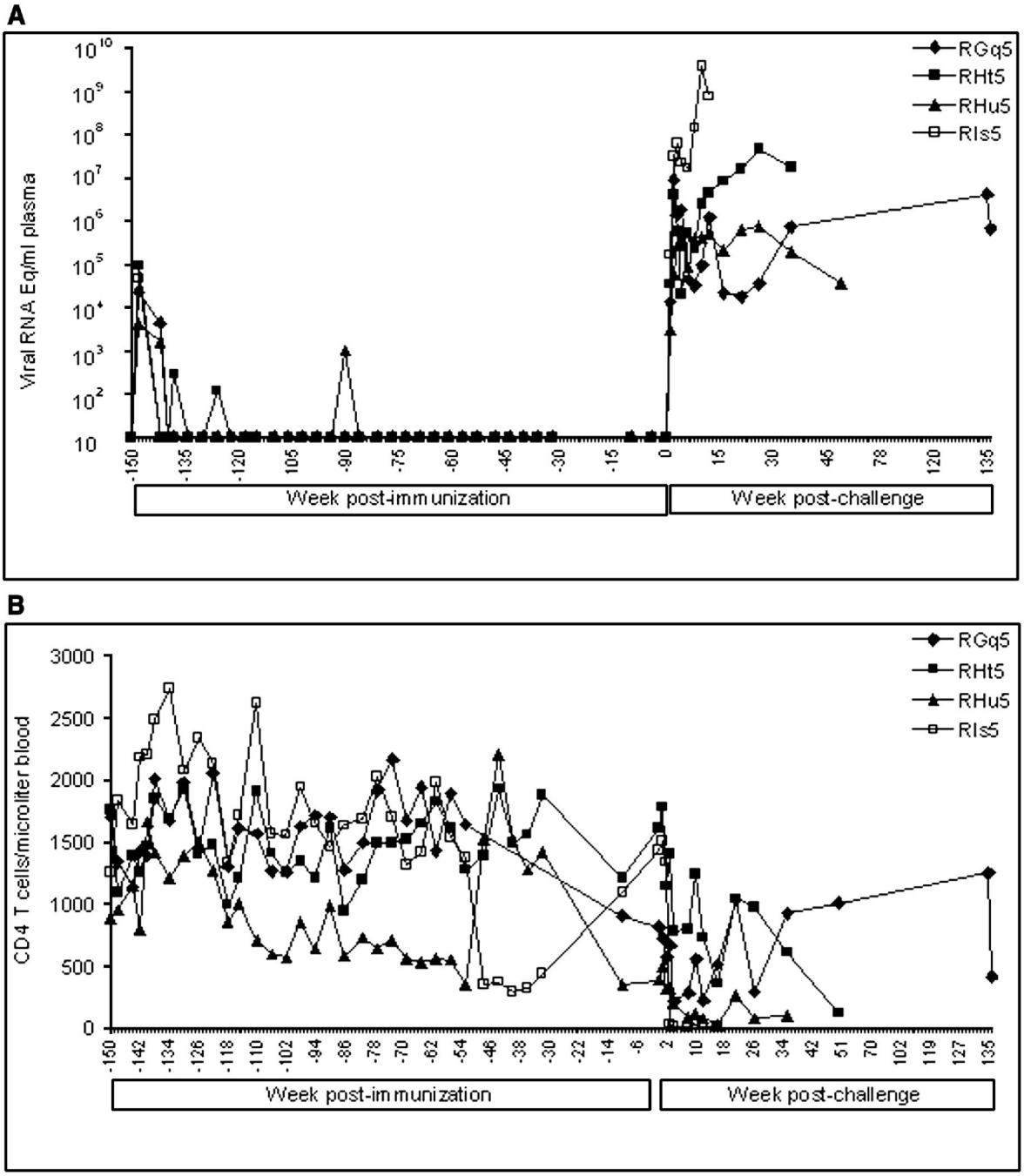

FIG. 1.

Plasma viral RNA concentrations and $CD4^+$ T cell counts in macaques immunized with Vacc-II and challenged with the three virus cocktail of $SHIV_{KU-2}$, $SHIV_{89.6}P$, and $SIV_{MAC}R71/17E$. Plasma samples were collected at different time points and total viral RNA concentrations was measured in real time RT-PCR using SIVgag primers. The viral RNA concentrations are expressed as RNA copy numbers per milliliter of plasma. The $CD4^+$ T cell counts were monitored by staining whole blood with a mixture of MAbs directed against CD3, CD4, and CD8 molecules. The absolute number of $CD4^+$ T cells/μl of blood was calculated by multiplying the percentage of the lymphocyte subset with the absolute number of lymphocytes/μl of blood from complete blood count (CBC).

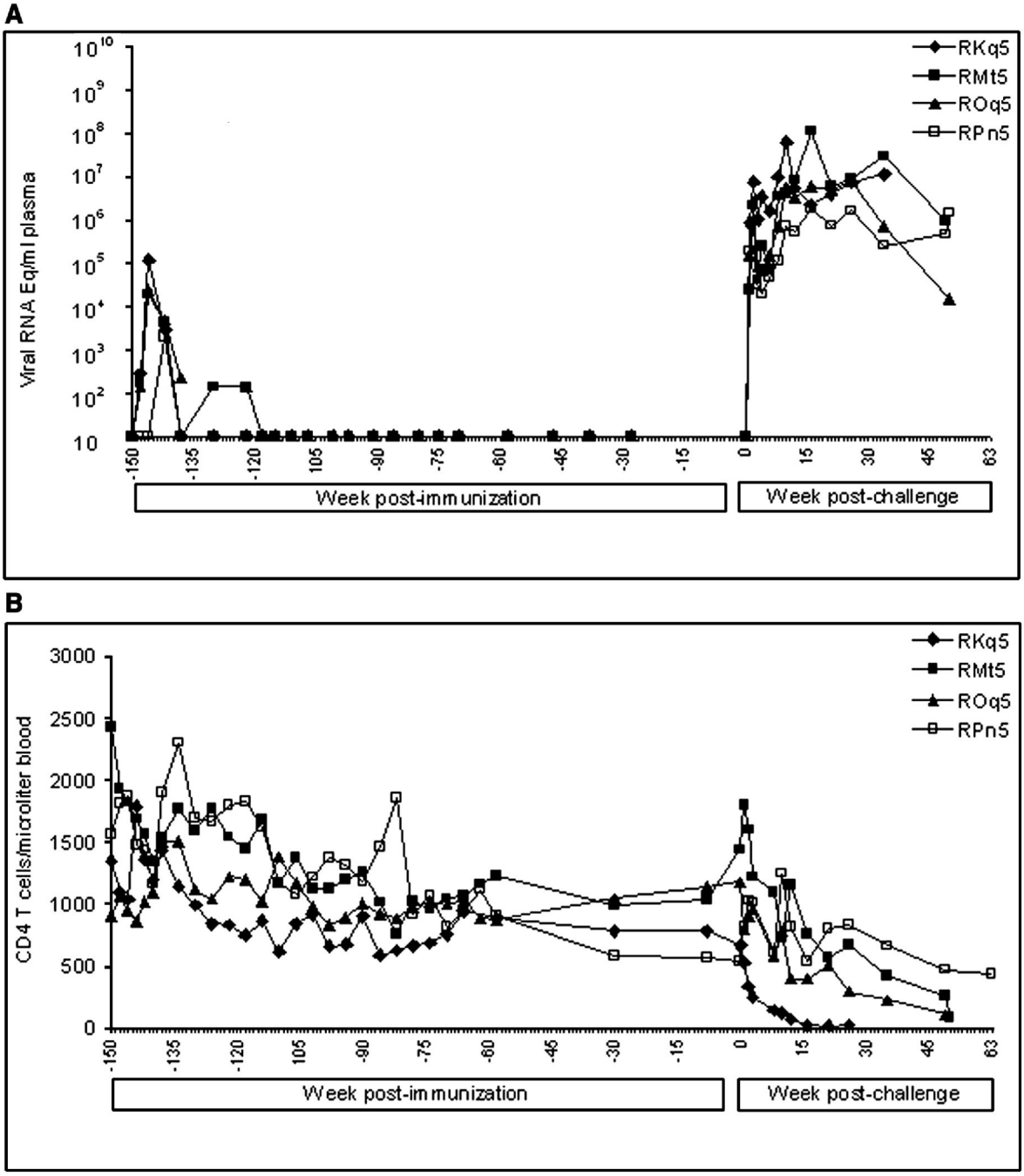

FIG. 2.

Plasma viral RNA concentrations and $CD4^+$ T cell counts in macaques immunized with Vacc-III and challenged with the three virus cocktail of $SHIV_{KU-2}$, $SHIV_{89.6}P$, and $SIV_{MAC}R71/17E$. Measurements identical to those summarized in Figure 1 were used here.

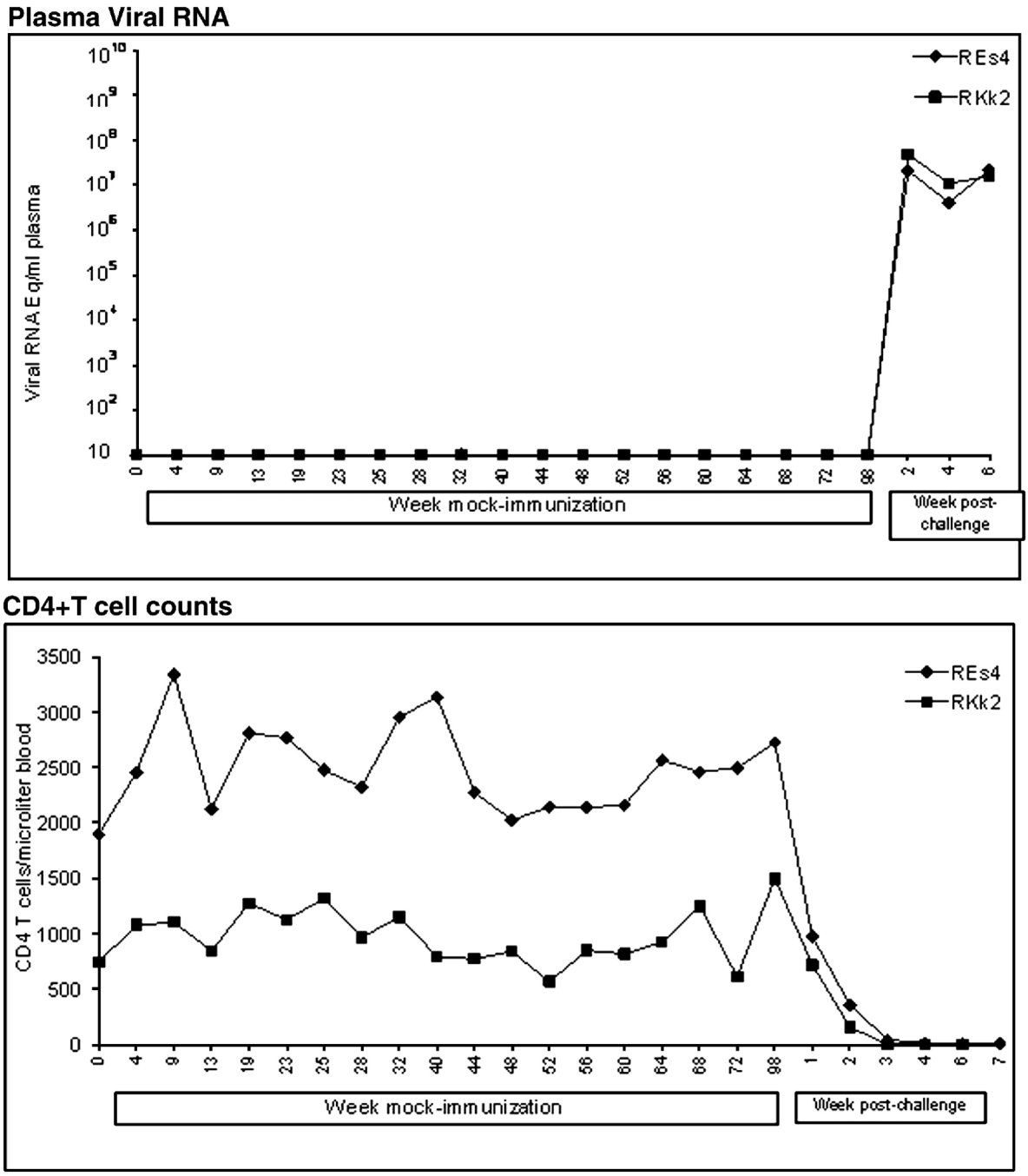

FIG. 3.

Viral RNA concentrations and $CD4^+$ T cell counts in two unvaccinated macaques that were challenged with the three-virus cocktail. Measurements were similar to those summarized in Figure 1.

Table-I

Virus-specific cellular response in macaques immunized with Vac-II[a] or Vac-III and challenged with cocktail virus

Macaque	Virus-specific T cells/106 PBMC on weeks post-challenge						
	0	**1**	**3**	**8**	**12**	**16**	**21**
Vac-II							
RGq5	0	10	235	530	760	1070	295
RHt5	0	220	20	30	110	75	0
RHu5	0	25	125	130	145	280	15
RIs5	0	15	125	35	5[b]		
Vacc-III							
RKq5	0	385	135	230	100	110	25
RMt5	0	35	425	85	160	415	540
ROq5	0	45	225	70	45	240	170
RPn5	0	70	485	350	205	325	215

[a]The ELISPOT results are presented as number of virus-specific cells/million PBMC

[b]The animal was euthanized at week 14, post-challenge

Table-II

Viral DNA distribution in plasma, lung, spleen and 3 brain regions of macaques immunized with Vac-III and challenged with pathogenic SHIVs and SIV

Macaque RKq5	**Challenge vpu**	**Vaccine vpu**	**KU2 env**	**89.6 env**	**SIV 17E env**
Virus in plasma	not done	not done	+	+	+
Lung	+	-	+	+	+
Spleen	+	-	-	+	+
Lymph Node	+	-	+	+	+
Occipetal Cortex	-	-	-	-	-
Parietal Cortex	+	+	-	-	-
Basal Ganglia	+	+	-	-	-
Macaque RMt5	Challenge vpu	Vaccine vpu	KU2 env	89.6 env	SIV 17E env
Virus in plasma	not done	not done	-	+	+
Lung	+	-	-	-	-
Liver	+	-	+	-	+
Spleen	+	-	+	+	+
Lymph Node	+	-	-	+	+
Occipetal Cortex	-	-	-	-	-
Parietal Cortex	-	-	-	-	-
Basal Ganglia	-	-	-	-	+

Table-III

Persistence of challenge viruses in the plasma of challenged macaques

Animal name	$SHIV_{KU-2}$	$SHIV_{89.6P}$	$SIV_{17E/71BR}$
RGq5	-	-	+
RHt5	+	-	+
RHu5	-	-	+
RIs5	+	+	+
RMt5	-	+	+
RKq5	+	+	+
RPn5	-	-	+
ROq5	-	+	+

The presence of individual challenge virus in the plasma of macaques at the time of death