Passive Immunization of Newborn Rhesus Macaques Prevents Oral Simian Immunodeficiency Virus Infection

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To determine if passively acquired antiviral antibodies modulate virus transmission and disease progression in human pediatric AIDS, the potential of pre- and postexposure passive immunization with hyperimmune serum to prevent oral simian immunodeficiency virus (SIV) infection or disease progression in newborn rhesus macaques was tested. Untreated neonates became infected after oral SIV inoculation and had high viremia, and most animals developed fatal AIDS within 3 months. In contrast, SIV hyperimmune serum given subcutaneously prior to oral SIV inoculation protected 6 newborns against infection. When this SIV hyperimmune serum was given to 3 newborns 3 weeks after oral SIV inoculation, viremia was not reduced, and all 3 infants died within 3 months of age due to AIDS and immune-complex disease. These results suggest that passively acquired anti–human immunodeficiency virus (HIV) IgG may decrease perinatal HIV transmission. However, anti–HIV IgG may not impart therapeutic benefit to infants with established HIV infection.

Perinatal infection with human immunodeficiency virus (HIV) occurs in ∼20%–40% of infants born to HIV-infected women. Although transmission can occur in utero and postnatally (through breast-feeding), evidence suggests that a large fraction of infants become infected around birth by contact with maternal blood and fluids, presumably by an oral route of infection [1, 2]. Infants infected with HIV often develop immunodeficiency and die sooner after infection than do HIV-infected adults. About one-third of HIV-infected infants have a rapidly fatal disease course and die within 1 year; the majority develop clinical disease more slowly and survive for >5 years (reviewed in [3, 4]).

Although zidovudine administration to HIV-infected pregnant women and their newborns can reduce the rate of vertical transmission by two-thirds [5], it is not 100% effective, and the prevalence of zidovudine-resistant HIV variants continues to increase. Accordingly, additional intervention strategies are being investigated, including those that are immune-based.

Because many variables may modulate transmission and disease progression (such as maternal virus load, immunologic status, the timing of vertical transmission, and the virulence and dose of the virus to which the newborn is exposed), it has been difficult to define the specific role of maternal HIV-specific antibody in vertical transmission and the pathogenesis of pediatric AIDS, and conflicting results have been reported [6–17]. Accordingly, clinical trials have been designed to determine whether increased levels of maternal anti–HIV antibodies, achieved either by passive administration of anti–HIV immunoglobulins or by active vaccination, can interrupt vertical transmission or alter disease progression in infected infants.

AIDS Clinical Trials Group (ACTG) protocol 185 was initiated to test the efficacy of zidovudine plus HIV hyperimmune globulin (HIVIG) administration versus zidovudine plus normal intravenous immunoglobulin administration to women with advanced HIV infection during pregnancy and to their infants at birth. The safety and pharmacokinetics of HIVIG in pregnant women and their newborns have already been assessed [18], but no data are available regarding the efficacy of HIVIG in reducing vertical HIV transmission. Because of the effectiveness of zidovudine in the ACTG 185 protocol, the role of HIVIG alone in preventing intrapartum HIV transmission could not be directly determined in this clinical trial. A preliminary analysis of the ACTG 185 trial recently indicated that due to an unexpectedly low rate of vertical transmission even in the zidovudine plus intravenous immunoglobulin control group, statistically valid comparisons between the two treatment arms were not possible. Accordingly, no definite conclusions could be made regarding the efficacy of HIVIG in reducing vertical

transmission, and the decision was made to discontinue the ACTG 185 trial [19].

It is still important to know, however, whether increased levels of anti–HIV antibodies can reduce vertical transmission, especially for developing countries, where prolonged chemo-prophylactic treatment with antiviral drugs is not feasible. An appropriate animal model can be very useful for studying the potential of passive immunization strategies for preventing transmission or disease.

Simian immunodeficiency virus (SIV) infection of rhesus macaques is an excellent animal model of HIV infection in humans [20, 21]. We have previously demonstrated that SIV_{mac} infection of newborn macaques is a useful animal model of human pediatric HIV infection that allows rapid evaluation of intervention strategies [22–26]. We have also demonstrated that vaccination of pregnant macaques against SIV resulted in transplacental antibody transfer that protected 2 of 3 newborn macaques against oral-conjunctival SIV infection at birth [2].

To further explore the role of passively acquired antiviral antibodies, the present study focused on determining whether passive immunization of newborn macaques with SIV hyperimmune serum (SIV-HIS) before or after oral SIV exposure could prevent infection or modulate the disease course.

Materials and Methods

Animals and sampling. Sixteen newborn rhesus macaques (*Macaca mulatta*) from simian type D retrovirus–free and SIV-seronegative dams at the California Regional Primate Research Center were removed from their mothers and reared in a primate nursery. When necessary, animals were immobilized with 10–15 mg/kg ketamine HCL (Parke-Davis, Morris Plains, NJ) injected intramuscularly. Samples were collected immediately before virus inoculation or serum administration and regularly thereafter for monitoring viral and immunologic parameters; 0.5–1 mL of blood (heparinized) was taken weekly for the first month, every 2 weeks for the next 6 months, and then monthly. Complete blood cell counts were done with EDTA-anticoagulated blood samples from all animals. Samples were analyzed by using an automated electronic cell counter (Baker 9000; Serono Baker Diagnostics, Bethlehem, PA); differential blood cell counts were done manually. Saliva samples were taken with Omni-Sal collectors (Saliva Diagnostic Systems, Vancouver, WA).

Virus inoculation of newborn macaques. Within 3 days of age, newborn macaques were inoculated orally with 1 mL of an un-cloned $SIV_{mac}251$ virus stock, administered atraumatically by dispensing the inoculum slowly into the mouth; animals were monitored to ensure that the inoculum was swallowed. The virus stock used in this study consisted of uncloned $SIV_{mac}251$ propagated on rhesus peripheral blood mononuclear cells (PBMC) with a titer of 10^5 $TCID_{50}$/mL [27]. This $SIV_{mac}251$ stock has been shown to cause persistent viremia and simian AIDS in neonatal, juvenile, and adult rhesus macaques at the California Regional Primate Research Center [2, 24, 27].

Preparation of SIV-HIS and normal control serum. The SIV-HIS was prepared by pooling sera from 2 juvenile and 4 adult rhesus macaques that had previously been immunized with live attenuated $SIV_{mac}1A11$ [28]; several of these animals had received a booster immunization with either live attenuated $SIV_{mac}1A11$ or whole inactivated SIV (which was prepared according to methods described previously [2]). After immunization, each of the 6 animals was challenged intravenously or intravaginally with $SIV_{mac}251$ propagated in rhesus PBMC and maintained low virus levels over 2 months to 4 years after challenge (unpublished data). These 6 macaques had no detectable plasma viremia, high anti–SIV antibody titers (by ELISA), and no clinical illness at the time serum was collected. Normal control serum was prepared by pooling serum from 5 healthy SIV-seronegative adult macaques. All sera were heat-inactivated for 1 h at 56°C before pooling. Pooled sera were then aliquoted and stored at −70°C until use. The absence of infectious SIV in the serum was confirmed by culturing a serum aliquot with CEMx174 cells and monitoring p27 core antigen according to methods previously described [29]. Total protein levels in the pooled sera were determined by the Coulter DART Total Protein Assay (a modified biuret method) run on the DACOS chemistry analyzer (Coulter, Hialeah, FL). Total IgG levels were determined by two techniques: a human IgG radial immunodiffusion kit (The Binding Site, Birmingham, UK) and a rhesus monkey–specific IgG radial immunodiffusion assay [30]. Levels of RANTES chemokine in sera were determined (Quantikine Human RANTES Immunoassay; R&D Systems, Minneapolis).

Virus neutralization assay. Neutralization assays were performed with CEMx174 cells using heat-inactivated (56°C, 1 h) plasma from preservative-free heparinized blood. Neutralization was measured by a reduction in virus-induced cell killing as described previously [31]. Briefly, cell-free virus (50 μL containing 0.5–1 ng of p27) was added to multiple dilutions of test plasmas in 100 μL of growth medium in triplicate wells of 96-well microtiter plates. Virus-plasma mixtures were incubated at 37°C for 1 h, and then CEMx174 cells (10^5 cells in 100 μL) were added to each well. Cell densities were reduced, and the medium was replaced after 3 days of incubation. Infection led to extensive syncytium formation and virus-induced cell killing in ∼6 days in the absence of antibodies. Neutralization was measured by staining viable cells with Finter's neutral red in poly-L-lysine–coated plates. Percent protection was determined by calculating the difference in absorption (A_{540}) between test wells (cells + plasma sample + virus) and virus control wells (cells + virus) and dividing this result by the difference in absorption between cell control wells (cells only) and virus control wells. Neutralization was measured when virus-induced cell-killing in virus control wells was >70% but <100%. Neutralizing titers are given as the reciprocal of the plasma dilution required to protect 50% of cells from virus-induced killing.

Virus stocks were grown in H9 cells or stimulated rhesus PBMC as described [31]. Virus-containing culture supernatants were made cell-free by low-speed centrifugation and filtration (0.45 μm) and stored in aliquots at −70°C. The concentration of virus was determined by p27 gag immunoassay as described by the supplier (Coulter). Seed stocks of laboratory-passaged and primary $SIV_{mac}251$ were obtained from Ronald Desrosiers (New England Regional Primate Research Center, Southborough, MA). Laboratory-passaged $SIV_{mac}251$ had been passaged multiple times in T cell lines. Primary

SIV$_{mac}$251 is a low-passaged virus that has been grown only in PBMC [32]. SIV Delta$_{B670}$ was obtained from Michael Murphy-Corb (Tulane Regional Primate Research Center, Covington, LA) and had been passaged multiple times in T cell lines. SIV$_{mac}$251-UCD is a single rhesus PBMC passage derived from the rhesus PBMC-grown SIV$_{mac}$251 stock used to challenge the animals in the present passive immunization experiment.

Measurement of SIV envelope glycoprotein–specific antibody end-point titer, conformational dependence, and antibody avidity by concanavalin A (ConA) ELISA. Serum samples were analyzed for their reactivity to viral envelope glycoproteins in the ConA ELISA [33]. Measurements of end-point titer, conformational dependence, and antibody avidity were determined as previously described [34].

In brief, for the measurement of end-point titer, preparations of density gradient–purified SIV/B7 were used as a source of viral envelope glycoproteins [35]. Immulon II 96-well plates (Dynatech, Chantilly, VA) coated with 2.5 μg/well ConA (Vector Laboratories, Burlingame, CA) were used to adsorb the native envelope glycoproteins from Triton X-100–disrupted SIV/B7. The ConA-adsorbed glycoprotein wells were then incubated with heat-inactivated SIV-HIS to capture envelope-specific serum antibodies, extensively washed with PBS, and incubated with a peroxidase-conjugated anti–monkey IgG (Sigma, St. Louis). Wells were again washed with PBS and incubated with TM Blue substrate (Intergen, Milford, MA) before color development was determined after the reaction was stopped by the addition of 1 N sulfuric acid. Antibody reactivity to the ConA-anchored glycoprotein was determined by measuring the optical density (OD) at 450 nm using an automated plate reader (Titertek; Flow Laboratories, Inglewood, CA). End-point titers were defined as the last 2-fold dilution with an OD twice that of normal monkey serum at the lowest dilution (1:50) or 0.100, whichever value was greater.

Serum samples were analyzed for conformational dependence by comparing the serum antibody reactivities to native and denatured viral envelope glycoprotein substrates in the ConA ELISA as previously described [34]. Briefly, native (Triton X-100–disrupted SIV/B7) or denatured (reduced and carboxyamidated SIV/B7) envelope substrates were captured onto 96-well microtiter plates using ConA. Sera (diluted to produce an OD at 450 nm of 1.0–1.5 in the end-point titer ConA ELISA procedure) were reacted in triplicate wells with either native or denatured glycoprotein substrates, washed extensively with PBS, incubated with peroxidase-conjugated anti–monkey IgG and then with TM Blue substrate, and developed as described above for the end-point titer ConA ELISA. A conformation ratio was then calculated from the ratio of antibody reactivities to native versus denatured envelope glycoprotein substrates. Thus, the conformation ratio is a direct measure of the conformational dependence of the binding of a particular antibody sample to SIV envelope. The larger the conformation ratio, the greater the requirement for native envelope glycoprotein structure for antibody binding.

The antibody avidity index values of serum antibodies to the native viral envelope were determined by measuring the resistance of serum antibody–envelope glycoprotein immune complexes in the ConA ELISA to 8 M urea as previously described [34]. For these avidity assays, ConA-anchored native glycoprotein was incubated with heat-inactivated SIV serum diluted to produce an OD

at 450 nm of 1.0–1.5 in the end-point titer ConA ELISA procedure and plated in two sets of triplicate wells. Following serum incubation, triplicate wells were treated in parallel three times for 5 min (each) with either PBS or a solution of 8 M urea in PBS. Wells were then incubated with peroxidase-conjugated anti–monkey IgG followed by incubation with TM Blue substrate; development proceeded as described above for the end-point titer ConA ELISA procedure. The avidity index was then calculated from the ratio of the absorbance value obtained with urea treatment to that observed with PBS treatment multiplied by 100%. Serum samples with avidity index values <30% were designated low avidity, those with index values of 30%–50% were designated intermediate avidity, and those with index values >50% were designated high avidity [36].

While measurements of antibody conformational dependence and antibody avidity were performed at dilutions producing an OD at 450 nm of 1.0–1.5 in the end-point titer ConA ELISA, experiments using several different dilutions within this linear range were performed to ensure that the variation in actual values was within 10%.

Quantitative virus isolation. Cell-associated and cell-free virus levels in peripheral blood were determined regularly by limiting dilution culture (four replicates per dilution) of PBMC and plasma, respectively, with CEMx174 cells in 24-well plates and subsequent p27 core antigen measurement, according to methods previously described [2, 29, 37, 38]. Virus levels were calculated according to the method of Reed and Muench [39] and expressed as TCID$_{50}$/10^6 PBMC or per milliliter of plasma [38]. In addition, for animals with low or undetectable virus load, 1×10^6 to 5×10^6 PBMC were cocultivated for 8 weeks with CEMx174 cells in tissue culture flasks, as described previously [37, 38].

Polymerase chain reaction (PCR) amplification. Nested PCR was carried out in a GeneAmp 9600 (Perkin-Elmer Cetus, Emeryville, CA). Two rounds of 30 cycles of amplification were performed on aliquots of plasmid DNA containing the complete genome of SIV$_{mac}$1A11 [28] (positive control) or aliquots of PBMC lysates using SIV$_{mac}$-specific *gag* primers and conditions described elsewhere [37, 40]. This nested PCR amplification procedure allows visual detection of a single copy of SIV *gag* sequences in as many as 200,000 PBMC [40].

Anti–SIV isotype–specific antibody determination in plasma and saliva. The anti–SIV isotype–specific antibody ELISAs have been described [2, 23, 25]. The SIV-specific IgG and IgA antibody ELISA uses microtiter ELISA plates (Falcon 3912; Becton Dickinson, San Jose, CA) coated with whole SIV$_{mac}$251 (Advanced Biotechnologies, Columbia, MD) at 500 ng of total protein per well. These plates were incubated with test or control plasma (4-fold dilutions starting at 1:100) or saliva (2-fold dilutions starting at 1:4) samples, washed, then incubated with 1:2000 diluted enzyme-conjugated goat anti–monkey IgG or IgA (Nordic, San Juan Capistrano, CA), washed, incubated with *o*-phenylenediamine (Sigma) substrate, and read spectrophotometrically. The SIV-specific IgM ELISA was performed on plates coated with a 21–amino-acid synthetic peptide derived from the SIV$_{mac}$ transmembrane glycoprotein [23]. Each plasma sample and the positive control plasma (from an SIV-infected animal) were assayed in duplicate, and mean values of OD were calculated. Plasma samples were considered positive if the mean OD value was >0.150 (IgG)

and exceeded two times the mean OD of plasma obtained before inoculation. Saliva titers were multiplied by 2 because the sample collection and processing procedures result in a 2-fold dilution of the whole saliva. Immunoblots were performed to detect specific SIV proteins, as described previously [41].

Necropsy collection and preparation of tissue samples. The criteria for euthanasia have been described [2]. A complete necropsy examination was performed on all animals that died during the course of the study. Tissues collected at necropsy were fixed in 10% buffered formalin, embedded in paraffin, cut into 6-μm sections, stained with hematoxylin-eosin, and examined by light microscopy.

Immunohistochemistry staining. A standard avidin-biotin peroxidase technique was used to detect IgG in sections of formalin-fixed, paraffin-embedded kidney from the 3 animals in the SIV-HIS therapy group (group F, table 1). Briefly, the sections were deparaffinized, rehydrated, and incubated with proteinase K (0.012 mg/mL; Sigma) for 15 min. The slides were blocked with horse serum, washed, and incubated with IgG specific antisera for 16 h at 4°C (rabbit anti−human IgG; Dako, Carpenteria, CA). Binding of the antisera was detected with anti−rabbit IgG-specific antisera (biotinylated-horse anti−rabbit IgG, Vector Laboratories). Endogenous peroxidase activity was quenched, and the slides were incubated with an avidin-biotin horseradish peroxidase complex (ABC

Table 1. Passive immunization of newborn macaques: summary of experimental design and outcome.

Group, description	n	Serum administration*	Oral SIV inoculation[†]	Animal no.	SIV IgG half-life[‡]	Growth rate[§] (95% CI)	Infection/disease status[‖]
A, SIV control (no serum)	4	None	2 days of age	28689	NA	5.4 (5.2−5.7)	Infected/SAIDS at 11 weeks
				28690		6.2 (5.6−6.8)	Infected/SAIDS at 11 weeks
				28972		6.6 (6.1−7.3)	Infected/slow progressor
				28981		6.4 (6.0−6.9)	Uninfected
B, normal serum control (prophylaxis and therapy)	1	Normal serum at birth, and at 1 and 2 weeks	2 days after first serum	28948	NA	5.6 (4.8−6.4)	Infected/SAIDS at 13 weeks
C, SIV-HIS safety and kinetics (3 doses)	2	SIV-HIS at birth and at 1 and 2 weeks	None	28760	2.47 (2.39−2.55)	5.6 (5.2−5.9)	Uninfected/healthy at 9 months
				28762	2.47 (2.39−2.55)	6.1 (5.8−6.4)	Uninfected/healthy at 9 months
D, SIV-HIS prophylaxis (1 dose)	2	SIV-HIS at birth	2 days after SIV-specific serum	28986	2.09 (1.96−2.24)	8.3 (7.6−9.0)	Uninfected/healthy at 8 months
				28987	3.18 (2.77−3.73)	7.3 (6.7−7.9)	Uninfected/healthy at 8 months
E, SIV-HIS prophylaxis (3 doses)	4	SIV-HIS at birth and at 1 and 2 weeks	2 days after first SIV-specific serum	28759	2.46 (2.15−2.87)	5.3 (4.9−5.7)	Uninfected/healthy at 9 months
				28761	2.47 (2.39−2.55)	5.1 (4.8−5.4)	Uninfected/healthy at 9 months
				28767	2.13 (1.89−2.44)	7.8 (7.4−8.1)	Uninfected/healthy at 9 months
				28768	2.13 (1.88−2.46)	8.1 (7.6−8.7)	Uninfected/healthy at 9 months
F, SIV-HIS therapy (1 dose)	3	SIV-HIS at 3 weeks	At birth	29119	1.22 (1.08−1.41)	4.8 (4.5−5.2)	Infected/SAIDS at 13 weeks
				29122	1.94 (1.58−2.51)	5.5 (4.9−6.1)	Infected/SAIDS at 12 weeks
				29126	1.32 (1.11−1.63)	4.0 (3.4−4.7)	Infected/SAIDS at 11 weeks

NOTE. NA, not applicable; SIV-HIS, SIV hyperimmune serum; CI, confidence interval; SAIDS, simian AIDS.

* Each dose = 20 mL/kg body weight, subcutaneously.

[†] 1 mL (10^5 TCID$_{50}$) of uncloned SIVmac251, grown in rhesus peripheral blood mononuclear cells, given orally to newborn rhesus macaques within 72 h after birth.

[‡] Half-life weeks of anti−SIV IgG in plasma ($\pm$ SE) determined by antibody ELISA.

[§] During 1st 10 weeks (g/day) was determined for each animal by regression analysis. Average growth rate of 50 uninfected controls was 6.2 g/day (95% CI, 6.1−6.3).

[‖] $P = .015$ for immunized animals (groups D and E) vs. controls (groups A and B) by 1-sided Fisher's exact test.

Elite; Vector Laboratories) according to the manufacturer's instructions. Diaminobenzidine (DAB; Zymed, South San Francisco) was used as the chromogen. The slides were lightly counterstained with Meyer's hematoxylin, and a coverslip was applied for microscopic examination. Hematoxylin-eosin–stained sections from each block of tissue were examined to detect morphologic abnormalities and inflammatory lesions. Control procedures included staining matched kidney sections from 1 animal in the SIV control group (group A, table 1), 1 in the normal serum control group (group B, table 1), and 1 in the SIV-HIS prophylaxis group (group D, table 1); substitution of the primary antibody with an irrelevant mouse monoclonal antibody of similar isotype; and staining known positive control tissue.

Statistical analyses. The rates of clearance for SIV-HIS among passively immunized neonates and the growth rates between uninfected and SIV-infected infant rhesus macaques were compared. Clearance rates of SIV-HIS ($\log_{10}$ antibody titers per day) were calculated by regression analysis (Excel, version 5.0; Microsoft, Redmond, WA). Growth rates (weight gained, g/day) during the first 10 weeks of life were calculated by performing regression analysis on daily body weight, measured in kilograms, during the first 10 weeks of age (Excel, version 5.0). Protection against SIV infection was statistically analyzed by comparing immunized and control animals with a one-sided Fisher's exact test (Stata, College Station, TX).

Results

Characterization of pooled normal and SIV hyperimmune sera. As described above, rhesus SIV-HIS was prepared by pooling sera collected from macaques that had previously been vaccinated against SIV and were healthy slow progressors following challenge with pathogenic $SIV_{mac}251$; normal control serum was prepared by pooling sera from healthy SIV-seronegative donor rhesus macaques. The characteristics of these two rhesus serum pools are presented in table 2. The SIV-HIS had high ELISA anti–SIV IgG titer (1:409,600), low anti–SIV IgA titer (1:400), and strong reactivity to all viral structural proteins (including envelope glycoprotein) by immunoblotting. The SIV-HIS demonstrated envelope-specific antibody reactivity that was high titer, conformationally dependent, and of high avidity (table 2). This SIV-HIS preparation contained a high titer of neutralizing antibodies against laboratory-passaged $SIV_{mac}251$ and SIV Delta$_{B670}$ but had a very low neutralization titer against primary $SIV_{mac}251$ and undetectable neutralization against our own rhesus PBMC–grown $SIV_{mac}251$ (here designated as $SIV_{mac}251$-UCD), which is derived directly from the $SIV_{mac}251$ challenge stock of the present passive immunization study (table 3). The levels of total protein and total IgG in SIV-HIS and in normal rhesus serum were similar (table 2). Both serum pools had very high levels of RANTES chemokine (>5000 pg/mL), presumably due to massive platelet activation during the serum collection. No infectious virus was detected by cocultivation of the heat-inactivated SIV-HIS with CEMx174 cells.

Table 2. In vitro characteristics of pooled sera used for passive immunizations.

	SIV hyperimmune serum	Normal serum
Total protein (g/dL)	6.4	7.2
Total IgG* (mg/mL)		
Human IgG RID	10.8	10.8
Rhesus IgG RID	24.3	25.3
Anti–SIV IgG titer (ELISA)[†]	409,600	<100
Anti–SIV IgA titer (ELISA)[‡]	400	<100
Western blot[§]	+++ (all viral proteins)	−
Anti–SIV *env* titer (ConA ELISA)[‖]	102,400	<50
Conformation ratio[¶]	1.6	−
Avidity index (%)**	53	−

* Measured by commercial anti–human IgG radial immunodiffusion (RID) kit and by in-house rhesus monkey IgG RID with rhesus IgG standard (see Materials and Methods).

[†] End-point titer determined using whole $SIV_{mac}251$ as capture antigen; goat anti–rhesus IgG secondary antibody. Titers are reported as reciprocal of highest dilution above assay cutoff.

[‡] End-point titer determined using whole $SIV_{mac}251$ as capture antigen; goat anti–rhesus IgA secondary antibody.

[§] Whole $SIV_{mac}251$ used as antigen.

[‖] Antibody end-point titers were measured to native envelope glycoproteins in ConA ELISA.

[¶] Conformation ratio was determined by comparing reactivity of native to denatured envelope glycoproteins in ConA ELISA.

** Avidity index was determined by measuring relative stability of native viral envelope antigen–serum antibody complexes to 8 M urea in ConA ELISA; values were calculated using equation (urea-washed cells/PBS-washed cells × 100). Serum samples with avidity index values <30% were designated low avidity, those with index values of 30%–50% were designated intermediate avidity, and those with index values >50% were designated high avidity.

Decay kinetics and safety of SIV-HIS in rhesus neonates. Pooled heat-inactivated SIV-HIS was administered subcutaneously (20 mL/kg body weight; ~490 mg total IgG [determined by rhesus IgG radial immunodiffusion assay] per kilogram of body weight) to 2 newborn macaques (animals 29760 and 29762; table 1, group C) at 0, 7, and 14 days of age. The SIV-HIS was rapidly absorbed, and a high level was detected in plasma samples of each neonate by 2 days after the first administration (figure 1A). No toxicity or adverse effects (such as serum sickness) were observed following subcutaneous administration of the three doses of SIV-HIS. In addition, no infectious virus was recovered from any samples of plasma or PBMC from these 2 infants. Following administration of the third dose of serum at 2 weeks, the plasma half-life of SIV-specific IgG in these infants was estimated by linear regression to be ~2.5 weeks (table 1). SIV-specific IgG was detected in plasma until ~6 months of age with moderate titers (1:400–1:1600) maintained for 12 weeks (figure 1A). Because of a low level of SIV-specific IgA in the SIV hyperimmune sera (1:400), SIV-specific IgA titers in newborn macaques following passive immunization remained ≤1:100 during the observation period (data not shown).

Table 3. Neutralizing antibody titer of serum pools.

| | SIV isolate* | | | | |
| | Laboratory-passaged SIV$_{mac}$251 | | | Primary | |
Sample	rhPBMC	H9	SIV Delta$_{B670}$ (H9)	SIV$_{mac}$251 (rhPBMC)	SIV$_{mac}$251-UCD (rhPBMC)
SIV hyperimmune serum	5697	1472	17,304	39	<30[†]
Negative control serum	<15	<15	<15	<15	<30
Serum 145−86[‡]	3645	2323	11,304	135	280

NOTE. PBMC, peripheral blood mononuclear cells. Titers are reciprocal dilution where 50% protection from cell killing was observed in CEMx174 cells.

* Virus stocks were grown in rhesus PBMC (rhPBMC) or H9 cells; laboratory-passaged SIV$_{mac}$251 is derived from SIV$_{mac}$251 stock grown multiple times in T cell lines and then passed once in H9 cells or rhPBMC. Primary SIV$_{mac}$251 is single rhPBMC-passaged virus derived from animal challenge stock virus [32]. SIV$_{mac}$251-UCD is single rhPBMC passage derived from rhPBMC-grown SIV$_{mac}$251 stock used to challenge animals in present passive immunization experiment.

[†] Plasma obtained from animals following administration of SIV hyperimmune serum (table 1, groups C−F) also failed to neutralize SIV$_{mac}$251-UCD (neutralization titer, <30).

[‡] From long-term surviving macaque infected with SIV$_{mac}$251.

Control neonates inoculated with SIV$_{mac}$251. Three SIV$_{mac}$-inoculated control infants (table 1, group A) and the 1 infant that received normal serum (group B) became infected following oral inoculation with 1 mL (10^5 TCID$_{50}$) of uncloned SIV$_{mac}$251 (grown in rhesus PBMC) and developed persistently high cell-associated viremia (figure 2A). All 4 infected animals had an anti−SIV IgM response within 4 weeks after virus inoculation (data not shown), but 3 infants (animals 28689, 28690, and 28948) made no SIV-specific IgG response by ELISA (figure 2C) or immunoblotting (data not shown). These 3 infants had persistently high plasma viremia (figure 2B) and died at 11−13 weeks of age, with clinical signs and gross and microscopic pathologic changes consistent with immunodeficiency and terminal SIV infection (table 4). This fulminant course of SIV infection is consistent with previous observations in newborn macaques inoculated intravenously or mucosally with uncloned SIV$_{mac}$251 [2, 22]. The 4th SIV-infected control infant (28972) had an antiviral IgG response, lower plasma viremia, and a slower disease course (simian AIDS at 18 months). One control newborn (28981) did not become infected.

Passive immunization of neonates prior to oral inoculation with SIV$_{mac}$251. Six newborns were given SIV-HIS (20 mL/ kg body weight) subcutaneously on the day of birth (day 0);
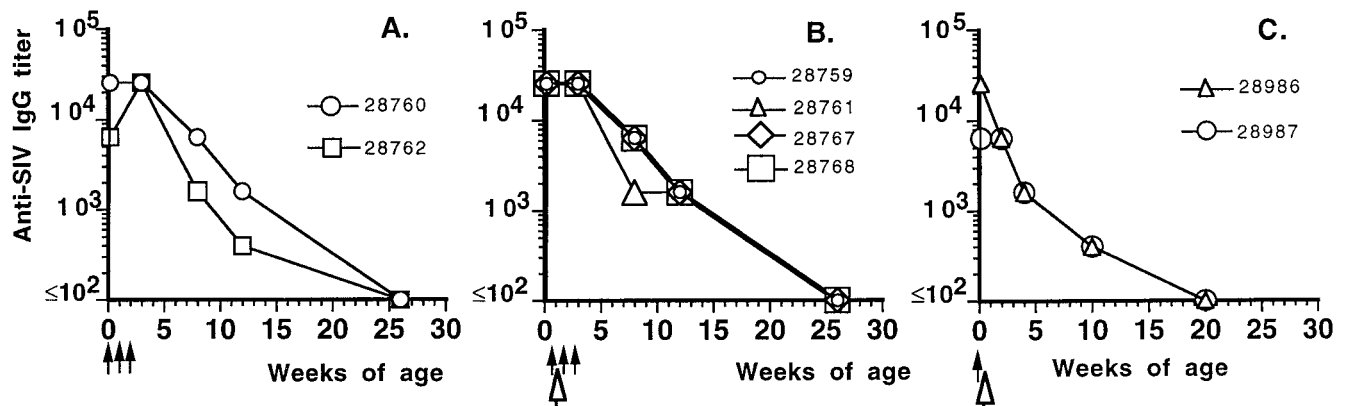

Figure 1. Passive immunization of newborn macaques with SIV hyperimmune serum (SIV-HIS) prior to oral SIV inoculation: SIV-specific IgG levels in plasma. **A**, **B**, **C**: animals from table 1, groups C, E, D, respectively. All 8 newborn rhesus macaques were given SIV-HIS subcutaneously at birth (small arrows). Animals in **B** and **C** were inoculated orally with SIV$_{mac}$251 2 days later (open arrow), and animals in **A** and **B** received another dose of SIV-HIS at 1 and 2 weeks of age. None of these 8 animals became SIV-infected. SIV-specific IgG levels in plasma were determined by ELISA and are expressed as reciprocal of highest dilution that gave positive optical density above cutoff value.

4 were given additional doses of SIV-HIS at 7 and 14 days of age (table 1, groups D–E). Two days after administration of SIV-HIS, all 6 animals were seropositive by ELISA (figure 1B, C) and had antibodies to all viral structural proteins detected by immunoblotting (data not shown). At 2 days of age, all 6 animals were inoculated orally with 1 mL (10^5 TCID$_{50}$) of uncloned SIV$_{mac}$251. None of these 6 infant macaques given SIV-HIS prior to oral SIV inoculation became infected. No virus was recovered from any samples of plasma or PBMC during the 8- to 9-month observation period. No proviral DNA could be detected by PCR in PBMC or in lymph node mononuclear cells obtained at 8–9 months of age (data not shown). SIV antibody titers in the 6 animals declined, with a half-life of 2–3 weeks (similar to those in the 2 infants that had only received SIV-HIS [table 1, group C]) and were undetectable by ELISA (figure 1B, C) and immunoblotting (data not shown) by 6 months after oral SIV inoculation. None of these 6 animals had clinical signs of immunodeficiency or serum sickness during the observation period and all were healthy at 8–9 months of age. When these 6 animals and the 2 that had received only the hyperimmune serum (table 1, groups C, D, and E) were rechallenged orally at 8–9 months of age (i.e., when passively acquired antibodies were undetectable) with 1 mL (10^5 TCID$_{50}$)

of uncloned SIV$_{mac}$251, all 8 became infected and showed persistent viremia; 2 developed fatal AIDS within 8 months of infection (data not shown).

SIV-specific antibodies in saliva of rhesus neonates after administration with SIV-HIS. Although only a limited number of saliva samples was collected during this study, SIV-specific IgG was detected in saliva sampled from neonates after subcutaneous administration of SIV-HIS. Titers measured by SIV-specific IgG ELISA ranged from 1:8 to 1:32. In some animals, SIV-specific IgG was detected in saliva as late as 10 weeks after administration of SIV-HIS (table 5).

Passive immunization of neonates 3 weeks after oral inoculation with SIV$_{mac}$251. To study the potential therapeutic effects of SIV-HIS for infant macaques with established SIV infection, 3 newborn macaques were infected orally with uncloned SIV$_{mac}$251 and then given a single dose of SIV-HIS (20 mL/kg body weight, administered subcutaneously) 3 weeks later. As in the SIV$_{mac}$251-infected untreated control infants, SIV-specific IgM was detected in the plasma of all 3 postexposure-immunized neonates within 2 weeks following infection (data not shown). One animal (29119) had a weak (1:100) SIV-specific IgG response at 3 weeks, while the other 2 had no detectable SIV-specific IgG 3 weeks after virus inoculation.

Figure 2. Passive immunization of newborn macaques with SIV hyperimmune serum (SIV-HIS) 3 weeks after oral SIV inoculation: time course of viral and immune parameters. All animals were inoculated orally within 3 days of age with uncloned SIV$_{mac}$251 (open arrow). Untreated control animals are represented by dashed lines. + indicates euthanasia. Control animal 28972 had de novo anti–SIV IgG and was slow progressor. Animal 28948 (table 1, group B), which received normal serum 2 days prior to virus inoculation and another dose of normal serum at 1 and 2 weeks of age, is represented by ○. Three animals (solid lines) received SIV-HIS at 3 weeks of age (solid arrow). Levels of peripheral blood mononuclear cell (PBMC)–associated virus (**A**) and virus in plasma (**B**) were determined by limiting dilution culture assay of PBMC and plasma, respectively. Anti–SIV IgG titers (**C**) were determined by ELISA and are expressed as highest of 4-fold dilutions (starting from 1/100 dilution) with 2 replicates per dilution that gave positive optical density above cutoff value. CD4:CD8 T lymphocyte ratio (**D**) was determined by flow cytometry.
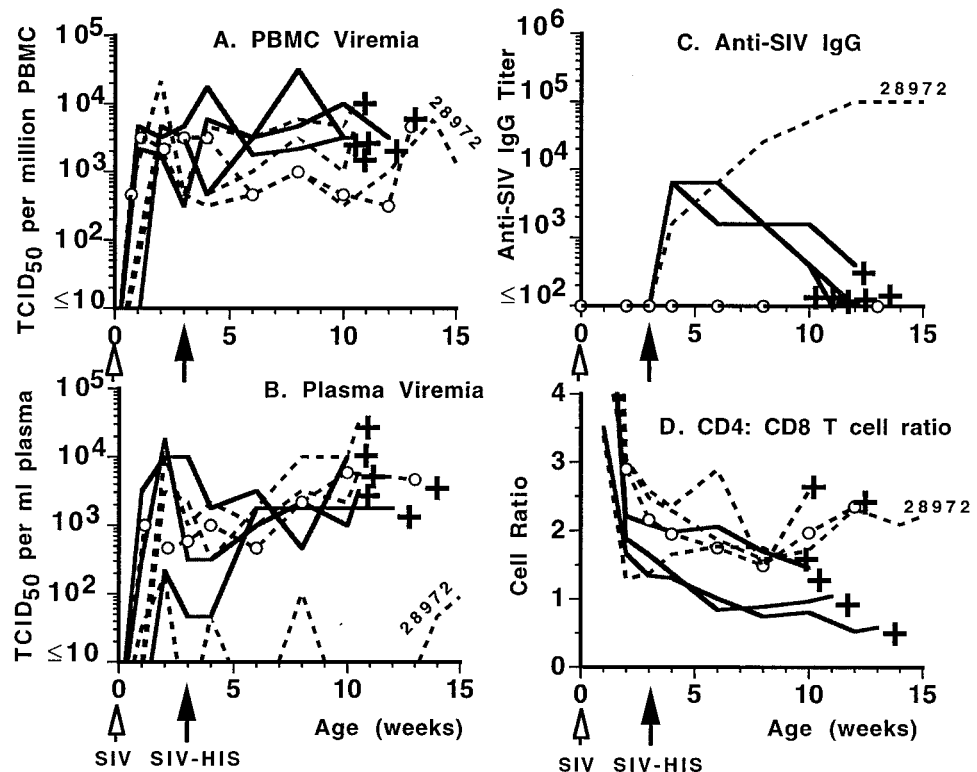
Table 4. Histopathologic findings of SIV-infected animals with simian AIDS after euthanasia.

Animal no.	Age (weeks) at death	Histopathologic and hematologic findings
28689	11	Colitis, dermatitis, cholangiohepatitis, lymphoid depletion, glomerulopathy, pylitis, hypoproteinemia (total protein, 4.6 g/dL)
28690	11	Cholangiohepatitis, enterocolitis, glomerulopathy, lymphoid depletion
28948	13	Pancreatitis, cholangiohepatitis, enteritis, glomerulopathy, adenoviral nephritis, thymic atrophy, interstitial pneumonia, hypoproteinemia (2.7 g/dL), icterus (total bilirubin, 9.8 mg/dL)
29119	13	Cryptosporidial bronchitis, enterocolitis, pancreatitis, and cholangiohepatitis; interstitial pneumonia; membranoproliferative glomerulonephritis; staphylococcal meningitis and myocarditis; lymphoid depletion; hypoproteinemia (total protein, 2.9 g/dL; albumin, 0.9 g/dL)
29122	12	Cryptosporidial tracheitis and enterocolitis; lymphofollicular hyperplasia of lymph nodes; chronic glomerulonephritis; hypoproteinemia (total protein, 3.3 g/dL; albumin, 2 g/dL)
29126	11	Cryptosporidial tracheobronchitis, enteritis, cholecystitis and cholangiohepatitis; pancreatitis; hypoproteinemia (total protein, 4.8 g/dL; albumin, 2.2 g/dL)

NOTE. Animals 28689 and 28690 were untreated controls; animal 28948 received 3 doses of normal serum (20 mL/kg body weight, at birth and 1 and 2 weeks of age); animals 29119, 29122, and 29126 received 1 dose of SIV hyperimmune serum at 3 weeks of age. Normal total protein levels for rhesus macaques: 6.9 g/dL (range, 5.9–7.9); normal albumin levels, 3.6 (range, 3.2–4.1).

Following SIV-HIS administration at 3 weeks after oral SIV inoculation, anti–SIV IgG levels of 1:6400 were detected at 4 weeks of age; after 4 weeks, plasma IgG levels decreased rapidly, with a half-life of 1–2 weeks (table 1), and de novo anti–SIV IgG synthesis was not detected in these 3 animals. Each of these 3 neonates maintained persistently high PBMC-associated and plasma viremia at levels similar to those of naive controls (figure 2). In addition, all 3 developed clinical signs consistent with serum sickness documented at 4 weeks of age (i.e., 1 week after SIV-HIS administration) and persisting until the animal's demise. Clinical signs included varying degrees of abdominal distention and peripheral edema with palpebral, facial, and scrotal edema. Deterioration of clinical condition was associated with severe hypoproteinemia (2.1–

Table 5. Anti–SIV IgG in saliva of infant macaques following administration of SIV hyperimmune serum (SIV-HIS).

Treatment group, animal no.	Days 4	Weeks 1	Weeks 4	Weeks 6	Weeks 8	Weeks 10
SIV-HIS at 0, 1, and 2 weeks						
28760	NA	NA	NA	NA	8	NA
28762	NA	NA	NA	NA	8	NA
SIV-HIS at 0, 1, and 2 weeks; oral SIV at day 2						
28759	NA	NA	NA	NA	16	NA
28761	NA	NA	NA	NA	8	NA
28767	NA	NA	NA	NA	<8	NA
28768	NA	NA	NA	NA	16	NA
SIV-HIS at 0 weeks; oral SIV at day 2						
28986	8	4	8	8	NA	<8
28987	32	16	8	32	NA	8

NOTE. SIV-HIS was given subcutaneously at 20 mL/kg body weight; SIV-specific IgG levels were determined by ELISA, and are given as reciprocal of highest dilution above assay cutoff. NA, not available.

5.4 gm/dL) and hypoalbuminemia (0.9–2.6 gm/dL). A transient normocytic normochromic anemia was also evident. Although no urinalysis data were available, blood loss presumptively resulted from hematuria, a common feature in glomerulonephritis. Two of the 3 animals (29119 and 29126) had poor weight gain (<5 g/day; table 1). All 3 infants developed clinical symptoms indicative of simian AIDS, which failed to respond to standard supportive fluid and antibiotic treatment, and were euthanatized by 11–13 weeks of age. Their clinical signs, plus gross and microscopic pathologic changes, were consistent with terminal SIV infection (table 4). Of interest, all 3 animals had widespread *Cryptosporidium* infection of the epithelia of the digestive and respiratory tracts; respiratory *Cryptosporidium* infection is a rare finding in HIV-infected humans and SIV-infected macaques, including newborn macaques ([42], unpublished data).

Histologic evidence of immune-complex disease. Of the 3 animals given SIV-HIS 3 weeks after SIV infection (table 1, group F), 2 had histologic lesions that were consistent with immune-complex glomerulonephritis, while no significant lesions were found in the 3rd animal. Animal 29119 had a moder-

ate to severe glomerulonephritis characterized by swollen hypercellular glomeruli with prominent, thickened basement membranes or shrunken glomeruli with indistinct cell borders and adhesions between the glomerular tuft and Bowman's capsule. Numerous tubules were dilated with a glassy, eosinophilic material that contained variable numbers of neutrophils (figure 3). In addition, there was edema, fibrosis, and a moderate lymphocytic infiltrate in the subcapsular and interstitial connective tissue of the kidney. Animal 29122 had moderate numbers of shrunken glomeruli with indistinct cell borders and adhesions between the glomerular tuft and Bowman's capsule. In addition, there was periglomerular fibrosis and edema, fibrosis, and a moderate lymphocytic infiltrate in the interstitial connective tissue of the kidney. These histologic changes are indicative of chronic kidney disease and are consistent with a prior episode of immune complex glomerulonephritis. By immunohistochemical analysis, IgG was detected in glomeruli in all 3 animals in this group. However, the IgG-positive glomeruli were rare or occasional (figure 3) and, on the basis of the number of IgG-positive glomeruli seen, the amount of tissue damage in the kidneys of 29122 and 29119 was greater than
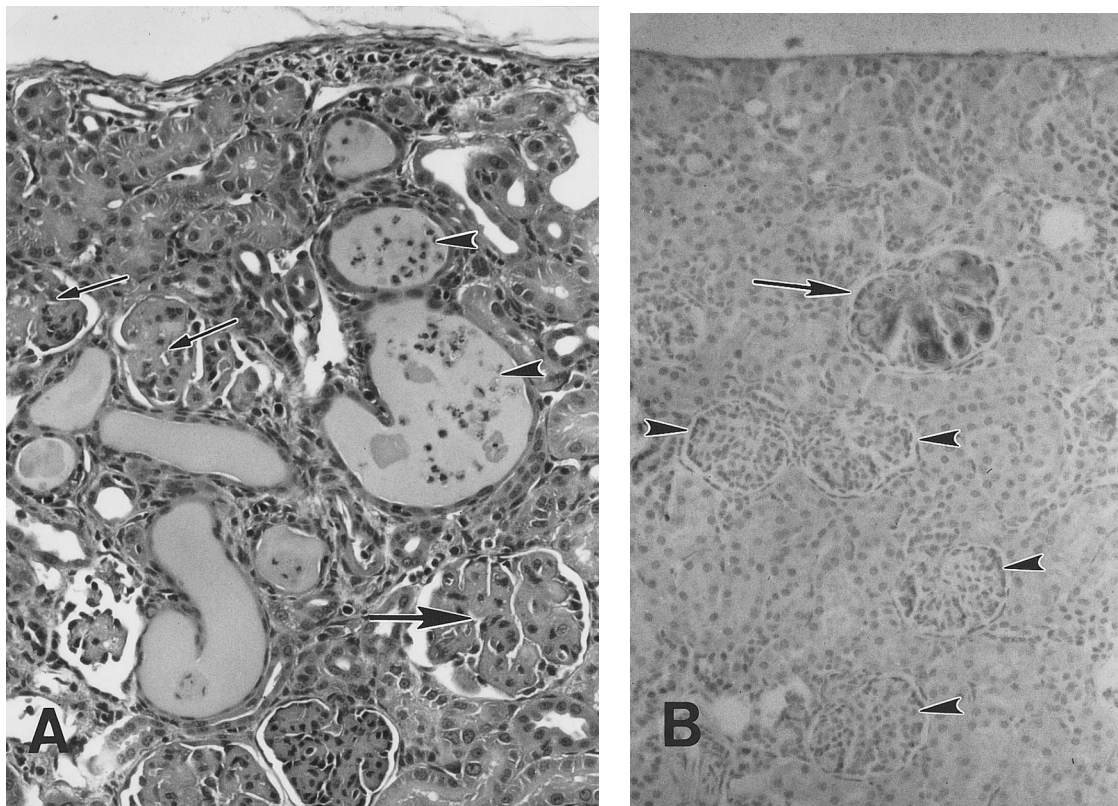

Figure 3. Histologic preparation (×200) of renal lesion in animal 29119. **A,** Note moderate to severe glomerulonephritis characterized by shrunken glomeruli with indistinct cell borders (small arrows) and swollen glomeruli with abundant acellular material (large arrow). There were also numerous dilated tubules with glassy, eosinophilic material containing neutrophils (arrowheads). There is edema, fibrosis, and moderate lymphocytic infiltrate in subcapsular and interstitial connective tissue of the kidney. **B,** By immunohistochemistry, IgG-positive glomerulus (arrow) could be detected, but most glomeruli were IgG negative (arrowheads).

expected. This discrepancy is likely related to the time interval (8–10 weeks) between the administration of the hyperimmune serum and the necropsies. Presumably much of the immune complex deposition and glomerular damage occurred soon after the SIV-HIS was administered, and most of the immune complexes had been eliminated by the time the animals were necropsied.

Discussion

The goal of our experiments was to determine if passive immunization of newborn macaques with SIV-HIS can modulate infection or disease progression following oral SIV inoculation. Our study demonstrated that multiple subcutaneous administrations of SIV-HIS at a dosage regimen of 490 mg total IgG (determined by rhesus IgG radial immunodiffusion) per kilogram of body weight (in the same range as the 200 mg of HIVIG per kg body weight used in ACTG 185) had no detectable side effects for newborn macaques. Following subcutaneous administration of SIV-HIS to uninfected newborn macaques, anti−SIV IgG was efficiently absorbed into the blood, and plasma levels of anti−SIV IgG declined with a half-life of 2–3 weeks, slightly shorter than the reported half-life of 3–4 weeks for HIV IgG in human infants [18, 43, 44].

Administration of SIV-HIS to 6 neonatal macaques 2 days prior to oral SIV inoculation resulted in absorption into the blood and protected all 6 newborns against SIV infection. This protection was associated with the presence of anti−SIV IgG in saliva. A single dose of SIV-HIS given prior to SIV exposure was sufficient to prevent oral SIV infection, so the two extra doses of SIV-HIS that were given to 4 of these 6 animals at 1 and 2 weeks of age were probably unnecessary.

We have previously demonstrated that vaccination of pregnant macaques against SIV resulted in transplacental antiviral IgG transfer, which protected 2 of 3 newborn macaques against oral-conjunctival SIV infection at birth [2]. The results of the experiment described herein further confirm the potential of anti−SIV IgG to protect newborn macaques against SIV infection following mucosal exposure. Previous studies investigating the potential of hyperimmune serum or purified immunoglobulin to protect macaques against intravenous challenge with SIV have shown conflicting results [45–48]. In addition, newborn macaques with maternally acquired anti−SIV antibodies (obtained through vaccination of their mothers with attenuated $SIV_{mac}239\Delta3$) became infected following oral exposure with $SIV_{mac}239\Delta3$; however, the challenge inoculum contained a higher dose of a different virus than we used in our study [49]. The precise reasons for the protection observed in the current passive immunization study are unknown; however, it is likely that the combination of an oral route of virus challenge, a high-quality hyperimmune serum, and a minimal virus challenge dose is responsible for the protection in our newborn macaques.

Protection against SIV infection by passive immunization may be more easily achievable following oral than intravenous virus challenge. It is well established that mucosal surfaces are barriers to SIV [27]. Mucosal infection may result in slow virus spread to submucosal tissues and delayed systemic dissemination; this delay may allow more time for immune effector mechanisms to operate than would be possible following intravenous virus inoculation. Following parenteral administration of SIV-HIS to newborn macaques, anti−SIV IgG transuded from the circulation into oral secretions; it is possible that this anti−SIV IgG reduced the dose of the challenge virus at the mucosal surface or interfered with the passage of SIV to submucosal tissues. Further investigation is required to identify which stage of the initial infection the anti−SIV IgG was effective in blocking. In addition, the identity and location of the initial cellular targets of SIV and HIV during oral infection are yet to be determined but may provide important clues regarding the exact mechanism of antibody protection against oral infection. Of importance, our passive immunization study indicates that antiviral secretory IgA was not required to protect newborn macaques against mucosal SIV infection but that anti−SIV IgG was sufficient. The critical role that IgG can play in limiting mucosal infection has also been shown in other virus systems, such as reovirus infection of mice [50].

The SIV-HIS in this study was collected from 6 macaques that had been immunized previously with live attenuated $SIV_{mac}1A11$ and that had controlled viral replication following challenge with virulent $SIV_{mac}251$; pooled serum from these animals is more likely to contain antibodies to a variety of SIV epitopes than serum from a single vaccinated or SIV-infected animal. The SIV-HIS used in this study had strong reactivity to all viral structural proteins by immunoblotting, high anti− SIV envelope antibody titer, and high avidity. It was able to neutralize laboratory-passaged strains of SIV rather well but had undetectable neutralization activity against the rhesus PBMC−grown $SIV_{mac}251$ stock derived from the challenge stock used in this experiment. Thus, a discrepancy exists between results obtained by in vitro neutralization assays and the observation of in vivo protection against oral infection with this virus. A simple explanation is that in vitro neutralization assays may not predict the efficacy of specific sera in preventing infection in vivo; this is also true in other retrovirus systems and other viral infections [51−58]. The observation that protection against mucosal SIV exposure is achievable without high in vitro neutralizing titers is encouraging for HIV vaccine development.

In the current study, SIV-HIS administration protected newborn macaques against oral SIV infection. These same infants, however, gradually cleared these passively acquired antibodies and became infected when rechallenged orally with SIV when the passively acquired anti−SIV IgG was undetectable. These results, suggesting that infants may become susceptible to oral HIV infection as maternally derived

antiviral antibodies decrease, are consistent with observations of breast-feeding human populations, where the risk of HIV transmission increases by prolonged breast-feeding practices [59–63].

In the present study, when the same SIV-HIS that had high prophylactic efficacy when given prior to SIV inoculation was given to SIV-infected newborn macaques 3 weeks after oral SIV inoculation, no therapeutic effects were detected. The 3 SIV-infected neonatal monkeys given postexposure passive immunization had a clinical disease course similar to that of control neonates infected orally with SIV$_{mac}$251. The half-life of SIV-specific IgG in these SIV-infected infants was shorter than that in the uninfected infants in this study, which is likely due to the formation of immune complexes that were cleared rapidly from the circulation, as indicated by presence of immune complexes in the kidneys. SIV-HIS given 3 weeks after oral SIV inoculation did not reduce viral replication, and these 3 infants developed clinical symptoms indicative of simian AIDS and were euthanatized within 3 months of age.

Haigwood et al. [64] showed that passive immunization of older macaques with purified anti–SIV IgG at 1 day and 2 weeks after intravenous inoculation with SIV$_{sm}$E660 inoculation was able to delay the disease course in 4 of 6 animals. The SIV$_{sm}$E660 challenge virus used by Haigwood et al. was sensitive to neutralization by its passively transferred anti–SIV IgG (Montefiori DC, unpublished results). Although these passively acquired antibodies delayed de novo antiviral immune responses, the development of these de novo antiviral immune responses following the decay of passively acquired antibodies appeared to be necessary in controlling virus burden and disease progression [64]. Although the different age groups (adults vs. neonates), the virulence of the challenge virus isolates, and the neutralizing activity of the anti–SIV IgG may be factors in determining the success of passive immunization, another explanation for the different outcome in the present study is that we waited 3 weeks before administering SIV-HIS to our SIV-infected infant macaques. The postinfection immunotherapy may have been more effective if it had been given sooner after viral infection. The presence of passively acquired antiviral antibodies (obtained either by passive immunization or transplacental maternal antibodies) during the first days of infection, when virus levels are still very low, is likely to be more efficient in changing the course of infection. This situation would also mimic that of infants born to HIV-infected women with high levels of maternally derived anti–HIV antibodies.

In addition, studies in HIV-infected human adults have demonstrated that patients in later stages of disease have a much poorer response to passive immunization than healthier patients (reviewed in [65]), which suggests that passive immunotherapy may be less effective in the presence of high virus levels and immunodeficiency. In the present study, SIV-infected newborn macaques were treated with SIV-HIS at 3 weeks of age, when newborn macaques infected with uncloned SIV$_{mac}$251 have high-level viremia and widespread systemic virus dissemination and already demonstrate signs of immunosuppression [2, 22, 23]. Administration of SIV-HIS when SIV antigen levels in the circulation are high apparently resulted in rapid formation of immune complexes and deposition of immune complexes in the tissues of our SIV-infected infant macaques. We have previously shown that antiviral drug treatment of newborn macaques starting 3 weeks after oral SIV$_{mac}$251 inoculation was able to drastically reduce virus levels, enhance antiviral immune responses, and delay disease progression [25]. Thus, it is likely that passive immunization in the presence of high-level viremia requires concurrent treatment with a potent antiviral drug to achieve greater safety and efficacy.

In conclusion, our current passive immunization study demonstrates that antiviral IgG protected newborn macaques against oral SIV infection; secretory IgA was not required for this protection against mucosal exposure. These results suggest that intervention strategies such as active immunization of HIV-infected pregnant women and anti–HIV IgG administration may decrease the rate of perinatal HIV transmission.

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