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Incomplete Protection, but Suppression of Virus Burden, Elicited by Subunit Simian Immunodeficiency Virus Vaccines

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We compared the efficacy of immunization with either simian immunodeficiency virus (SIV) Env glycoprotein (Env), Env plus Gag proteins (Gag-Env), or whole inactivated virus (WIV), with or without recombinant live vaccinia vector (VV) priming, in protecting 23 rhesus macaques (six vaccine and two control groups) from challenge with SIVmac251 clone BK28. Vaccination elicited high titers of syncytium-inhibiting and anti-Env (gp120/gp160) antibodies in all vaccinated macaques and anti-Gag (p27) antibodies in groups immunized with WIV or Gag-Env. Only WIV-immunized macaques developed anticell (HuT78) antibodies. After homologous low-dose intravenous virus challenge, we used frequency of virus isolation, provirus burden, and change in antibody titers to define four levels of resistance to SIV infection as follows. (i) No infection ("sterilizing" immunity) was induced only in WIV-immunized animals. (ii) Abortive infection (strong immunity) was defined when virus or provirus were detected early in the postchallenge period but not thereafter and no evidence of virus or provirus was detected in terminal tissues. This response was observed in two animals (one VV-Env and one Gag-Env). (iii) Suppression of infection (incomplete or partial immunity) described a gradient of virus suppression manifested by termination of viremia, declining postchallenge antibody titers, and low levels (composite mean = 9.1 copies per 10^6 cells) of provirus detectable in peripheral blood mononuclear cells or lymphoid tissues at termination (40 weeks postchallenge). This response occurred in the majority (8 of 12) of subunit-vaccinated animals. (iv) Active infection (no immunity) was characterized by persistent virus isolation from blood mononuclear cells, increasing viral antibody titers postchallenge, and high levels (composite mean = 198 copies per 10^6 cells) of provirus in terminal tissues and blood. Active infection developed in all controls and two of three VV-Gag-Env-immunized animals. The results of this study restate the protective effect of inactivated whole virus vaccines produced in heterologous cells but more importantly demonstrate that a gradient of suppression of challenge virus growth, reflecting partial resistance to SIV infection, is induced by subunit vaccination. The latter finding may be pertinent to studies with human immunodeficiency virus vaccines, in which it is plausible that vaccination may elicit significant suppression of virus infection and pathogenicity rather than sterilizing immunity.

Simian immunodeficiency virus (SIV) infection is valuable as a model for human immunodeficiency virus (HIV) vaccine development because vaccine-induced immunity can be challenged experimentally. Previous studies have established that whole inactivated virus (WIV) SIV vaccines can prevent infection (3, 4, 8, 13, 18, 19, 25, 26, 31, 32); however, the protection induced may in good part reflect immunization against contaminating heterologous (human) major histocompatibility complex proteins present in all WIV vaccines (1, 5, 24, 32). While immunization with purified viral subunit proteins could obviate the latter issue, subunit vaccines have heretofore proven much less effective (24, 26). Nevertheless, Hu et al. (16, 17) have reported protection against homologous SIV_{mne} challenge by priming with an SIV Env-expressing live vaccinia vector followed by boosting with baculovirus-derived $gp160^{Env}$. Girard and colleagues (12) have demonstrated that chimpanzees immunized with recombinant HIV-1 $gp120^{Env}$, alone or in combination with Env peptides, resisted HIV challenge (12). However, others have found that recombinant SIV Env vaccines provided no protection against infection (10, 23). More recently, Daniel et al. (6) reported that chronic infection of macaques with a replication-competent *nef* gene-deleted SIV induced very low-level infection but also protected against subsequent high-dose virus challenge.

While complete vaccine protection ("sterilizing immunity") against HIV infection remains the ideal, this may not be realistic under conditions of natural exposure in which multiple challenges and virus strains may be involved. Moreover, most virus vaccines protect not by completely preventing initial virus infection, but by priming the host immune response to contain and suppress virus replication. Thus, the evaluation of candidate lentivirus vaccines for their abilities to aid the vaccinated individual in suppressing viral replication and its consequent effects on the immune system remains pertinent. Here, we

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assess the capacity of WIV and recombinant subunit SIV vaccines to protect against homologous viral challenge. We demonstrate several levels of resistance ranging from apparently complete protection to partial resistance manifest by termination of viremia and substantial reduction of provirus burden to extremely low levels.

MATERIALS AND METHODS

Animals. Twenty-three juvenile rhesus macaques (*Macaca mulatta*) between 1 and 2 years old and weighing 2.0 to 3.5 kg each were obtained from the New Mexico Regional Primate Research Laboratory. All were negative for SIV and simian type D retrovirus.

Vaccine preparations. SIVmac251BK28 was grown in HuT78 cells, concentrated by ultrafiltration, purified on a metrizamide gradient, and inactivated with 1% formalin (0.00125 μl of 37% formalin per μg of virus protein) at 4°C overnight. Inactivation was confirmed by inoculation of HuT78 and human peripheral blood mononuclear cells (PBMC) with the 100 μl of undiluted, formalin-treated virus (infectious titer of untreated virus stock = 8×10^3 50% tissue culture infective doses per ml in HuT78 cells). Each WIV dose consisted of 800 μg of protein. Recombinant vaccinia viruses (VV) expressing respectively Gag and Env proteins of SIVmac251BK28 were those described by Delchambre et al. (7), and Horth et al. (15). Recombinant VV expressing *pol* was obtained by inserting the reverse transcriptase and integrase domains of the *pol* gene of SIVmac251BK28 downstream to the P7.5 promoter in the VV genome (Lister strain). A translation initiation codon and a glycine codon were inserted at the presumed junction between the protease and the reverse transcriptase coding sequences. The VV recombinant expressed the fused reverse transcriptase and integrase as a protein of 90-kDa molecular mass. The three recombinant VV used have been deposited in the Medical Research Council AIDS Reagent Repository (reference numbers: ADP261, ADP262, and ADP263). Stocks of recombinant VV were grown in CV-1 cells.

SIVmac251BK28 $Pr57^{Gag}$ was produced by a baculovirus recombinant virus grown on *Spodoptera frugiperda* (Sf-9) cells (7). The complete SIV *gag* gene was introduced into the baculovirus genome under the control of the polyhedrin promoter. The resultant Gag particles budded from Sf-9 cells were collected by centrifugation and detergent disrupted and soluble $Pr57^{Gag}$ was purified by lectin chromatography and then subjected to immunoaffinity chromatography.

Full-length SIV $gp160^{Env}$ was obtained from lysates of CV-1 cells infected with recombinant VV (15) by lectin chromatography and immunoaffinity. Each dose of Env and Gag contained 122 and 133 μg of the proteins, respectively.

The adjuvant threonylmuramoyl dipeptide (250 μg) was dissolved in the emulsion vehicle SAF-1 (both provided by Noleen Byars at Syntex, Inc., Palo Alto, Calif.). Adjuvant was mixed with immunogen immediately before immunization.

Vaccine and vaccine groups. The 23 macaques were apportioned into six vaccine groups of three animals each as follows. Group 1 (WIV) animals were immunized three times with WIV without VV priming. Group 2 (VV-WIV) animals were primed by intradermal vaccination with three live recombinant VV expressing, respectively, SIVmacBK28 Gag, Env, and Pol proteins and boosted three times with WIV (SIVmacBK28). Group 3 (VV-Gag-Env) animals were primed with VV expressing SIVmacBK28 Gag and Env proteins and boosted three times with SIVmac $p57^{Gag}$ and $gp160^{Env}$. Group 4 (Gag-Env) was immunized with $p57^{Gag}$ and $gp160^{Env}$ four times. Group 5 (VV-Env) macaques were primed once with VV-Env and boosted three times with purified $gp160^{Env}$. Group 6 (Env) animals were immunized four times with purified $gp160^{Env}$. Three control animals were immunized with adjuvant alone (Group 7 [adjuvant]), and two were immunized with wild-type VV in adjuvant (Group 8 [VV-adjuvant]). Vaccinia priming was done by inoculation of 10^8 PFU by intradermal scarification. All other immunizations were by intramuscular injection of the immunogen in adjuvant. The animals were immunized at 0, 3, 13, and 52 weeks, except for group 1 (WIV only) which received three immunizations, at 0, 4, and 24 weeks.

Challenge, observation, and necropsy. All animals were challenged by intravenous injection of 7 to 10 50% animal infectious doses pretitered in naive rhesus macaques of SIVmac251BK28 3 weeks after the last vaccine boost (55 weeks after primary immunization for groups 2 through 8 and 27 weeks after primary immunization for group 1). Physical examinations were performed on all challenged macaques at monthly intervals, and heparinized blood was collected at 0, 1, 2, 4, 8, 12, 18, 21, 29, and 40 weeks postchallenge. The study was terminated at 40 weeks postchallenge for groups 2 through 7 and at 18 weeks postchallenge for group 1. All macaques were necropsied and examined for macroscopic lesions. Samples of bone marrow, spleen, inguinal lymph node, and mesenteric lymph node were collected for virus and/or provirus assay.

Neutralizing antibodies. Antibody titers against SIVmac251 were determined after each vaccination as described by Langlois et al. (22). This assay uses 90% inhibition of syncytium induction by uncloned SIVmac251 as the end point.

Anti-HuT78 antibodies. Anticellular antibodies were assayed after each vaccination by observing clumping of uninfected HuT78 cells in the presence of serial serum dilutions as described by Langlois et al. (21).

Antibodies to Gag and Env by enzyme-linked immunosorbent assay (ELISA). Microtiter plates (Immulon-2; Dynatech Laboratories, Inc., Chantilly, Va.) were coated either with 3E8 (100 ng per well), a monoclonal antibody (MAb) to the SIV $p27^{Gag}$ (gift from Niels Pedersen, Davis, Calif.); MAb 101.1 (200 ng per well), which detects SIV gp120 and gp160; or MAb 43.1 (200 ng per well), which detects antibody to gp40 TM (the last two MAbs were from SmithKline Beecham). The wells were blocked with TEN buffer (0.05 M Tris base, 0.001 M EDTA, 0.15 M NaCl [pH 7.2 to 7.4]), containing 2% bovine serum albumin (Boehringer Mannheim, Indianapolis, Ind.) before the addition of 100 μl of a reference SIV stock to permit virus binding. Following plate washing, twofold dilutions of test sera were incubated for 30 min and, after the plates were washed, incubated with peroxidase-conjugated rabbit anti-monkey immunoglobulin G serum (Cappel Laboratories, Durham, N.C.) and developed with TMB substrate (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.). The plates were read at 450 nm, with a purified preparation of SIV $Pr57^{Gag}$ for calibration. The detection limit of the assay was 2 pg/ml of antigen.

Immunoblotting. Concentrated SIVmacBK28 produced in HuT78 cells (2 μg per well) was electrophoresed in each well of a 12% polyacrylamide gel at 200 V and then transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, Mass.) at 90 V for 90 min. Plasma or serum samples were diluted 1:200 (in 0.01 M phosphate-buffered saline [PBS; pH 7.2], 18% glucose, 10% fetal bovine serum, 0.2% Tween 20, and 10% glycerol) and incubated with membrane strips for 1 h at room temperature before a 90-min washing (with 0.01 M PBS–0.5% Tween 80–150 mM NaCl, pH 7.4). The strips were then incubated for 1 h at room temperature with peroxidase-

conjugated goat anti-monkey serum (Cappel Laboratories) diluted 1:500 in 0.01 M PBS containing 10% nonfat milk powder. After a 1-h washing, the strips were developed with TMB substrate (Kirkegaard and Perry Laboratories, Inc.).

Virus isolation. Virus was recovered from PBMC, bone marrow, mesenteric lymph node, or spleen of challenged macaques by coculture with either HuT78 cells or phytohemagglutinin-stimulated human PBMC from HIV-seronegative donors. Four human T-cell lines (HuT78, MDLT4, CEMx174, and SupT1) were tested for susceptibility to clone BK28. HuT78 proved the most sensitive of the four and was thus used in parallel with human primary PBMC for virus isolation. PBMC and bone marrow mononuclear cells were separated by Ficoll-Hypaque (Organon Teknika, Durham, N.C.) gradient centrifugation, and isolations were performed in 12-well plates (Nunc, Inc., Naperville, Ill.) containing 5×10^6 macaque cells and 5×10^6 coculture cells per well. Lymph nodes (mesenteric and inguinal) and spleen were minced and passed through 1-mm stainless steel mesh, and 5×10^6 of the cells were cocultured with 5×10^6 human PBMC in 25-cm^2 tissue culture flasks. Cultures were incubated at 37°C in a humidified 5% CO_2 incubator, the medium was replaced every 3 to 5 days, and culture supernatants were assayed for SIV p27^{Gag} by antigen-capture ELISA. Cultures were maintained for 40 days before being considered negative.

The medium for PBMC and HuT78 cocultures was RPMI 1640 (Irvine Scientific, Santa Ana, Calif.) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, Utah), 2 mM L-glutamine (JRH Biosciences, Lenexa, Kans.), 55 μg of gentamicin (GIBCO-BRL Laboratories, Grand Island, N.Y.) per ml, and 2 μg of hexadimethrine bromide (Sigma Chemical Co., St. Louis, Mo.) per ml. For human PBMC cocultures, interleukin-2 (8.6 U/ml) (Cetus Corporation, Emeryville, Calif.) and 2×10^6 to 5×10^6 phytohemagglutinin-stimulated normal human PBMC were added to the cultures every 10 days.

Lymphocyte proliferation assays. PBMC from vaccinated and control macaques were cryopreserved in RPMI 1640 containing 10% dimethylsulfoxide (Sigma Chemical Co.) and 25% fetal bovine serum. Thawed cells were washed and suspended at a concentration of 5×10^6 cells per ml in RPMI containing 10% heat-inactivated human serum (HIV antibody negative and AB positive) and 2 mM L-glutamine. Aliquots of 5×10^5 cells in 100 μl of medium were seeded in duplicate wells and incubated with 100 μl of antigen or mitogen. Phytohemagglutinin (50 μg/ml) was used as a positive control. The antigens used were purified SIVmac251BK28 gp160^{Env} or WIV (10 μg/ml inactivated by boiling for 3 min). Proliferative activity against viral antigens was measured at day 7 (day 4 for phytohemagglutinin) with 1 μCi of [^{3}H]methylthymidine (Amersham, Arlington Heights, Ill.) per ml. The stimulation index

TABLE 1. Frequency of virus isolation from PBMC and antibody data postchallenge in macaques vaccinated with SIVmac251BK28 subunit proteins and WIV

Group no. and vaccine type	Animal no.	Virus isolation[a]	Antibody titer (10^2)[b]								
			Gag			Env			Neutralizing		
			Challenge	4 wks PC	29 wks PC	Challenge	4 wks PC	29 wks PC	Challenge	4 wks PC	17 or 18 wks PC
1, WIV	131D	0/7	512	512	54***	6	8	8***	ND	ND	ND
	113D	0/7	258	128	32***	16	<4	<4***	ND	ND	ND
	135D	0/7	512	128	32***	4	<4	<4***	ND	ND	ND
2, VV WIV	120D	0/10	1,024	1,024	64	64	32	32	290?	100	180
	133D	0/10	1,024	1,024	128	32	32	32	34?	?	?
	153D	2/10	1,024	512	64	64	64	32	60?	?	?
3, VV Gag-Env	123D	1/10	1,024	2,048	32	32	32	8	130	400	125
	117D	6/10	1,024	512	8	64	128	512	400	440	1,400
	146C	6/10	1,024	1,024	256	64	256	32	66	800	460
4, Gag-Env	10D	0/10	64	1,024	64	64	128	32	135	220	135
	15D	4/10	32	1,024	256	64	64	64	106	170	70
	23D	2/10	16	128	6	64	64	32	23	205	56
5, VV-Env	117C	1/10	2	4	<4	64	32	8	210	70	21
	125D	2/10	2	8	4	64	64	16	68	110	82
	164D	2/10	8	16	128	64	128	32	600	1,000	120
6, Env	114D	2/10	32	32	<4	64	64	16	120	500	32
	121D	3/10	16	84	8	128	64	32	480	670	200
	124D	2/10	8	16	8	64	64	8	76	220	62
7, cont adjuvant[c]	96D	7/10	<1	64	>512	<1	<1	128	<0.20	130	135
	178D	8/10	<1	16	>512	<1	<1	128	<0.20	100	110
	169D	5/7	<1	<1	8***	<1	<4	4	ND	ND	ND
8, cont VV	137D	3/10	<1	2	8	<1	<1	<4	<0.20	7.2	1.15
	221D	7/10	<1	64	512	<1	<1	256	<0.20	70	400

[a] Cumulative number of positive/total number of attempts during the postchallenge (PC) period.
[b] ***, 10 weeks PC for group 1 only. ?, assay not interpretable because of indicator cell (HuT78) clumping activity of sera. ND, not done.
[c] Cont, control.

was the ratio of radioactivity incorporated by test PBMC in the presence versus that incorporated in the absence of antigen.

SIV capture ELISA. MAb 3E8 was used to capture SIV $p27^{Gag}$ from culture media by the same methodology described above for Gag antibody detection. The test culture supernatant replaced the virus stock, and polyclonal macaque antiserum was used to recognize immobilized p27. Peroxidase-conjugated anti-monkey immunoglobulin and TMB substrate were used to develop the assay. Purified SIV $Pr55^{Gag}$ was used for assay calibration.

Provirus detection by PCR. Qualitative nested PCR was performed on DNA extracted from lysed PBMC (20) of challenged macaques. Two rounds of amplification were performed with primers from the *pol* gene of SIVmacBK28.

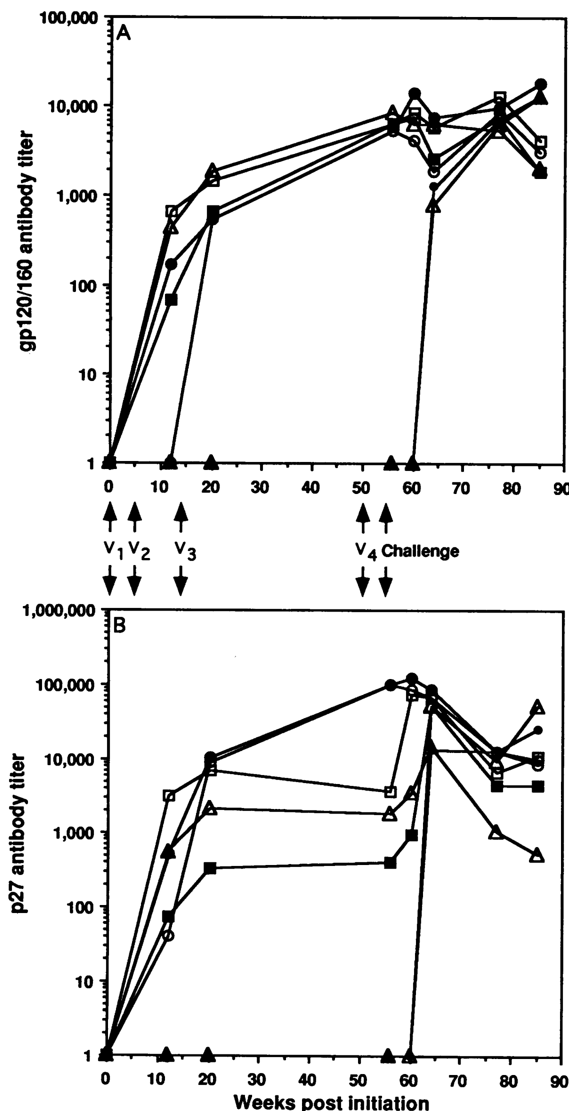

FIG. 1. Serum antibodies against SIVmac251BK28 gp120 and $gp160^{Env}$ (A) and SIV $p27^{Gag}$ (B) generated by vaccinated, challenged macaques. Symbols: •, VV-WIV (group 2); ●, VV-Gag-Env (group 3); □, Gag-Env (group 4); ■, VV-Env (group 5); △, Env (group 6); ▲, adjuvant only (group 7); ○, VV only (group 8). The first (priming) immunization (V_1) and subsequent (booster) immunizations V_2, V_3, and V_4 are indicated.

Primers used for round 1 were SD29 (3′-GTACCTGCAC ATAAAGGAATAG, corresponding to positions 4431 to 4454 of the SIVmac251BK28 genome) and SD46 (TAGCTTTCC TCCTTGGTACTACCT, positions 5300 to 5276). Primers for round 2 were SD25 (TATAGTTGCAGTACATGTGG CTAGTG, positions 4724 to 4748) and SD44 (TCTCTGTT CTCTGTAATAGAC, positions 5200 to 5177). Primers used for amplifying the intracellular TM region of the Env gene were for round 1: PExtm6 (GGATCTGGTATGCTCTCG ATAGCAA, positions 8977 to 8953) and UP3 (AGACTGC AGATGTGAAGAGGTACAC, positions 8114 to 8130). Round 2 primers were PExtm7 (GATACTGCAGCAACAGC AACAGCTG, positions 8265 to 8286) and UP5 (CTTCTCTG GTTGGCAGTG, positions 8827 to 8810). Thirty-five cycles of amplification were performed for both rounds (94°C for 45 s, 55°C for 1 min, and 72°C for 2 min, followed by a final extension at 72°C for 10 min). The amplification mixture consisted of 50 mM KCl, 10 mM Tris HCl (pH 8.3), 2 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP [Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.]), 15 pmol of each primer, and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Corp., Norwalk, Conn.), and it contained 45 μl of DNA in a volume of 100 μl. Five microliters from each reaction product of round 1 was added to 95 μl of the reaction mixture and amplified. DNAs from PBMC of uninfected macaques were run in parallel as negative controls.

Quantitative nested PCR was performed on serial twofold dilutions of genomic DNA prepared by standard proteinase K digestion-phenol-chloroform extraction (28) and by using two sets of primers from the *nef* gene. As controls for the possible introduction of contaminating SIV sequences during sample preparation and PCR setup, DNA was extracted in parallel from samples of uninfected human placenta, and 1 μg of control DNAs was amplified as described below, along with reactions containing only reagents to which no template DNA was added. Primers used for round 1 were PEnef-1 (5′-GGCCTTGGCAGATAGAATAT, positions 8869 to 8889) and SD12 (GCATGCGAATTCTTATTACCGAGTACCG AGTTGACCAG, positions 10160 to 10188). Thirty-five cycles of amplification were performed in 50-μl reaction mixtures, as described above except that 1.75 mM $MgCl_2$ and 1.25 U of *Taq* polymerase were used; the reactions were supplemented with 1% dimethylsulfoxide, and the extension time at 72°C was limited to 1.5 min. Two microliters from each round 1 reaction product was transferred to fresh tubes containing 98 μl of PCR reaction mix and 100 nM primers PEnef-2 (GCGGGATCCT GATACGCCTCTTGACTTGGCTAT, positions 8912 to 8936) and PEnef-3 (GCGGGATCCGTGGAGCCACTCT GCCCAGCAC, positions 10056 to 10077) and amplified for 35 cycles. Amplified DNA products were detected by ethidium bromide staining after electrophoresis in 1% agarose gels (28).

Copy number standard titrations were used to determine that the above conditions produced the maximum quantity of product synthesized when single or greater numbers of viral molecule templates were employed. These titrations were conducted in parallel with each set of amplifications from macaque tissues.

The minimum number of proviral DNA copies present per 10^6 cells was estimated in terminal tissues by serial dilution from a subset of animals. The proviral load was estimated by assuming that each positive reaction contained one provirus template and scoring all samples from the lowest dilution that contained at least one positive and one negative reaction and all subsequent dilutions. The provirus load was calculated by dividing the number of positive reactions by the total number

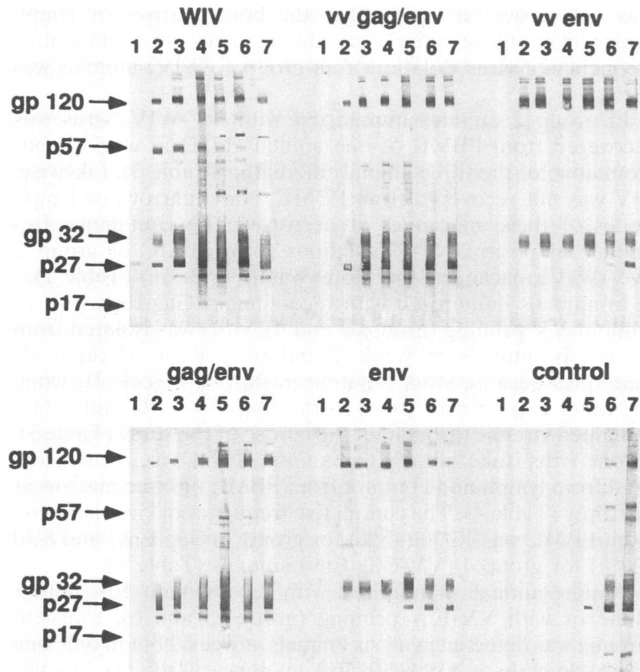

FIG. 2. Antibody responses of SIVmac251BK28-vaccinated, challenged macaques assessed by immunoblotting. Results from randomly selected macaques from vaccine groups 2 through 7 are shown. Lane 1, preimmunization; lane 2, 4 weeks after second vaccination; lane 3, 4 weeks after third vaccination; lane 4, 3 weeks after fourth vaccination (prechallenge); lane 5, 4 weeks after challenge; lane 6, 12 weeks after challenge; lane 7, 21 weeks after challenge.

of cell DNA equivalents examined. Initially, from 5 to 13 replicate reactions were conducted at the lowest dilution (2 μg, 3×10^5 cells). Five or ten replicates at this dilution were used to confirm each negative sample (reported as <0.7 or <0.3 proviruses per 10^6 cells). When any of these reactions were positive, serial twofold dilutions of extracted DNA were carried out and then amplified by nested PCR until at least the highest one to three dilutions were consistently negative. Each dilution was tested in 2 to 13 replicates, and a range of 9 to 29 (mean 18.5) reactions was conducted to titrate each positive sample. We confirmed that sporadic positives obtained at high dilution were not due to imprecise pipetting of high-molecular-weight DNA, since these occasional positive reactions occurred whether or not the template DNA was cleaved by restriction endonuclease digestion prior to dilution.

RESULTS

Antibody responses induced by vaccination. Titers of antibodies to SIV $p27^{Gag}$ and $gp120^{Env}$ were measured after each immunization. Macaques in all vaccine groups developed antibodies to Env beginning 10 weeks after initiation of the vaccine regimen (Table 1). Mean peak titer for all groups (1:5,483 ± 2,845) was attained by 55 weeks postinitiation (prechallenge) (Fig. 1A). In groups 1 through 4, immunization also elicited antibodies to Gag (mean peak titer = 1:63,822 ± 48,016 at 55 weeks postinitiation) (Fig. 1B). Postimmunization antibody responses also were detected to SU and TM in all vaccine groups by immunoblot analysis and to $p17^{Gag}$, $p27^{Gag}$, and $Pr57^{Gag}$ in groups 1 through 4 (WIV- or Gag-immunized animals) (Fig. 2). Although groups 5 and 6 were immunized

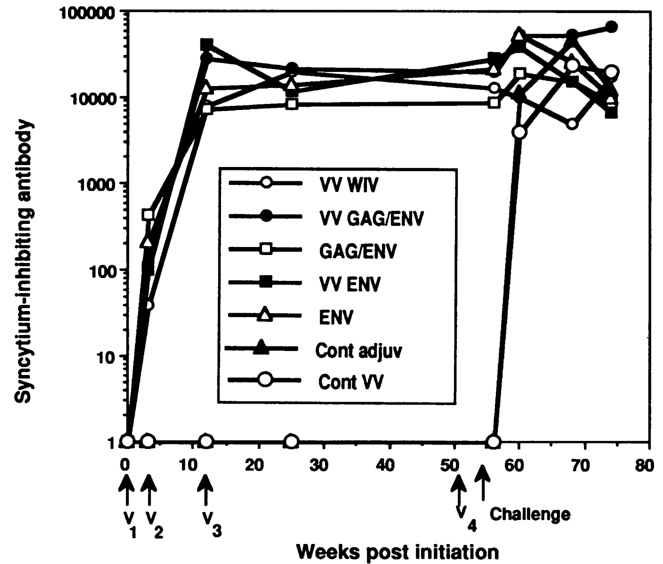

FIG. 3. Anti-SIVmac251 neutralizing antibodies measured by the syncytium inhibition assay of Langlois et al. (22) in SIVmac251BK28-vaccinated, challenged macaques. Intervals are the same as for Fig. 1. Cont adjuv, control adjuvant.

only with Env protein, a low-level Gag-reactive response was detected by ELISA but not by immunoblot assay (Fig. 1B and 2). Similar low-level Gag-like reactivity after gp160 immunization has been reported by Hu et al. (16).

SIV syncytium-inhibiting (neutralizing) antibody responses coincided with the Env and Gag responses, reaching a mean peak titer of 1:18,653 prior to challenge (Fig. 3). Anti-HuT78-cell antibody responses were observed only in macaques immunized with WIV (group 1) or VV-WIV (group 2) (Fig. 4). No macaques in control groups 7 or 8 (adjuvant or VV-

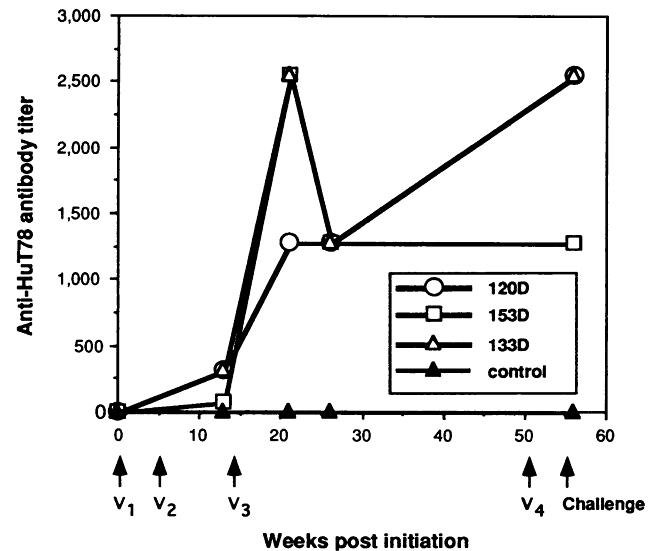

FIG. 4. Anti-HuT78 antibody titers in SIVmac251BK28 VV-WIV-vaccinated macaques (group 2). Vaccinated animals 120D, 153D, and 133D and control animal 96D are represented. Intervals are the same as for Fig. 1.

TABLE 2. Proliferative response of lymphocytes from vaccinated macaques to SIVmac251BK28 WIV and gp160^{Env} after the second vaccination (7 weeks after initiation of the study)

Group and vaccine	Animal	Test antigen SI[a]	
		WIV	Env gp160
1, WIV	131D	0.5	1
	113D	**3.2**	0.7
	135D	0.4	1.2
2, VV-WIV	120D	1.2	ND
	133D	**4.8**	**3.6**
	153D	0.8	2.3
3, VV-Gag-Env	123D	1	1
	117D	**5.5**	**7.5**
	146C	0.7	2.3
4, Gag-Env	10D	2.3	**10.1**
	15D	0.8	0.6
	23D	1.4	**3**
5, VV-Env	117C	**3.3**	**4**
	125D	1.2	**5.7**
	164D	**8.7**	**14.7**
6, Env	121D	**3.4**	**4.5**
	114D	**3.4**	**3.4**
	124D	0.7	2.1
7, Adjuvant	96D	1.2	0.6
	178D	0.7	2.3
	169D	ND	ND
8, VV-adjuvant	137D	2.5	2.1
	221D	**7.6**	**7.8**

[a] Entries in boldface have a stimulation index (SI) of >3.0. ND, not done.

adjuvant) developed SIV antibodies detectable by any assay prior to challenge.

Lymphocyte proliferative responses induced by vaccination. PBMC from 7 of the 18 vaccinated macaques exhibited proliferative responses to heat-treated whole virus 7 weeks after the first immunization (Table 2). Despite one intervening booster immunization, responses discernible above background level (stimulation index, >3.0) remained detectable in only two of these animals at 18 weeks (macaques 10D and 113D) and one animal at 27 weeks (macaque 10D). Proliferative responses to recombinant Env were observed in 9 of 18 immunized macaques at 7 weeks (Table 2). At 27 weeks, responses were detectable in five of these nine animals and in two other vaccinated macaques. However, proliferation of PBMC from two control macaques also was evident at 27 weeks, thus diminishing the significance of the proliferative reactions seen in the virus-immunized animals.

Postchallenge virus isolation. Coculture with both HuT78 and mitogen-pulsed human PBMC was used to recover SIV from the PBMC of challenged macaques. In the initial postchallenge period (2 to 4 weeks), isolations were obtained with both host-cell systems. With time, however, PBMC proved decidedly more sensitive, so that positive isolations from vaccinated macaques at 12 weeks postchallenge and thereafter were obtained only by using human PBMC. These results are consistent with the expected shift from the inoculated strain harboring a truncated TM and HuT78 tropism to a mutant form of the virus with an extended TM and reduced HuT78 growth (14).

Virus was recovered by PBMC coculture from all five adjuvant and wild-type VV-inoculated control animals by 2 to 4 weeks postchallenge. Four of the five animals remained consistently viremic thereafter. By contrast, virus was never isolated from animals in group 1 (WIV) at any time postchallenge (1, 2, 4, 6, 8, 11, or 18 weeks) (Table 3). In addition, virus was not recovered from either the bone marrow or lymph nodes from these animals at death. Thus, the cumulative frequency of virus isolation from group 1 (WIV) animals was zero.

In group 2 animals immunized with VV-WIV, virus was recovered from PBMC of one animal (153D at week 4) but from none of the three animals thereafter (Table 3). Likewise, SIV was not recovered from PBMC, bone marrow, or lymph nodes of these macaques at necropsy. The cumulative frequency of positive virus isolations from PBMC of group 2 (VV-WIV) macaques, therefore, was 1/30 (3.3%) (Table 1).

In animals immunized with recombinant Gag-Env, with or without VV priming (groups 3 and 4), virus was isolated from six of six animals at week 2 and/or 4. Four of these six macaques became virus isolation negative by week 21, while the other two animals (both from group 3—117D and 146C) remained viremic throughout the course of the study (Table 3). In one virus isolation-negative animal (15D), virus was recovered from lymph node but not from PBMC or bone marrow at necropsy (Table 4). The cumulative frequency of virus isolation from PBMC was 13/30 (43%) for group 3 (Gag-Env) and 6/30 (20%) for group 4 (VV-Gag-Env) animals (Table 1).

Among animals immunized with recombinant Env, either alone or with VV-Env priming (groups 5 and 6), transient viremia was detected in all six animals at week 2 but in only one of six animals at week 12 and in none of the six animals thereafter (Table 3). The cumulative frequency of virus isolation from PBMC cocultures was 5/30 (17%) in group 5 (VV-Env) and 7/30 (23%) in group 6 (Env) (Table 2). At termination, SIV was not recovered from PBMC or bone marrow from any of the six macaques, but was isolated from lymph node in three of these six nonviremic animals (Table 4).

Postchallenge provirus burden. To assess the presence of circulating (PBMC) provirus in challenged macaques, qualitative nested PCR amplification of SIV *env*, *pol*, and *nef* sequences was performed with DNA obtained from uncultured PBMC collected at 10, 12, and 29 weeks and at termination. To determine provirus burden in tissue compartments, quantitative nested PCR was performed on DNA from uncultured PBMC, spleen, and mesenteric lymph node obtained at necropsy. Nested PCR detection of SIV *nef* under the conditions employed was sensitive to single copies of template DNA so that the presence of any target molecules should result in maximum synthesis of the product (8a, 14).

By performing multiple parallel *nef* PCRs on uncultured cellular DNA equivalents of 1.5×10^5 to 7.5×10^5 cells, we determined the frequency of SIV provirus-positive and -negative assays (Table 3). The results of each replicate assay are expressed as percent positive assays in Table 4. A similar analysis of proviral load in terminal tissues employed multiple replicates and serial twofold dilutions corresponding to template cellular DNA equivalents ranging from 3×10^5 to 3×10^2 cells (see Materials and Methods). Determination of the minimum number of DNA equivalents which gave a positive PCR signal permitted expression of provirus burden as the number of provirus copies per 10^6 cells (Tables 4 to 6). The results of these assays (Tables 4 to 6) revealed four groupings of descending provirus burden as follows. (i) As would be expected, the highest burden (composite mean = 198.1 copies per 10^6 cells) was found in viremic animals, i.e., the five VV-adjuvant controls and two of the three VV-Gag-Env-vaccinated animals. (ii) A 10- to 200-fold gradient of reduced provirus burden (composite mean = 9.1 copies per 10^6 cells) was found in 11 of the 15 macaques comprising the VV-WIV and recombinant subunit vaccine groups 2 through 6 (classified in the virus suppression-incomplete immunity response group

TABLE 3. Serial detection of virus and provirus in PBMC of SIVmac251BK28-vaccinated, challenged macaques[a]

			Time post challenge (in weeks)												Summary at 29 weeks	
			0	1	2	4	6	8	10-11	18		21	29			
Group no.	Vaccine type	Animal no.	VI	VI	VI	VI	VI	VI	PCR	VI	PCR	VI	VI	PCR	VI	PCR
		131D	□	□	□	□	□	□	○○○○○	□	○○○○○ *					
1	WIV	113D	□	□	□	□	□	□	○○○○○	□	○○○○○ *					
		135D	□	□	□	□	□	□	○○○○○	□	○○○○○ *				0/6**	2/6**
		120D	□	□	□	□	□	□	◇	□	●●○○○○○○○	□	□	○○○○○○○		
2	VV-WIV	133D	□	□	□	□	□	□	◇	□	●●○○○○	□	□	●●○○○○○		
		153D	□	□	□	■	□	□	◇	□	●●●○○○○○	■	□	●●●○○○○		
		123D	□	□	□	■	□	□	◆	□	●●●○○○○○	□	□	●●		
3	VV-GAG + VV-ENV	117D	□	□	□	■	□	□	◆	■	●●	■	■	●●		
		146C	□	□	■	■	■	□	◆	□	●●	■	■	●●	3/6	5/6
		10D	□	□	□	□	□	□	◇	□	●○○○○○○○○○	□	□	○○○○○○○		
4	GAG + ENV	15D	□	■	■	■	□	□	◆	□	●●●●●●○	■	■	●●●●●○		
		23D	□	□	■	□	■	□	◇	□	●●●●●●●○	□	□	●●		
		117C	□	□	■	□	□	□	◇	□	○○○○○○○	□	□	○○○○○○○		
5	VV-ENV	125D	□	□	■	■	□	□	◇	□	●●●●●●●○○	□	□	●●●●○○○		
		164D	□	□	■	□	■	□	◇	□	●●●●●●○○	□	□	●●	0/6	5/6
		114D	□	□	■	■	□	□	◆	□	○○○○○	□	□	●●●●●●●○○○○○		
6	ENV	121D	□	□	■	■	□	□	◇	□	○○○○○○○○	□	□	●●●●○○○		
		124D	□	□	■	■	□	□	◇	□	●●●●●○	□	□	●●		
		96D	□	□	■	■	□	■	◆	■	●●	■	■	●●		
7	adjuvant	178D	□	■	■	■	■	■	◆	□	●●	■	■	●●		
		169D	□	□	□	■	■	■	nd	■	●●●●● *				4/5	5/5
		137D	□	□	■	■	■	□	◆	□	●●	□	□	●●		
8	VV-adjuv.	221D	□	■	■	■	■	□	◆	□	●●	■	■	●●		
	N-PCR Neg. Controls								○○○○		○○○○○○○○○○ ○○○○○			○○○○○○○○○○		
	N-PCR Pos. Controls								●		●●●●●●●●●● ●●			●●●●		

[a] *, terminal; **, number of animals positive/total; VI, virus isolation; □, negative virus isolation; ■, positive virus isolation; ◇/◆, negative/positive provirus detection by nested PCR (N-PCR) detecting *env* gene sequences; ○/●, negative/positive provirus detection by nested PCR (N-PCR) detecting *nef* gene sequences. Neg., negative; pos., positive; nd, not done.

[see Discussion]). (iii) No proviral DNA could be detected at termination in any of the three WIV animals or in two other subunit-immunized macaques (10D and 117C). These analyses also revealed significant differences (P = 0.002) among provirus burdens found in the three tissue compartments studied. The highest provirus load was found in the lymph nodes of 10 of the 12 animals studied (Tables 4 to 6).

Postchallenge antibody responses. Anti-Gag and anti-Env titers increased in four of five control macaques and in two of three macaques in group 3 (VV-Gag-Env), each of which were also persistently viremic postchallenge (Table 2; Fig. 1 and 3). By contrast, antibody titers decreased in all three macaques in group 1 (WIV). In the majority of animals in groups 2, 4, and 5 (VV-WIV, Env, and VV-Env), in which virus suppression was evident after challenge, a transient postchallenge rise in antibody titers was followed by a progressive decrease until the time of termination.

Postchallenge clinical findings. No evidence of clinical disease was observed in any of the macaques during the study period. This was not surprising given the long (mean > 2 years) incubation period to disease induction for SIVmac251 molecular clone BK28 (8a). Splenomegaly was detected in four macaques: two in group 3 (VV-Gag-Env), one in group 2 (VV-WIV), and one in group 8 (VV control). Three of these four animals were viremic. Axillary and inguinal lymphadenopathy was also detected in two of the three viremic macaques.

DISCUSSION

The results of this study confirm those of other investigators (3, 6, 8, 13, 19, 24, 31) in demonstrating that WIV vaccines confer protection against challenge with homologous virus. Immunization with SIVmac251BK28 gp160^{Env} provided discernible yet incomplete protection against homologous virus challenge. In this respect, the present study extends the work of Hu et al. (16, 17) and Shafferman and colleagues (29), who demonstrated, respectively, that despite early virus replication after challenge, SIV$_{mne}$ infection was suppressed in animals immunized with homologous recombinant Env (gp160) or Env peptides. A consistent feature of Env vaccination in the present study was the suppression of virus replication postchallenge as reflected by transition from positive to negative virus isolation from PBMC and a >10- to 200-fold decrease in provirus load compared with those of wild-type VV- and/or adjuvant-immunized controls. Neither concurrent immunization with recombinant Gag nor priming with VV expressing Gag or Env enhanced this response. In comparing two of three vaccine regimens in which VV priming was used with regimens in which it was not used (i.e., WIV versus VV-WIV and VV Gag-Env versus Gag-Env), the VV-primed animals appeared less able to suppress virus replication.

The frequency of virus isolation from PBMC, the provirus burden, and the postchallenge antibody titer were used to

TABLE 4. Summary of response categories for macaques immunized with SIVmac251BK28 vaccines, using the criteria of cumulative frequency of virus isolation from PBMC, provirus burden in PBMC and terminal tissues, and postchallenge antibody response pattern

Vaccine type	Animal no.	Postchallenge period: 1–4 wks VI (% pos)	6–29 wks VI (% pos)	6–29 wks PCR (% pos)	0–40 wks Ab titer Gag	0–40 wks Ab titer Env	Termination (40 wks) Blood (PBMC) VI	Blood (PBMC) PCR (% pos)	Blood (PBMC) Proviral load (per 10^6 cells)	Spleen PCR (% pos)	Spleen Proviral load (per 10^6 cells)	Lymph nodes VI	Lymph nodes PCR (% pos)	Lymph nodes Proviral load (per 10^6 cells)	Category of infection
WIV	131D	0	0	0	↓	↓	○	0	<0.7	0	<0.7	○	0	<0.7	None detected
WIV	113D	0	0	0	↓	↓	○	0	<0.7	0	<0.7	○	0	<0.7	None detected
WIV	135D	0	0	0	↓	↓	○	0	<0.7	0	<0.7	○	0	<0.7	None detected
Gag-Env	10D	0	0	6	↓	↓	○	0	<0.7	0	<0.7	○	0	<0.7	Abortive
VV-Env	117C	25	0	0	↓	↓	○					○			Abortive
VV-WIV	133D	0	0	31	↓	↓	○	0	<0.7	33	0.7	○	40	7.9	Suppressed
Env	121D	50	0	25	↓	↓	○	0	<0.7	0	<0.3	○	33	6.8	Suppressed
VV-WIV	120D	0	0	13	↓	↓	○	20	0.5	57	5.3	○	100	12	Suppressed
VV-Env	125D	50	0	65	↑	↓	○					●			Suppressed
Env	114D	50	0	44	↓	↓	○	43	1.3	22	1.4	●	100	20	Suppressed
VV-WIV	153D	25	20	40	↓	↓	○	60	2.1	57	1.3	○	100	13	Suppressed
VV-Gag-Env	123D	25	0	55	↓	↓	○					○			Suppressed
Gag-Env	15D	75	40	86	↑	↔	○	80	4.8	100	21	●	100	14	Suppressed
Gag-Env	23D	25	20	82	↓	↓	○	87	2.9	86	3.7	○	100	28	Suppressed
VV-Env	164D	25	20	73	↑	↓	○					●			Suppressed
Env	124D	50	0	82	↔	↓	○	36	1.5	46	1.6	○	100	28	Suppressed
VV-Gag-Env	117D	25	60	100	↓	↑	●					○			Active
VV-Gag-Env	146C	50	60	100	↓	↓	●					○			Active
Adjuvant	96D	50	80	100	↑	↑	●	100	32	100	56	●	100	155	Active
Adjuvant	178D	75	80	100	↑	↑	●	100	39	100	70	●	100	569	Active
Adjuvant	169D	25	100	100	↑	↑	●	100				●			Active
VV only	137D	50	20	100	↑	↔	○	100	12	100	18	○	100	53	Active
VV only	221D	75	60	100	↑	↑	●	100	853	100	412	○	100	110	Active

[a] Virus isolation (VI) positive (●) and negative (○) results and PCR results (nested and performed on serial PBMC) expressed numerically as the percent parallel assays that were positive are shown. Quantitative PCR (nested and performed on terminal tissues) results are expressed as the number of provirus copies per 10^6 cells. Postchallenge antibody (Ab) responses: ↑, increasing; ↓, decreasing; ↔, unchanged.

classify degrees of virus suppression in vaccinated animals as follows (Tables 4 to 6). (i) For animals with no infection (sterilizing immunity), neither virus nor proviral DNA were detected throughout the postchallenge period. Only animals in group 1 (WIV) met this criterion. (ii) With abortive infection (strong immunity), either virus or provirus was detected once but not thereafter during the postchallenge period. Provirus was undetectable in terminal PBMC or tissues (Table 4). Antibody titers declined postchallenge. One animal from group 4 (Gag-Env) and one animal from group 5 (VV-Env) were so classified. (iii) With suppressed infection (incomplete or partial immunity), 9 of the 11 animals (82%) in this responder class developed transient cell-associated viremia (positive virus isolation from PBMC) primarily in the early (2- to 4-week) period postchallenge (however, virus was recovered as late as week 21 in 2 animals) (Table 3). Subsequently, infection was suppressed so that virus was no longer demonstrable by PBMC coculture; however, low levels of provirus (composite mean = 9.1 copies per 10^6 cells; range = 1.6 to 16.2 copies per 10^6 cells) were detectable at termination. This response occurred in 8 of the 12 animals immunized with Env or Env-Gag and two of the three VV-WIV-immunized ma-

TABLE 5. Vaccine responder classification in relationship to vaccine type and proviral burden[a]

Response category	No. of animals with response[b]: WIV (3)	VV-WIV (3)	Gag + Env (3)	VV-Gag+Env (3)	Env (3)	VV-Env (3)	Adjuv. control (3)	VV-adjuv. control (2)	Total	Terminal provirus load: PBMC	Spleen	Lymph node	Composite mean
Sterilizing immunity (no infection)	3								3	<0.7	<0.7	<0.7	<0.7
Strong immunity (abortive infection)			1			1			2	<0.7	<0.7	<0.7	<0.7
Incomplete immunity (suppressed infection)		3	2	1	3	2			11	1.6	9.6	16.2	9.1
No immunity (active infection)				2			3	2	7	233.8	138.7	221.8	198.1

[a] Proviral burden is expressed as the number of provirus copies per 10^6 cells.
[b] Numbers in parentheses are the number of animals in each group. Adjuv., adjuvant.

TABLE 6. Terminal proviral load in tissues in relation to vaccine type

Tissue assayed	Terminal proviral load in animals vaccinated with[a]:							
	WIV (n = 3)	VV-WIV (n = 3)	Gag-Env (n = 3)	VV-Gag-Env (n = 3)	Env (n = 3)	VV-Env (n = 3)	Adjuv. control (n = 3)	VV-adjuv. control (n = 2)
PBMC	<0.7	1.1	2.8	ND	1.2	ND	35.2	432.3
Spleen	<0.7	2.4	8.3	ND	1.1	ND	62.8	215.1
Lymph node	<0.7	10.8	14.5	ND	18.5	ND	362.0	81.7
Composite mean	<0.7	4.8	8.5		6.9		153.3	243.0

[a] Proviral burden is expressed as the number of provirus copies per 10^6 cells. Adjuv., adjuvant; ND, not done.

caques. The latter 2 animals demonstrated the greatest degree of virus suppression among the 11 macaques classified in this response group. A gradient of apparent virus suppression-resistance as reflected in the frequency of PBMC virus isolation and the magnitude of provirus burden was evident among animals in the virus suppression-partial immunity response group (Tables 4 to 6). The overall composite mean provirus burden was 21.8-fold less than that in control animals (Table 5). However, in 4 of the 11 PBMC virus isolation-negative macaques in this group, virus was recovered by cocultivation of lymph node cells at death, consistent with the greater reservoir of provirus-bearing cells in this tissue compartment (Table 4). (iv) With active infection (no immunity), the response group was distinguished by persistently positive virus isolation, high proviral load in PBMC and lymphoid tissues (composite mean = 198.1 copies per 10^6 cells; range = 138.7 to 233.8 copies per 10^6 copies) (Table 5), and increasing postchallenge antibody titers (Tables 1 and 4). Active infection was present in all five control macaques (groups 7 and 8) and in two animals immunized with VV-Gag-Env (group 3).

In agreement with the previous observations of others (17, 19, 24, 26, 29), we found that SIV recombinant proteins elicited a strong antibody response. Somewhat in contrast to the studies of Mills and colleagues (24) and Giavedoni et al. (11), we found that while recombinant Env immunization produced incomplete immunity against viral challenge, vaccination clearly enhanced resistance to fulminant SIV infection. Differences among studies evaluating recombinant SIV Env vaccines probably reflect (by the use of a homologous, molecularly cloned versus an uncloned virus for challenge) the duration of the postchallenge observation period and the presence or absence of a quantitative assessment of proviral load.

Generation of SIV-neutralizing antibodies detected by syncytium inhibition (22) was concordant with the appearance of antibodies to Gag and Env detected by enzyme immunoassay or immunoblot but did not correlate with the strength of virus suppression observed. Neutralizing antibodies have been found to be the best correlate of protection in chimpanzees immunized with HIV subunit proteins (2, 9, 11) but have usually not been detected in macaques immunized with SIV subunit vaccines (18, 29). The relationship between syncytium-inhibiting antibodies and resistance to challenge in SIV-immunized macaques remains unclear (3, 4, 11, 13, 24, 25, 32–34).

In agreement with other investigators (1, 4, 23, 24, 27, 32), we found that in addition to antibodies directed against Gag and Env, sera from macaques immunized with WIV SIV contained antibodies reactive with human (HuT78) cells. We found no similar anticell reactivity in animals vaccinated with VV vectors and/or recombinant SIV proteins. These findings are consistent with the work of Stott, Cranage, and other investigators (1, 4, 6, 25, 32) who have demonstrated a role for immunity raised against heterologous cell antigens, probably HLA proteins (1), in the protection elicited by WIV vaccines.

Five of the six immunization regimens used in this study elicited lymphocyte proliferative responses to SIV antigens within 7 weeks. By 18 and 27 weeks postinitiation, however, antigen-driven lymphoproliferation was demonstrable in only one of three completely protected animals and one of two animals in which infection was aborted. In addition, we detected pre- and postimmunization proliferative responses in one of five control (VV-adjuvant) macaques. The cause of this response in this seronegative animal was not determined; however, it reduces the significance of the results obtained in virus antigen-immunized animals. Thus, whereas Shen et al. (30) reported SIV-specific T-helper cell immunity in VV-SIVmac-immunized monkeys, and Hu et al. (17), Mills et al. (24), and Voss et al. (35, 36) detected lymphoproliferative responses in all protected macaques, we were not able to discern a clear relationship between T-cell proliferative responses and resistance to SIV challenge in the present study.

Frequency of virus isolation, provirus burden, and postchallenge antibody titers were correlated. Declining antibody responses were detected in animals that remained virus isolation- and PCR-negative (e.g., the WIV group). Macaques that developed abortive or suppressed infections (the majority of the subunit-immunized animals) in general experienced a transient increase followed by a progressive decrease in antibody titer. By contrast, persistently viremic animals demonstrated increasing antibody titers, consistent with observations of other investigators which indicate that active SIV infection is manifested by strong and escalating antibody responses (16, 25, 26, 29, 31, 35).

Suppression of virus burden in animals vaccinated with SIV recombinant Env may be relevant to the evaluation of recombinant HIV vaccines. Historically, successful viral vaccines generally have not prevented initial virus replication. Thus, heightening of host resistance to produce persistent suppression of virus expression may also be a more realistic goal for lentivirus vaccines. Demonstration of degrees of resistance to SIV infection in vaccinated macaques may therefore be pertinent to studies employing analogous HIV vaccines in high-risk populations. Quantitative assessment of viral and proviral burden may permit recognition of a category of HIV-vaccinated people in which viral infection is suppressed, prognosis is better, and transmission rate is lower.

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