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Quintuple Deglycosylation Mutant of Simian Immunodeficiency Virus SIVmac239 in Rhesus Macaques: Robust Primary Replication, Tightly Contained Chronic Infection, and Elicitation of Potent Immunity against the Parental Wild-Type Strain

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We previously generated a mutant of simian immunodeficiency virus (SIV) lacking 5 of a total of 22 N-glycans in its external envelope protein gp120 with no impairment in viral replication capability and infectivity in tissue culture cells. Here, we infected rhesus macaques with this mutant and found that it also replicated robustly in the acute phase but was tightly, though not completely, contained in the chronic phase. Thus, a critical requirement for the N-glycans for the full extent of chronic infection was demonstrated. No evidence indicating reversion to a wild type was obtained during the observation period of more than 40 weeks. Monkeys infected with the mutant were found to tolerate a challenge infection with wild-type SIV very well. Analyses of host responses following challenge revealed no neutralizing antibodies against the challenge virus but strong secondary responses of cytotoxic T lymphocytes against multiple antigens, including Gag-Pol, Nef, and Env. Thus, the quintuple deglycosylation mutant appeared to represent a novel class of SIV live attenuated vaccine.

Apparently potent humoral and cytotoxic T lymphocyte (CTL) responses are elicited in humans following infection with human immunodeficiency virus type 1 (HIV-1) (3, 23). However, these responses can only partially control viral replication, allowing the establishment of a long-term persistent infection, in which vigorous viral replication and elimination of the virus by the host responses take place (7). Essentially the same feature is shared with a simian counterpart, simian immunodeficiency virus (SIV), and appears to be the background underlying the difficulty in developing HIV-1 vaccines of sufficient protective efficacy (9, 18). One possible factor contributing to viral ability to evade host responses and to cause persistent infection may be heavy glycosylation of the gp120 external envelope glycoproteins, because viral surface glycans are thought to help shield the potential epitopes from immune recognition. In addition, the glycans tend to protect the proteins from proteolysis, suggesting that heavily glycosylated proteins are less efficiently antigen-presented than those with fewer glycans. On the other hand, enveloped viruses causing acute infection, which is eradicable naturally or by vaccination, are generally sparsely glycosylated. For instance, there are 26 and 23 potential N-linked glycosylation sites in the gp120s of HIV-1 strain SF2 and SIV strain mac239, respectively, whose polypeptide backbones are 482 and 503 amino acids long, respectively (11, 16, 17, 22). On the other hand, measles virus (Edmonston strain), also targeting cells constituting the immune system but readily eradicable by immune responses, contains only 5 potential N-linked glycosylation sites on its 617-residue-long receptor-binding protein H (22).

In a series of mutagenesis experiments to silence the potential N-glycosylation sites of SIVmac239 individually and in combination, we found that 22 of the 23 potential sites are actually glycosylated and that 18 are dispensable for viral infectivity, while 4 are essential (22). Two of the dispensable glycans appeared to be a strong downmodulater of viral replication ability, because removal of either markedly enhanced viral infectivity and replication. The remaining 16 were regarded as neutral, because their removal neither increased nor decreased infectivity (22). There was a striking position specificity for these three functionally different glycans, because most of the neutral sites were mapped to the N-terminal half of gp120, while the downmodulating sites mapped to the C terminus and the essential sites mapped to the central portion. In addition, we were able to remove up to five glycans in combination (22). Thus, a panel of deglycosylation mutants are now available to define the role of each glycan as well as glycans in combination in the context of SIV replication and pathogenesis in vivo in rhesus monkeys (22). In this study, we have used a mutant with five glycans removed, here termed Δ5G, and show that it is fully capable of replication in the acute phase but greatly, albeit not completely, controlled in the subsequent

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chronic phase in rhesus macaques. This quintuple deglycosylation mutant was also found to behave as a live attenuated vaccine in macaques, conferring potent protective immunity against wild-type SIV.

MATERIALS AND METHODS

Viruses. SIVmac239 and its derivative Δ5G were used. The Δ5G variant was created by mutagenesis of the parental infectious DNA clone so that the asparagine residues for N-glycosylation at positions 79, 146, 171, 460, and 479 were converted to glutamine residues (22). Actual removal of the five glycans was biochemically confirmed (22). The stocks of SIVmac239 and Δ5G were obtained by DNA transfection of the respective infectious DNAs into COS1 or SW480 cells. For monkey infection studies, these virus stocks were propagated in phytohemagglutinin-stimulated peripheral blood mononuclear cells (PBMCs) from rhesus monkeys and their titers (50% tissue culture-infective doses [$TCID_{50}$]) were determined utilizing herpes saimiri virus-transformed cynomolgus $CD4^+$ T cells (CyfT/HSV) (21).

SIV infection of rhesus macaque PBMCs. PBMCs (5×10^6) were stimulated with 5 ml of 1-μg/ml phytohemagglutinin in RPMI medium supplemented with 10% fetal calf serum, penicillin (50 U/ml), and streptomycin (50 μg/ml) (R10 medium) for 2 days. PBMCs were infected with Δ5G or SIVmac239 virus stock containing 5 ng of p27 Gag antigen prepared by DNA transfection of SW480 cells. PBMCs were maintained in R10 medium supplemented with human interleukin-2 (100 U/ml). Culture supernatant collected every 3 to 4 days was subjected to the SIV p27 Gag antigen assay (Coulter, Tokyo, Japan).

Animal infection studies. Juvenile rhesus macaques seronegative for SIV, simian T-lymphotropic virus, B virus, and type D retroviruses were used. All animals were housed in individual cages and maintained according to the rules and guidelines for experimental animal welfare of our institution. Infection was initiated intravenously with 100 or 500 $TCID_{50}$ of the viruses.

Determination of plasma viral load by real-time PCR. Viral RNA was isolated from plasma with a commercial viral RNA isolation kit (Boehringer Mannheim, Tokyo, Japan). SIV *gag* RNA was amplified and quantified with a commercial RNA reverse transcription-PCR kit (TaqMan EZ RT-PCR; PE Applied Biosystems, Urayasu, Japan) with the *gag* forward primer 1224F (5′-AATGCAGAGC CCCAAGAAGAC-3′) and reverse primer 1326R (5′-GGACCAAGGCCTAA AAAACCC-3′) and TaqMan probe 1272T (FAM-5′-ACCATGTTATGGCCA AATGCCCAGAC-3′-TAMRA). Purified viral RNA (10 μl) was reverse transcribed and amplified in a MicroAmp optical 96-well reaction plate (PE Applied Biosystems) according to the manufacturer's instructions and the following thermal cycle conditions: one cycle of three sequential incubations (50°C for 2 min, 60°C for 30 min, and 95°C for 5 min) and 50 cycles of amplification (95°C for 5 s and 62°C for 30 s) was performed in a 7700 Prism sequence detection system (PE Applied Biosystems). In vitro RNA transcripts were quantified by optical density at 260 nm (OD_{260}) measurement and b-DNA assay for SIV viral RNA (Bayer Diagnostics, Tarrytown, NY). RNA equivalent to 10^7 to 10 copies per reaction was used as a standard for each assay. The detection sensitivity of plasma viral RNA by this method was 1,000 copies/ml.

Quantification of viral DNA in PBMCs by serial dilution and nested PCR and their sequencing. PBMCs were isolated from citrate-treated blood by standard Ficoll-Hypaque gradient centrifugation. Cellular DNA was extracted from 10^6 cells and dissolved in 200 μl of elution buffer with a commercial DNA purification kit (Qiagen, Tokyo, Japan). Serial 10-fold diluted DNA (5 or 10 μl) was subjected to nested PCR with the Ex-Taq PCR kit (Takara, Tokyo, Japan). The first PCR was done with the first primer pair env-1F (nucleotides 6537 to 6559) (5′-CACGAAAGAGAAGAAGAACTCCG-3′) and env-1R (nucleotides 7324 to 7304) (5′-GCAAAGCATAACCTGGAGGTG-3′). The numbering is for SIVmac239 (24). The *env* sequence encompassing the V1 to V2 region, where two deglycosylation mutations, at amino acids (aa) 146 and 171, reside, was amplified with the second primer pair, F6875 (nt 6875 to 6863) (5′-GGCAACT CTTTGAGACCTC-3′) and B7164 (nt 7164 to 7140) (5′-CCAAGTTTCATTG TACTCTTTTTTC-3′). This technique detects 1 copy per 50,000 cells. The number of PBMCs was determined with a blood cell counter (Sysmex, Kobe, Japan). PCR-amplified DNA was detected by regular agarose gel electrophoresis. The cloned *env* DNAs were sequenced to define whether the viral DNA was derived from the Δ5G virus or the challenge virus SIVmac239.

Determination of viral load in PBMCs by coculture with monkey $CD4^+$ T cells. Fourfold serial dilutions of PBMCs (starting at 10^6 in 0.5 ml) obtained from the infected monkeys were added to 2×10^5 CyfT/HSV cells (in 0.5 ml) in duplicate in 24-well plates and cultured for 4 weeks. After 3 to 4 weeks, the wells were scored for the development of cytopathic effect due to SIV infection. Although CyfT/HSV cells are very sensitive to cytopathic effect, SIV infection was confirmed by the presence of p27 Gag antigen in culture supernatants with a commercial SIV p27 assay kit (Coulter, Tokyo, Japan).

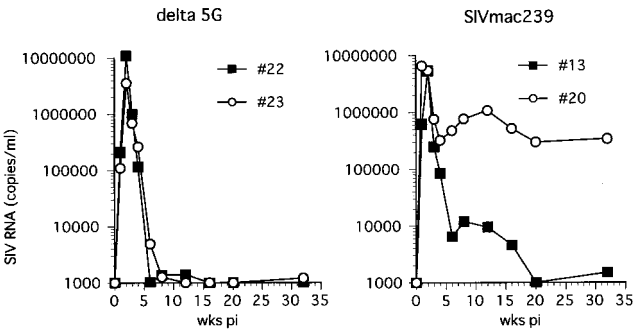

FIG. 1. Replication of the Δ5G deglycosylation mutant and the parent SIVmac239 in rhesus macaques. At various weeks (wks) times after intravenous injection of 500 $TCID_{50}$ of each virus, viral loads in plasma were determined.

Anti-SIV ELISA. A 1:100 dilution of each plasma sample in phosphate-buffered saline (pH 7.4) containing a blocking reagent (Dainippon Seiyaku, Osaka, Japan) was assayed for SIV-specific antibody using a standard enzyme-linked immunosorbent assay. (ELISA) technique in 96-well plates coated with SIVmac239 virion lysate. The OD_{492} was recorded and used as a relative measure of antibody titer.

Neutralization assay. The method originally described by Means et al. (19) was employed for neutralization. Heat-inactivated (56°C for 30 min) plasma was serially twofold diluted and tested for inhibition of SIV infection in CEMx174 cells harboring the SIV long terminal repeat (LTR)-driven secreted alkaline phosphatase (SEAP) reporter gene (CEMx174 SEAP cells). Diluted plasma (25 μl) was incubated with SIV (100 μl) in a 96-well plate at room temperature for 1 h. CEMx174 SEAP cells (20,000 cells) were added to the mixture and incubated for 3 days (SIVmac239 infection) or 5 days (Δ5G infection). SEAP activity in the culture supernatant was assayed using the Tropix Phospha-Light assay kit (PE Applied Biosystems). Chemiluminescence was measured with a Wallac Microbeta plate reader (Amersham Pharmacia Biotech, Tokyo, Japan).

CTL assay. The CTL assay method used was previously described (29). PBMC samples stored at −150°C were thawed and cultured in RPMI medium with concanavalin A (5 μg/ml) at 10^6 cells per ml for 3 days, washed, and then maintained for another 3 days in medium supplemented with human interleukin-2 (2 ng/ml). Target cells, autologous herpesvirus papio-transformed B-LCL cells, were infected with recombinant vaccinia virus (VV) expressing the SIV proteins SIVmac251 Gag-Pol, SIVmac239 Nef, SIVmac239 Env, or Δ5G Env or the parental VV (NYCBH strain) at 37°C for 16 h. They were labeled with ^{51}Cr and then incubated with effector cells at 37°C for 5 h. Specific lysis was calculated as percent SIV-specific lysis minus percent lysis of control VV (NYCBH)-infected target cells.

RESULTS

Viral loads following Δ5G infection. The mutant Δ5G lacks specific sites for N glycosylation at the asparagine residues at amino acid positions 79, 146, 171, 460, and 479. The first three are neutral, and the last two downmodulate viral infectivity in cell culture (22). The Δ5G mutant replicated as efficiently as parental SIVmac239 in T-cell lines. Both the mutant and wild-type viruses were propagated in rhesus monkey PBMCs. They replicated with similar kinetics and reached comparable peak titers, 45 and 30 ng/ml, respectively, of p27 antigen on day 21. We then intravenously infected two rhesus macaques with 500 $TCID_{50}$ of the Δ5G virus and another two with the same dose of the parental SIVmac239 and determined viral RNA levels in the plasma. SIVmac239 and Δ5G were both found to replicate vigorously in the early acute phase, the viremia peaking at 2 weeks postinfection (p.i.) with comparably high titers (Fig. 1). Thus, Δ5G did not appear to be attenuated at all, at least in

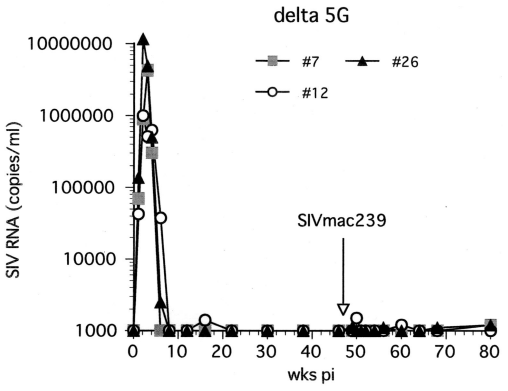

FIG. 2. Challenge infection of Δ5G-infected monkeys with wild-type SIVmac239. Three animals (7, 12, and 26) were initially infected with 100 $TCID_{50}$ of Δ5G. After 48 weeks, they were challenged with SIVmac239 at an input dose of 500 $TCID_{50}$. Plasma viral loads were determined throughout the period of the initial infection and the challenge infection. The time of challenge infection is indicated by an arrow.

this early stage of infection. At 3 weeks p.i., the viral loads began to decrease sharply in both cases, but dramatic differences in the set point and subsequent viral load in the chronic phase were seen. In both monkeys infected with Δ5G, the set points were mostly below the level of detection (1,000 copies/ml) and low loads or loads just above the detection level persisted for up to 32 weeks p.i. Compared with these, both the set points of wild-type infection and the chronic viral loads were remarkably higher for monkeys infected with SIVmac239, except that one of the monkeys displayed a low viral load at 20 weeks p.i.

The replication patterns suggested the possibility that Δ5G totally lacked the ability to replicate in the chronic phase or was totally controlled by host immune responses. To address this issue, we attempted to isolate the virus from PBMCs of monkeys 22 and 23. At 8 weeks p.i., 40,000 and 8,000 PBMCs were required to isolate the virus from the controls, monkeys 13 and 20, respectively. These values were well within the range usually seen for SIVmac239 infection (13). In contrast, no virus could be isolated even from 2,000,000 PBMCs from either of the Δ5G-infected monkeys 22 and 23, suggesting virtually complete virus clearance at this stage (8 weeks p.i.). However, we did recover the viruses at 16 weeks from 2,000,000 (monkey 22) and 1,000,000 (monkey 23) PBMCs. Sequencing of the *env* genes from the recovered virus, encoding the V1 to V2 region, unequivocally demonstrated that the recovered viruses were Δ5G and not revertants. Thus, we concluded that Δ5G persisted, albeit greatly limited in replication, without being cleared from the bodies.

Taken together, these results demonstrate that one or more of the five N-glycans play a luxury function required to establish and maintain a full chronic infection but are totally dispensable for primary acute infection.

Challenge infection of Δ5G-infected monkeys with wild-type SIVmac239. As shown in Fig. 2, another set of thee monkeys (7, 12, and 26), which received 100 $TCID_{50}$ of Δ5G, displayed essentially the same replication pattern as seen for the two Δ5G-infected animals shown in Fig. 1. Probably because of the outbred nature of the monkey populations used, we sometimes observed a Δ5G-type replication pattern in about 30% of the monkeys even following infection with SIVmac239 (1). However, it has to be emphasized that with the wild type we have never experienced such low levels of viremia in all five arbitrarily chosen monkeys as seen for the Δ5G mutant. The above three Δ5G-infected animals (7, 12, and 26) were challenged with 500 $TCID_{50}$ of SIVmac239 at 48 weeks after the initial Δ5G infection and then assayed for plasma viral loads. Remarkably, no viremia was detected during the observation period starting at 2 weeks following challenge for two (7 and 26) of the monkeys, and only transient viremia of a marginal level for the remaining one was found at 2 weeks (Fig. 2). Serving as controls in this challenge experiment were the two naive monkeys (13 and 20), which were described above and are shown in Fig. 1. A comparison with these naive cases (Fig. 1) indicated that the immunity induced by Δ5G was potent enough to contain the viral load in the acute phase from as much as about 10,000,000 to 1,000 copies/ml or less.

TABLE 1. Detection frequency of Δ5G and SIVmac239 in PBMCs from monkeys 7, 12, and 26 after challenge infection with SIVmac239

Wk postchallenge	No. of cloned viral DNAs/5 × 10^5 PBMCs in monkey:					
	7		12		26	
	Δ5G	mac239	Δ5G	mac239	Δ5G	mac239
−2	3	0	5	0	2	0
2	1	0	4	3	0	0
4	3	0	0	10	0	0
6	1	0	5	1	1	0
16	2	0	1	0	1	0
20	3	0	3	0	1	0

PBMCs were obtained from the monkeys 2 weeks before and at various times after the challenge. They were dispensed into 10 tubes so that each tube contained 50,000 cells, and the viral DNA encoding the V1 to V2 region was amplified. When successfully amplified, the DNAs were sequenced to determine whether they were of Δ5G or wild-type origin. As shown in Table 1, proviral sequences could be amplified in 3, 5, and 2 of the 10 tubes for monkeys 7, 12, and 26, respectively, which altogether contained 500,000 PBMCs in total, 2 weeks before challenge. Without exception, the mutations introduced to remove the glycans were retained, and no additional mutations were found in any of the amplified sequences. These data indicated that Δ5G virus persisted over 46 weeks in all three monkeys after the initial infection, though at a very limited level. After the challenge, proviral sequences were almost consistently detected at frequencies similar to those before challenge in monkeys 7 and 26. Their sequences were again of Δ5G origin. Thus, protection against the wild type was apparently nearly complete in these two monkeys. However, the results did not rule out the possibility that a transient superinfection had taken place during the period from 0 to 2 weeks after challenge, when no attempt at virus isolation was made. Superinfection was not ruled out, either, at a level lower than was detectable by the methods employed (use of 500,000 PBMCs and detection level above 1,000 copies/ml). Indeed, superinfection was clearly present in monkey 12, as 3 of the 7 sequences at week 2, all 10 at week 4, and 1 of the 5 at week 6

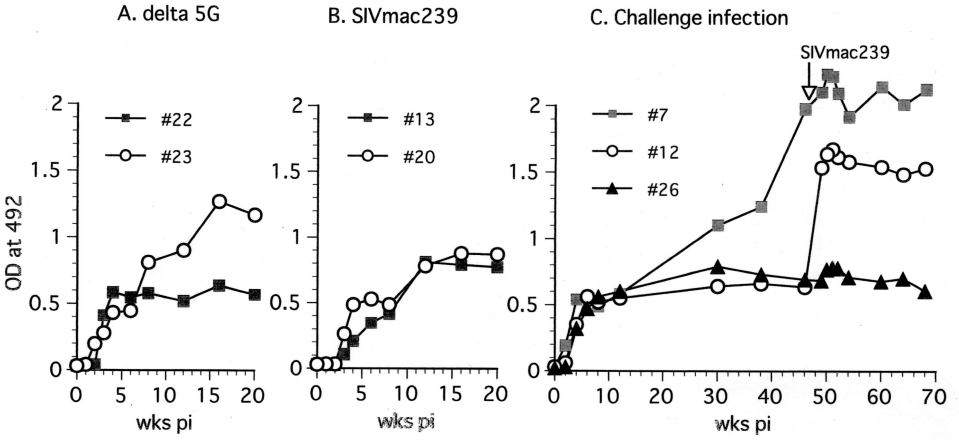

FIG. 3. Anti-SIV ELISA. The OD_{492} was used as a relative measure of antibody titer. (A) Δ5G infection. (B) SIVmac239 infection. (C) Before and various times after challenge infection of Δ5G-infected animals with SIVmac239 (arrow).

were of wild-type origin (Table 1). The superinfection appeared to be transient and controllable, as no wild-type clone became detectable by week 16. Taken together, these results suggested that the Δ5G mutant could confer on rhesus macaques not complete but remarkable protective immunity against SIVmac239.

Host responses following Δ5G infection. Humoral responses were compared between the Δ5G-infected (22 and 23) and wild-type-infected (13 and 20) monkeys by assaying binding ELISA antibody titers to wild-type SIVmac239 virion proteins and neutralizing antibody titers against both the Δ5G and wild-type viruses. As shown in Fig. 3A and B, no appreciable difference was seen between the two monkey groups in either the developmental sequence or the maximum titer of binding antibody.

We used essentially the same protocol for the neutralization assay as used in previous macaque infection studies with deglycosylated SIVs (19, 26), which involved infection of CEMx174 cells containing a SEAP gene under the control of a Tat-responsive LTR. As shown in Table 2, both of the Δ5G-infected monkeys displayed considerably high neutralization titers (100 to 400) to the homologous virus at 20 weeks. Neutralizing activity became detectable at 4 or 8 weeks p.i. in these animals (Table 2), before the time that set points were reached (Fig. 1). Sera from the same animals possessed no appreciable neutralization titer against the counterpart SIVmac239. Both of the SIVamc239-infected monkeys displayed low levels of neutralization against the homologous and heterologous strains for up to 20 weeks. These results may suggest that greater containment of Δ5G was due in part to better humoral response to the homologous virus.

We then compared CTL responses against Gag-Pol, Nef, and Env from both Δ5G and wild-type SIVmac239 between the monkeys infected with Δ5G (22 and 23) and those infected with the wild type (13 and 20). We found no temporal or quantitative difference in the CTL response patterns between the two monkey groups. However, slight increases (5 to 9%) in lysis were seen against Δ5G Env compared to wild-type Env when effector cells from both Δ5G-infected and wild-type-infected monkeys were used (data not shown).

Host responses following challenge infection. To find the immunological correlates of protection induced by Δ5G, ELISA antibody titers, neutralization antibody titers, and CTL responses were measured before and after challenge infection of Δ5G-infected monkeys 7, 12, and 26 with wild-type SIVmac239. As shown in Fig. 3C, upon challenge, ELISA titers immediately doubled in monkey 12, while no such vigorous secondary response was seen for either 7 or 26. These results were in agreement with the fact that transient superinfection with the wild type was clearly detectable in monkey 12 but not in the other two (Table 1). As shown in Table 3, neutralization antibodies against the homologous strain were present in two of the three Δ5G-infected monkeys at a relatively high (200,

TABLE 2. Neutralizing titers in rhesus macaques infected with SIVmac239 (13 and 20) or Δ5G (22 and 23) against homologous and heterologous viruses

Wks p.i.	Titer[a] in monkey:							
	13		20		22		23	
	mac239	Δ5G	mac239	Δ5G	mac239	Δ5G	mac239	Δ5G
Preinfection	<25	<25	<25	<25	<25	<25	<25	<25
1	<25	<25	<25	<25	<25	<25	<25	<25
2	<25	<25	<25	<25	<25	<25	<25	<25
4	<25	<25	<25	<25	<25	50	<25	<25
6	<25	<25	<25	<25	<25	50	<25	<25
8	<25	<25	<25	<25	<25	200	<25	25
20	50	25	<25	25	<25	400	50	100

[a] Reciprocal of the dilution of plasma giving 50% neutralization.

TABLE 3. Neutralizing titers against SIVmac239 and Δ5G in rhesus macaques 7, 12, and 26 infected with Δ5G and challenged with SIVmac239

Wk postchallenge	Titer[a] in monkey:					
	7		12		26	
	mac239	Δ5G	mac239	Δ5G	mac239	Δ5G
−2	<25	200	<25	25	<25	<25
1	<25	400	<25	50	<25	<25
2	<25	400	<25	50	<25	<25
4	<25	400	<25	25	<25	<25
6	<25	100	<25	25	<25	<25

[a] Reciprocal of dilution of plasma giving 50% inhibition of SIV replication.

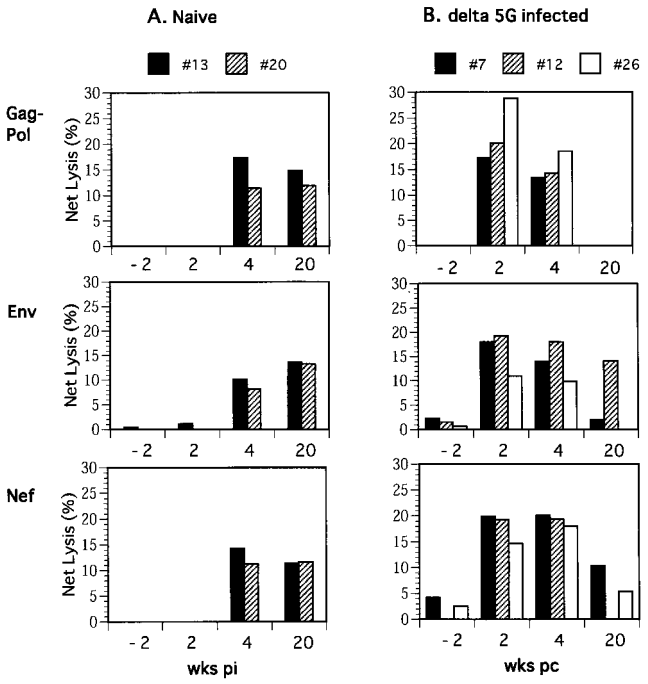

FIG. 4. CTL responses at various times after infection of naive monkeys with SIVmac239 (A) and at 2 weeks before and various times after challenge (pc) of Δ5G-infected monkeys with SIVmac. Wild-type Env was used.

monkey 7) or marginal (25, monkey 12) level and undetectable in monkey 26 2 weeks before challenge infection. The levels of neutralization were not much altered after SIVmac239 challenge in any monkey. On the other hand, neutralization activities against the challenge virus SIVmac239 were undetectable not only before but also after challenge for up to 6 weeks (Table 3). These results strongly suggested that the striking containment of the challenge virus was not due to neutralization antibodies.

Figure 4 compares the CTL responses against multiple antigens following challenge with SIVmac239 between naive and Δ5G-infected monkeys. In naive control animals 13 and 20, CTL against all these targets became detectable 4 weeks after challenge and persisted for 20 weeks (Fig. 4A). CTL activities were undetectable immediately before challenge for all three Δ5G-infected monkeys 7, 12, and 26 (Fig. 4B). However, it was remarkable that CTL became clearly detectable by 2 weeks after challenge in all of them, 2 weeks earlier than in control monkeys (Fig. 4B). Moreover, the titers were obviously higher in the former than in the latter, and remained so up to 4 weeks. These results strongly suggested that Δ5G had mounted sufficient memory to recruit vigorous secondary CTL responses against multiple antigens quickly upon challenge infection.

Superinfection is a prerequisite of immunological secondary responses. Thus, the strong CTL responses observed here for all three Δ5G-infected monkeys support the above-described notion that we have failed to detect actual superinfection for two of the monkeys (7 and 26). The CTL responses tended to decline rapidly in all three immune monkeys by 20 weeks (Fig. 4B), suggesting efficient control of the superinfecting viruses before 20 weeks.

DISCUSSION

Our deglycosylated mutant-Δ5G retained full replication ability in rhesus macaques in the primary acute phase, but its set point and subsequent replication in the chronic period were greatly, though not completely, contained (Fig. 1 and 2). Kimata et al. compared the phenotypes in macaques and the Env sequences of a pair of strains, SIVMneCL8 and its derivative, SIVMne35wkSU (4, 14, 27). The former was found to be strongly contained, just like our Δ5G, in the chronic phase, whereas the latter was as active as SIVmac239. The former raised appreciable neutralization titers against the homologous strain, whereas the latter failed to do so. These phenotypic differences were closely related to the absence (former) or presence (latter) of glycans in the V1 region. Thus, this pair of strains are closely similar to our pair, Δ5G and SIVmac239. Reitter et al. created a series of mutants with two glycans deleted in the V1 region and between the V1 and V2 regions (25, 26). These mutants were also attenuated in the chronic phase and induced extremely high titers of neutralization antibodies. Probably because of such high titers, the antibodies were able to neutralize not only the homologous deglycosylated mutants but also the fully glycosylated wild type. Moreover, when they reverted to greater virulence during the infection, they always mutated to restore the glycans (26). Selection of neutralization resistance of a simian-human chimeric virus in vivo was found to occur by acquisition of N glycans in V1 and V3 (5). Thus, it now appears to be firmly established that SIV and HIV strains with fewer glycans are better controlled and elicit better humoral response.

As noted above, acquisition of N-glycans and reversion to a more pathogenic phenotype were frequently seen for the mutants with one or two glycans deleted in the region encompassing the V1 to V2 region (26). This was not seen for Δ5G during the entire observation period. This stability was attributable to mutation at as many as five sites. Reitter et al. also generated a mutant with five potential N-glycosylation sites silenced (25, 26). According to our criteria, four of the sites are neutral, little affecting replication ability, if silenced individually, while the remaining one did not appear to be actually glycosylated (22). Significant attenuation of this mutant at acute phase (a reduction in the peak titer of 10- to 100-fold) (26) suggests that simultaneous deletion of four neutral sites somehow impairs replication in vivo. Robust replication in acute phase of Δ5G may be related to the fact that two (460 and 479) of the sites deleted are downmodulating, in that removal of either greatly enhances infectivity (22).

Strikingly low set points and strong containment in the chronic phase were the characteristics shared by these multiple deglycosylation mutants. The better humoral responses to deglycosylated mutants may be responsible. This view may be supported by detection of neutralizing antibodies to Δ5G before set points were reached. In another case, however, the set point appeared to be reached well before neutralization antibodies became detectable (26), suggesting other responsible factors. Specific CTLs and helper T cells could be significantly involved, as was seen in controlling wild-type virus replication (2, 3, 21). However, as noted above, we observed little difference in CTL response patterns between Δ5G- and wild-type-infected monkeys. Alternatively, the apparently better contain-

ment of deglycosylated mutants might have little to do with host responses but could be caused by some impairment of functional and structural integrity of Env due to removal of multiple glycans.

The Δ5G mutant was regarded as a novel class of live attenuated vaccine because it was able to elicit not complete but remarkably potent protective immunity in rhesus macaques against wild-type SIVmac239. The protection appeared to correlate with strong secondary CTL responses in the absence of neutralizing antibodies, joining a list of accumulating data that indicate a major role for CTL as an immunological correlate of protection by live SIV and HIV vaccines. In view of these previous data (8, 20, 28; for a review, see reference 12), CTL responses not to Env but to multiple other antigens, including Gag-Pol and Nef, could be important for the good protective immunity elicited by Δ5G.

Without doubt, T-cell precursor frequencies depend on the initial viral load or burst size. They may be maintained at fairly stable levels in an antigen-independent manner (10, 15). However, more recent reports provided evidence suggesting that a low level of viral persistence is critical for maintaining the functional status of immune memory cells even in mice acutely infected with and then rendered immune to certain viruses, such as lymphocytic choriomeningitis virus (6 and references therein). Thus, infection of monkeys with Δ5G is closely similar to lymphocytic choriomeningitis virus persistence in immune mice, suggesting that the robust primary replication followed by a marginal level of persistent infection in the chronic phase underlie the strong secondary CTL responses against multiple SIV antigens.

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