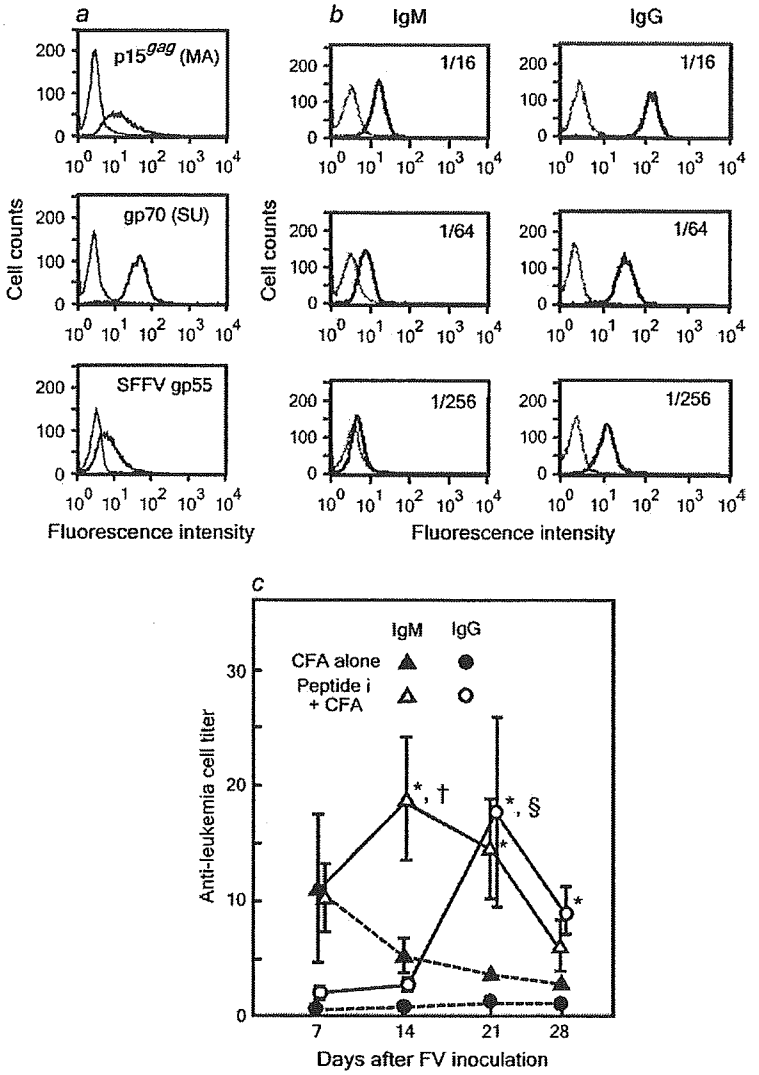

Fig. 6. Detection of serum antibody reactive to the surfaces of FV-induced leukemia cells in FV-infected $CB6F_1$ mice. (a) Expression of FV antigens on the surfaces of Y57-2C cells used as the indicators for FACS analyses. Thick lines represent the binding of each mAb reactive to the indicated FV gene product, while thin lines represent the binding of each isotype-matched control antibody. (b) Representative patterns showing the binding of serum IgM and IgG onto Y57-2C cells. A serum sample obtained from a peptide-immunized $CB6F_1$ mouse at PID 21 was diluted serially and incubated with Y57-2C cells. FACS patterns of IgM and IgG binding at the indicated serum dilution are shown, with thin lines representing the binding of the pooled control serum prepared from uninfected $CB6F_1$ mice at the same indicated dilution. Similar results were obtained when $H2^{a/a}$ AA-41 leukemia cells were used as indicator cells. (c) Changes in the titers of IgM and IgG anti-leukemia cell antibodies detectable in the sera of $CB6F_1$ mice after FV infection. $CB6F_1$ mice were either immunized once with 10 μg per mouse peptide i in CFA or given CFA alone and challenged with 150 SFFU FV 4 weeks later. Each data point shows mean ± SEM calculated by using five to six mice per group. *, titers in the immunized mice are significantly higher than those in unimmunized mice at $P < 0.05$; †, the IgM titer is significantly higher than IgG titer at $P < 0.05$; §, the average IgG titer at PID 21 is significantly higher than that at PID 14, $P < 0.01$.

serum anti-leukemia cell antibodies were detectable at as early as PID 7, but average titers of these antibodies decreased in the following 3 weeks of infection, and no class switching to IgG was observed in the unimmunized animals (Fig. 6c). In contrast, anti-leukemia cell IgM titers were significantly higher in the peptide-immunized than in the unimmunized control mice at PID 14, and IgG class of anti-leukemia cell antibodies were detectable at PID 21 and 28 in the peptide-immunized animals.

Role of CD8⁺ T cells in the induction of Ig class switching of virus-neutralizing and anti-leukemia cell antibodies

Kinetics of the production and class switching of virus-neutralizing and anti-leukemia cell antibodies in the genetically

modified animals were analyzed between PID 7 and 28. Serum titers of FV-neutralizing IgM and IgG in unimmunized $\beta_2m^{-/-}$ mice were not significantly different from those in the unimmunized wild-type mice (compare Figs 1d and 7a). As in the case of peptide-immunized wild-type mice, production of virus-neutralizing IgM was detected at PID 14 in the serum of peptide-immunized $\beta_2m^{-/-}$ mice genetically lacking $CD8^+$ T cells. However, in contrast to the peptide-immunized wild-type mice, neutralizing IgG titers in the peptide-immunized $\beta_2m^{-/-}$ animals were not significantly higher than their IgM titers even at PID 28, suggesting some roles of $CD8^+$ T cells in facilitating class switching of virus-neutralizing antibodies in FV-infected mice. The role of $CD8^+$ T cells in the induction of IgG class virus-neutralizing antibodies was further confirmed by transferring purified $CD8^+$ T cells from peptide-immunized to unimmunized mice. Unimmunized mice did not possess detectable levels of virus-neutralizing antibodies in their serum at PID 10, and the antibodies were IgM-dominant at PID 20 (Fig. 7b), confirming the results of the kinetic analyses shown in Fig. 1(d). As expected, the recipients of $CD4^+$ T cells from the peptide-immunized and FV-infected mice showed the production of neutralizing IgG at PID 20. Interestingly, the recipients of highly purified $CD8^+$ T cells from peptide-immunized and challenged mice also showed the production of virus-neutralizing IgG, the level of which was comparable to that in the recipients of the $CD4^+$ T cell transfer. As controls, transfer of purified $CD4^+$ or $CD8^+$ T cells from unimmunized control mice into FV-infected $CB6F_1$ mice did not induce significant class switching of virus-neutralizing antibodies even at PID 20 (data not shown). No neutralizing antibodies were detectable in FV-infected *μMT/μMT* mice regardless of whether they were immunized with peptide i or not.

When anti-leukemia cell antibodies in the sera were tested, unimmunized $\beta_2m^{-/-}$ mice possessed anti-leukemia cell IgM at PID 7 and their titers decreased toward PID 14 as observed in the umimmunized wild-type mice (Fig. 7c). High titers of anti-leukemia cell IgM were also detectable in the peptide-immunized $\beta_2m^{-/-}$ mice at PID 7; however, in contrast to the peptide-immunized wild-type mice, $\beta_2m^{-/-}$ mice did not show a significant increase in the IgG titers between PID 14 and 21, confirming inefficient class switching of both neutralizing and anti-leukemia cell antibodies in the $\beta_2m^{-/-}$ animals.

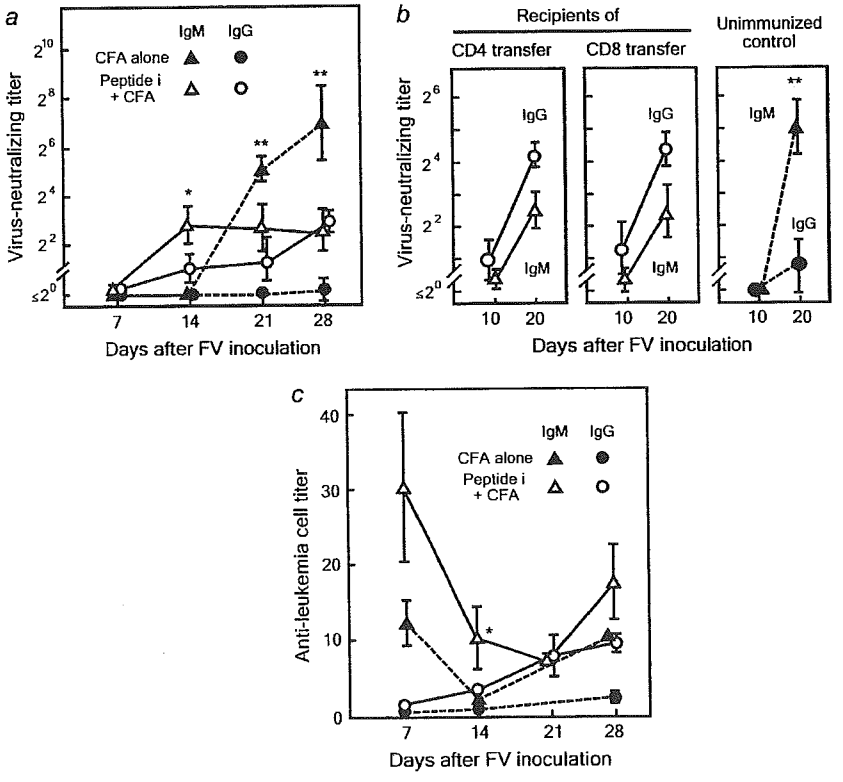

Fig. 7. Titers of virus-neutralizing and anti-leukemia cell IgM and IgG antibodies in sera from the $CD8^+$ T cell-deficient $CB6F_1$ mice and the effect of immune T cell transfer on the production of virus-neutralizing antibody. (a) $CB6F_1$-$\beta 2m^{-/-}$ mice were either immunized with peptide i or given CFA alone and challenged with FV as described. Sera were collected at the indicated time points and their neutralizing IgM and IgG titers were determined. Each data shows mean ± SEM calculated from five to nine serum samples, with statistically significant differences between the paired IgG and IgM titers indicated with $*P < 0.03$; $**P < 0.001$. (b) $CD4^+$ and $CD8^+$ T cells were purified from the spleen of peptide-immunized $CB6F_1$ mice at PID 7 and separately transferred into unimmunized control mice at PID 7. Sera were collected 3 (PID 10) and 13 days (PID 20) after cell transfer. Data shown here are mean ± SEM calculated from 4 to 5 serum samples at each time point. Differences between neutralizing IgM and IgG titers were compared by paired *t*-test: $**P < 0.005$. (c) Changes in the titers of IgM and IgG anti-leukemia cell antibodies detectable in the sera after FV infection. Each data point shows mean ± SEM calculated by using four to seven mice per group. *, the indicated titer in the immunized mice is significantly higher than that in unimmunized mice at $P < 0.05$.

Discussion

In the present study, we attempted to unravel the different roles of $CD8^+$ T and B cells in peptide-induced immune protection against FV-induced disease development by using genetically modified animals of the highly susceptible $CB6F_1$ background. $CB6F_1$ mice immunized only once with the peptide that contained a single $CD4^+$ T cell epitope were protected against fatal FV disease, and the development of early erythroid cell proliferation was prevented by the peptide immunization. The minimum sequence of the peptide required for *in vivo* protection against FV challenge, VYSQFEKSYRHKR, was the same as that required for $CD4^+$ T cell stimulation *in vitro*, indicating a close correlation between the peptide's ability to stimulate $CD4^+$ T cells and its efficacy in inducing protective immunity against FV-induced disease development. The requirement of $CD4^+$ T cells for the peptide-induced immune protection against FV infection was further confirmed by the lack of protection in the vaccinated animals after antibody-induced depletion of $CD4^+$ T cells.

In contrast to the bursting of TER-119^+ erythroid cells in the spleen of the unimmunized mice starting from PID 7, the reduction in the number of erythroid cells was observed between PID 5 and 7 in the peptide-immunized $CB6F_1$ mice, before virus-neutralizing antibodies became detectable in the serum (Fig. 1), and the numbers of spleen and bone marrow infectious centers were significantly smaller in the immunized than in the unimmunized control mice at PID 8 (Fig. 4). Although IgM antibodies reactive to the surfaces of FV-induced leukemia cells were detectable at as early as PID 7 (Figs 6 and 7), these antibodies are unlikely to play major roles in the observed suppression of the early growth of FV-infected cells in the vaccinated animals because anti-leukemia cell titers were not significantly different between the immunized and unimmunized groups at PID 7 (Fig. 6). This notion is consistent with the significantly reduced numbers, in comparison with those in unimmunized animals, of infectious centers in the spleen and bone marrow of the vaccinated mice at PID 8 even in the absence of B cells (Fig. 4c and f), and suggests the possibility that cellular immune responses, rather than antibodies, are involved in the regulation of SFFV-induced erythroid cell proliferation. In this regard, significantly larger populations of both $CD4^+$ and $CD8^+$ T cells were activated in the peptide-immunized, B cell-deficient mice in comparison with those in the unimmunized control mice at PID 7 (Fig. 5), and cytotoxic effector functions exerted by $CD4^+$ and $CD8^+$ T cells and those exerted more efficiently by NK cells have been demonstrated at as early as PID 7 in FV-infected $CB6F_1$ mice (14). These non-B effector cells might be involved in the control of SFFV-induced erythroid cell proliferation through direct killing of infected target cells. On the other hand, the numbers of virus-producing cells detected after PID 14 in the spleen and bone marrow were not different between the immunized and unimmunized groups of the B cell-deficient mice (Fig. 4c and f). This is again consistent with the detection of significantly higher titers of virus-neutralizing and anti-leukemia cell antibodies in the serum of vaccinated than in the unimmunized wild-type and $\beta_2m^{-/-}$ animals starting from PID 14 (Figs 1d, 6 and 7). These data suggest that the observed elimination of FV-producing cells in the peptide-immunized mice after PID 14 may depend mainly on the production of antibodies. Thus, early prevention of erythroid cell proliferation apparently depends mainly on cellular immune responses, but humoral responses seem to play crucial roles in the elimination of virus-producing cells in the later stage.

As to the relationship and relative importance between virus-neutralizing and anti-leukemia cell antibodies, tempos of the production and class switching of these antibodies after PID 14 were similar in the vaccinated wild-type animals (Figs 1d and 6c). However, in unimmunized animals, significant production of virus-neutralizing IgM was detectable after PID 21 when anti-leukemia cell titers were low and diminishing. Similarly, paradoxical production of virus-neutralizing antibodies in the presence of low anti-leukemia cell antibody titers has been observed in $H2^{a/b}$ (A.BY × A/WySn)F_1 mice at 20 days after FV infection (Miyazawa, M., Ishihara, C., and Takei, Y. A., unpublished results). It should be noted that although the anti-leukemia cell IgM titers at PID 21 and 28 were low, mean fluorescence intensities were four to five times higher than that obtained with the control serum, which reflects significant shifting of the peaks of fluorescence (Fig. 6b). Thus, it is conceivable that only a small proportion of serum antibodies detectable as anti-leukemia cell antibodies exhibit virus-neutralizing capability especially in the early stage of FV infection, and the proportion of neutralizing antibodies among anti-FV antibodies increases in the later stage. This interpretation also suggests that the production of antibodies reactive to virus-neutralizing epitopes, but not just any anti-FV antibody, depends on T cells. Further studies are required to elucidate the molecular and epitope specificities of neutralizing and anti-leukemia cell antibodies. It can be pointed out that the presence of virus-neutralizing IgM at PID 21 is not effective to reduce the number of FV-infected cells as clearly shown in the case of unimmunized, wild-type $CB6F_1$ mice (Figs 1d and 4). Further, class switching to IgG of virus-neutralizing and anti-leukemia cell antibodies may not be a requisite because the number of FV infectious centers were reduced in the absence of efficient class switching in the peptide-immunized $\beta_2m^{-/-}$ mice (Figs 4 and 7). Thus, the presence of virus-neutralizing and/or anti-leukemia cell IgM at around PID 14 might be crucial in preventing the spread of FV infection to a large enough number of target cells to support progressive infection.

Using partially FV-resistant (B10.A × A.BY)F_1 mice and an N-tropic F-MuLV as an attenuated vaccine, Dittmer, Brooks, and Hasenkrug (11) dissected different roles of the immune cell components in protection against FV-induced disease development. Their demonstration of the effectiveness in inducing the recovery from initial splenomegaly of cell transfer from vaccinated to naive animals after depletion of immune $CD8^+$ T cells is in agreement with our results showing the peptide-induced reduction of FV-producing cells in the spleen and bone marrow in the absence of $CD8^+$ T cells (Fig. 4). However, the development of splenomegaly was prevented only when the whole spleen cells or all three sub-populations ($CD4^+$, $CD8^+$ and $CD19^+$) of lymphocytes were transferred from the vaccinated to naive animals in the above live vaccine experiments, while in the present study the development of early splenomegaly was prevented in >70% of the $CB6F_1$-$\beta_2m^{-/-}$ mice after immunization with peptide i. This apparent discrepancy may be due to the difference in challenge dose of

FV (10 000 versus 150 SFFU), or might also be explained by the exaggerated and earlier production of IFN-γ from $CD4^+$ T cells and persistent activation of NK cells in $\beta_2m^{-/-}$ mice in comparison with those in their wild-type counterparts (30), as discussed below.

Dittmer, Brooks, and Hasenkrug (11) also showed that passive immunization with a virus-neutralizing mAb prior to FV challenge resulted in a significant reduction in the number of virus-producing cells at PID 10 and recovery from the initial development of splenomegaly, although 2.9×10^6 infectious centers on average were still detectable in the spleen. This result is partly consistent with our demonstration that B cells are required for the elimination of virus-producing cells from the spleen and bone marrow, especially after PID 14. However, the reported lack of protection after the transfer of B cells from vaccinated to naive mice contrasts with our demonstration of the crucial role of B cells. As they discussed later (12), transferred B cells might not have produced a sufficient level of FV-reactive antibodies until they were re-stimulated upon FV challenge of the recipients. In this regard, the production of virus-neutralizing antibodies in FV-infected mice is dependent on $CD4^+$ T cells (31). Further, we have shown in our previous (13, 17) and the present experiments that peptide-induced priming of $CD4^+$ T cells facilitates both production and class switching of virus-neutralizing and anti-leukemia cell antibodies upon FV infection. Thus, the lack of protection by the transfer of purified B cells alone from vaccinated to naive mice does not necessarily contradict our demonstration of the requirement of B cells for peptide-induced immune protection, especially because the titers of anti-FV antibody had not been determined in the above-reported B cell-transferred animals. The same authors (32) have shown that the presence of virus-neutralizing antibodies at the time of infection is crucial for a vaccine-induced protection of naturally resistant C57BL/6 mice against FV infection.

The observed lack of protection in the *μMT/μMT* mice might also be caused by the lack of or inefficient priming and/or re-activation of $CD4^+$ T cells due to the absence of B cells as APC. Although successful priming of $CD4^+$ and $CD8^+$ T cells with protein as well as cellular antigens and effective induction of CTL responses have been reported in the *μMT/μMT* mice (33, 34), the effect of the homozygous *μMT* mutation on T cell priming can be variable (35). However, since we were using as immunogen the 18-mer peptide which has been shown to directly bind onto the MHC class II $E^{b/d}$ molecule (23), the uptake and processing of the given antigen by B cells were not required. In such cases where already processed peptide is used as an immunogen, B cells are regarded as unnecessary for the priming of T cells (35). In fact, $CD4^+$ cells purified from the peptide-immunized *μMT/μMT* mice showed potent proliferative responses upon re-stimulation with peptide i, and larger numbers of $CD4^+$ T cells were activated upon FV infection in the peptide-immunized than in the unimmunized B cell-deficient mice (Fig. 6). Thus, the *μMT/μMT* mice were not protected most conceivably because they lacked antibody production.

In previous reports, $CD8^+$ T cells have always been associated with spontaneous and vaccine-induced immune resistance against FV infection: antibody-induced depletion of $CD8^+$ T cells abrogated spontaneous recovery from FV-induced splenomegaly in highly resistant (C57BL/10 × A.BY)F_1 mice (9), and recovery from splenomegaly was induced by transferring purified $CD8^+$ T cells from (B10.A × A.BY)F_1 mice previously vaccinated with the live N-tropic F-MuLV into naive animals (11). These results apparently contradict the present results showing successful protection of the $\beta_2m^{-/-}$ mice with the peptide vaccine. However, in the case of spontaneous recovery observed in the strain (C57BL/10 × A.BY)F_1, $CD4^+$ T cells were not primed prior to FV infection, and mice were infected with 1500 SFFU of B-tropic FV, a ten times higher dose than we used in the present study. Thus, infection-induced priming of $CD4^+$ T cells might have been inefficient or too slow in inducing effector mechanisms other than $CD8^+$ T cells, which might be required to confine the rapid spread of inoculated FV. In this regard, $CD8^+$ T cells are required not solely as cytotoxic effector cells but are also involved in the generation of T helper type 1 cells in FV-infected mice (36, 37). Thus, antibody-induced depletion of $CD8^+$ T cells quite likely had also affected $CD4^+$ T cell functions in the experiment reported by Robertson *et al.* (9), and adoptive transfer of immune $CD8^+$ cells must have induced the activation of $CD4^+$ effector cells in the recipients in the experiment performed by Dittmer, Brooks, and Hasenkrug (11). The role of $CD8^+$ T cells in inducing class switching of virus-neutralizing and anti-leukemia cell antibodies (Fig. 7) might reflect the reported influence of $CD8^+$ T cells on helper functions of $CD4^+$ T cells. In fact, the effect of $CD8^+$ T cell transfer from vaccinated to naive animals along with passive immunization with the neutralizing mAb was dependent on the presence of endogenous $CD4^+$ T cells in the recipients (11). Thus, the reported requirement of $CD8^+$ T cells for spontaneous resistance and vaccine-induced protection against FV infection might be compensated, at least partly, by the peptide-induced priming of $CD4^+$ T cells. It has been shown that the effect of $CD8^+$ T cells on the induction of protective $CD4^+$ T cell responses is blocked by neutralizing anti-IFN-γ antibody (37). Likewise, intravenous administration of neutralizing anti-IFN-γ antibody on the day of FV challenge and at PID 7 abrogated the effect of peptide vaccine in six of seven injected $CB6F_1$ mice in our preliminary experiment (data not shown). Thus, all these data indicate that the apparent requirement of $CD8^+$ T cells for spontaneous resistance and observed effectiveness of $CD8^+$ T cell transfer in vaccine-induced protection against FV infection might have been actually mediated through the effect of $CD8^+$ T cells on $CD4^+$ T cell functions, and peptide-induced priming of $CD4^+$ T cells may have bypassed the $CD8^+$ T cell functions and induced protection in the $\beta_2m^{-/-}$ mice.

Actual effector mechanisms involved in the observed elimination of FV-infected erythroid cells in the absence of $CD8^+$ T cells (Fig. 4) may include the previously described $CD4^+$ CTLs and NK cells (14), as well as FV-reactive, cytotoxic antibody (38). However, anti-leukemia cell IgMs were detectable in both immunized and unimmunized animals at PID 7 (Figs 6 and 7), excluding the role of these antibodies in controlling the growth of FV-infected cells in the early stage. It should be emphasized that killing activities of $CD4^+$ cytotoxic and NK cells were detectable in the peptide-immunized $CB6F_1$ mice at as early as PID 7, and NK cells were much more efficient than $CD8^+$ CTLs in killing FV-induced leukemia

cells (14). Further, it has been shown that activation of NK cells after lymphocytic choriomeningitis virus infection is prolonged in $\beta_2m^{-/-}$ mice than in the wild-type mice (30). Thus, similarly enhanced activation of NK cells, along with the exaggerated production of IFN-γ from vaccine-primed $CD4^+$ T cells, might have compensated otherwise indispensable $CD8^+$ T cell functions in the peptide-immunized $CB6F_1$-$\beta_2m^{-/-}$ mice. In this regard, the incidences of splenomegaly in unimmunized $CB6F_1$-$\beta_2m^{-/-}$ mice at PID 14 and 21 were significantly lower than those in the unimmunized wild-type mice ($P < 0.001$), although their survival curves were not significantly different ($P > 0.05$) (Fig. 4). These observations are consistent with the previously observed activation and killing efficacy of NK cells in FV-infected $CB6F_1$ mice without prior immunization (14), and with the reported enhancement of virus-induced NK cell activity in $\beta_2m^{-/-}$ mice (30).

Taken together, the present study has demonstrated that for efficient immune protection against FV infection with the single-epitope peptide, B cells are more important than $CD8^+$ T cells, but B cell-independent responses, probably exerted by previously demonstrated $CD4^+$ CTLs and NK cells, do play some roles in the earlier stage of FV-induced disease development in suppressing the expansion of FV-infected erythroid cells. Careful comparison of these results and other reports may suggest the possibility that priming of $CD4^+$ T cells with the peptide vaccine might allow the bypassing of the $CD8^+$ T cell functions that have been reported to induce $CD4^+$ T_h1 effector cells. These observations may contribute to the development of efficient vaccine strategies against other virus infections.

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Abbreviations

7-AAD	7-aminoactinomycin D
APC	antigen-presenting cell
B6	C57BL/6
$CB6F_1$	(BALB/c × C56BL/6)F_1
ED_{50}	dose required to induce 50% of the maximum response
FBS	fetal bovine serum
F-MuLV	Friend murine leukemia virus
FV	Friend retrovirus complex
β_2m	β_2-microglobulin
μMT	Ig μ-chain membrane exon-targeted
PBBS	phosphate-buffered balanced salt solution
PID	post-infection day
SFFU	spleen focus-forming unit
SFFV	spleen focus-forming virus

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Influence of Glycosylation on the Efficacy of an Env-Based Vaccine against Simian Immunodeficiency Virus SIVmac239 in a Macaque AIDS Model

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The envelope glycoprotein (Env) of human immunodeficiency viruses (HIVs) and simian immunodeficiency viruses (SIVs) is heavily glycosylated, and this feature has been speculated to be a reason for the insufficient immune control of these viruses by their hosts. In a macaque AIDS model, we demonstrated that quintuple deglycosylation in Env altered a pathogenic virus, SIVmac239, into a novel attenuated mutant virus (Δ5G). In Δ5G-infected animals, strong protective immunity against SIVmac239 was elicited. These HIV and SIV studies suggested that an understanding of the role of glycosylation is critical in defining not only the virological properties but also the immunogenicity of Env, suggesting that glycosylation in Env could be modified for the development of effective vaccines. To examine the effect of deglycosylation, we constructed prime-boost vaccines consisting of Env from SIVmac239 and Δ5G and compared their immunogenicities and vaccine efficacies by challenge infection with SIVmac239. Vaccination-induced immune responses differed between the two vaccine groups. Both Env-specific cellular and humoral responses were higher in wild-type (wt)-Env-immunized animals than in Δ5G Env-immunized animals. Following the challenge, viral loads in SIVmac239 Env (wt-Env)-immunized animals were significantly lower than in vector controls, with controlled viral replication in the chronic phase. Unexpectedly, viral loads in Δ5G Env-immunized animals were indistinguishable from those in vector controls. This study demonstrated that the prime-boost Env vaccine was effective against homologous SIVmac239 challenge. Changes in glycosylation affected both cell-mediated and humoral immune responses and vaccine efficacy.

Primate lentiviruses, human immunodeficiency viruses (HIVs), and simian immunodeficiency viruses (SIVs) share common genetic and biological properties. As SIVmac, originally isolated from macaques in primate research centers in the United States, causes AIDS in macaques with remarkable similarities to HIV type 1 (HIV-1) infection in humans, this AIDS monkey model has been utilized to study vaccine development and the pathogenesis of HIV infection (for reviews, see references 10, 14, 17, 43, and 47).

HIV/SIV infection in the host consists of two phases, the primary infection and chronic infection. During the primary infection, extensive viral replication and dissemination of the infection occur. In chronic infection, viral replication continues for a long period, eventually leading to AIDS. Due to the host immune response against the infection, these two phases are separated by a set point at which the viral load reaches its lowest level. The viral loads of the set point and chronic infection are inversely correlated with the control of SIV/HIV infection and predict disease progression (25, 31); however, it remains unclear which host responses determine the viral loads of the set point and chronic infection. Nevertheless, virus-specific immune responses have been implicated in the host's control of the infection. Cellular immunity, such as that shown by cytotoxic T lymphocytes (CTL) and helper T cells, has been reported to correlate with the control of HIV/SIV infection (for reviews, see references 2, 24, 28, and 39). The role of the neutralizing antibody (NAb) in the control of infection and the

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emergence of escape mutants has also been reported previously (7, 16, 51).

Despite these immune responses against HIV/SIV infection, humans and macaques fail to contain the infection due to the virus properties. HIV/SIV infects major target cells, such as $CD4^+$ T cells and macrophages, by binding viral envelope glycoproteins (Env) to cellular surface proteins and CD4 and chemokine receptors (CCR5, CXCR4, or others) on target cells (5, 32). Since viral entry consists of multiple steps (virion binding to these viral receptors, conformational change of Env, and fusion between the virion and the cellular membrane) and the critical parts of Env used in these steps are exposed only during each step, naturally generated antibodies are only partly effective in preventing HIV/SIV infection in their hosts (7, 8). Primary isolates can be neutralized to various degrees by HIV-infected patient serum but not by contemporaneous autologous samples. Consequently, escape mutants against preexisting NAb are selectively replicated (51). Thus, effective NAb is rarely induced in HIV/SIV infection (8, 10). This could partly explain the failure of Env-based vaccine trials against HIV-1 (8, 50).

The heavy glycosylation of Env is a unique feature of HIV/SIV that is distinctive from features of other enveloped viruses and is significantly related to their neutralization-resistant property (8, 29, 44). We therefore assumed that the insufficient immune containment of HIV/SIV might be due to heavy glycosylation in Env and that the removal of some glycans might allow the host to mount a protective immune response against the infection. Thus, we studied the influence of deglycosylation on the replication of SIVmac239 in a T-cell line and created a quintuple deglycosylation mutant of SIVmac239 (Δ5G), which has maximal removal of N-glycans at amino acid residues 79, 146, 171, 460, and 479 in Env and retains a replication capability similar to that of SIVmac239 in phytohemagglutinin-stimulated rhesus peripheral blood mononuclear cells (PBMCs) (36, 40). We then examined the infection of rhesus macaques with Δ5G; although Δ5G was replicated as extensively as SIVmac239 during the primary infection, the subsequent Δ5G infection was restricted to a level less than the detection sensitivity of a plasma viral load assay by 8 weeks postinfection (p.i.), in contrast to high chronic viral replication in SIVmac239 infection. Furthermore, an almost sterilizing immunity against SIVmac239 was induced in Δ5G-infected animals (36). Interestingly, another quintuple-deglycosylation-mutation strain with mutations at amino acid residues 146, 156, 184, 244, and 247 in Env was created (44) and was demonstrated to share common features with Δ5G in viral replication in animals and in functions as an attenuated vaccine (20). Since these two viruses share only one deglycosylation mutation and other mutations distributed differently in surface envelope protein gp120 (SU), these two studies suggest that heavily glycosylated Env determines the pathogenicity of HIV/SIV.

To dissect the mechanism for notable containment of Δ5G infection after primary infection, we hypothesized that the Env of Δ5G, a viral protein that differs from that in SIVmac239, might elicit protective immunity against SIVmac239, because deglycosylation in Env might alter antigenic properties such as B-cell and T-cell epitopes and enhance the protective immunity against SIVmac239. For this purpose, we immunized animals with Env of Δ5G (Δ5G Env) or Env of SIVmac239 (the wild type; wt Env), and examined the effect of these vaccinations against SIVmac239 infection.

MATERIALS AND METHODS

Generation of SU DNA vaccines. DNA vaccine plasmids expressing SIV mac239 SU or Δ5G SU, pJWSUmac239 and pJWSUmacΔ5G, were constructed using the expression vector pJW4303 (45). To produce secreted SU efficiently, the native signal sequence in the SIVmac239 SU gene was replaced with the human tissue plasminogen activator signal in plasmid pJW4303, and a termination codon was created at the cleavage site for SU transmembrane (TM) protein (9). An SIVmac239 SU or Δ5G SU DNA sequence was amplified with a pair of primers, SUmacA (5′-TGTGCTAGCTATGTCACAGTCTTTTATGGTGTAC-3′) and SUmacB (5′-CCAGGATCCTATTACCTCTTCACATCTGTGGGGG C-3′). The SUmacA primer consisted of nucleotides (nt) 6923 to 6955 of the SIVmac239 sequence (GenBank accession number M33262) and the boldface nucleotides, which were changed to create a NheI site; primer SUmacB consisted of nt 8412 to 8381 and the boldface nucleotides, which were changed to create a BamHI site, and the underlined nucleotides, which generated tandem termination codons. The PCR-amplified fragments were digested with NheI and BamHI and cloned into the NheI- and BamHI-digested eukaryotic expression vector pJW4303 to yield pJWSUmac239 and pJWSUmacΔ5G. These plasmids were prepared using a Plasmid Mega kit (QIAGEN, Tokyo, Japan).

Generation of Env vaccinia vaccines. Recombinant vaccinia viruses expressing Env of SIVmac239 or Δ5G, WRvvmac239 or WRvvΔ5G, respectively, were constructed using a vaccinia virus WR strain (WRvv) as described previously (15). To excise the entire coding region of the *env* gene from the cloned SIV plasmid, BamHI and SmaI sites were introduced by in vitro mutagenesis at 5′- and 3′-end-flanking sites of the *env* gene, respectively. Primer B-6808 (5′-GAA AGAGAAGAAGGATCCCGAAAAAGG-3′) consisted of nt 6796 to 9822 and the underlined mutations of the BamHI site; S-9537 (5′-TATGAATACTCCC GGGAGAAACCC-3′) consisted of nt 9527 to 9550 and the underlined mutations of the SmaI site. DNA fragments containing the *env* gene of SIVmac239 or Δ5G were isolated by digesting the mutated plasmids with BamHI and SmaI and were cloned into the SmaI- and BamHI-digested vaccinia virus vector plasmid pNZ68K2. To transfer the *env* gene from a recombinant plasmid to WRvv, the standard homologous recombination method using CV-1 cells was performed. Env expression in the recombinant vaccinia virus was confirmed by immunoprecipitation. The function of Env was confirmed by CD4- and CCR5-dependent fusion activity. The recombinant Env-expressing vaccinia viruses obtained were propagated and titrated in CV-1cells. The two recombinant viruses were propagated with similar kinetics in CV-1 cells.

Expression of SU-expressing plasmids and Env-expressing vaccinia virus in vitro. CV-1 cells were transfected with equal amounts of the following SU-expressing plasmids: pJWSUmac239, pJWSUmacΔ5G, or the vector pJW4303. Secreted SU metabolically labeled with ^{35}S protein labeling mix (PerkinElmer, Boston, MA) in culture supernatant was concentrated, immunoprecipitated with plasma from SIVmac239-infected monkeys, and then analyzed by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) as described previously (40). To examine Env-expressing vaccinia viruses, CV-1 cells were infected with WRvvmac239, WRvvΔ5G, or WRvv at a multiplicity of infection of 10, metabolically labeled with ^{35}S protein labeling mix overnight, lysed, immunoprecipitated with plasma from SIVmac239-infected monkeys, and then analyzed by SDS-PAGE as described for the expression of SU-expressing plasmids.

Animals, immunization, and challenge. Twelve juvenile rhesus macaques from Myanmar or Laos that were seronegative for SIV, simian T-cell lymphotropic virus, B virus, and type D retroviruses were used. As the polymorphism of major histocompatibility complex (MHC) genes influenced cellular immune responses against SIV/HIV infection, MHC II haplotypes and alleles of the macaques were determined (data not shown). All animals were housed in individual cages and maintained according to the rules and guidelines for experimental animal welfare stated by the National Institute of Infectious Diseases. As shown in Fig. 1, the 12 animals were divided into three immunization groups of four animals each: the SIVmac239 (wt)-Env immunization group (Mm0005, Mm0007, Mm0010, Mm0012), the Δ5G Env immunization group (Mm0001, Mm0002, Mm0003, Mm0009), and the vector control immunization group (Mm0004, Mm0006, Mm0008, Mm0011). All animals were inoculated with 1 mg of plasmid DNA in 1 ml of saline, one into each quadriceps femoris at 0, 4, and 8 weeks after the initial prime immunization (weeks p.p.). The boost consisted of 5×10^7 PFU of vaccinia virus in 1 ml of phosphate-buffered saline (PBS), administered in two 0.1-ml intradermal inoculations, one into the skin of each femur, and two 0.4-ml inoculations, one into each quadriceps femoris at 21 weeks p.p. All animals were

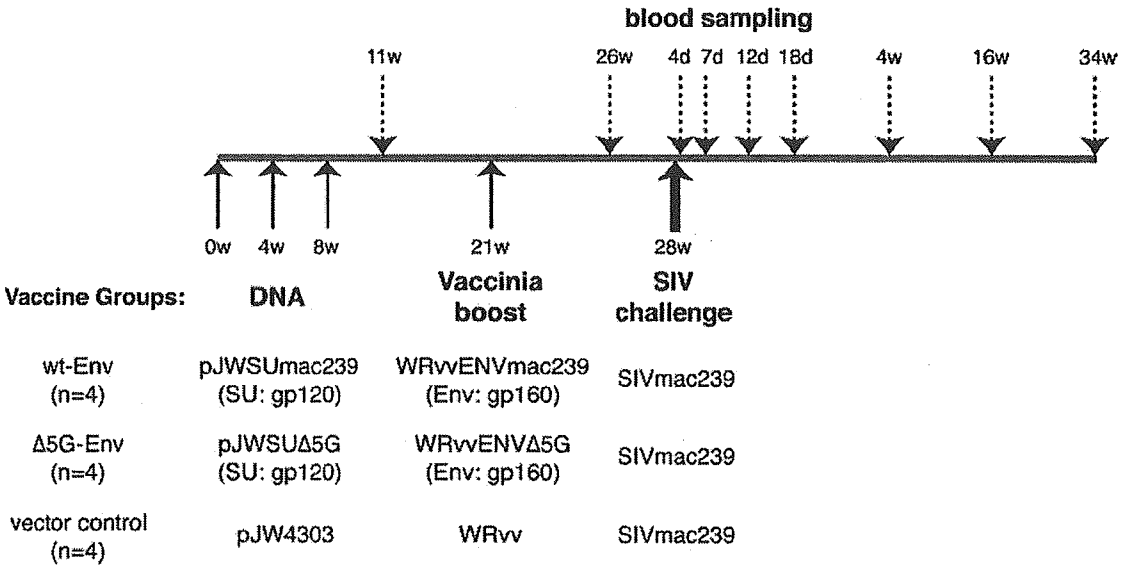

FIG. 1. Outline of immunization, challenge infection, and blood sampling. Twelve juvenile rhesus macaques were divided into three immunization groups of four animals each: the wt-Env immunization group (Mm0005, Mm0007, Mm0010, and Mm0012), the Δ5G Env immunization group (Mm0001, Mm0002, Mm0003, and Mm0009), and the vector control immunization group (Mm0004, Mm0006, Mm0008, and Mm0011). Animals were inoculated with a DNA vaccine (pJWSUmac239 for the wt-Env vaccine group, pJWSUΔ5G for the Δ5G Env vaccine group, and pJW4303 for the vector control group) at 0, 4, and 8 weeks p.p. The boost vaccine consisted of vaccinia virus (WRvvENVmac239 for the wt-Env vaccine group, WRvvENVΔ5G for the Δ5G Env vaccine group, and the WR strain for the vector control group) administered at 21 weeks p.p. All animals were challenged with 10 $TCID_{50}$ of SIVmac239 intravenously at 28 weeks p.p. w, weeks; d, day.

challenged with 10 50% tissue culture infective doses ($TCID_{50}$) of SIVmac239 intravenously at 28 weeks p.p.

Viral load measurement. To monitor SIV infection, the plasma viral load was measured by the real-time-PCR method described previously (36). Viral RNA was isolated from plasma from the infected animals using a commercial viral-RNA isolation kit (PE Applied Biosystems, Urayasu, Japan). SIV *gag* RNA was amplified and quantified using a commercial RNA reverse transcription (RT)-PCR kit (TaqMan EZ RT-PCR; PE Applied Biosystems) with the two *gag* primers, namely, the forward primer 1224F (5′-AATGCAGAGCCCCAAGAA GAC-3′), the reverse primer 1326R (5′-GGACCAAGGCCTAAAAAACCC-3′), and TaqMan probe 1272T (6-carboxyfluorescein-5′-ACCATGTTATGGCC AAATGCCCAGAC-3′-6-carboxymethylrhodamine). 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 with the following thermal cycle conditions: 1 cycle of three sequential incubations (50°C for 2 min, 60°C for 30 min, and 95°C for 5 min) and then 50 cycles of amplification (95°C for 5 s, 62°C for 30 s) in a 7000 Prism sequence detection system (PE Applied Biosystems). In vitro RNA transcripts were quantified by optical density at 260 nm (OD_{260}) measurement and branched DNA assay for SIV viral RNA (Bayer Diagnostics, Tarrytown, N.Y.). RNA equivalent to 10 to 10^7 copies per reaction was used as the standard for each assay. The detection sensitivity of plasma viral RNA using this method was 1,000 copies/ml.

Flow cytometry. CD4 depletion was monitored by measuring the percentage of $CD4^+$ T cells, memory cells (CD29 high $CD4^+$) T cells (48) in PBMCs. PBMC samples were purified from a citrate anticoagulant containing blood using standard Ficoll-Hypaque gradient centrifugation. For flow cytometry, 2×10^5 PBMCs were reacted with fluorescein isothiocyanate or phycoerythrin-labeled antibodies (anti-human CD4, Nu-Th/I [Nichirei, Tokyo, Japan]; anti-human CD8, Leu2a [Becton Dickinson, San Jose, CA]; anti-human CD29, 4B4 [Coulter, Miami, FL]; anti-monkey CD3, FN-18 [Biosource, Camarillo, CA]; and anti-human CD20, Leu16 [Becton Dickinson, San Jose, CA]) as previously described (36, 37, 48).

Peptides. Overlapping peptides were synthesized by Emory University, Microchemical Facility, Winship Cancer Center (Atlanta, GA.). All SIVmac239 viral proteins except Env, Gag, Pol, Vif, Vpr, Vpx, Tat, Rev, and Nef were covered by consecutive 20-mer peptides overlapped by 12 amino acids. Env of SIVmac239 was covered by 72 consecutive 25-mer peptides overlapped by 13 amino acids. Peptides were dissolved in PBS with 10% dimethyl sulfoxide (Sigma Chemical, St. Louis, Mo.).

rSeV. Recombinant Sendai viruses (rSeV) expressing SIVmac239 Gag, SU, or Δ5G SU were used to infect herpesvirus papio-transformed B-lymphoblastoid cell lines (B-LCLs) to prepare autologous B-LCLs presenting these viral antigens. rSeV Gag expressing unprocessed SIVmac239 Gag and p55 (22, 23) and rSeV SU and rSeV/Δ5G SU expressing wt SU and Δ5G SU were constructed as described previously (52) and were also used to infect autologous B-LCLs.

Anti-SIV ELISA. A 1:100 dilution of each plasma sample in PBS (pH 7.4) containing a blocking reagent (Dainippon Seiyaku, Osaka, Japan) was assayed for SIV-specific antibody by using a standard enzyme-linked immunosorbent assay (ELISA) technique with 96-well plates precoated with SIVmac239 virion lysate. The OD_{492} was measured using a microplate reader (range of absorbance with linearity, 0 to 3.0; Tecan Japan, Tokyo, Japan) and utilized as a relative measurement of the antibody titer.

ELISPOT assay. Virus-specific $CD4^+$ T cells and $CD8^+$ T cells in PBMCs were measured using a monkey γ-IFN ELISPOT assay kit (U-CyTech, Utrecht, The Netherlands).

Cryopreserved PBMCs were thawed and cultured overnight in R-10 medium (RPMI 1640 [Sigma] supplemented with 10% heat-inactivated, defined fetal bovine serum [HyClone, Logan, Utah], 55 μM 2-mercaptoethanol, 50 U/ml penicillin, and 50 μg/ml streptomycin). PBMCs were subjected to the depletion of $CD4^+$ cells with magnet beads coated with anti-human CD4 Ab (Dynal ASA, Oslo, Norway) or subjected to the depletion of $CD8^+$ cells with magnet beads coated with anti-human CD8 Ab (Miltenyi Biotec, Bergisch Gladbach, Germany). Depletion of $CD4^+$ or $CD8^+$ cells from PBMCs was confirmed by flow cytometry. Using this depletion method, more than 95% of $CD4^+$ or CD8 cells were removed from PBMCs. These PBMCs were used for ELISPOT assay for virus-specific $CD8^+$ T cells and virus-specific $CD4^+$ T cells. Virus-specific stimulation of T cells was performed with autologous B-LCLs pulsed with pooled peptides for Pol, Vif, Vpx, Vpr, Tat, Rev, and Nef or B-LCLs infected with an rSeV for Gag, wt Env, and Δ5G Env. B-LCLs were incubated with pooled peptides corresponding to each viral protein at a final concentration of 2 μg/ml or infected with rSeV at a multiplicity of infection of 10 at 37°C overnight. Peptide-pulsed or infected B-LCLs were inactivated with long-wave UV irradiation (19) in the presence of 10 μg/ml psoralen (Sigma) for 10 min at a distance of 3.5 cm from a UV light, washed three times with R-10, and then used as stimulators in an ELISPOT assay. $CD4^+$ or $CD8^+$ cell-depleted PBMCs were cultured with these stimulators in an anti-γ-IFN Ab-coated ELISPOT plate (U-CyTech) overnight according to the protocol for the kit. Spots on the ELISPOT plate were imaged using an Olympus model SZX12 microscope

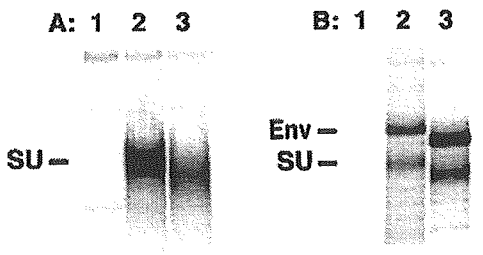

FIG. 2. Expression of SU and Env by SU-expressing DNA vaccines and Env-expressing vaccinia viruses. A: SU secreted in supernatant from CV-1 cells transfected with SU-expressing plasmids. Lane 1, pJW4303 vector; lane 2, pJWSUmac239; lane 3, pJWSUmacΔ5G. B: Env in cell lysates of CV-1 cells infected with recombinant vaccinia viruses. Lane 1, WRvv; lane 2, WRvvmac239; lane 3, WRvvΔ5G.

(Olympus, Tokyo, Japan) equipped with a digital camera, PDMCIe/OL (Polaroid, Cambridge, MA), and analyzed using a personal computer with MAC SCOPE version 2.61 (Mitani Corporation, Toyama, Japan). The results were calculated as numbers of spot-forming cells (SFC) per million PBMCs after subtraction of the background.

Neutralization assay. The original protocol of this neutralization assay was reported by Means et al. (29). Plasma that was heat inactivated at 56°C for 30 min was serially diluted and incubated with a fixed concentration of SIVmac239, Δ5G, or a macrophage-tropic SIV, 239/envMERT, at room temperature for 1 h. CEMx174/SIVLTR-SEAP cells were added to the mixture and then incubated at 37°C for 3 days. Secreted alkaline phosphatase activity in the culture supernatant was measured using a Phospha-Light System (Applied Biosystems). Chemiluminescence was detected with a Wallac Microbeta plate reader.

Statistical analysis. Statistical analysis was based on the Mann-Whitney test and performed using GraphPad Prism 4.0 software.

RESULTS

Experimental design. We adopted a DNA prime-vaccinia virus boost regimen to immunize rhesus macaques with wt Env or Δ5G Env as shown in Fig. 1. Twelve macaques were immunized at 0, 4, and 8 weeks after the initial prime immunization (weeks p.p.) with one of three different DNA expression plasmids (n = 4): pJWSUmac239 expressing SU of SIVmac239, pJWSUΔ5G expressing SU of Δ5G, or the vector pJW4303. At 21 weeks p.p., all animals were boosted with recombinant WR vaccinia viruses expressing the respective Env proteins: vaccinia virus expressing Env of SIVmac239, vaccinia virus expressing Env of Δ5G, or vaccinia virus (Fig. 1).

Expression of SU DNA plasmids and Env vaccinia viruses in vitro and in animals. Although Δ5G replicated similarly to wild-type SIVmac239 in animals (36), quintuple deglycosylation might affect the expression of SU in a plasmid vector and the expression of Env in the vaccinia virus vector. Thus, we examined the expression of these vaccines in CV-1 cells. SU expressions in the wild-type plasmid (pJWSUmac239) and in the deglycosylated SU plasmid (pJWSUmacΔ5G) were at similar levels (Fig. 2A). The expression and processing of Env in the wild type (WRvvENVmac239) and in the deglycosylated Env mutant vaccinia virus (WRvvENVΔ5G) were also at similar levels (Fig. 2B). The reduced molecular size of the proteins due to deglycosylation was confirmed by PAGE (Fig. 2). As the amount of secreted SU in the supernatant by DNA transfection was comparable to that of Env in the cell lysate from CV-1 cells infected with WRvvEnv, a high expression of SU was achieved in a *rev*-independent manner by the pJW403 expression plasmid as described previously (9).

The expression of Env vaccines in the immunized animals was indirectly estimated by Env-specific antibody responses measured by a peptide ELISA using overlapping Env peptides. Env peptide-specific Ab was detected from 11 weeks p.p. after immunization with DNA vaccines, whereas there was no significant difference in the titers and the specificity of the responses between the two vaccine groups (data not shown), suggesting similar amounts of Env expressed in animals immunized with either Env vaccine. To examine the protective effect of the Env vaccines, all animals were challenged with 10 $TCID_{50}$ of SIVmac239 intravenously at 28 weeks p.p.

Cellular immune responses elicited by Env vaccines. The DNA prime-vaccinia virus boost regimen has been used in many studies, has successfully induced a high frequency of virus-specific $CD8^+$ T cells in macaques, and has conferred protective immunity against chimeric simian/human immunodeficiency virus (SHIV) (3, 27, 45). We therefore examined the vaccine-induced Env-specific T-cell responses by IFN-γ ELISPOT assay. Since deglycosylation in Env might change T-cell epitopes in SIVmac239, we measured the wt-SU and Δ5G SU-specific T-cell response by using autologous B-LCLs infected with recombinant Sendai viruses expressing either wt SU and/or Δ5G SU, respectively.

Although there was a tendency for more ELISPOT-positive cells to be observed by homologous SU than heterologous SU, comparable results were obtained by both assays (Fig. 3A and B). As vaccinated animals were challenged with SIVmac239, the results from the wt-SU assay were subsequently used to assess the SU-specific immune response. Immunization with the DNA vaccine induced only marginal SU-specific $CD8^+$ T cells or $CD4^+$ T cells at 11 weeks p.p.; however, boost immunization with recombinant WR vaccinia virus significantly increased SU-specific $CD8^+$ T cells and $CD4^+$ T cells in PBMCs at 26 weeks p.p. (Fig. 3A, B, and C). Notably, SIVmac239 Env (wt Env) induced twofold more SU-specific CD8 T cells (mean, 770 SFC per million PBMCs; range, 540 to 880) responding to wt SU than Δ5G Env (mean, 320; range, 110 to 400) (P = 0.029) (Fig. 3A and C). Similarly, twofold more SU-specific $CD4^+$ T cells were observed in wt-Env vaccinees (mean, 1,260; range, 840 to 1,710) than in Δ5G Env vaccinees (mean, 680; range, 150 to 1,260) at 26 weeks p.p. (P = 0.11) (Fig. 3B and C). Thus, a twofold-greater number of both SU-specific $CD4^+$ T cells and $CD8^+$ T cells were induced in SIVmac239 Env vaccinees than in Δ5G Env vaccinees at 26 weeks p.p. In vector controls, only negligible SU-specific $CD4^+$ T cells and $CD8^+$ T cells were detected in PBMCs at 26 weeks p.p. (Fig. 3A and B).

Humoral immune response elicited with Env vaccines. The anti-Env Ab titer was examined by SIVmac239 virion lysate ELISA. Anti-SIV Ab was detected in both wt-Env vaccinees and Δ5G Env vaccinees after an rVV boost (Fig. 4) (26 weeks p.p.). Anti-SIV Ab titers were comparable between the two vaccine groups.

Next, we examined the NAb against either SIVmac239, Δ5G, or a macrophage-tropic mutant, 239env/MERT (33, 35), in the two vaccine groups. Macrophage-tropic SIVs were highly susceptible to neutralization by plasma from most SIV-infected macaques (29), whereas SIVmac239 was highly resistant to neutralization as were most clinical isolates of HIV-1

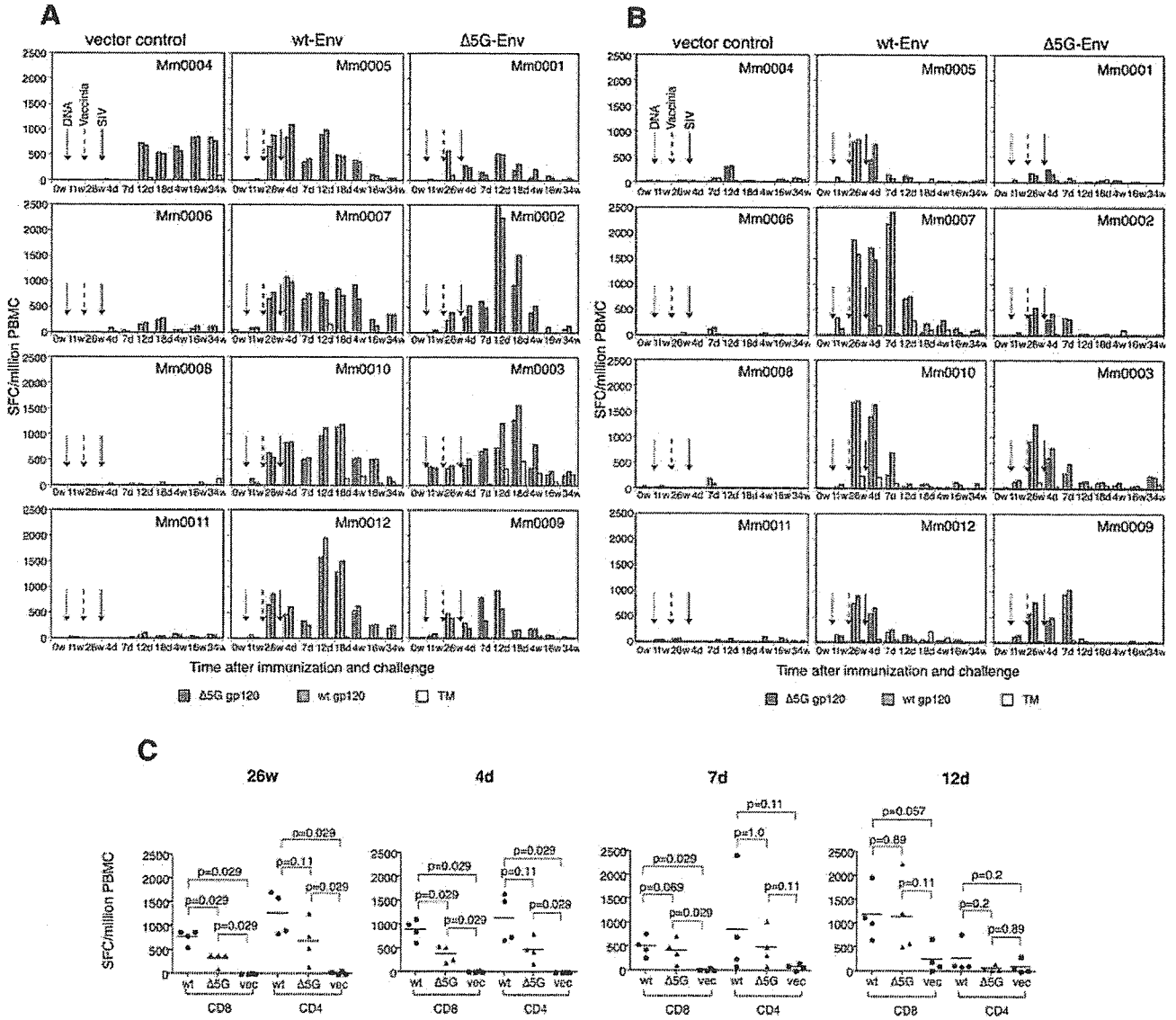

FIG. 3. Env-specific $CD4^+$ T-cell and $CD8^+$ T-cell responses in 12 macaques. A: Env-specific $CD8^+$ T cells in PBMCs were measured by ELISPOT assay for IFN-γ in three groups. B: Env-specific $CD4^+$ T cells in PBMCs were measured by ELISPOT assay for IFN-γ in three groups. ELISPOT results are colored as follows: Δ5G SU-specific T cells (red), wt-SU-specific T cells (green), and TM-specific T cells (yellow). Arrows with a dotted line, arrows with broken line, and arrows with a solid line indicate the time of the third DNA vaccination at 8 weeks p.p., the time of the vaccine boost at 21 weeks p.p., and the time of SIVmac239 challenge at 28 weeks p.p., respectively. C: Comparison of SU-specific $CD8^+$ T cells and $CD4^+$ T cells in PBMCs among the wt-Env vaccine group, the Δ5G Env vaccine group, and the vector control group at 26 weeks p.p. and 4, 7, and 12 days p.i. The numbers of SFC responding to SIVmac239 SU were used to compare the effects of the two vaccines. w, weeks; d, days.

(21, 29, 30). Plasma at 26 weeks p.p. from all immunized animals failed to neutralize not only SIVmac239 but also a multiple-deglycosylation-mutation strain, Δ5G (Table 1); in contrast, these plasma specimens did neutralize 239env/MERT. Furthermore, a marked difference was observed between the two vaccine groups. The NAb titer in the wt-Env vaccine group was eightfold higher than in the Δ5G Env vaccine group (Table 1). The difference of this immune response between the two vaccine groups was significant ($P = 0.029$).

SIV replication in Env-immunized animals. As described above, wt-Env vaccine and Δ5G Env vaccine induced different magnitudes of virus-specific cellular and humoral immunity in macaques. To examine the effect of the two vaccines, we challenged the vaccinated animals with SIVmac239. Viral loads in vector controls were mostly consistent with our previous results with SIVmac239-infected rhesus macaques (36, 48). The mean peak viral load at 2 weeks p.i. was 1.4×10^7 copies/ml, with a range of 0.5×10^7 to 2.2×10^7 copies/ml. Viral loads in chronic infection diverged into two patterns (Fig. 5A). Subsequent to the set point at 20 weeks p.i., the viral loads in three animals increased more than 10^4 copies/ml. In contrast, viral loads in one animal (Mm0011) remained as low as 1,000 copies/ml up to 45 weeks p.i.

Compared with the vector controls, viral loads in wt-Env

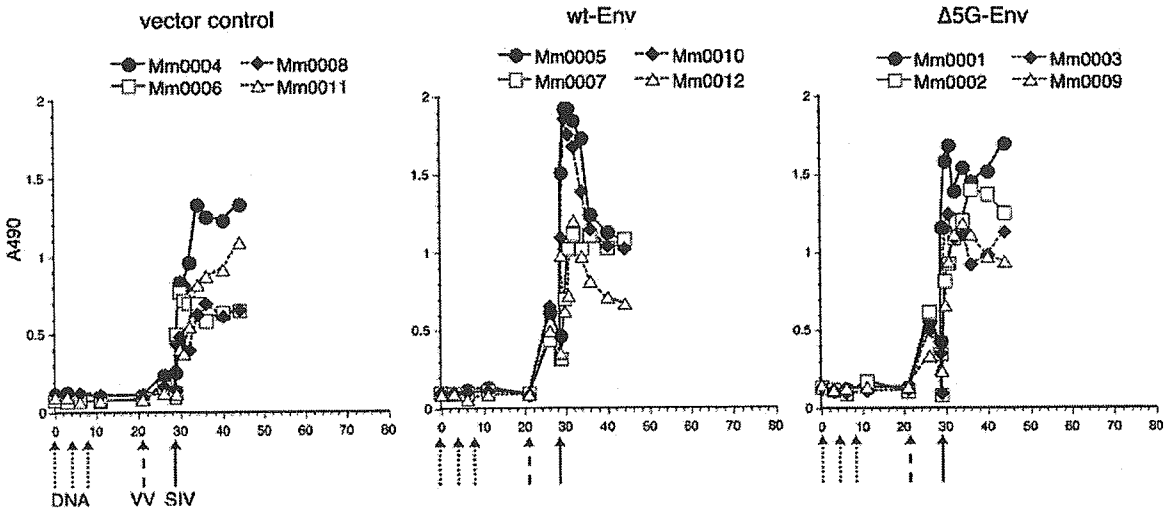

FIG. 4. Humoral immune response during immunization and after challenge infection. The OD_{492} was used as a relative measurement of anti-SIV ELISA antibody titer.

vaccinees were markedly reduced (Fig. 5B). Peak viral loads at 2 weeks p.i. (mean, 1×10^6 copies/ml; range, 0.8×10^6 to 1.2×10^6 copies/ml) were 1-log lower than those in the vector controls. Furthermore, viral loads decreased to as low as 1,000 copies/ml by 8 to 20 weeks p.i., remaining low until autopsy at 45 weeks p.i.

Unexpectedly, viral loads in the Δ5G Env vaccine group resembled those in vector controls (Fig. 5C). Peak viral loads (mean, 2.4×10^6 copies/ml; range, 0.9×10^6 to 4.2×10^6 copies/ml) were slightly lower than those in vector controls. Set points and viral loads in the chronic phase were similar to those of vector controls.

In summary, as shown by the mean viral loads in primary and chronic infection (Fig. 5D) and statistical analysis (Fig. 5E), the effects of vaccination differed between the wt-Env vaccine and Δ5G Env vaccine. In the effect on primary infection (up to 6 weeks p.i.), wt-Env vaccination decreased viral loads more extensively and significantly than Δ5G Env vaccination (P = 0.029 versus P = 0.057); however, in chronic infection (viral loads after 8 weeks p.i.), significant reductions in viral loads compared with those in vector controls were seen only in the wt-Env vaccine group and not the Δ5G Env vaccine group (Fig. 5E). Collectively, wt-Env vaccination induced significantly effective immunity to control SIVmac239 infection, whereas Δ5G Env vaccination induced a marginal effect seen only in primary and not in chronic infection.

TABLE 1. Neutralizing-antibody titers in the vaccinated macaques at 26 weeks p.p.

Vaccine	Animal	Neutralizing-antibody titer[a]			Mean[b]
		SIVmac239	Δ5G	239/envMERT	
wt-Env	Mm0005	<20	<20	800	
	Mm0007	<20	<20	400	
					400
	Mm0010	<20	<20	400	
	Mm0012	<20	<20	200	
Δ5G-Env	Mm0001	<20	<20	100	
	Mm0002	<20	<20	20	
					50
	Mm0003	<20	<20	100	
	Mm0009	<20	<20	50	

[a] Reciprocal of the dilution of plasma giving 50% inhibition of SIV replication.
[b] The difference in NAb levels between the two vaccine groups was significant (P = 0.0029).

$CD4^+$ T-cell subsets in PBMCs. CD4 cell depletion is a primary manifestation indicating immune disorder in HIV/SIV infection. As CD4 depletion results from HIV/SIV infection in lymphatic tissue, it correlates with the extent of viral replication. Accordingly, viral loads were correlated mostly with CD4 depletion (Fig. 5 and 6A). Despite fluctuations due to immunizations and the challenge infection, the percentage of $CD4^+$ T cells in wt-Env-immunized animals in the chronic phase recovered to the levels at the initiation of the experiment. By contrast, in vector controls and Δ5G Env vaccinees, the percentage of $CD4^+$ T cells decreased in the chronic phase. Among them, an extensive decrease in $CD4^+$ T cells occurred in animals with high viral loads in the chronic phase (Mm0001, Mm0008, and Mm0009) (Fig. 5 and 6A). However, in the other animals, the levels of $CD4^+$ T cells remained as before the challenge (Mm0003, Mm0011).

A subset of $CD4^+$ CD29 high cells, approximately corresponding to memory $CD4^+$ T cells, is useful for diagnosing a deterioration in the immune function in animals with AIDS (26, 38, 48). Although this parameter usually correlates with the percentage of $CD4^+$ T cells, remarkable differences were noted between two Env vaccine groups after the challenge infection. First, all animals in the wt-Env vaccine group showed an increased percentage of this subset in the chronic phase (Fig. 6B). Second, three of the Δ5G Env vaccinees had a marked decrease after the challenge infection (Mm0001, Mm0002 and Mm0009), whereas the remaining animal (Mm0003) showed an increased percentage of this subset. In

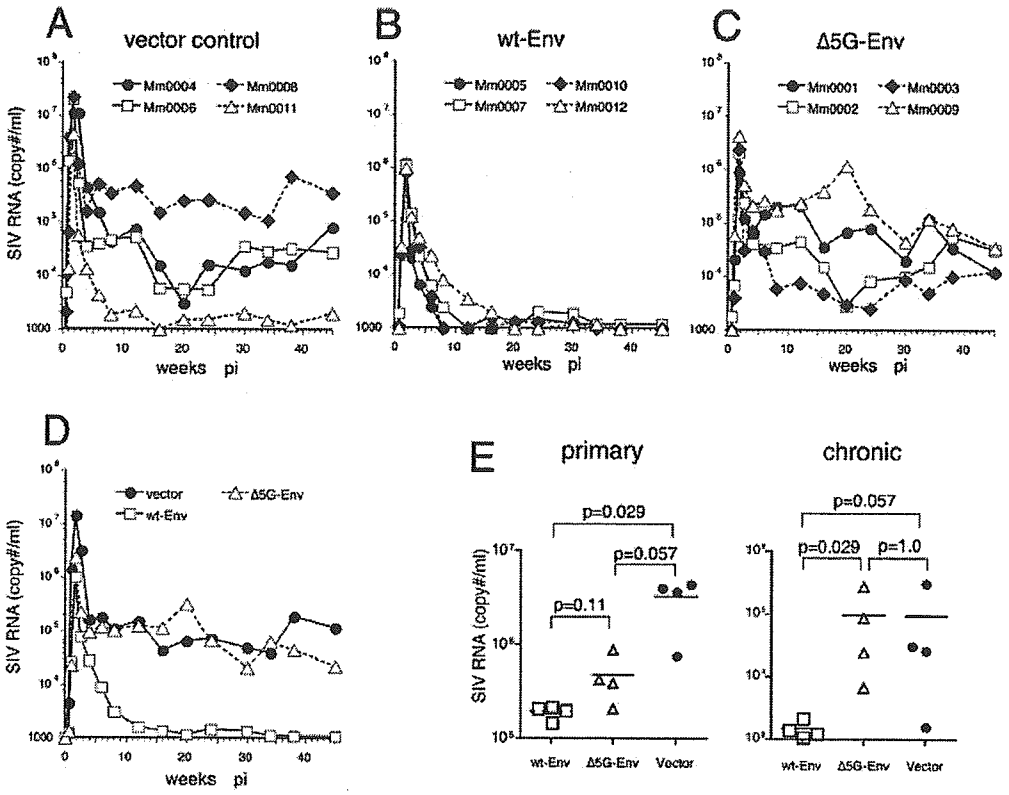

FIG. 5. Plasma viral loads after SIVmac239 challenge infection. Plasma viral load was measured by real-time PCR with a detection limit of 1,000 copies/ml. A: wt-Env vaccine group; B: Δ5G Env vaccine group; C: vector controls; D: comparison of viral loads among three groups; E: comparison of viral loads during the primary infection (5 days to 6 weeks p.i.) and chronic infection (8 weeks to 45 weeks p.i.) among three groups. Viral load was determined by averaging over a period of time.

vector controls, this subset remained in the range before the challenge infection in all animals but one (Fig. 6B).

Env-specific-T-cell immunity after the challenge infection. The magnitude of Env-specific T cells after the challenge infection is assumed to be influenced not only by vaccination but also by viral replication. Namely, SU-specific T cells at 4 days p.i. and those at 12 days p.i. were likely influenced by the former and the latter respectively. The magnitudes of SU-specific $CD4^+$ T cells and $CD8^+$ T cells at 4 days p.i. were comparable to those before challenge at 26 weeks p.p. (Fig. 3A and B); therefore, twofold-more SU-specific $CD8^+$ T cells and $CD4^+$ T cells were present in wt-Env vaccinees than in Δ5G Env vaccinees up to 4 days p.i. (Fig. 3C). However, this difference in the magnitudes of SU-specific $CD8^+$ T and $CD4^+$ T cells was not sustained at 7 and 12 days p.i. (Fig. 3C). Present with robust viral replication in primary infection, SU-specific $CD4^+$ T cells immediately decreased to an undetectable level at 12 days p.i. In contrast, SU-specific $CD8^+$ T cells increased (Fig. 3A and B). Subsequently, SU-specific $CD8^+$ T cells gradually decreased to very low or undetectable levels by 34 weeks p.i. (Fig. 3A). Thus, vaccine-induced SU-specific $CD8^+$ T and $CD4^+$ T cells were sustained only for a short period of time after challenge infection in both Env vaccine groups.

SIV-specific T-cell immunity after challenge infection. Despite an Env vaccination, robust SIV infection occurred shortly after the challenge infection (Fig. 5B and C). Consequently, SIV-specific $CD8^+$ T cells and $CD4^+$ T cells were elicited not only in vector controls but also in Env vaccine groups (Fig. 7A and B). To examine the effect of these SIV-specific T cells on the control of SIV infection, all animals were divided into SIV infection-controlled (controlled) and SIV infection-uncontrolled (uncontrolled) animals. Viral loads in chronic infection and the percentage of $CD4^+$ cells in PBMCs were used to classify the animals as controlled or uncontrolled (Fig. 6A). All animals in the wt-Env vaccine group, Mm00011 in vector controls, and Mm0003 in the Δ5G Env vaccine group were grouped as control animals. The remaining animals, Mm0004, Mm0006, and Mm0008 in vector controls and Mm0001, Mm0002, and Mm0009 in the Δ5G Env vaccine group were grouped as uncontrolled animals. Notably, SIV-specific $CD4^+$ T cells as well as the percentage of $CD4^+$ CD29H cells remained high in the chronic phase in controlled animals (Fig. 7B and 6B, respectively).

Although overall SIV-specific $CD8^+$ T cells were high in Env-vaccinated controlled animals, such correlation was not seen in vector controls grouped as uncontrolled animals (Fig. 7A). Therefore, to examine the relevance of virus-specific T cells to the control of SIV infection, the magnitudes of every viral-protein-specific T cell in controlled and uncontrolled animals were compared. As shown in Fig. 7C, Gag-specific $CD8^+$ T cells and $CD4^+$ T cells, and Tat/Rev-specific $CD4^+$ T cells

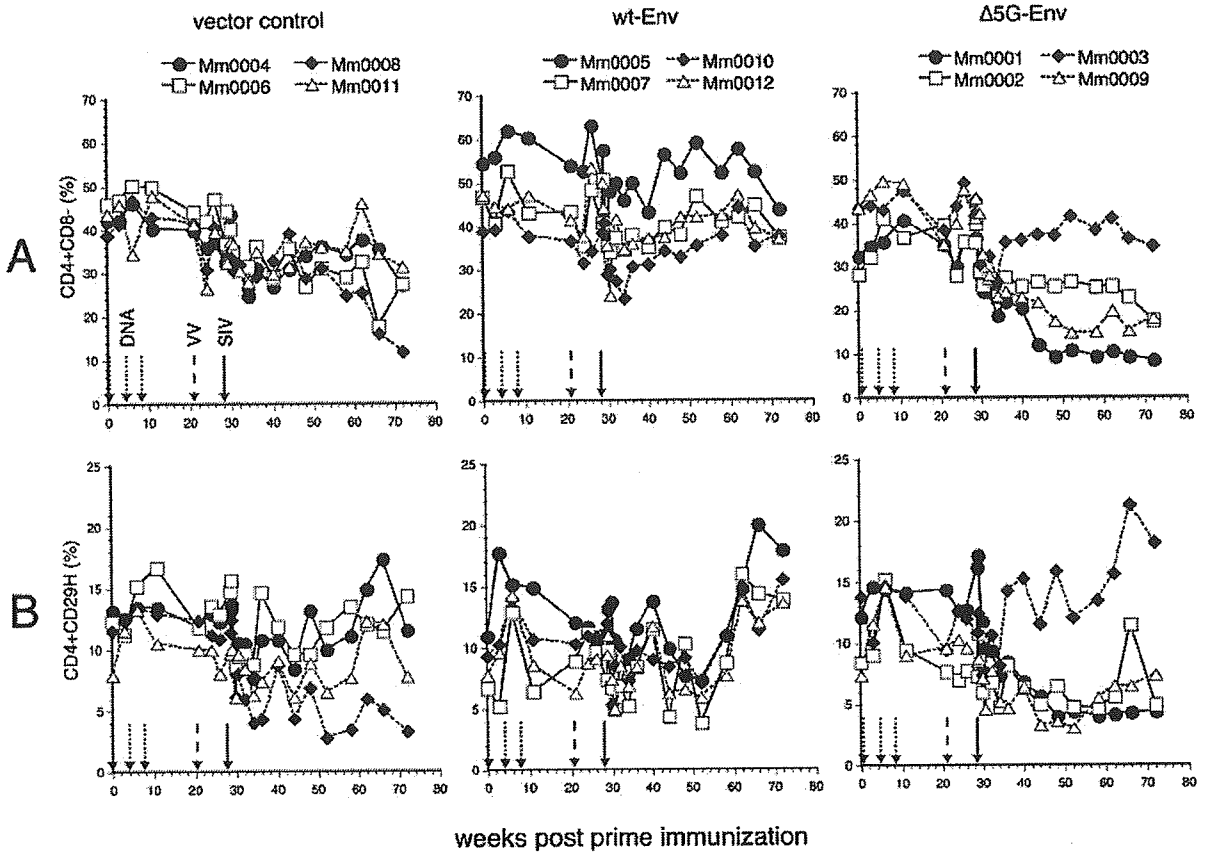

FIG. 6. $CD4^+$ T cells in PBMCs from rhesus macaques during immunization and after the challenge infection. A: Percentage of $CD4^+$ T cells in PBMCs; B: percentage of $CD4^+$ CD29high T cells in PBMCs.

were induced, with statistical significance ($P < 0.05$), in the control animals.

DISCUSSION

The heavily glycosylated structure of Env has been considered a main cause of chronically persistent viral replication and the pathogenicity of HIV/SIV, primarily because it potentially interferes with the development of the host immune response associated with protective immune functions, such as NAb and CTL (10, 36, 44). This characteristic constitutes the primary reason for the difficulty of developing effective vaccines. We therefore examined the efficacy of a deglycosylated-Env vaccine and compared it with the wt-Env vaccine. This study showed that quintuple deglycosylation neither improved the immunogenicity of the wt-Env vaccine nor elicited NAb against SIVmac239. This was in contrast to what occurred with Δ5G infection in rhesus macaques, because the host response elicited by Δ5G infection not only contained Δ5G infection but also protected the animals from SIVmac239 challenge infection (36). This study therefore suggested that an almost sterilizing immunity against SIVmac239 induced in Δ5G-infected animals could not be explained by the immunogenicity of Δ5G Env; instead, it is likely associated with the property of Δ5G as an attenuated virus. In fact, Δ5G was more neutralization-sensitive than SIVmac239 (36). Alternatively, the immunogenic property of Env in Δ5G could not successfully be duplicated by immunization with a Δ5G Env DNA prime-vaccinia virus boost regimen. Therefore, another immunization regimen might be able to elicit the protective immune response induced by Δ5G infection.

The Env vaccine is superior to other vaccines containing other viral proteins with respect to the induction of NAb; however, both the Δ5G Env vaccine and the wt-Env vaccine could not induce detectable NAb against either SIVmac239 or Δ5G. Instead, the wt-Env vaccine induced higher NAb against macrophage-tropic SIV than the Δ5G Env vaccine. Notably, this parameter most significantly correlated with the efficacies of the two Env vaccines. As Ab neutralized the macrophage-tropic variant 239/envMERT, which has only four separate amino acid substitutions distributed in *env* of SVmac239 (34), it might recognize unknown epitopes conserved between SIVmac239 and 239/envMERT. On the other hand, Δ5G Env may not sufficiently present this epitope due to mutations. Regarding the role of nonneutralizing Ab for the control of SIVmac239 infection, it is assumed that, as the neutralization assay did not necessarily reflect in vivo conditions, such nonneutralizing Ab with potential virus-binding ability may interfere with SIVmac239 infection in animals. Alternatively, Ab

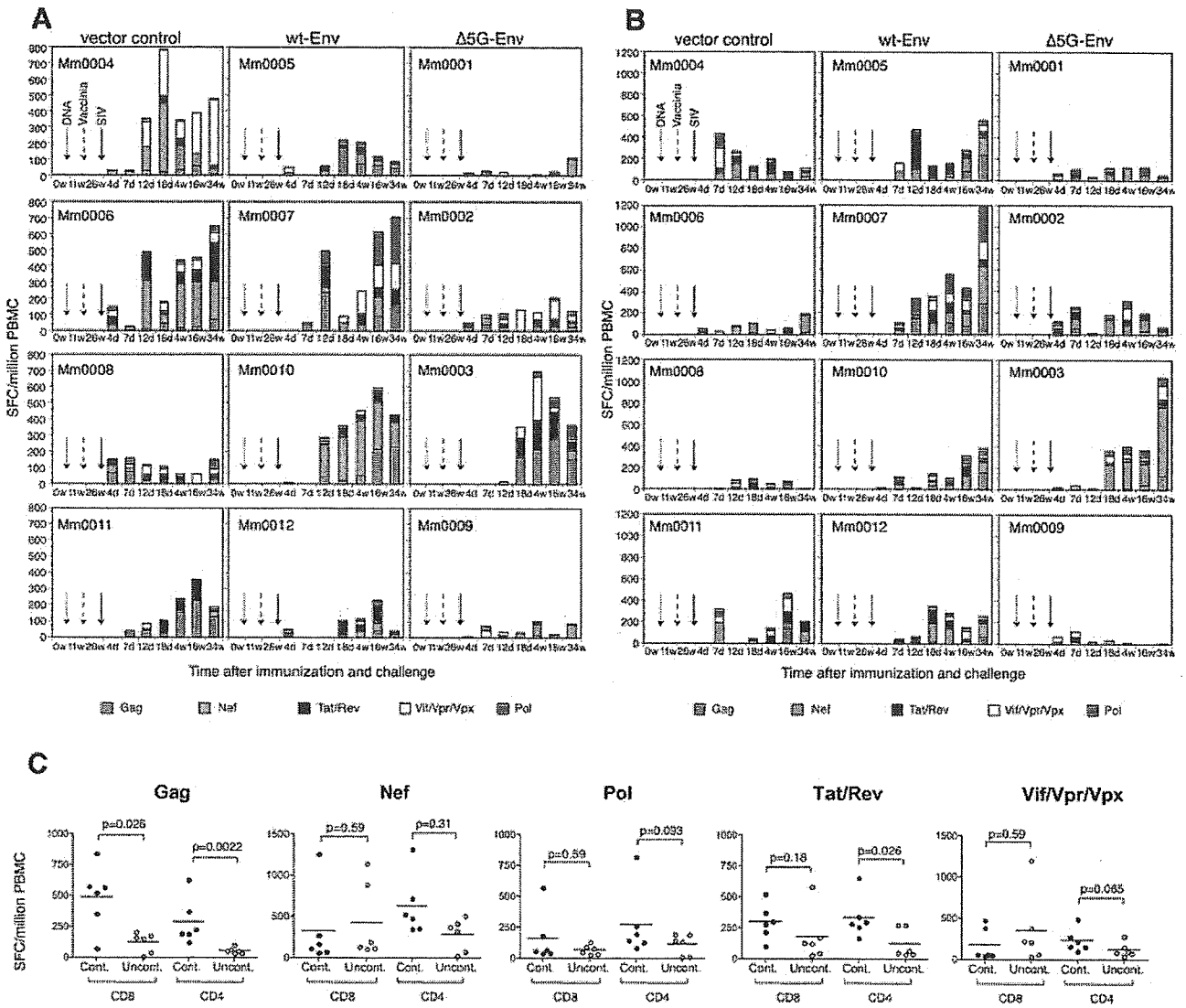

FIG. 7. SIV-specific $CD8^+$ T-cell and $CD4^+$ T-cell responses in 12 animals. A: SIV viral-protein-specific $CD8^+$ T cells in PBMCs were measured by ELISPOT assay for IFN-γ in three groups: vector controls, wt-Env vaccine group, and Δ5G Env vaccines. B: SIV viral-protein-specific $CD4^+$ T cells in PBMCs were measured by ELISPOT assay for IFN-γ in three groups. ELISPOT results of individual SIV proteins are colored as follows: Gag (red), Nef (green), Tat/Rev (blue), Vif/Vpr/Vpx (yellow), and Pol (pink). C: Comparison of cumulated $CD8^+$ T cells or $CD4^+$ T cells specific to the viral proteins Gag, Pol, Nef, Tat/Rev, and Vif/Vpr/VpX between SIV infection-controlled and uncontrolled animals. w, weeks; d, days.

might play a role in other effector functions, such as antibody-dependent cell-mediated cytotoxicity to eliminate the infected cells. The antibody-mediated enhancement of viral antigen processing and cross presentation is also a mechanism potentially related to the control of SIV infection in vivo (49).

Reduced immunogenicity in the Δ5G Env vaccine was also noted in cellular immunity. The levels of stimulation of antigen-specific $CD8^+$ T cells and $CD4^+$ T cells are MHC I and MHC II dependent, respectively. As the macaques in this study have different MHC haplotypes (data not shown), the magnitude and breadth of SIV-specific T cells should vary among the animals. Nevertheless, the magnitude of SU-specific $CD8^+$ T cells and $CD4^+$ T cells in PBMCs was greater in the wt-Env vaccine group than in the Δ5G Env vaccine group. Although the expression of SU by expressing plasmids and that of Env by the vaccinia virus vector elicited by either the wt-Env vaccine or Δ5G Env vaccine were indistinguishable in cultured cells (Fig. 2), wt-Env might persist longer than Δ5G Env in vaccinated animals. T-cell epitopes in the wt-Env vaccine might therefore be more efficiently presented on MHC molecules in antigen-presenting cells than in the Δ5G Env vaccine. Differences in glycosylation levels might also affect some processes in antigen-presenting cells associated with the presentation of T-cell epitopes in Env.

Taking all results together, Env glycosylation might affect the presentation of B-cell epitopes and T-cell epitopes required for Ab-mediated and T-cell-mediated immunities related to the control of SIV infection.

As seen in viral loads and SU-specific T cell levels after challenge infection (Fig. 3 and 5), the effect of vaccination was limited. That seemed related to the development of escape mutants. Therefore, distinctive cellular immune responses after the challenge infection were also implicated in the control of SIVmac239 replication. The magnitude of virus-specific $CD8^+$ T cells did not always correlate with the suppression of viral replication as reported previously (1, 6), particularly in vector controls (Fig. 5 and 7A); however, selected epitope-specific CTL responses might be associated with infection control. Gag-specific CTLs are such candidates, because a high magnitude of Gag-specific $CD8^+$ T cells was significantly elicited in five control animals (Fig. 7C). The magnitude of Gag- or Tat/Rev-specific $CD4^+$ T cells was statistically correlated with infection control (Fig. 7C). This may simply indicate a lower depletion of virus-specific $CD4^+$ T cells in animals with lower viral loads as reported previously (11). Alternatively, these virus-specific $CD4^+$ T cells may play an important role in protective immunity (39). Taken together, these results implicated the dominant role of selected epitope-specific $CD4^+$ T cells and $CD8^+$ T cells for the control of SIVmac239 infection.

The challenge virus that should be used has been an important issue in AIDS vaccine studies (8, 10, 12). Many studies have reported impressive efficacy in a pathogenic-SHIV macaque model (3, 4, 45, 46); however, pathogenic SHIVs use CXCR4 as a coreceptor, whereas the majority of clinical isolates of HIV-1 use CCR5 (13, 27). Therefore, the challenge virus for an AIDS vaccine study should be an R5 virus, such as SIV (10). Consistent with this concern, a DNA prime–modified-vaccinia virus Ankara boost regimen, inducing broad SIV-specific T-cell responses, reduced the initial viral replication but did not prevent disease progression against SIVmac239 challenge (18). Thus, vaccine studies using pathogenic SHIV should be reevaluated by using an R5 virus (10).

Matano et al. reported that a DNA prime-Sendai virus boost regimen induced the CTL-based control of SIVmac239 in rhesus macaques (27). This study demonstrated that a DNA prime-vaccinia virus WR boost regimen expressing only Env controlled the chronic infection of SIVmac239 in rhesus macaques. The relatively lower viral loads in macaques from Myanmar or Laos than in those of Indian origin might contribute to the control of SIVmac239 infection. Nevertheless, it is important that these two studies demonstrated the efficacies of the two vaccine regimens against highly pathogenic SIVmac239. In earlier studies, other R5 SIVs were used as a challenge virus for an efficacy study of vaccine candidates. An Env-based vaccine in vaccinia virus vector priming and subunit protein boosting protected cynomologous macaques against homologous SIVmne clone E11S (42). In recombinant modified vaccinia virus, Ankara viruses expressing Gag-Pol and/or Env exhibited vaccine efficacy because of reduced viremia and the increased survival of rhesus macaques infected with uncloned SIVsmE660 (41). Accordingly, the efficacy of vaccine candidates might be influenced by the experimental conditions. Thus, well-defined animal models with detailed virological, immunological, and genetic information and suitable challenge viruses are required for the evaluation of vaccine candidates and the development of an AIDS vaccine.

This study demonstrated the importance of Env as a component of the AIDS vaccine, and Env-specific $CD8^+$ and $CD4^+$ T cells and nonneutralizing Env-specific Ab were suggested as protective immunity components. Quintuple deglycosylation in Env reduced vaccine efficacy and Env-specific immune responses. Env may therefore be comprised of appropriate antigenic properties to elicit humoral and cellular immune responses required for protective immunity against homologous or allele-specific target SIV/HIV. These properties could be modified by the alteration of glycosylation.

In conclusion, although Env is an important immunogen for the AIDS vaccine, Env properties, including glycosylation, should be carefully considered to design vaccines specific to the targeted viruses.

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Genotypes at chromosome 22q12-13 are associated with HIV-1-exposed but uninfected status in Italians

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Objective: Despite multiple and repeated exposures to HIV-1, some individuals possess no detectable HIV genome and show T-cell memory responses to the viral antigens. HIV-1-reactive mucosal IgA detected in such uninfected individuals suggests their possible immune resistance against HIV. We tested if the above HIV-1-exposed but uninfected status was associated with genetic markers other than a homozygous deletion of the *CCR5* gene.

Methods: Based on our mapping in chromosome 15 of a gene controlling the production of neutralizing antibodies in a mouse retrovirus infection, we genotyped 42 HIV-1-exposed but uninfected Italians at polymorphic loci in the syntenic segment of human chromosome 22, and compared them with 49 HIV-1-infected and 47 uninfected healthy control individuals by a closed testing procedure.

Results: A significant association was found between chromosome 22q12-13 genotypes and a putative dominant locus conferring anti-HIV-1 immune responses in the exposed but uninfected individuals. Distributions of linkage disequilibrium across chromosome 22 also differed between the exposed but uninfected and two other phenotypic groups.

Conclusions: The data indicated the presence of a new genetic factor associated with the HIV-1-exposed but uninfected status.

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Keywords: HIV-1, exposed seronegatives, genetic background, chromosome 22, neutralizing antibody, synteny, association study

Introduction

The absence of clinical progression in some HIV-1-infected individuals and the lack of a detectable HIV-1 genome despite multiple and repeated exposures to this virus in some groups of people are noteworthy phenomena when considering the development of preventative and therapeutic means to HIV infection [1–3]. There are individuals who show strong HIV-1 antigen-specific T-lymphocyte responses and HIV-1-reactive mucosal IgA production despite the absence of detectable plasma HIV-1 RNA and HIV-1 cDNA from peripheral blood mononuclear cells (PBMC) [4–6]. HIV-1-neutralizing activity exerted by the IgA isolated from some HIV-1-exposed but uninfected individuals (EUI) [7–9] has suggested a possible contribution of the

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host immune responses to the resistance against HIV infection. However, genetic factors that may influence the observed T-cell priming and the production of anti-HIV-1 IgA without the establishment of HIV replication are currently unknown.

Host genetic factors influencing viral entry and replication and antiviral immune responses have been extensively studied in mouse models of retroviral infections, among which the best analyzed is Friend mouse leukemia virus complex (FV) [10–13]. Host gene loci that control the entry and replication of FV in the target cells have been identified [14–17]. In addition, MHC class II loci directly restrict the T-helper cell recognition of the viral antigens [18–20], while a class I locus influences the production of cytokines from virus-specific T cells [21]. Another locus that has been mapped to chromosome 15 strongly influences the persistence of viremia after FV infection [12,22–25]. However, the possible relationship between the above persistence of viremia and production of virus-neutralizing antibodies has not been directly examined. Here we have performed linkage analyses on a mouse locus that influences the production of virus-neutralizing antibodies upon FV infection. An extension of this mouse study unexpectedly led us to find human chromosomal markers that are associated with the presence of HIV-1-reactive immune responses in HIV-uninfected individuals.

Methods

Mice, virus, and assays for neutralizing antibodies

B10.A and A/WySn mice were purchased from Japan SLC, Inc., Hamamatsu, Japan and The Jackson Laboratory, Bar Harbor, Maine, USA, respectively. The F_1 crosses and backcross mice were bred and maintained at Rakuno Gakuen University and Kinki University School of Medicine under specific pathogen-free conditions. The following experiments were approved by and performed under guidelines of each university. FV was prepared and inoculated as described [18,20–27]. FV-neutralizing antibodies were titered by immunoenzymatically visualizing foci of virus infection as described [20,26,27].

Analyses of simple sequence length polymorphisms (SSLP) and linkage mapping in mice

Genomic DNA was prepared from the tail tip of each mouse using DNeasy Tissue Kit (QIAGEN GmbH, Hilden, Germany). A pair of oligonucleotide primers for each microsatellite locus was designed based on the sequence information listed in the Genetic and Physical Maps of the Mouse Genome site (http://www-genome.wi.mit.edu/cgi-bin/mouse/) and ordered from QIAGEN GmbH. Fifty nanogrmas of each template DNA was subjected to 35 cycles of PCR amplification using a recombinant *Taq* polymerase (Invitrogen Life Technologies, Carlsbad, California, USA). PCR products were separated by electrophoresis in 4% agarose gel and visualized by ethidium bromide staining. Correlation between genotypes at each examined locus and the presence or absence of virus-neutralizing antibodies was analyzed by Pearson's χ^2 test. Map orders of the chromosomal loci and log-of-the-odds (LOD) scores were determined by multipoint analyses using MAPMAKER/EXP version 3.0b (The Whitehead Institute, Massachusetts, USA).

EUI and HIV-1-infected individuals

Forty-two heterosexual couples discordant for HIV-1 serostatus were enrolled. The female partner was HIV-1-infected in 32 couples, whereas the male partner was HIV-1-infected in the remaining 10 couples. The diagnosis of HIV-1 infection was made based on the detection of plasma HIV-1 RNA before the initiation of antiretroviral drug treatment and significant titers of serum anti-HIV-1 IgG antibody as described in the following section. The inclusion criteria for the EUI group were a history of multiple unprotected sexual episodes for >4 years with an average of eight reported unprotected sexual contacts per year (range 5 to >40) at the time of inclusion, and at least three episodes of at-risk intercourses within 4 months prior to the study point. Forty-two of the 49 HIV-1-infected individuals studied here are the steady and reportedly monogamous partners of the above EUI individuals. In all the infected individuals the diagnosis of HIV-1 infection was made during their chronic phase, and thus unprotected sexual intercourses had been initiated long before their diagnosis. Mean CD4 cell count of the infected partners at the time of this study was 370×10^6/l (range 36×10^6–850×10^6/l). Seven additional age- and sex-matched HIV-1-infected individuals were added to the study, and their HIV-related phenotypes were within the ranges of the above infected partners. All the EUI and HIV-1-infected individuals and 47 uninfected, age- and sex-matched healthy volunteers were enrolled from the Santa Maria Annunziata Hospital, Firenze, and the Luigi Sacco Hospital, Milano. All of the enrolees are Caucasians from the Toscana region. The ethics committees of the above hospitals have approved the research protocols. The genotyping analyses were approved by Kinki University School of Medicine. Written informed consent was obtained from all enrolees, and samples were anonymized and analyzed in a blinded fashion.

Phenotype definitions

Plasma HIV-1 load was quantified by using the AMPLICOR HIV Monitor test (Roche Diagnostic Systems, Nutley, New Jersey, USA) as described [4,6]. Possible presence of HIV-1 cDNA in PBMC and in cells

isolated by urethral swabbing or uterine cervical brushing was analyzed by a reverse transcription-PCR method [4,6]. For the detection of mucosal anti-HIV-1 IgA, 500 μl of mucus was collected from each enrolee by swabbing from the urethra or vagina [4,6]. Titers of serum anti-HIV antibodies were determined by an enzyme-linked immunosorbent assay (EIA) using Abbot HIV-1/2^+ test (Abbott Laboratories, Abbott Park, Illinois, USA). This assay detects HIV-specific IgG and IgM [7]. Titration of HIV-1-specific IgA in the mucosal secretions was performed by an isotype-specific EIA using the HIV EIA test (Calypte Biomedical Corp., Berkeley, California, USA) with modifications [4–9]. HIV-1-reactive memory T cells in the peripheral blood were enumerated by an enzyme-linked immunospot assay for interferon (IFN)-γ as described [6].

Analyses of human SSLP markers

Five-hundred nanograms of genomic DNA extracted from PBMC of each examined individual was used as the template for 40 cycles of PCR amplification using the flanking primer sets designed based on the sequence data compiled in the Ensembl Genome Browser (http://www.ensembl.org/) and ordered from QIAGEN GmbH. Each forward primer was labeled with a florescent dye, and 50–100 fmol of PCR amplified fragments were applied onto an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, California, USA) with appropriate size markers. Peak identification and size measurements were done with the GeneScan software (Applied Biosystems). To determine absolute fragment sizes, PCR products obtained from two or more homozygotes for each locus were cloned into pCR2.1-TOPO vector (Invitorogen Life Technologies) and sequenced by using the M13 forward primer until six or more identical clones were observed for each allele.

Population genetic analyses and detection of linkage disequilibrium (LD)

Genotypic data were analyzed for possible population differentiation and LD between pairs of loci by using the Arlequin 2.001 (Genetics and Biometry Laboratory, University of Geneva, Switzerland). A population-pairwise genetic distance test using pairwise F_{ST} and extended exact test were performed to examine possible population differentiation. A likelihood ratio test was performed to examine the possible LD between pairs of loci. A total of 100,172 permutations on 10 initial conditions were performed by the expectation maximizing algorithm for each pair of loci.

Statistical analyses

Distributions of allele frequencies at each examined locus were compared between each pair of the three phenotypic groups by a Monte-Carlo approach using the CLUMP software [28]. T2 statistic was chosen because many cells in the contingency tables contained values ≤ 2.

To examine the possible presence of a dominant allele having different frequencies between the three phenotypic groups, mathematical analyses were performed based on the assumption that the number of individuals possessing each genotype had a multinominal distribution. Since the number of candidate dominant alleles was more than one, multiple comparisons were taken into consideration. The test statistics for alleles i and j, t_i and t_j, respectively, can be strongly correlated especially when most of the individuals having allele i or j are of the genotype i/j. Therefore, the typically used Bonferroni correction may be too conservative. A universally applicable method for overcoming this problem is a closed testing procedure [29], where t_i is based on a well-acquainted variance stabilizing transformation and the test statistic for a common hypothesis is based on the maximization of t_i's. To calculate the P-values, we applied a parametric bootstrap [30] based on the asymptotic null distribution of t_i's. Details of the mathematical methods are described in the Appendix section.

Results

Linkage mapping of a mouse locus controlling FV-neutralizing antibodies

When (B10.A × A/WySn)F_1 and A/WySn mice that share FV-susceptible *Fv-1*$^{b/b}$, *Fv-2*^{s}, and H2$^{a/a}$ genotypes were tested for their production of virus-neutralizing antibodies, none possessed a detectable level of neutralizing antibodies at post-infection day (PID) 10. Neutralizing antibodies remained undetectable at PID 15 and 20 in parental A/WySn mice. In contrast, all the infected (B10.A × A/WySn)F_1 mice possessed a significant neutralizing titer at PID 15, and the titers increased toward PID 20 (Fig. 1a). Therefore, possible segregation of neutralizing titers in (B10.A × A/WySn) × A/WySn backcross mice was examined by testing them at PID 15, 17, and 21. Virus-neutralizing antibodies were not detectable in 63 (44%) of the 143 backcross mice at PID 15 (Fig. 1b), suggesting that a single locus is involved in the production or lack of production of neutralizing antibodies. For linkage analyses, we concentrated genotyping on chromosome 15, because initial analyses performed by using 43 separate backcross individuals showed significant correlation between virus-neutralizing titers at PID 17 and genotypes at four loci in chromosome 15 (data not shown). The results of linkage analyses performed by using the 143 backcross mice indicated a strong correlation between genotypes at marker loci in chromosome 15 and titers of virus-neutralizing antibodies at PID 15, with the strongest correlation ($\chi^2 = 74.0$, $P = 1.17 \times 10^{-7}$) observed at the D15Mit71 locus (Fig. 2). Linkage mapping with MAPMAKER/EXP located a single locus determining the presence or absence of virus-neutralizing antibodies at PID 15