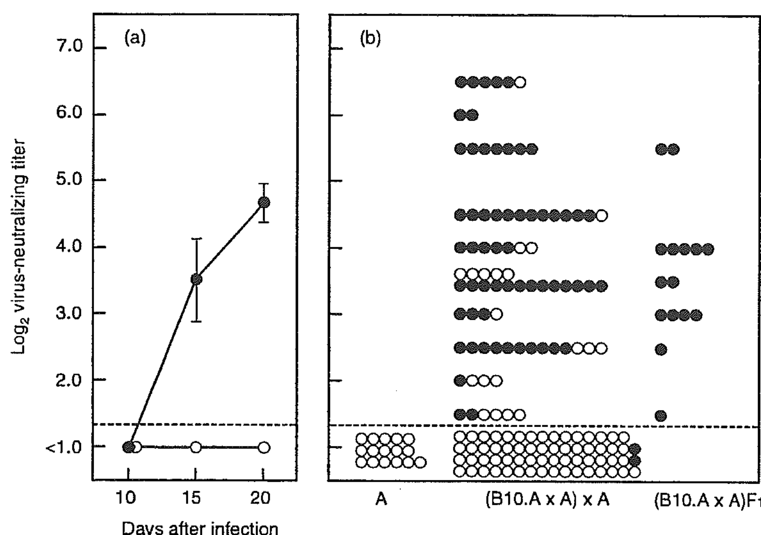

Fig. 1. Titers of virus-neutralizing antibodies in FV-infected mice. (a) Changes in the average titer ($n = 11-16$) of virus-neutralizing antibodies in (B10.A × A/WySn)F_1 (●) and A/WySn (○) mice at different time-points after infection with 150 spleen focus-forming units of FV. SEM. are shown with the bars. The dashed line indicates the limit of detection. (b) Titers of virus-neutralizing antibodies in each individual mouse tested at PID 15. Genotypes at the D15Mit71 locus are either homozygous for the A/WySn-derived allele (○) or heterozygous for the B10.A-derived and A/WySn-derived alleles (●).

between the D15Mit71 and D15Mit171 loci. Further mapping was performed by genotyping the backcross animals that possessed a critical recombination between the D15Mit28 and D15Mit171 loci. As a result, eight backcross mice that possessed reciprocal recombination within this region were identified (Fig. 2). Since a significant correlation ($P = 0.029$ by two-tailed Fisher's exact test) between genotypes at the D15Mit71, D15Mit2, D15Mit214, D15Mit69, and D15Mit70 loci and the production of virus-neutralizing antibodies at PID 15 was observed in these recombinant animals, it is conceivable that the locus controlling the production of FV-neutralizing antibodies is located within the region telomeric to the D15Mit1 and centromeric to D15Mit118 loci at the widest estimation.

Genetic analyses of HIV-1-exposed and uninfected Italians

The above region of mouse chromosome 15 harbors previously mapped genes that are known or likely to affect immune cell development and/or activation and retroviral replication. Therefore, we next explored the possibility that a putative ortholog of the above mouse locus might influence immune responsiveness in HIV-1 infection. Because of the route of transmission of HIV-1 and resultant rarity of multicase families, linkage analyses comparing affected and unaffected siblings are impossible. Thus, we performed a simple association study by comparing genotypes between the exposed but uninfected and HIV-1-infected individuals, hypothesizing that efficient anti-HIV-1 immune responses are associated with the presence of a dominant genetic factor which might be an ortholog of the above mouse locus conferring the ability to produce FV-neutralizing antibodies. Thus, the three phenotypically distinct groups of individuals (Table 1) were genotyped at the loci shown in Fig. 2. The examined groups were not different to each other when tested for population-pairwise genetic distance ($P > 0.17$), in accordance with their all being Caucasians enrolled from the Toscana region of Italy. When allele frequencies were compared among the three phenotypic groups, their distribution at the D22S277 locus differed between the EUI and healthy control groups at $P = 0.0396$. No significant difference was observed at the other loci. When likelihood ratio tests were performed for all possible pairs of the examined loci, a highly significant LD of an exact $P < 0.0004$ level was observed in all three groups between the D22S284, D22S423, and D22S299 loci, reflecting their close physical locations (Fig. 2 and Fig. 3). A similarly significant LD ($P < 0.00002$) was observed between the D22S418 and D22S1166 loci in all three groups, confirming their close genetic locations. Interestingly, a highly significant LD ($P < 0.00002$) was observed between the D22S276 and the above surrounding loci in both the HIV-1-infected and healthy control groups, but this was not observed in the EUI group (Fig. 3).

When frequencies of individuals possessing a particular allele at a given locus were compared among the three phenotypic groups by adopting a dominant model, objective mathematical analyses revealed multiple loci with significant differences (Table 2). These individual

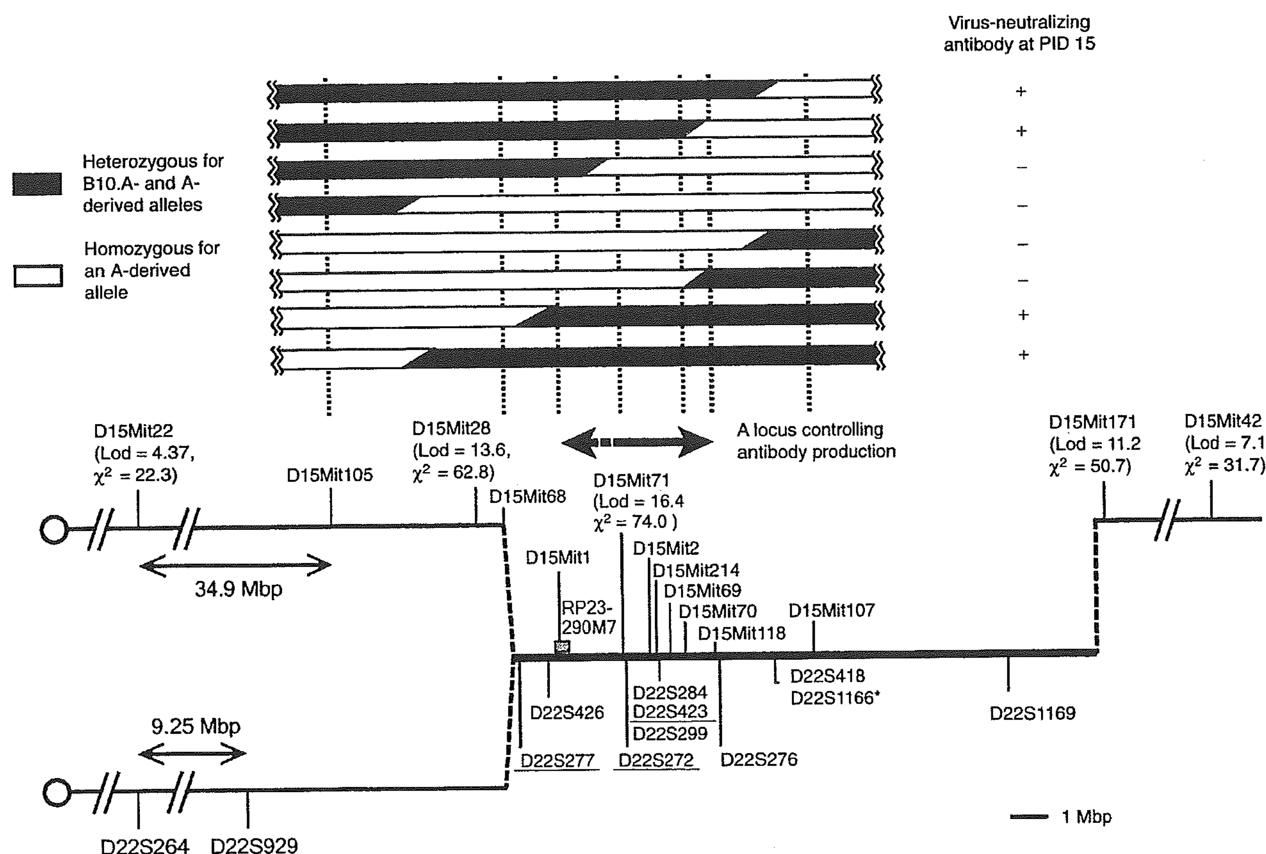

Fig. 2. The order of and distance between microsatellite markers located within the syntenic region of mouse chromosome 15 (the upper half of the diagram) and human chromosome 22 (the bottom half). Physical mapping and synteny data are based on those compiled in the Ensembl Genome Browser (http://www.ensembl.org/). Centromeres (o) are placed on the left. The mouse marker D15Mit28 has not been physically mapped, but genetic linkage data archived in the Mouse Genome Informatics site (http://www.informatics.jax.org) indicate that this locus, mapped at 43.7 cM, is closely linked to the D15Mit68 locus at 44.1 cM. Note that, although D15Mit1 is currently not included in the physical map of the mouse genome archived in the Ensembl Genome Browser, we identified the flanking primer and repeat sequences of this microsatellite marker (base numbers 47915-48097) within a clone of bacterial artificial chromosome, RP23-290M7, harbouring the segment of mouse chromosome 15 (shown with the hatched box). Lod scores and χ^2 values indicating degrees of genetic association between the identified genotypes and virus-neutralizing titers at PID 15 for each examined mouse locus are shown in parentheses. The estimated location of the putative mouse locus controlling the production of FV-neutralizing antibodies is shown with the bidirectional arrow. Human loci at which genotypes were compared between the EUI, HIV-infected, and healthy control groups are also shown, and the loci at which significant genetic differences were observed by the closed testing procedures are underlined. *The D22S1166 locus has not been physically mapped, but the genetic maps archived at the Center for Medical Genetics, Marshfield Clinic Research Foundation (http://research.marshfieldclinic.org/genetics/) indicate that this and the D22S418 loci are closely linked.

differences were further examined for possible false rejection of a single equal-frequency hypothesis due to multiple comparisons by using the closed testing procedure. As a result, frequencies of individuals possessing either the allele 156 or 158 at the D22S277 locus were significantly different between the EUI and two other groups, those of the individuals possessing the allele 134 at the D22S272 locus were significantly different between the EUI and healthy control groups, and those of individuals possessing the allele 229 at the D22S423 locus also differed significantly between the EUI and HIV-infected individuals.

Discussion

In the present study we have demonstrated that the presence or absence of virus-neutralizing antibodies in FV-infected (B10.A × A/WySn) × A/WySn backcross mice at PID 15 is closely associated with their genotypes at the chromosome 15 loci. The linkage mapping data indicated that a single gene controlling the production of virus-neutralizing antibodies was located near the D15Mit71 locus, colocalizing with the previously mapped *Rfv-3* locus [23,24]. Since the *Rfv-3*-associated phenotypes were defined by clearance of viremia by

Table 1. HIV-1-related phenotypes of the three groups genetically analyzed in the present study.

Group	Age	Plasma HIV load (copies/ml)	Urethral/ vaginal anti-HIV-1 IgA ($A_{405\ nm}$)	Serum anti-HIV-1 IgG ($A_{405\ nm}$)	HIV-1 envelope-reactive IFN-γ ELISPOT[a] (/10^6 cells)
HIV-1-exposed but uninfected ($n = 42$)	40.1 ± 1.4	Not detectable (all)[b]	0.556 ± 0.047[c] (0.12–1.14)[d]	0.004 ± 0.0006 (0.00–0.02)	131.2 ± 11.3[e] (5–280)
HIV-1-infected ($n = 49$)	40.8 ± 1.9	<40 – 750,000 (median: 400)[f]	0.360 ± 0.039 (0.11–0.87)	0.793 ± 0.069 (0.11–1.25)	63.4 ± 9.2 (<5–120)
Healthy control ($n = 47$)	37.8 ± 3.6	Not detectable (all)[b]	0.002 ± 0.0006 (0.00–0.03)	0.002 ± 0.0001 (0.00–0.01)	<5 (<5–15)

Numbers shown are mean ± SEM except for the Plasma HIV load.
[a]PBMC were stimulated with a mixture of five synthetic peptides representing the immunodominant and promiscuous epitopes identified in the HIV-1 gp160 [6], and spots of secreted interferon (IFN)-γ were visualized and counted by using a biotin-conjugated anti-IFN-γ antibody.
[b]All enrolees were tested for the presence of HIV genome by measuring plasma HIV-1 RNA and by detecting HIV-1 cDNA from total RNA of peripheral blood mononuclear cells. In the case of the exposed uninfected individuals (EUI), possible presence of HIV-1 cDNA was also tested in mucosal cells by reverse transcription–PCR. All the individuals in the EUI and healthy control groups were negative for all these tests.
[c]Significantly higher than the average for the HIV-1-infected individuals at $P = 0.0022$ by Welch's t test. A non-parametric analysis by Mann–Whitney's U-test also showed a significant difference, $P = 0.021$.
[d]Ranges of observed values are shown in parentheses for IgA and IgG titers and enzyme-linked immunospot (ELISPOT) foci.
[e]Significantly higher than the average for the HIV-1-infected individuals at $P = 0.015$ by Welch's t test, and $P = 0.0002$ by Mann–Whitney's U-test.
[f]All the HIV-1-infected individuals were enrolled during their chronic phase of infection before the initiation of antiretroviral drug treatment, but 32 of the 42 infected partners and the seven additional HIV-1-infected enrolees were receiving the highly active anti-retroviral therapy at the time of this study. The range of plasma viral load in the 13 currently untreated individuals in the infected group was 5900 to 750 000 (median, 65 000) copies/ml.

35–40 days after FV infection [22–25], and neutralizing antibodies were detectable at as early as PID 15 in mice possessing the B10.A-derived dominant allele (Fig. 1), it is conceivable that early production of virus-neutralizing antibodies is associated with early clearance of viremia.

It is intriguing that genotypes at microsatellite loci located within the segment of human chromosome 22 syntenic to mouse chromosome 15 differed between the HIV-1-exposed but uninfected and HIV-1-infected groups of individuals. The strongest association was observed at the D22S423 locus where the frequency of individuals possessing the allele 229 was significantly higher in the EUI group than in the HIV-1-infected one even after corrections for multiple comparisons were made. This marker locus is located in the middle of the chromosomal segment corresponding to the region of mouse chromosome 15 that harbors the gene locus controlling the production of virus-neutralizing antibodies (Fig. 2). It may also be worth noting that the alleles 156 and 158 at the D22S277 locus that are rare (5.6 and 9.3% per haploid chromosome, respectively) among the Caucasian CEPH

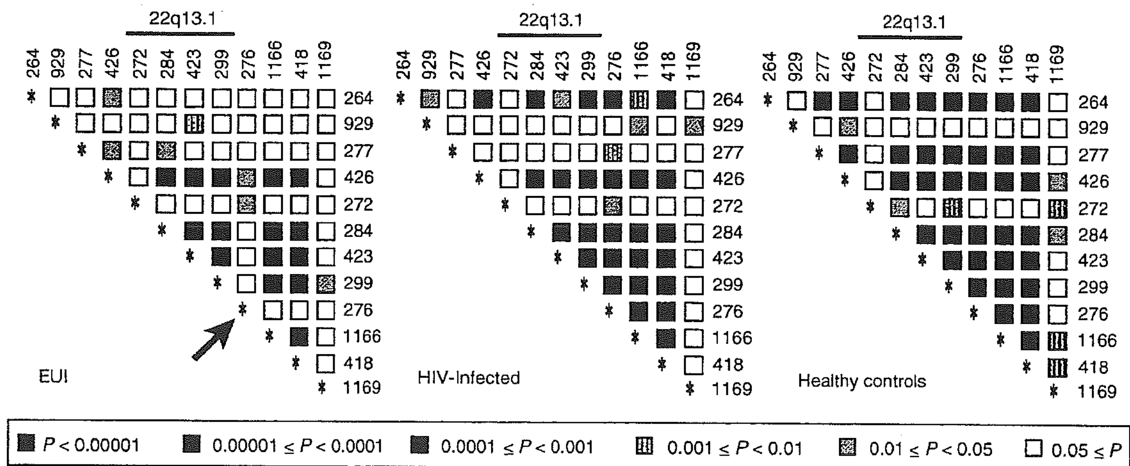

Fig. 3. Distributions of LD across chromosome 22. LD is plotted for the 12 microsatellite loci examined against each other, each represented by a small square. Locus names are abbreviated by omitting D22S. The pattern of distribution of LD is shown for each of the three phenotypic groups for comparison. Filling patterns reflect the significance level in exact P values as indicated at the bottom. The arrow indicates observed disruption within the segment of multilocus LD in the EUI group. The allele distributions in the three examined groups did not deviate from the Hardy-Weinberg equilibrium at any examined locus, except only at the D22S418 locus in the healthy control group, when exact tests using a Markov chain (100,172 chain length and 1,000 dememorization steps) were performed.

Table 2. Chromosome 22q association analyses.

Cytogenetic location	Locus	Allele size (bp)	Frequency in the EUI group[a]	Compared to[b]	Odds ratio	*P* value	
						Individual hypothesis	Common hypothesis
22q11.21	D22S264	188	0.405	HIV	3.276	0.0142	ns[c]
		198	0.238	HC	0.306	0.0128	ns
22q12.2	D22S277	158	0.310	HIV	3.766	0.0152	ns
		156 or 158	0.429	HIV	3.656	0.0066	0.0378[d]
				HC	3.875	0.0079	0.0448[d]
		162	0.262	HC	0.379	0.0371	ns
22q13.1	D22S272	134	0.667	HC	0.308	0.0243	0.0466[d]
22q13.1	D22S423	229	0.333	HIV	4.200	0.0087	0.0317[d]
22q13.2	D22S418	145	0.605	HC	2.520	0.0475	ns
	D22S1166	130	0.571	HC	2.816	0.0266	ns
22q13.32	D22S1169	126	0.643	HC	2.528	0.0313	ns

[a]Frequencies of individuals possessing the indicated allele either heterozygously or homozygously.
[b]HIV, HIV-1-infected individuals; HC, Healthy controls.
[c]Not significant (ns) at the $P < 0.05$ level.
[d]These differences are also significant ($P < 0.05$) after conventional Bonferroni correction.

population [31,32] were more frequently observed in the EUI group (9.5 and 17.9%, respectively). There were significant differences in the frequency of the allele 156 and that of the allele 158 ($P = 0.035$ and 0.0061, respectively, by two-tailed Fisher's exact test) when the HIV-1-infected and healthy control groups were combined and compared with the EUI group. The rates of microsatellite mutation are much higher than those of point mutation at coding genes [33], and the most common stepwise mutation is biased toward the reduction of repeat numbers for microsatellites of >20 repeats [34]. Thus, we can justifiably hypothesize that the alleles 156 and 158 at the D22S277 locus (25 and 26 dinucleotide repeats, respectively) are both linked to the same putative allele that is associated with the presence of immune responses to HIV-1 in the uninfected individuals. In this regard, the variance stabilizing analyses performed by assuming that the alleles 156 and 158 are both linked to a single dominant genetic factor resulted in the demonstration of significant differences between the EUI and HIV-1-infected, and the EUI and healthy control groups, and these individual null hypotheses were also rejected (significant difference validated) after the correction for multiple comparisons was made (Table 2). Further, when the same comparison was made between a combined group of the HIV-1-infected and healthy control individuals and the EUI group, the frequency of individuals possessing either the allele 156 or 158 was significantly higher among the EUI ($P = 0.0019$), and this was highly significant even after the correction for multiple comparisons was made ($P = 0.0121$). The combination of the HIV-1-infected and healthy control groups was justifiable because neither allele frequency distributions nor frequencies of individuals possessing the allele 156 or 158 were significantly different between the two groups. Thus, genotypes at multiple loci within the segment of human chromosome 22 that is syntenic to mouse chromosome 15 are significantly associated with the presence of strong mucosal and T-cell immune responses against HIV-1 (Table 1) in HIV-uninfected Italians. Furthermore, the multilocus LD spanning from D22S284 to D22S418 which is observed in both the HIV-infected and healthy control groups is disrupted at the D22S276 locus in the EUI group (Fig. 3). This observation is consistent with the hypothesis that in the ancestors of the EUI individuals a possible recombinational or mutational event might have happened in the chromosomal segment surrounding this locus.

Production and class-switching of virus-neutralizing antibodies in FV-infected mice are dependent on CD4 T-cell functions [26,27,35], and the priming of CD4 T-helper cells with a singe-epitope peptide resulted in the early production and class-switching of virus-neutralizing antibodies and strong protection against FV infection [20,27]. Likewise, EUI individuals enrolled into the present study possessed significantly higher amounts of mucosal anti-HIV-1 IgA and larger numbers of HIV-1 envelope-reactive T cells in the peripheral blood in comparison with the HIV-infected individuals (Table 1). IgA antibodies isolated from some EUI individuals have been shown to inhibit the replication of primary HIV-1 isolates [7] and HIV-1 transcytosis across the epithelial cells *in vitro* [8,36]. Thus, it is possible that efficient priming of T cells with HIV antigens might have resulted in rapid production of HIV-1-reactive IgA antibodies which, in turn, might have been involved in the possible immune protection in the EUI individuals. In this regard, it is noteworthy that IFN-γ production is required for the control of viremia and class-switching of virus-neutralizing antibodies in FV infected mice [37].

It has been shown that CD4 T cell-dependent early IgA responses against influenza virus infection can be generated in the absence of virus-specific IgM and IgG [38], and costimulatory signals required for mucosal IgA production are strikingly different from those needed for systemic antibody responses [39]. Similarly, mucosal IgA

responses to T-dependent HIV-1 antigens might be stimulated without inducing serum IgG production, and putative human homolog of the mouse gene influencing the T cell-dependent production of FV-neutralizing antibodies might be involved in the above activation of mucosal IgA-production in EUI individuals. In fact, the segment of mouse chromosome 15 between the D15Mit1 and D15Mit118 loci and the corresponding segment of human chromosome 22 harbor several genes that are known to be involved in T- and B-cell growth and activation. Expression analyses of these candidate genes both in the mouse model and in humans are currently underway.

None of the previously reported human genes that affect the risk of HIV acquisition are located in chromosome 22, *CCR5* and *CCR2* being located at 3p21, *SDF1* and *MBL2* at 10q11.1 and 10q11.2, respectively, *HLA* including the polymorphic *TNF* and *MIC* loci at 6p21.3, *KIR*s at 19q13.4, *IL10* at 1q31-32, and *SLC11A1 (NRAMP1)* at 2q35 [3,40–52]. In addition, the homozygous *CCR5-Δ32* mutation, which results in the lack of the HIV coreceptor [3,40–42], is known to be rare among the EUI individuals in Italy and Thailand [4,9,49], and was not found in the enrolees of the present study, although three of the 42 EUI individuals were heterozygous for this mutation (data not shown). In a very recent analysis of a separate cohort of repeatedly exposed but HIV-1-seronegative individuals in the USA, Liu *et al.* [53] demonstrated the lack of association between genotypes at the *CCR2*, *SDF1*, and *RANTES* loci and the uninfected status. The homozygous *CCR5-Δ32* mutation was also rare (3.2%) among the seronegative individuals. The same authors also noted a significant difference in the frequencies of heterozygosity at the polymorphic *DC-SIGN (CD209)* locus at 19p13.2 between the exposed but seronegative and HIV-1-infected groups: however, the observed frequency of heterozygotes was 3.2% (3/94), and thus, this genetic skewing could not explain the possible mechanisms that confer HIV resistance to the majority of the seronegative individuals. Altogether, our results have indicated the possible presence in human chromosome 22 of a novel genetic factor that is associated with strong T-cell and mucosal immune responses to HIV-1 antigens.

Acknowledgements

We thank M. P. Gorman and S. Tsuji-Kawahara for critically reading the manuscript.

Sponsorship: This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, including the High-Tech Research Center Project (2002), from the Ministry of Health, Labor and Welfare of Japan, from the Japan Health Science Foundation, from the Istituto Superiore di Sanita' "Programma Nazionale di Ricerca sull' AIDS", from Centro di Eccellenza CISI, from the EMPRO and AVIP EC WP6 Projects, and from the Tuscancy Region, General Direction, Right to Health and Solidarity Policy.

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Appendix

The possible presence of a dominant allele having different frequencies between the phenotypic groups was examined as follows: Define x_{ij} as the number of individuals having the genotype i/j ($i \leq j$) for the EUI group, where $n = \sum_{i \leq j} x_{ij}$ is the total number of individuals belonging to this group. Assume that $x = (x_{ij})_{i \leq j}$ has a multinomial distribution with the parameter $a = (a_{ij})_{i \leq j}$, where $\sum_{i \leq j} a_{ij} = 1$. For convenience, let $a_{ji} = a_{ij}$. Similarly, define the notations y, b and z, c for the HIV-1-infected and healthy control groups, respectively. The frequency of the individuals having the allele i for the EUI group is expressed as $a_i = \sum_k a_{ik}$. Similarly, define b_i and c_i. The hypothesis where the frequencies of the individuals having the allele i for the EUI and HIV-infected groups is the same is expressed

as H_i: $a_i = b_i$. Similarly, consider the hypothesis $a_i = c_i$ to compare the EUI with healthy control groups.

We tested whether or not the frequency of individuals possessing a certain dominant allele in the EUI group was different from those in other groups. Since there are multiple candidate alleles at each locus, we must take multiple comparisons into consideration. Let t_i be the test statistic for allele i. t_i and t_j 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 can 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 values.

Let $\overline{H}$ be the closed set consisting of all the intersections of the hypotheses' H_i values. Assume that we can make the reject region with common significance level α for any hypothesis $H \in \overline{H}$. The closed testing procedure says that we can reject $H \in \overline{H}$ only after we reject all the hypotheses including H, using the corresponding reject region. Let t_i be the standardized test statistic for the hypothesis H_i. The corresponding reject region becomes $W_i = \{|t_i| > e_i\}$. Consider a common hypothesis H. For example, let H be the intersection of $H_1, \ldots, H_I$. The corresponding reject region can be defined by $W = \{\max_{i=1,\ldots,I}|t_i| > e\}$. We used the following variance stabilizing type as the standardized test statistic:

$$t_i = \frac{\left(\sin^{-1}\sqrt{x_i/n_x} - \sin^{-1}\sqrt{y_i/n_y}\right)}{\sqrt{1/4n_x + 1/4n_y}}$$

where $n_x = \sum_{i \leq j} x_{ij}$ and $n_y = \sum_{i \leq j} y_{ij}$. As an advantage over the commonly used likelihood ratio and Pearson's χ^2 tests, the above type enables us to infer that the smaller a P value is the stronger the rejection of the corresponding null hypothesis, because the variances of the arcsine are constant independent of the samples. In view of the closed testing procedure, if the maximal intersection hypothesis $H \in \overline{H}$ is rejected, the individual hypothesis corresponding to the minimum P value can automatically be rejected. In addition, if the hypothesis corresponding to the minimum P value alone is rejected among the individual hypotheses, it is the only rejected hypothesis.

The joint distribution of t_i values can be approximated by the multivariate normal distribution under the null hypothesis, and the corresponding approximated P values can easily be calculated for the individual hypotheses. The approximated P values for a common hypothesis can be calculated by using the central limit theorem and the parametric bootstrap [30] based on the asymptotic null distribution of t_i values. To avoid unnecessary disturbances, we tested only the hypotheses having the estimated frequency ≥ 0.1 when considering the common hypotheses, because alleles with a frequency < 0.1 cannot explain the phenotype of the whole group. Calculations were performed by drawing 100 000 random samples from the approximated multivariate normal distribution for each hypothesis.

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Recent Res. Devel. Virol., 6(2004): 105-118 ISBN: 81-7895-127-4

7 Host genes that influence immune and non-immune resistance mechanisms against retrovirus infections

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Abstract

Several host genes control retroviral replication and pathogenesis. These include host genes that directly affect the replication of retroviruses in target cells of infection and those affecting the host immune responses to the viral antigens. Host genetic factors that affect both retroviral replication and immune responses to the viral antigens have been best studied in mouse models of Friend leukemia virus (FV) infection. The presence or absence of an appropriate receptor restricts the attachment and entrance of viral particles into target cells, and the product of the Fv-4 gene blocks a receptor on mouse cells. APOBEC

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enzymes mutate reverse-transcribed viral DNA, and the Fv-1 gene product interferes with the proviral integration. Growth induction of infected erythroid progenitor cells is affected by the Fv-2 mutation of a hematopoietic cell growth factor receptor, STK. Several genes located within the major histocompatibility complex (MHC) and a separate gene not linked to MHC control the host immune responses. The latter, Rfv-3, controls the production of virus-neutralizing antibodies, and thus affects the duration of viremia. Both MHC class I-restricted CD8$^+$ and class II-restricted CD4$^+$ T cells are required for spontaneous and vaccine-induced immune resistance against Friend virus infection. Further, natural killer (NK) cells are activated soon after the viral infection, and are indispensable for vaccine-induced immune protection of susceptible mice. Single-epitope peptides that prime CD4$^+$ T cells are effective in inducing protective immunity against FV, and effector mechanisms including NK cells, CD4$^+$ and CD8$^+$ cytotoxic cells, as well as virus-neutralizing antibodies are involved in protection in the vaccinated animals. The above knowledge of the host genes that influence resistance against mouse retroviral infection may be useful when considering preventative and therapeutic means to human retroviral infections.

Introduction

Upon entrance into target cells retroviruses reverse-transcribe their RNA genome into double-stranded DNA, and the resultant viral DNA integrates into cellular chromosomes as proviruses. Integrated proviral DNA replicates through the process of normal cell division along with the cellular genome, and is transferred to daughter cells as a part of the cellular chromosomes. Since most retroviruses do not show cytopathic effects, productively infected cells continue to divide, supplying ever-increasing sources of infectious virus particles. Thus, for the elimination of retroviruses, the destruction of extracellular virions is insufficient. Rather, the eradication of the host cells that harbor integrated proviruses is required, making the control of retroviral infections more difficult than that of other viruses. In addition, the destruction of retrovirus-infected cells inevitably results in the loss of their functions, potentially causing harmful outcomes in the host body. Such harmful complications of the destruction of retrovirus-infected cells by the host's immune system are known as retrovirus-induced immunopathology, which is implicated in the development of acquired immunodeficiency syndrome (AIDS) in human immunodeficiency virus (HIV) infection, human T-cell leukemia virus type-I (HTLV-I)-associated myelopathy, a neurological complication of HTLV-I infection, and some autoimmune diseases associated with chronic retroviral infections [1-3]. In addition, if a retrovirus infects germ cells, the integrated provirus is transmitted through generations as a constituent of the host genome, resulting in the presence of viral genome in all the cells of each

progeny. Such germ line-transmitted proviruses are called endogenous retroviruses, and they are inherited in a Mendelian manner. More than 1% of the whole human genome is occupied by endogenous retroviruses or their remnants. There is no way for the host immune system to distinguish the products of endogenous retroviruses from those of other chromosomal genes, and thus the immune system has to ignore the endogenous retroviral proteins through the mechanism of self-tolerance.

Integration of a provirus into chromosomes can also cause a physical disruption of functional cellular genes, which may result in the loss of their functions. In addition, since viral long terminal repeats (LTR) are strong promoters of gene expression, proviral integration can cause the activation of adjacent cellular genes, which may lead to their overexpression and resultant gain of cellular functions. These mechanisms are known to be involved in retrovirus-induced tumorigenesis and to cause physically and/or functionally discernible mutations [4]. Thus, retroviruses are most threatening to the genetic integrity of all animal species. In this regard, it stands to reason that the existing mammals have evolved by acquiring a battery of mechanisms that restrict the infectious entry and replication of retroviruses. These include cellular mechanisms that interfere with the attachment and fusion of virions to target cells, including the presence or absence of cell-surface receptors, the mechanisms that affect the uncoating of viral nucleocapsid or reverse transcription, and those that interfere with the process of proviral integration. In addition, there are other mechanisms that work at the level of the whole body, particularly the immune responses directed toward the viral proteins and infected cells.

Friend murine leukemia virus and host genes that affect its replication and pathogenicity

Host mechanisms that affect the retroviral replication and pathogenicities have been most extensively studied in the mouse model of Friend retrovirus infection [5, 6]. Friend mouse retrovirus complex (FV) is composed of replication-competent Friend murine leukemia helper virus (F-MuLV) and defective spleen focus-forming virus (SFFV), the latter of which induces rapid growth and terminal differentiation of infected erythroid progenitor cells. FV is known to induce fatal erythroleukemia associated with severe immunosuppression when inoculated into immunocompetent adult mice of susceptible strains. One of the advantages of the FV system in analyzing host factors that affect retroviral replication and pathogenesis is that FV causes the rapid development of readily detectable splenomegaly associated with severe polycythemia, and ultimately causes the development of fatal leukemia within a few months after inoculation into adult mice. Most other retroviruses cause

neoplastic and/or neurological diseases only when they are inoculated into neonatal mice, and it usually takes more than 6 months for the virus-induced disease to develop. Inoculation of neonatal mice with an exogenous retrovirus causes immunological tolerance, and this induction of immune unresponsiveness and resultant persistence of viremia are crucial in the development of the above pathologies. On the contrary, FV can cause splenomegaly and fatal leukemia even when the virus is inoculated into immunocompetent adult mice, making the analyses of host immune responses and their modifications by preventative and therapeutic modalities possible. In addition, the presence of inbred strains has made the genetic analyses of host factors that affect FV infection much easier than retroviral infections of any other species. In fact, the FV system has led the genetic and molecular analyses of host factors in retroviral infections for decades, which have recently been applied and expanded to human retroviral infections. In the following sections, cellular factors that directly interfere with FV replication and FV-induced disease development are summarized, followed by a detailed description and discussion of the host genes that influence the immune responses against FV antigens. The possible implications of the knowledge acquired through the analyses of FV infection for the development of antiretroviral vaccines are also discussed.

Cellular genes that directly influence the replication of FV

Retroviral infection starts with the attachment of virions to the surface of target cells and the fusion of the viral envelope with the cellular membrane. These processes are mediated by the interaction between the viral envelope glycoproteins and cellular receptors. The presence or absence of appropriate receptors that can interact with the incoming viral envelope protein thus determines the infectivity of retroviruses to target cells. There are four known groups of mouse retroviruses that differ in their ranges of host specificities: ecotropic viruses infecting only mouse and rat cells, xenotropic infecting non-mouse cells, amphotropic infecting both mouse and non-mouse cells, and polytropic showing variable infectivities to mouse and non-mouse cells. Infectious polytropic viruses are recombinants between infectious ecotropic and endogenous polytropic viruses that carry varying segments of an endogenous polytropic viral *env* gene [7]. Viral envelope proteins of retroviruses that belong to the same host range group interfere with each other: thus, cells productively infected with an ecotropic virus cannot be superinfected by another ecotropic virus because of the receptor blockade. Xenotropic and polytropic viruses partly interfere with each other because they share the same polymorphic receptors. The cell surface receptor molecule for ecotropic viruses is mCAT1, a cationic amino acid transporter [8-10]. The receptor for

xenotropic and polytropic retroviruses is XPR1, and its polymorphism through different species affects the infectivities of recombinant polytropic viruses to host cells of different species [11-14]. The receptor for amphotropic viruses is separate from the above ecotrpoic and xenotropic viral receptors, and its molecular identity is that of an inorganic sodium phosphate transporter, Pit2 [15, 16]. A related transporter, Pit1, serves as the receptor for Gibbon ape retrovirus and feline leukemia viruses of cats [17]. One of the host genes that restrict the infection with FV, *Fv-4*, is an endogenous ecotropic viral *env* gene, the product of which directly interacts with mCAT-1 and blocks the attachment of incoming ecotropic virus [18, 19].

Once the virion attaches on an appropriate cellular receptor and the viral envelope fuses with the cell membrane, the process of reverse transcription starts immediately through the activation of the viral RNA-dependent DNA polymerase by the changes of pH and Mg^{++} or Mn^{++} concentrations and through the supply of nucleotide precursors. At this stage, cellular cytidine deaminases that are members of the apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like (APOBEC) family show broad antiretroviral activity. Thus, after the penetration of a retrovirus into target cells and the initiation of reverse transcription of the RNA genome into DNA, APOBECs can induce the conversion of cytosine to uracil in minus strand viral cDNA, leading to a failure of reverse transcriptase and to a very high number of G-to-A mutations in the integrated proviral genome that greatly reduces viral efficiency in its replication [20, 21]. Human APOBEC3G and APOBEC3F are shown to restrict the replication of HIV-1 as well as mouse retroviruses. HIV-1 Vif protein counteracts the activity of APOBEC3G by forming a complex with it in the cytoplasm and by impeding its packaging into virions, thus preventing editing mutations upon infection of the newly generated viral particles [20-22]. The interaction with Vif also stimulates APOBEC3G degradation by the ubiquitine-proteasome pathway, resulting in the increase in viral replication [23, 24].

The process of the integration of double-stranded viral DNA into cellular chromosomes is mediated by a complex of the double-stranded viral DNA and viral core proteins. The preintegration complex composed of the viral DNA, *gag*- and *pol*-encoded proteins, as well as some cellular proteins, is transported into the nucleus, and viral integrase mediates the nick formation in the chromosomal DNA and proviral integration. The product of host resistance gene *Fv-1* interferes with the process of proviral integration through the interaction with the preintegration complex [25]. *Fv-1* is the *gag* gene of a polymorphic endogenous retrovirus that belongs to a new member of mouse endogenous viruses (MuERV) similar to human endogenous retroviruses (HERV). Ecotropic mouse retroviruses are divided into two major groups depending on their infectivity to cells of two different strain groups: one

represented by cells derived from NIH Swiss mice (N-type), and the other represented by cells of BALB/c origin (B-type). N-tropic mouse retroviruses infect N-type cells more efficiently, while B-tropic viruses infect B-type cells more efficiently. The resistance is dominant, and thus (BALB/c × NIH Swiss)F_1 mice are resistant to both N-tropic and B-tropic viruses. Some tissue culture-adapted viruses can infect both N-type and B-type cells with comparable efficiencies, and are called NB-tropic. The exact molecular mechanisms of the action of *Fv-1* gene product are still unknown; interestingly, however, the same mechanism seems to work in human cells restricting the integration of mouse retroviruses [26].

Molecular mechanisms of Friend virus-induced disease development

Upon inoculation of FV complex, the virus replicates first in vascular endothelial cells, and then reaches hematopoietic cells of the bone marrow and spleen [27]. The product of the SFFV *env* gene, gp55, makes a complex with erythropoietin receptor (EpoR), and this interaction induces the growth and terminal differentiation of erythroid progenitor cells (Fig. 1). EpoR is expressed in erythroid cells at the stages later than burst-forming unit of erythroid (BFU-e) and is mainly effective in the growth regulation of the colony-forming unit of erythroid (CFU-e). The transduction of growth signals from the gp55-EpoR

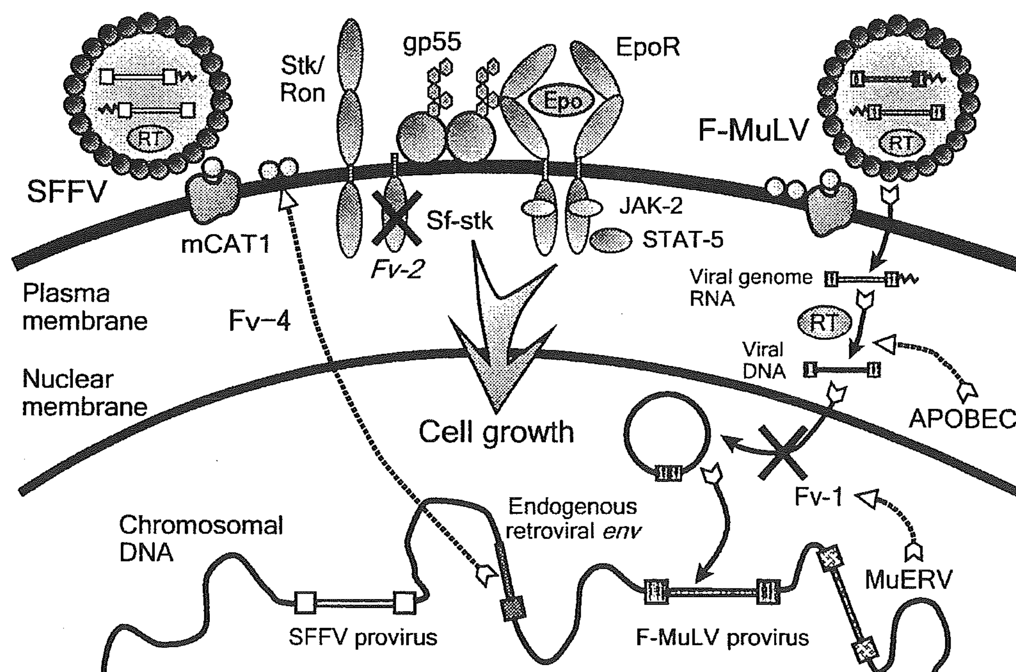

Figure 1. Schematic summary of the mechanisms of FV-induced erythroid cell proliferation and cellular factors that interfere with FV infection.

complex also requires the involvement of another molecule, sf-STK, which is a short form of a kinase-type hematopoietic growth factor receptor, STK [28, 29]. Due to mutations in the intron of the *STK* gene, mice of C57BL strains lack the expression of sf-STK, although they express the full-length STK. Due to this lack of sf-STK, C57BL/6 (B6) and C57BL/10 (B10) mice are resistant to FV-induced disease development. This genetic factor found in C57BL mice had been described as an FV-resistance gene, *Fv-2*. The recessive resistant allele *Fv-2*^{r} is actually the above mutated *STK* that results in the lack of sf-STK.

It has been widely accepted that the SFFV-induced erythroid cell proliferation is crucial for the development of FV-induced erythroleukemia. That scheme of FV-induced leukemogenesis is as follows: upon infection of susceptible strains of mice with FV, SFFV gp55 induces rapid proliferation of erythroid progenitor cells. This leads to the development of splenomegaly and polycythemia readily detectable at as early as two weeks after FV inoculation. However, since the stimulation of erythroid progenitor cells through EpoR induces not only their growth but terminal differentiation, the process of erythroid cell expansion is self-limited, and progenies of SFFV-infected erythroid cells ultimately stop dividing and mature into red blood cells. Thus, even continuous infection of erythroid progenitor cells cannot result in the development of leukemia. For the development of malignant transformation, changes in the expression of host genes due to proviral integration into specific chromosomal locations are involved. Rapid expansion of erythroid progenitor cells is believed to provide target cells for repeated integration of SFFV and F-MuLV proviruses, and resultantly increases the chances for the proviruses to "hit" cellular genes by the promoter insertion adjacent to an oncogene or by disruption and silencing of a tumor suppressor gene [30, 31]. In fact, activation of several oncogenes and disruption of a few tumor suppressor genes have been documented in FV-induced leukemia cells. However, the proposed relationships between SFFV-induced early expansion of erythroid progenitor cells and malignant transformation due to proviral integration still remain circumstantial: for instance, it has not been proven if the SFFV-induced proliferation of erythroid cells is a prerequisite to allow repeated proviral integration resulting in a higher frequency of disease-inducing "hits." In this regard, it has been shown that even *Fv-2*$^{r/r}$ B6 mice can develop FV-induced leukemia when T lymphocytes are absent [32]. Further, our recent data suggest that SFFV-induced early splenomegaly is not a requisite for FV-induced leukemogenesis in a certain immunodeficient condition (Kawabata, H., *et al.*, manuscript in preparation). Thus, there are definitely interactions between host immune responses to FV-infected cells and the progression from the SFFV-induced erythroid cell proliferation to malignant transformation of virus-infected erythroid cells.

Host genes that control immune responses to FV infection

Even when mouse strains that share the same susceptible genotypes at the above host genes regulating FV replication and SFFV-induced cell growth are infected, the rate and tempo of the development of splenomegaly and leukemic death can change drastically depending on the host's genotypes at other genetic loci that regulate immune responses to FV. Fig. 2 shows examples of the effects of such immnunoregulatory genes. (B10 × A.BY)F_1 and (B10.D2 × A.BY)F_1 mice share the same (B10 × A)F_1 background, and thus share the *Fv-1*$^{b/b}$, *Fv-2*$^{r/s}$, and *Fv-4*$^{s/s}$ genotypes regarding FV infectivity. Nevertheless, (B10 × A.BY)F_1 mice are highly resistant to FV infection, and only <10% die within 100 days

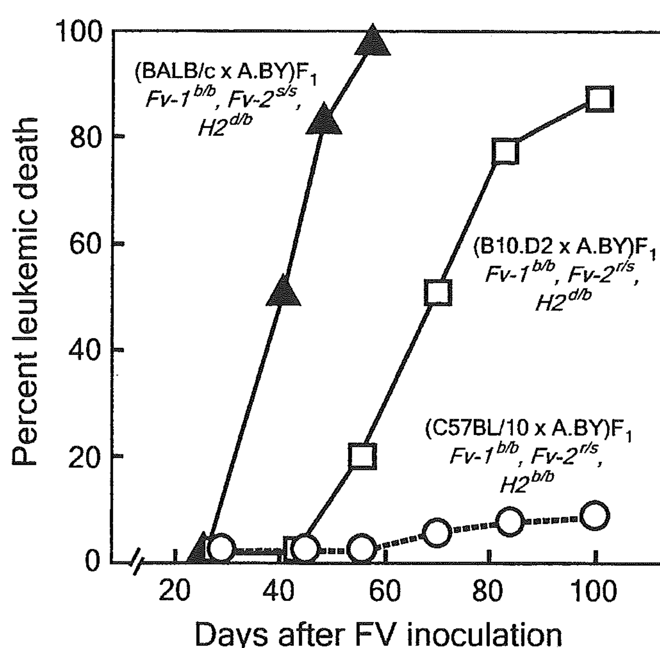

Figure 2. Comparison of tempos of leukemic death after FV infection in three different strains of mice.

after inoculation of a high dose of FV in the range of 1,500 to 30,000 spleen focus-forming units. These two strains differ only at the major histocompatibility complex (MHC) loci: (B10 × A.BY)F_1 possessing the homozygous *H2*$^{b/b}$ haplotype, and (B10.D2 × A.BY)F_1 being *H2*$^{d/b}$. Genes within mouse MHC that influence the incidence and tempo of the development of FV-induced splenomegaly and leukemic death have been mapped by utilizing H2 congenic and recombinant mouse strains [33-36], and are summarized in Table 1. Both class I-restricted $CD8^+$ and class II-restricted $CD4^+$ T cells are required for full resistance against FV-induced disease

Table 1. Host genes that influence immune response to FV infection.

Genes	Chromosomal location	Resistant allele	Susceptible allele	Phenotype influenced
Rfv-3	15	$Rfv\text{-}3^r$ (C57BL)	$Rfv\text{-}3^s$ (A/WySn)	Recovery from viremia, Kinetics of neutralizing Ab production
Rfv-1	17, *H2D*	D^b	D^d, D^k, D^q, D^{dm14}	Cytokine production from $CD8^+$ and $CD4^+$ T cells
Rfv-2	17, *Q/TL*	$Qa\text{-}1^a$	$Qa\text{-}1^b$	NK killing?
H2A	17	A^b	A^d, A^k, A^{bm12}	T helper cell responses to viral antigens
H2E	17	E^b (hybrid)	E^k, E^d	T helper cell responses to viral antigens

development. The requirement of both $CD4^+$ and $CD8^+$ T cell subsets for the spontaneous immune resistance of $H2^{b/b}$ mice was further substantiated by the results of antibody (Ab)-mediated T cell depletions [37]: the otherwise resistant mice depleted of $CD8^+$ T cells developed rapid splenomegaly, while the same strain of mice depleted of $CD4^+$ T cells showed initial recovery from splenomegaly, but ultimately developed leukemia and died at a high frequency. Further, administration of Ab reactive to the class II E molecule, but not those reactive to the A molecule, abrogated the spontaneous resistance against FV infection [38].

Interestingly, mice lacking the resistance-associated *b* allele at the class II loci as well as those depleted of $CD4^+$ T cells nevertheless showed spontaneous recovery from the initial development of splenomegaly. These results suggest that functions of $CD8^+$ T cells are required in the earlier, and those associated with $CD4^+$ T cells in the later stages of the infection, together exerting immune resistance against FV infection.

Epitopes recognized by $CD4^+$ and $CD8^+$ T cells have been identified in the *env* and *gag* gene products of F-MuLV [39-46], and are summarized in Fig. 3. Among these, an *env*-encoded, class II $E^{b/d}$-restricted peptide VYSQFEKSYRHKR and a *gag*-encoded class II-restricted peptide IVTWEAIAVDPPP identified within the matrix protein (MA) were effective in inducing protective immunity against FV challenge when given as a vaccine to $H2^{a/b}$ or $H2^{d/b}$ strains of susceptible mice [44, 47, 48]. In cases of both these peptides, virus-neutralizing Ab were not detectable before FV challenge, although non-neutralizing anti-MA Ab were detectable in mice immunized with the gag peptide. Rapid production and IgM-to-IgG class-switching of virus-neutralizing Ab were observed in peptide-immunized mice after FV challenge, indicating an advantage of $CD4^+$ T cell-priming vaccines in facilitating virus-reactive Ab production. Effector cells including $CD8^+$ and $CD4^+$ cytotoxic T lymphocytes (CTL) and natural

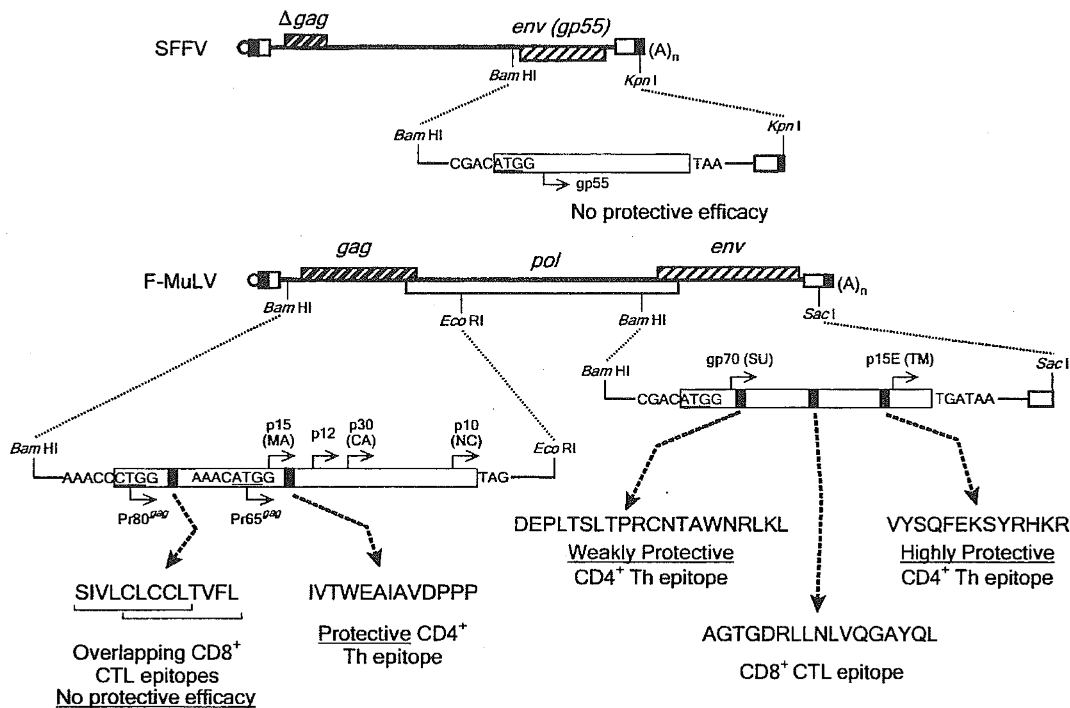

Figure 3. Amino acid sequence and location of the epitopes recognized by T cells in FV gene products.

killer (NK) cells are activated in mice immunized with the *env*-encode peptide within a few days after FV inoculation [48]. The $CD4^+$ CTL was only detectable in the peptide-immunized mice, but $CD8^+$ CTL and NK cells are activated both in immunized and unimmunized animals after FV infection. Further, NK cells were most efficient in killing FV-infected target cells, and Ab-mediated depletion of NK cells totally abrogated the protective efficacy of the *env*-encoded peptide vaccine [48]. These results indicate that peptide-induced priming of $CD4^+$ T cells augments the activation of multiple effector mechanisms upon FV infection. There has so far been no evidence showing that immunization with a single $CD8^+$ T cell epitope induces protection against FV infection. Further, immunization of mice with a recombinant vaccinia virus that expresses the SFFV *env* gene did not induce protective immunity [49], suggesting the lack of immunoprotective epitopes within the SFFV *env* gene products.

Another MHC-associated host gene that influences the development of FV-induced splenomegaly has been mapped in the class Ib Q/TL loci [35]. The exact molecular nature of this resistance gene, *Rfv-2*, has not been identified, but given the involvement of a Q-locus gene product, Qa-1^b, in the negative regulation of the target cell killing by NK cells [50], and the observed importance of NK cells in protective immunity against FV, it is possible that *Rfv-2* is actually *Qa-1*, and is regulating the susceptibility of FV-infected cells to NK killing.

Rfv-3 gene that controls the production of virus-neutralizing antibodies

The results shown in Fig. 2 also indicate that, although (B10.D2 × A.BY)F_1 and (BALB/c × A.BY)F_1 strains of mice share the same *$H2^{d/b}$* haplotype, they differ completely in their tempo of leukemia development after FV infection. In fact, (BALB/c × A.BY)F_1 mice die within 2 months after FV infection, while nearly half of (B10.D2 × A.BY)F_1 mice survive through post-infection day (PID) 70. The association between genotypes at a non-MHC host gene and the development of FV-induced disease was first described for the persistence of viremia after FV infection [51]: A/WySn mice persisted to be viremic at > 30 days after FV infection, while B10.A mice had cleared viremia by PID 30. Since F_1 crosses between these two strains were not viremic at PID 30, and about half of the (B10.A × A/WySn) × A/WySn backcross mice showed viremia at the same time-point, the presence of a recessive host gene was postulated in association with the persistence of viremia and was designated as *$Rfv\text{-}3^s$*. Thus, B10 mice possess a dominant allele, *$Rfv\text{-}3^r$*, conferring the early clearance of viremia. The *Rfv-3* locus was later mapped within chromosome 15 [52, 53]. To clarify the relationships between the *Rfv-3* genotypes and the production of virus-neutralizing Ab, we performed genetic mapping experiments by using > 200 (B10.A × A/WySn) × A/WySn backcross mice (Kanari, Y., et al., submitted for publication). A single gene determining the production of F-MuLV-neutralizing serum Ab at PID 15 was mapped in chromosome 15, and the strongest association was observed between the neutralizing titers at PID 15 and genotypes at the D15Mit71 locus, colocalizing with the previously mapped *Rfv-3* locus (Fig. 4). There are a few candidate genes that might be responsible for the observed regulation of neutralizing Ab production. Of note, A/WySn, but not A/J, mice are known to possess a mutation in the B cell-activating factor belonging to the TNF family receptor (BAFF-R) gene that results in the attenuation of germinal center responses of antigen-simulated B lymphocytes [54]. The structural gene for the BAFF-R is located in chromosome 15, a few megabase pairs (Mbp) telomeric to the D15Mit71 marker. In addition, the structural gene for mouse APOBEC3, the anti-retroviral enzyme, is also mapped in the same chromosome, located a few Mbp centromeric to the same D15Mit71 locus. Although there is no documented direct influence of APOBEC molecules on the functions of B lymphocytes, a presumably reduced replication of FV in the early stages of infection in the presence of a resistant APOBEC genotype, if any, might allow the host immune system to mount stronger Ab responses while infectious virions are still small in number. The possible presence of polymorphisms in the APOBEC3 locus between the resistant and susceptible strains must be demonstrated before making any further assumptions on the

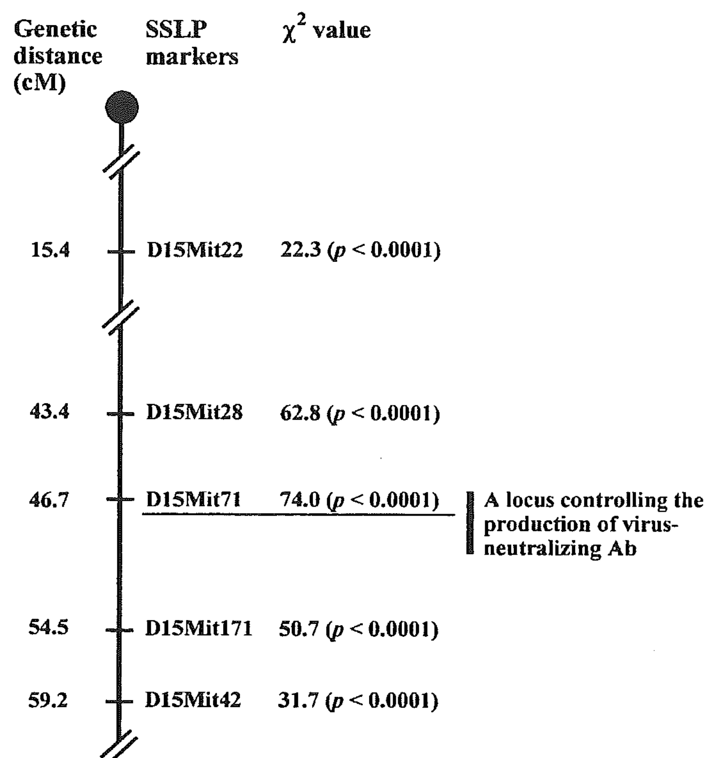

Figure 4. Genetic mapping of the host gene that controls serum titers of virus-neutralizing Ab in FV-infected mice.

role of this gene in the regulation of neutralizing Ab production. Additionally, the possible direct effect of the known polymorphism of the *BAFF-R* gene on the production of FV-neutralizing Ab can be assessed in the near future by establishing transgenic mice that express the B10.A-derived BAFF-R gene on the background of A/WySn strain.

Acknowledgements

The author's work documented in this review has been partly supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology, and the Ministry of Health, Labor, and Welfare of Japan, and those from the Japan Health Science Foundation. The author thanks Mr. M. Patrick Gorman for his critical review and correction of the manuscript.

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