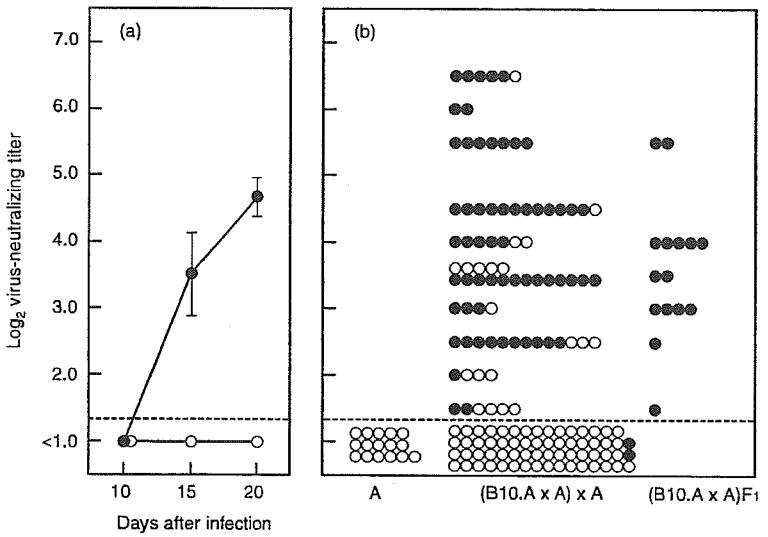

Fig. 1. Titers of virus-neutralizing antibodies in FV-infected mice. (a) Changes in the average titer ($n = 11–16$) of virus-neutralizing antibodies in (B10.A × A/WySn)F_1 (●) and A/WySn (○) mice at different time-points after infection with 150 spleen focus-forming units of FV. SEM. are shown with the 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

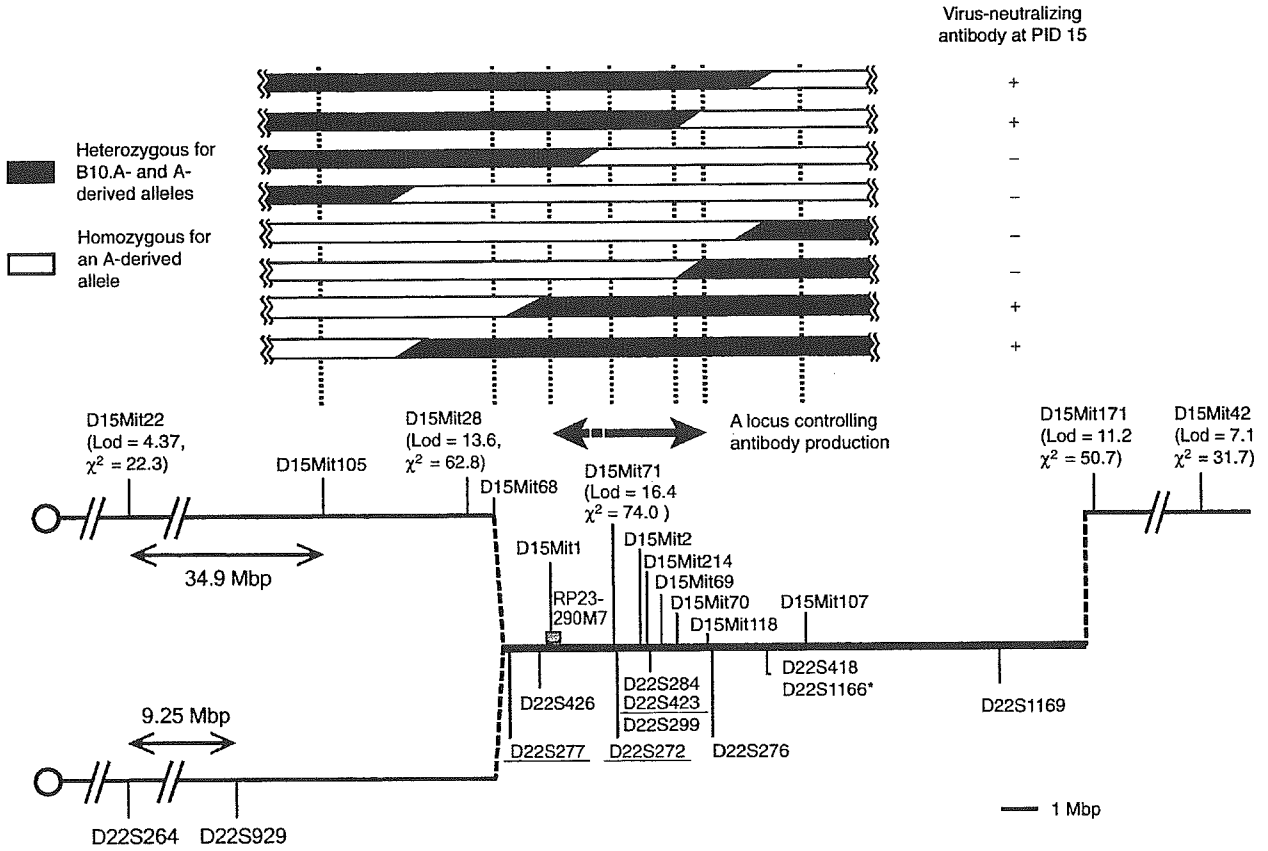

Fig. 2. The order of and distance between microsatellite markers located within the syntenic region of mouse chromosome 15 (the upper half of the diagram) and human chromosome 22 (the bottom half). Physical mapping and synteny data are based on those compiled in the Ensembl Genome Browser (http://www.ensembl.org/). 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

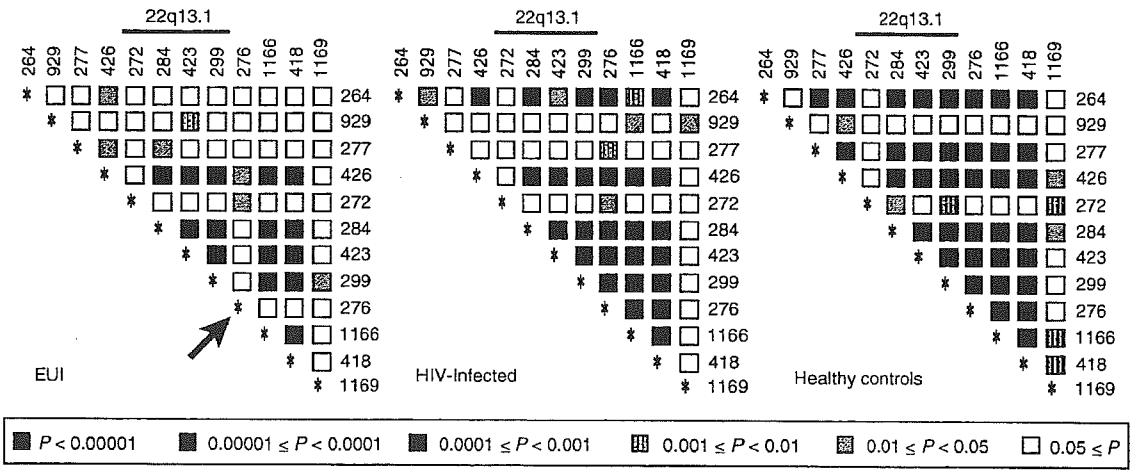

Fig. 3. Distributions of LD across chromosome 22. LD is plotted for the 12 microsatellite loci examined against each other, each represented by a small square. Locus names are abbreviated by omitting D22S. The pattern of distribution of LD is shown for each of the three phenotypic groups for comparison. 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.

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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 $n_x = \sum_{i \le j} x_{ij}$ and $n_y = \sum_{i \le j} y_{ij}$. 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.

特集：HIV と免疫

HIV に対する細胞傷害性 T 細胞の免疫応答

Cytotoxic T Lymphocyte-mediated Immune Responses to HIV

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はじめに

HIV の感染防御および HIV に感染後の病態制御では，CD8 陽性の細胞傷害性 T 細胞（$CD8^+$ cytotoxic T lymphocyte；CTL）の働きが重要であることが，さまざまな側面から明らかにされてきた。しかしながら CTL を中心とする免疫応答では，HIV を排除することはできず，慢性持続感染が成立して病態が進行する。HIV による CTL 免疫応答からの逃避機序の解明は重要な課題であるばかりでなく，その成果は有効なワクチンの開発研究にとって極めて有用である。本編では，HIV 特異的な CTL の性質について，特に HIV 感染制御に CTL とはどのようなものかという点に焦点を絞って，最近の知見を中心に述べる。

1. HIV 感染症の病態進行と HLA 遺伝子型

HIV 感染症の病態進行には個体差が認められる。遺伝学的な解析から *CCR5* の変異を始め，さまざまな遺伝学的要因が報告されている[1]。このうち獲得免疫系に関わる要因は HLA 遺伝子型との相関である。

HIV に感染した細胞では，HIV 由来の蛋白質はプロテアソームを始めとする蛋白質分解系によって消化されて，15 アミノ酸程度の短いペプチド断片となる。こうしたペプチド断片の一部は，小胞体で MHC（ヒトの場合は HLA）クラス I 分子と結合して，やがて細胞表面に運ばれ，CTL の抗原として提示される。HLA クラス I は多型性の著しい分子（A, B, C 合わせて 1,000 種類以上）で，それぞれの HLA 分子は異なった構造を持ったペプチドと結合する。たとえば *HLA-A** 02 を持つ人と *HLA-A** 24 を持つ人では，たとえ HIV の同じ蛋白質に由来しても，異なったペプチドが抗原として提示されて CTL に認識される。したがって，HIV 抗原に応答する CTL のレパートリーは，その人がどの HLA クラス I を持っているかに依存する。

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HLA 遺伝子型と HIV 感染症の病態進行の相関を表 1 にまとめた。ヒトの HLA クラス I は *HLA-A, B, C* と大きく 3 種類のアリルで構成され，それぞれが二対ずつあるため最大 6 種類の異なった HLA アリルを持つ。この種類が多いほど，提示する抗原ペプチドの構造多様性が大きく，より広範な CTL 応答が期待できるため，宿主免疫応答にとっては優位であると考えられている。しかしながら，ときに *HLA-A** 24 や *HLA-A** 02 など同一の HLA を両染色体に持つ人（homozygote）がいるが，こうした homozygote では異なった HLA アリルを持つ人（heterozygote）に比べて，エイズへの病態進行が統計学的に早いことが報告されている（表 1）。このことは，HLA 分子が仲介する CTL 応答が HIV 感染症の制御に大きな役割を担うことを示す。また，*HLA-B** 57 が病態進行の遅延と *HLA-B** 35 が病態の早期進行と相関するなど個別の HLA アリルが病態進行に影響することは，提示される抗原ペプチドの構造や種類によって HIV 特異的な CTL 応答の有効性が異なることを示唆している。我々は，病態進行の遅延と相関する HLA のうち，日本人に比較的頻度の高い HLA-B* 51 に拘束性の CTL による抗ウイルス免疫応答の解析から，HIV 感染症の病態進行にかかわる HLA あるいは免疫系の役割の解明を目指している。

最近 HLA クラス I アリルの抗 HIV 免疫応答の関与について興味深い報告がなされた。大規模な HIV 感染者のコホートを対象として，HLA 遺伝子型と病態進行の集団遺伝学的解析と，HIV に対する CTL 応答の機能的解析を組み合わせたところ，*HLA-A, B, C* アリルのうち *HLA-B* アリルに拘束される CTL 応答が最もよく病態進行の遅延と関連するという報告である[9]。実際，アリル多型性では *HLA-B* アリルが 600 種類以上の多型性を持っていて最大である。HLA がアリル多型性を得る進化学的要因は，数多くの病原体による選択圧だと考えられており，このことは HIV 感染症で *HLA-B* アリルが最も重要であるという結果と矛盾しない。しかし，この統計解析の結果は，*HLA-B** 27 や *HLA-B** 57 など個別のアリルで有意に HIV 感染症の病

表 1　HIV 感染症の病態と HLA 遺伝子型の相関

相関する HLA	HIV 感染症に与える影響	文献
HLA-A, B, C homozygosity	エイズへの進行促進	2, 3
*HLA-B**35	エイズへの進行促進	4, 5
*HLA-B**57, *HLA-B**27	病態進行の遅延	6
*HLA-A**01-*B0**8-*DRB1**03	エイズへの進行促進	7, 8

態遅延と相関する効果によって，見かけ上 *HLA-B* アリルの重要性がクローズアップされたのかもしれない。また，HIV がヒトからヒトへ伝播することを考えると，*HLA-A**02 のように遺伝子頻度の高い *HLA* アリルに対してはすでに HIV は適応しているため，免疫応答をうけにくいのかもしれない[10,11]。いずれにしても，HIV 感染者の体内で HIV 感染制御に最も有効性の高い CTL 応答の実体の解明が待たれる。

2.　HIV ゲノムの変異による CTL 応答からの逃避

HIV はさまざまな変異を獲得して抗 HIV 阻害剤に耐性を示すことが知られている。CTL による免疫応答に対しても，CTL エピトープあるいはその周辺領域に変異を獲得して，CTL から逃避することが明らかになってきた[12]。数百人の HIV 感染者を用いた大規模な HIV の遺伝子配列を感染者の HLA 遺伝子型に沿って調べたところ，各 HLA 分子に共通した変異が認められた。こうした変異は，その HLA 分子が提示する CTL エピトープの内部の配列であったり，ごく近傍に位置していた。機能的な解析から，実際に CTL による傷害活性やサイトカイン産生活性を示さなくなることが明らかとなった。こうした変異のうち，CTL エピトープ内部の変異は HLA 分子との結合や TCR との相互作用に影響して，CTL による免疫応答から逃れると考えられる。また最近，CTL エピトープの近傍の変異によって，ペプチド断片を生成する蛋白質のプロセッシングパターンを変えてしまうことにより，エピトープペプチドが生成されなくなるという新しい逃避メカニズムが報告された[13,14]。こうしたことを考え合わせると，HIV ゲノムの変異による CTL 免疫応答からの逃避は，当初考えられていたよりもかなり広範囲にわたって起きていることが強く示唆される。

それではこうした CTL エスケープ変異は，HIV 感染症の病態進行にどのようにかかわるのだろうか？　HLA-B*27 拘束性の Gag エピトープは，ドミナントなエピトープで非常に強い CTL 応答を誘導することが知られている。HIV 感染後数年にわたって血中ウイルス量が低く維持されていた患者では，このエピトープに特異的な CTL 応答が認められた[15]。そうした患者の一部では，このエピトープ領域に一つの変異を認めたが，それとほぼ同時期に急激なウイルス量の増加が観察された。この変異は，HLA-B*27 分子との結合を失わせる変異であったため，変異獲得後は CTL による免疫応答がほぼ完全に失われていた[15]。こうしたことから，HIV が変異を獲得して CTL による免疫応答から逃れる結果，HIV 感染症の病態が進行することは明らかである。しかしながら，CTL エスケープ変異と病態進行の相関を示す症例の報告はいまだ限られており，具体的にどんな，あるいはどの CTL エスケープ変異が病態進行と関連するのか，エスケープ変異に対して獲得免疫系はどのように対応するのかなど明らかになっていない問題は多い。

HIV ゲノムの変異にかかわる大きな因子の一つとして，HIV の複製効率への影響がある。もし CTL エピトープの領域が HIV の複製にとって必須であった場合，CTL エスケープ変異は HIV の複製効率を低下させ，結果として HIV の増殖抑制に働くかもしれない（図 1）。こうした事例が SIV 感染モデルを用いた研究から示された[16]。さらに，ウイルス複製能を低下させる CTL エスケープ変異を持ったウイルスが MHC の異なる他の個体に伝播するケースでは，変異はもとのアミノ酸に戻りウイルス複製能が回復することが SIV 感染モデルおよび HIV 感染症患者で明らか

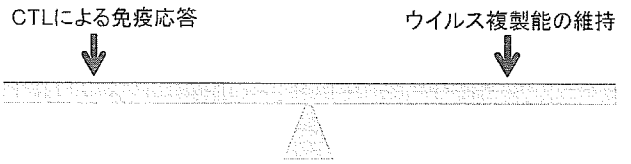

図 1　CTL エスケープ変異にかかわる要因
HIV に対する CTL の免疫応答の選択圧とウイルス複製能を維持する選択圧のバランスが，CTL エスケープ変異の出現に影響する。他にもさまざまな要因が考えられるが，それらがどの程度 HIV によるエスケープ変異獲得に関与するか現在さかんに研究されている。

にされた[17,18]。一方，HIV あるいは SIV が MHC を共有する他の個体に伝播したケースでは，エスケープ変異は保存されることから[17,18]，CTL 免疫応答による選択圧とウイルス複製能のバランスが，CTL エスケープ変異の出現を決める因子であると示唆された(図1)。しかしながら，$CD4^+$ T 細胞による細胞性免疫，抗体や自然免疫系あるいは他の生体内抗ウイルス因子など，CTL 以外の要因による抗ウイルス効果も，CTL エスケープ変異の出現に影響すると考えられる。実際，強い CTL 応答を示すにもかかわらず，エピトープおよびその周辺に変異が認められない例も多く(上野，未発表データ)，宿主が持つ HIV に対する選択圧については防御免疫系以外の要因も含め，今後の詳細な解析が待たれる。HIV による CTL エスケープ変異の出現要因や，変異によるエスケープ後の抗ウイルス免疫応答の解明という課題は，ワクチン開発に重要な情報を提供するだけでなく，持続的に感染する病原体に対するヒト免疫応答の性質を明らかにする上で，非常に興味深いモデルである。

3. CTL による抗ウイルス活性の効率

MHC テトラマーやサイトカイン産生細胞の染色法を用いて HIV 特異的な $CD8^+$ T 細胞の頻度を測定すると，慢性 HIV 感染者では HIV 特異的な CTL は十分に存在するが，抗ウイルス機能を測定するとその機能は減弱している。その原因として，$CD8^+$ T 細胞が機能的な CTL として十分に成熟できないというモデルが提唱されたが[19]，未だこの全容は解明されていない。我々は，HIV 特異的 $CD8^+$ T 細胞中には，エピトープ特異性は示すが HIV 感染細胞に作用できないサブセットがいるのではないかと考え，これまでに樹立した HIV 特異的 CTL クローンの抗ウイルス活性を再検討した。その結果，HLA-B*35 拘束性で逆転写酵素由来のペプチド（IPLTEEAEL）に特異的な CTL 55 は，MHC テトラマーに結合し，合成ペプチドをパルスした標的細胞を殺傷するにもかかわらず，HIV 感染細胞に対して全く機能しないことを見出した[20]。さらに興味深いことに，この患者の末梢リンパ球を数年にわたり経時的に解析したところ，CTL 55 タイプの CTL の頻度が増しており，逆に同一エピトープに特異的だが抗ウイルス活性に優れた CTL サブセットの頻度が減少していた。このことは，個々の CTL の機能が経時的に減少するのではなく，抗ウイルス活性機能が劣った別の CTL サブセットが増えてしまうために，個体全体の CTL による抗ウイルス機能が減弱化するという新しいモデルを示唆している（上野ら，投稿準備中)。

また，オックスフォード大学のグループは *HLA-B*08* を持つ長期未発症者（long-term nonprogressor ; LTNP）の Nef エピトープ（FLKEKGGL）に応答する CTL を解析した。その結果，$V\beta$ 13.2 という TCR を持つ CTL は，他の TCR を持つ CTL に比べて増殖能に優れ，エピトープ変異にも応答できる機能を有していた[21]。

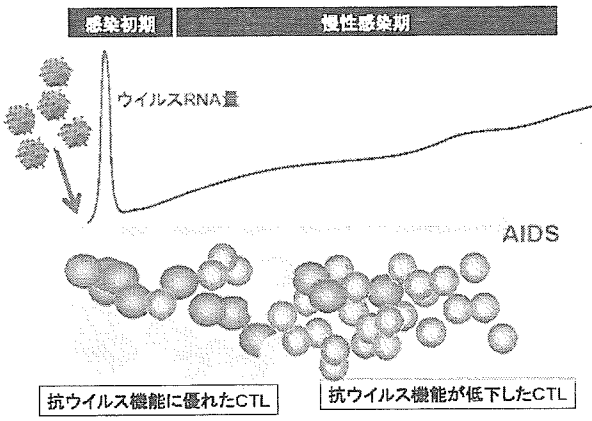

図 2 CTL による抗ウイルス免疫応答の経時変化
CTL による免疫応答は感染初期には効率的に HIV の複製を制御するが，HIV の排除には至らない。慢性感染成立後の HIV に対する CTL 応答を解析すると，HIV 特異的 CD8 T 細胞数は十分認められるにもかかわらず，CTL の抗ウイルス機能は低下している。抗ウイルス機能に優れた初期の CTL が時間とともにその機能を喪失するのか，あるいは機能が低下した CTL が後から誘導されるのだろうか。慢性感染ウイルスに対するヒト CTL 応答には未解明の問題が多く残されている。

こうした結果は，抗原特異性が同一であっても，T 細胞レセプター（TCR）が異なる CTL サブセット間では，抗ウイルス活性に差があることを示している。したがって，HIV 感染者の抗ウイルス免疫応答の活性を評価するには，抗原特異的な T 細胞の数よりもむしろ CTL の機能を包括的に解析する方法が必要である。一方，TCR 依存的に抗ウイルス活性に優れた CTL サブセットが一部の感染者で実在することを考えると，抗ウイルス活性に優れた TCR をコードする遺伝子を他の感染者の CD8 T 細胞に導入することにより，効率的な抗ウイルス機能を持った CTL を大量に作り出すことが可能である[22]。こうした情報を基に，抗ウイルス活性に優れた CTL の実体の解明や，こうした CTL を効率的に誘導するためのワクチン開発研究が期待される。

4. CTL による抗ウイルス活性のエピトープ依存性

CTL は HIV 由来の複数のエピトープペプチドに応答する。一つの HLA アリルあたり平均しておおよそ 10 種類程度のペプチドが CTL エピトープとして認識されている。

表 2　HLA-B51 拘束性エピトープに特異的な CTL の抗ウイルス活性

蛋白質	エピトープ	ウイルス増殖阻害	細胞傷害活性	サイトカイン産生
IN	LPPVVAKEI	＋＋＋	＋＋＋	＋＋
RT	TAKTIPSI	＋＋＋	＋＋＋	＋＋＋
Gag	NANPDCKTI	＋＋	＋	＋＋
Rev	VPLQLPPLERL	＋	−	＋

エピトープ特異性は，CTL の抗ウイルス活性に影響するのだろうか。個々のエピトープは，それが由来する蛋白質の発現量，プロテアソームなど蛋白質分解酵素による消化効率，HLA 分子との結合活性などが異なるため，最終的に感染細胞表面で提示される量はエピトープごとに異なっている。実際 Yang らは，CTL の抗ウイルス活性は，その CTL が抗原ペプチドを認識する感度よりも，どの抗原を認識するかという抗原特異性の影響が大きいことを報告した[23]。

また我々は，HLA-B*51 拘束性の四つの異なるエピトープについて，CTL の抗ウイルス活性を比較した（表 2）。これまでは CTL 活性を測定する標的細胞には B 細胞由来の細胞株が頻繁に用いられていたが，この実験では HIV-1 を感染させる標的細胞にヒト末梢血から分離した $CD4^+$ T 細胞を用いた。また Nef 蛋白質は HLA クラス I 分子の細胞表面での発現量を低下させ，CTL による傷害活性から逃避することが知られている。Nef 陽性の HIV-1 株（NL432）を用いることにより，CTL にとってより厳しい条件下で抗ウイルス機能を評価する実験系を確立した[24]。その結果，逆転写酵素やインテグレース由来エピトープに特異的な CTL では顕著な抗ウイルス活性を認めたが，Rev 特異的な CTL は抗ウイルス活性をほとんど示さなかった（表 2）。*HLA-B** 51 はエイズ病態進行の遅延と相関していることを考えると，HLA-B* 51 拘束性の CTL が Nef による HLA 分子の発現低下にもかかわらず効率的な抗ウイルス活性を示したことは，こうした CTL が病態進行を遅延させる要因となっているのかもしれない。

おわりに

これまでウイルス感染症に対する免疫応答の研究は，主に急性感染ウイルスをモデルを中心として進められてきた。HIV 感染症のように，長期にわたって持続的に抗原が存在する感染症に対して，ヒト獲得免疫系がどのように応答するのか，未解明な問題が多く残されている。ウイルス特異的 CTL の抗ウイルス機能が一様ではなく，ウイルス複製を制御できない CTL サブセットが長期に渡って存在するなど，急性感染症とは異なり，慢性 HIV 感染症に際立った感染免疫学的現象が見出されてきている。今後は，クロスプレゼンテーションなど抗原提示系や自然免疫系との相互作用を通じて，HIV の抗原変異がヒト免疫応答に与える長期的な影響や抗ウイルス機能に優れた CTL の実体とその成立メカニズムなどが統合的に解析されることにより，獲得免疫系による HIV の制御機構と破綻メカニズムの解明，合わせてそれを修復する新たな方法論の開発が期待される。

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Spontaneous recovery of hemoglobin and neutrophil levels in Japanese patients on a long-term Combivir® containing regimen

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Abstract

Objective: In order to evaluate long-term toxicity of Combivir, we retrospectively reviewed clinical records of HIV-1 infected cases under treatment with Combivir-containing regimen and we analyzed the clinical data compared to other NRTIs-containing regimens.
Study design: A total of 55 patients who were on Combivir and 39 on a control regimen were examined.
Results: After starting treatment with Combivir-containing regimens viral load and $CD4^+$ T-cell count improved as well as the control group. Rates of adverse events in Combivir group and ZDV (400 mg/day) + 3TC group were 50.9% (28/55) and 60% (12/20), respectively. Some of these Japanese patients who started Combivir regimen as a first-line HAART (primary Combivir group) showed some decrease in hemoglobin levels or neutrophil counts within 6 months. However, a significant recovery of these indices of hematological toxicities occurred in patients who continued the regimen for 18–24 months.
Conclusion: Our findings suggest that the safety of 600 mg of ZDV is similar to 400 mg/day of ZDV and the existence of mechanisms that compensate for anemia and for the neutropenia associated with long-term use of Combivir.

Keywords: Combivir; Zidovudine; Lamivudine; Hemoglobin; Neutrophil; Long-term treatment

1. Introduction

Prognosis of HIV infections dramatically improved after introduction of highly active anti-retro viral therapy (HAART). However, the occurrence of adverse events and drug resistance during long-term use of anti-retrovirals are now big issues (Yeni et al., 2002; Dieleman et al., 2002). Present HAART also has a problem to maintain a high adherence because of the pill burden and patients' quality of life is affected. Combivir® is a fixed dose combination tablet containing zidovudine (ZDV) and lamivudine (3TC) (Eron et al., 2000). Each tablet contains 300 mg of ZDV and 150 mg of 3TC and has been widely used as a nucleoside reverse transcriptase inhibitor (NRTI) component of HAART against HIV-1 infection.

HIV infection and AIDS are known to be associated with a significant hematological toxicity, including anemia, neutropenia, and thrombocytopenia (Moses et al., 1998). In addition, studies with zidovudine have shown that this drug may compound the hematological toxicity of HIV and lead to an independent development of anemia and neutropenia (Wilde and Langtry, 1993). Consistent with these observations, the incidence of anemia or neutropenia in mildly or asymptomatic adults treated with zidovudine was between 1.1% and 9.7%, whereas in adults with AIDS or the AIDS related complex it ranged from 15% to as high as 61% (Wilde

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and Langtry, 1993). In Japan, many physicians prescribe low dose ZDV such as 400 mg/day to avoid drug-induced anemia and neutropenia even though the standard dose of ZDV is 500–600 mg/day in United States (Kimura et al., 1992, 1998). Given the dose-dependent nature of these adverse effects, they are concerned about increased risk of hematological toxicity using Combivir that contains 600 mg of ZDV as the daily dose for Japanese patients who have lower body weights compared to patients in United States. Moreover, long-term consequence of the hematological toxicity resulting from continuous use of Combivir has not been well defined. We retrospectively reviewed clinical records of HIV-1 infected cases under treatment of Combivir-containing regimen used in three hospitals in Japan and we analyzed clinical data cross-sectionally to evaluate long-term toxicity of Combivir.

2. Patients and methods

HIV-1 positive Japanese patients were recruited from Kumamoto University Hospital, Osaka National Hospital and International Medical Center of Japan from June 1999 (after the Combivir launch) until June 2003. The clinical record was investigated in a retrospective manner. All collected cases were separated into four groups, as follows;

Primary Combivir Group (PCV): started Combivir as a first-line HAART.
Secondary Combivir Group (SCV): changed to Combivir from other NRTIs.
Primary Control Group (PCO): started NRTIs (except for Combivir) as a first-line HAART.
Secondary Control Group (SCO): changed to NRTIs except Combivir from other NRTIs.

We checked hemoglobin levels and neutrophil counts to examine the influence on hematological toxicity of ZDV every 6 months. We analyzed the data that could be followed over 18 months for removing various biases such as drop out cases with abnormal laboratory test values. Moreover, we also checked the HIV-RNA, $CD4^+$ T-cell counts and other laboratory test data every 6 months. We also checked any adverse events. This study was done under the approval of the Institutional Review Board of the Kumamoto University Hospital, Japan. All participants provided written informed consent.

3. Results

3.1. Patients' characteristics

Of the 94 data on subjects were 55 who were on Combivir (PCV: 27, SCV: 28) and 39 were on control regimens (PCO: 29, SCO: 10). The NRTIs used in the control group included of 20 cases of ZDV (400 mg/day) + 3TC, 18 cases of stavudine (d4T) + 3TC and one case of d4T + didanosine (ddI). Patients' characteristics are shown in Table 1. A couple of factors are statistically different such as the sex ($p < 0.01$: Fisher's exact test), weight ($p < 0.05$: Student's t-test) and Karnofsky score ($p = 0.0062$: Student's t-test) between Combivir group and control group. Combivir was likely to be used for patients with a higher baseline weight and the males. The mean viral load at baseline in Combivir group was $10^{3.9}$ copies/mL and for the control group was $10^{4.1}$ copies/mL. There was no statistical difference between the groups. The baseline $CD4^+$ T-cell counts in Combivir group were higher than in the control group significantly (393/mm^3 versus 263/mm^3; $p = 0.0101$: Student's t-test). Most patients were prescribed efavirenz (EFV) or nelfinavir (NFV) as a concomitant drug. Fifty-two percent of all patients were on EFV and 16% were taking NFV. The Combivir group had more combination cases with EFV than did the control group, because these two drugs approved for use in Japan at the same period have similar characteristics such as small pill counts and frequency of ingestion.

3.2. Effects on hemoglobin levels

To avoid biases in the data resulting from inclusion of patients with a shorter time follow up, including drop out cases, we focused on the patients that could be followed for over 18 months. Mean hemoglobin levels at baseline of Combivir group (PCV group: 13.9 g/dL, SCV group: 14.2 g/dL) were higher than for the control group (PCO group: 13.1 g/dL, SCO group: 13.7 g/dL) (Fig. 1A). It seems Combivir was likely to give to those with a lesser risk of anemia. We divided patients in PCV group into two sub-groups such as hemoglobin level decreased (sub-group A; $n = 10$) and not changed or increased (sub-group B: $n = 8$) at 6 months after starting Combivir. Fig. 1B shows a trend of hemoglobin levels in sub-group A. Each hemoglobin level at 6, 12, 18 and 24 months after starting treatment decreased significantly compared to baseline ($p < 0.005$, $p < 0.005$, $p < 0.005$ and $p < 0.05$, respectively; Wilcoxon matched pairs signed rank test). However, the decreased hemoglobin levels at 6 months gradually recovered to the baseline level despite continuation of the same regimen. The hemoglobin level at 18, 24 months increased significantly compared to 6-month values ($p < 0.05$ and $p < 0.005$, respectively). On the other hand, the hemoglobin level of sub-group B did not decrease for 18–30 months of follow up period (data not shown). The difference of background between sub-groups A and B was baseline level of hemoglobin and hematocrit. These levels in sub-group A were higher than for sub-group B statistically (14.9 ± 1.2 versus 12.6 ± 0.7; $p < 0.001$, 44.4 ± 3.2 versus 37.4 ± 2.0; $p < 0.001$, Student's t-test).

3.3. Effects on neutrophil counts

The trend of mean neutrophil counts was similar to counts for hemoglobin levels. Mean neutrophil counts of all groups

Table 1
Baseline characteristics

	Combivir group (PCV + SCV) ($n = 55$)	Control group (PCO + SCO) ($n = 39$)	p-value
Sex (male/female)	54/1	32/7	0.00815[a]
Age	35.9 ± 9.5 (22–68)	38.6 ± 10.7 (23–78)	0.2117[b]
Weight (kg)	64.6 ± 10.8 (47.0–91.6)	59.6 ± 11.2 (36.4–81.0)	0.0303[b]
Hemophilia			
Non	48	32	0.562[a]
A	5	7	
B	2	0	
Baseline VL (log)			
<2.6	19	11	0.4432[b]
2.6–3	1	1	
3–4	6	4	
4–5	11	13	
>5	15	10	
Unknown	3	0	
Mean ± S.D.	3.9 ± 1.2	4.1 ± 1.2	
Range	2.6–5.9	2.6–5.9	
Baseline CD4 count			
<200	14	14	0.0101[b]
200–500	25	19	
>500	13	5	
Unknown	3	1	
Mean ± S.D.	393 ± 265	263 ± 179	
Range	1–1132	5–607	
CDC class			
A1	5	3	0.8064[c]
A2	22	17	
A3	6	13	
B1	2	0	
B2	3	0	
B3	2	5	
C1	3	0	
C3	12	11	
Karnofsky score			
20%	0	1	0.0062[b]
40%	0	2	
50%	0	1	
60%	1	0	
70%	0	1	
80%	4	6	
90%	11	8	
100%	39	20	
Mean ± S.D.	95.8 ± 7.9	87.7 ± 19.4	

[a] Fisher' exact test.
[b] Student's t-test.
[c] Wilcoxon 2-sample test.

were over 2000/mm^3 and did not have statistically change from the baseline during the follow up period (Fig. 1C). We separated subjects in the PCV group into two sub-groups as well as for hemoglobin levels to examine the toxicity of Combivir to neutrophils. In the sub-group C ($n = 10$) those with mean neutrophil counts decreased and the sub-group D ($n = 7$) included subjects with no changes or increased neutrophil counts at 6 months after being on Combivir. Fig. 1D shows the trend of the neutrophil counts in sub-group C. Neutrophil counts at 6, 12, 18 and 24 months after starting the treatment decreased significantly compared to baseline ($p < 0.005$, $p < 0.05$, $p < 0.05$ and $p < 0.05$, respectively; Wilcoxon matched pairs signed rank test). However, the decreased neutrophil counts gradually recovered as did hemoglobin levels. The mean neutrophil counts at 18 months increased significantly compared to data at 6 months ($p < 0.05$; Wilcoxon matched pairs signed rank test).

3.4. Effects on other laboratory test value

MCV values at baseline for the secondary treatment group such as SCV group and SCO group were higher than for pri-

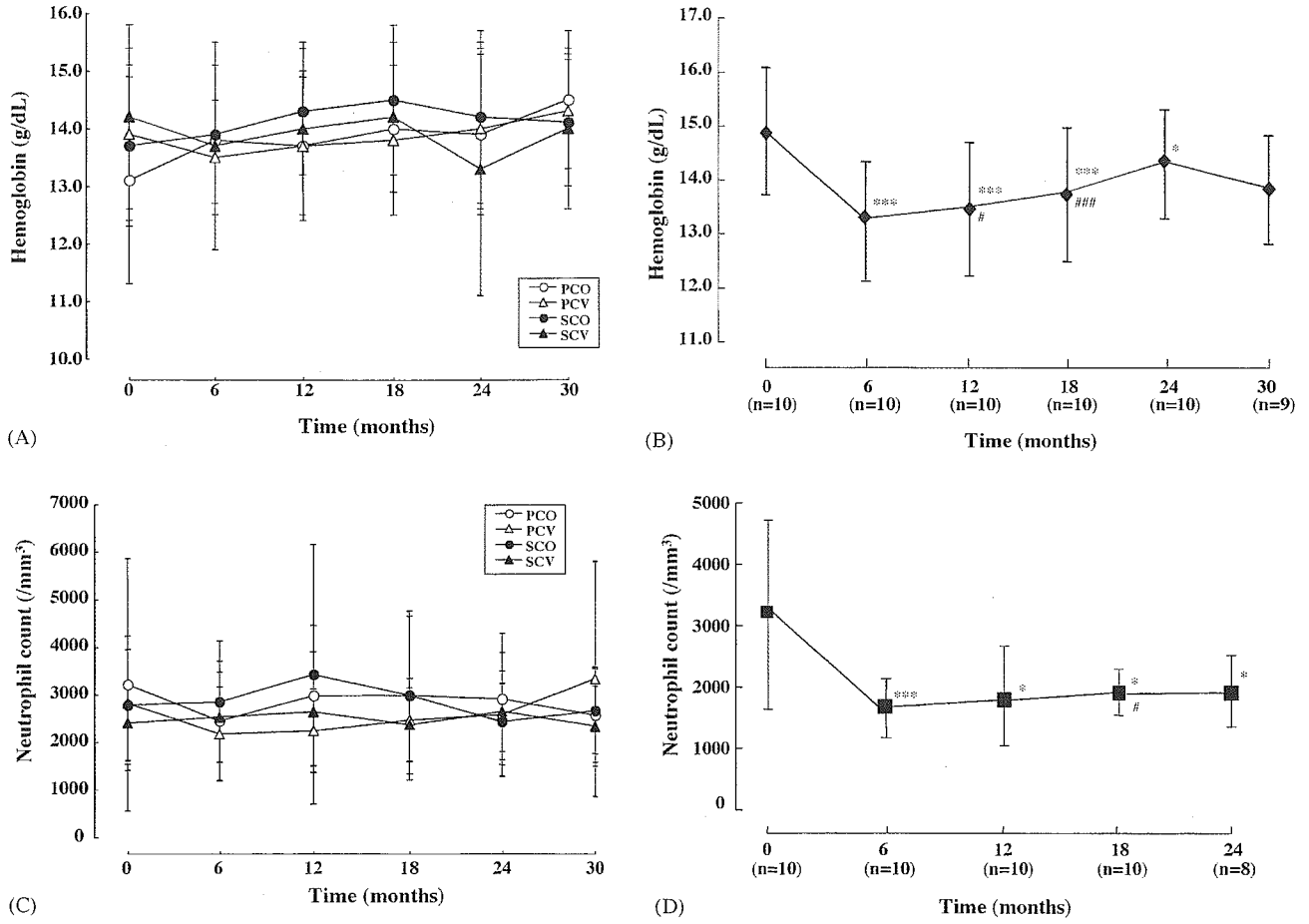

Fig. 1. Recovery after transient suppression of hemoglobin and neutrophil levels in patients with long-term use of Combivir. (A) Mean hemoglobin levels did not change significantly in all groups during each treatment. The baseline hemoglobin level in the Combivir group (PCV + SCV) was higher than in controls (PCO + SCO). (B) Mean hemoglobin levels at 6, 12, 18 and 24 months after start of treatment decreased significantly compared to baseline in the subgroup A of PCV group ($n = 10$). However the decreased hemoglobin level gradually reverted to the baseline levels despite continuation of the same regimen. Hemoglobin levels at 12 and 18 months were significantly high compared to findings at 6 months. (Wilcoxon matched pairs signed rank test; $^{*,\#}p < 0.05$, $^{***,\#\#\#}p < 0.005$). (C) Mean neutrophil counts did not change significantly in all groups during each treatment. (D) Mean neutrophil counts at 6, 12, 18 and 24 months after beginning treatment decreased significantly compared to baseline in sub-group C of PCV group ($n = 10$). However, the neutrophil counts gradually reverted to baseline levels despite continuation of the same regimen. The neutrophil counts at 18 months was significantly high compared to that of 6 months (Wilcoxon matched pairs signed rank test; $^{*,\#}p < 0.05$, $^{***}p < 0.005$).

mary treatment groups such as PCV group and PCO group. It seems ZDV or d4T in the secondary treatment group affected red blood cell counts. However, after starting each treatment, MCV values increased and became high at around 110/mm^3 in all groups (Fig. 2A). Other laboratory test values did showed no notable changes (data not shown).

3.5. Adverse events

The most common adverse events in each group were nausea/vomiting, dizziness and malaise. Anemia was observed in two in the Combivir group and one in the control group. Discontinuing each treatment led to elimination of these adverse effects. The anemia in two in the Combivir group was observed 2 months after their starting treatment, and that in one in the control group was evident as early as the eighth day. The occurrence of anemia in the control group was on ZDV 400 mg/day + 3TC. The frequency of anemia in the Combivir group was 3.6% (2/55) and similar to that in the control group {2.6% (1/39)}. The 20 in the control group on ZDV + 3TC regimen were on a ZDV 400 mg/day. We compared the safety profile of ZDV 600 mg/day to ZDV 400 mg/day. Adverse events rate of Combivir was 50.9% (28/55) and 60.0% (12/20) of AZT + 3TC group. Moreover, the number who discontinued Combivir group was 7 (12.7%) and that in ZDV + 3TC group was 5 (25.0%). In the SCV group, nineteen were changed to Combivir from ZDV 400 mg/day + 3TC. There were six with some adverse events and these were similar to other groups' events. These observations suggest that increasing the ZDV dose to 600 mg/day does not affect the incidence of adverse events. In addition there were no concomitantly used drugs that could affect pharmacokinetic parameters of ZDV and enhance its toxicity.

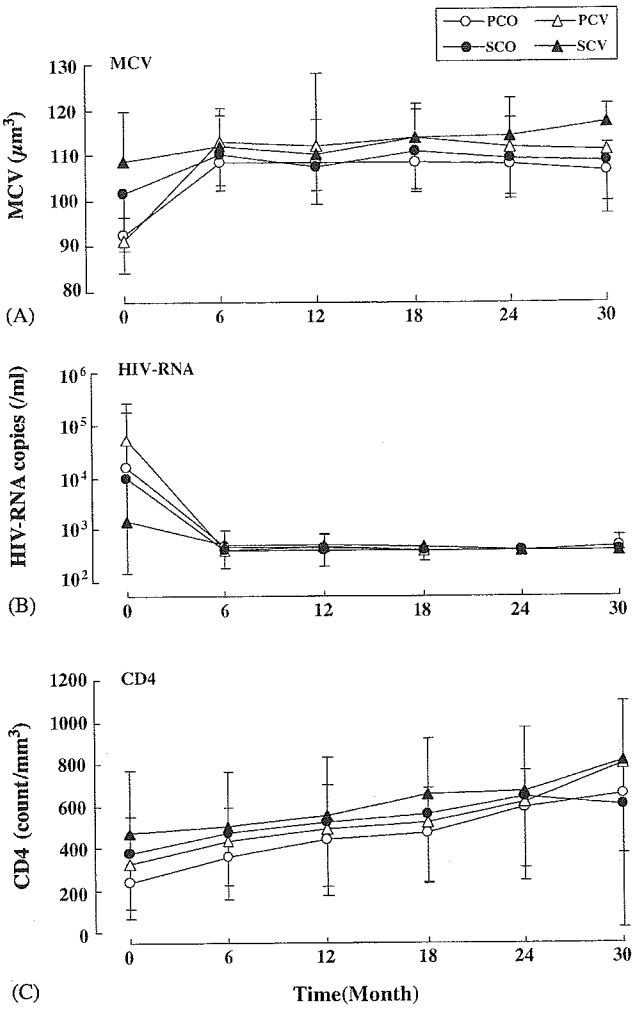

Fig. 2. Changes in MCV values, HIV RNA level and CD4$^+$ T cell counts in each group of patients. (A) MCV values for the secondary treatment group such as SCV and SCO group were higher than for primary treatment groups such as PCV and PCO group at baseline. However, after starting each treatment, MCV values increased and became high at around 110/mm^3 in all groups. (B) Mean HIV RNA level in all groups of treatment decreased compared to baseline significantly ($p < 0.05$–$p < 0.001$; Wilcoxon matched pairs signed rank test). (C) Mean CD4$^+$ T cell counts in all groups of treatment increased significantly compared to the baseline ($p < 0.05$–$p < 0.001$; Wilcoxon matched pairs signed rank test).

3.6. *Effects on viral load and CD4$^+$ T-cell counts*

Baseline viral load in the primary treatment group (PCV + PCO) was higher than in the secondary treatment group (SCV + SCO). Mean baseline viral loads of each group were $10^{4.6}$ copies/mL (PCV), $10^{4.0}$ copies/mL (PCO), $10^{3.0}$ copies/mL (SCV) and $10^{3.7}$ copies/mL (SCO), respectively. However, after starting each treatment, HIV RNA was not detectable in serum samples from in each group (VL < 50 or <400 copies/mL) (Fig. 2B). Baseline CD4$^+$ T-cell count in the SCV group was 518/mm^3 and higher than other groups (PCV: 304/mm^3, SCO: 345/mm^3, PCO: 277/mm^3) significantly ($p < 0.001$; Student's t-test) (Fig. 2C). This result suggests effective treatment with the previous combination for the SCV group. CD4$^+$ T-cell counts during each treatment increased significantly ($p < 0.05$–$p < 0.001$; Wilcoxon matched pairs signed rank test) and reached over 600/mm^3 at 30 months in all groups (Fig. 2C).

4. Discussion

The nucleoside reverse transcriptase inhibitor (NRTI) was first developed as an anti-HIV drug. However, the appropriate dosage was unclear because this type of drug is only active after being phosphorylated inside cells. A daily dose of 400 mg of ZDV has been widely used in Japan because anemia and neutropenia occurred frequently in cases of ingesting a higher dose (800 mg/day) than did 400 mg/day of ZDV in a clinical trial conducted in Japan (Kimura et al., 1992). Bone marrow toxicity associated with AZT such as macrocytic anemia and neutropenia has been frequently reported for the patients treated with a higher dose of ZDV mono therapy (Richman et al., 1987). Given the dose-dependent nature of these adverse effects, Japanese health care providers have some hesitance to prescribe Combivir that contains 600 mg of ZDV, as the daily dose. Data on four patients with severe anemia associated with Combivir have also been reported (Sibery et al., 2003). To evaluate the long-term toxicity of Combivir, we reviewed clinical records of HIV-1 infected Japanese patients on treatment with Combivir-containing regimen.

The results in this retrospective study showed that anemia and adverse events occurred at comparable frequency in each group of patients. Consistent with previous reports (Hester and Peacock, 1998; Tseng et al., 1998) these adverse events occurred in less than a few months after starting each treatment. The frequency of anemia in the Combivir group was only 3.7% (2/54), and it was similar to that for ZDV 400 mg/day + 3TC group (5.0%) group. In other words there was no difference in these groups with respect to the frequency of anemia by the difference in the dose of ZDV. It is also of note that the efficacy of Combivir was comparable to that of 400 mg of ZDV of four times a day with a twice a day dosing of 3TC. However, we have to take into account the fact that Combivir was prescribed for heavy weight patients. And such may mask the occurrence of adverse events as well as the difference in efficacy.

We observed a certain degree of decrease in hemoglobin levels and neutrophil counts in the subgroups of patients in PCV (subgroups A and C, respectively). Interestingly, a gradual recovery of these hematological toxicities occurred despite the continuation of Combivir containing regimens. The mechanism whereby the risk of hematological toxicity associated with increasing ZDV dosages may be related to the intracellular accumulation of the toxic metabolite zidovudine monophosphate (AZTMP) (Tornevik et al., 1995). AZTMP interferes with both cellular DNA synthesis and exonuclease-catalyzed removal of ZDV from host cell DNA (Sommadossi et al., 1989; Harrington et al., 1993). In addition, at clinically

relevant concentrations, AZTMP acts as a potent inhibitor of the transport of pyrimidine nucleotide sugars into the Golgi complex, thereby inhibiting protein glycosylation and altering glycosphingolipid synthesis (Yan et al., 1995). Therefore, AZTMP may elicit cytotoxic effects on rapidly growing erythrocytes and neutrophil precursors, both by interfering with nuclear DNA replication and by compromising the function of membrane receptors involved in receiving of extracellular stimuli required for cell growth and differentiation. From these observations it seems reasonable to speculate that either decrease in the intracellular concentration of AZTMP or compensatory mechanisms that improve the signal transduction for erythropoiesis and myelopoiesis mediated by cytokines contributed the recovery from hematological toxicities.

Two mechanisms may be related to the decrease in the concentration of AZTMP: altered metabolism of nucleoside analogues due to impaired nucleoside phosphorylation and increased efflux of the compounds by membrane transport mechanisms (Schuetz et al., 1999; Wijnholds et al., 2000). These mechanisms have been considered to contribute to the cellular drug-resistance (Dianzani et al., 1994; Groschel et al., 1997; Fridland et al., 2000; Turriziani et al., 2000). However, there was no evidence of treatment failure for patients in our PCV group as we found an increase in $CD4^+$ cell counts and an undetectable HIV-RNA load. Furthermore, the MCV level which is associated with the intracellular increase of AZTMP was kept high. These observations suggest that decrease in the level of AZTMP in the course of long-term treatment is unlikely although we must determine longitudinal changes of intracellular AZTMP level in precursors of blood cells in patients on Combivir treatment. Other compensatory mechanisms against the hematological toxicity may occur. An increase in erythropoietin or granulocyte-colony stimulating factor (G-CSF) levels in compensation for chronic anemia or neutropenia is another notion.

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Generation of High-Affinity Antibody against T Cell-Dependent Antigen in the *Ganp* Gene-Transgenic Mouse[1]

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Generation of high-affinity Ab is impaired in mice lacking germinal center-associated DNA primase (GANP) in B cells. In this study, we examined the effect of its overexpression in *ganp* transgenic C57BL/6 mice ($Ganp^{Tg}$). $Ganp^{Tg}$ displayed normal phenotype in B cell development, serum Ig levels, and responses against T cell-independent Ag; however, it generated the Ab with much higher affinity against nitrophenyl-chicken gammaglobulin in comparison with C57BL/6. To further examine the affinity increase, we established hybridomas producing high-affinity mAbs and compared their affinities using BIAcore. C57BL/6 generated high-affinity anti-nitrophenyl mAbs ($K_D \sim 2.50 \times 10^{-7}$ M) of IgG1/λ1 and contained the $V_H186.2$ region with W33L mutation. $Ganp^{Tg}$ generated much higher affinity ($K_D > 1.57 \times 10^{-9}$ M) by usage of $V_H186.2$ as well as noncanonical V_H7183 regions. $Ganp^{Tg}$ also generated exceptionally high-affinity anti-HIV-1 (V3 peptide) mAbs ($K_D > 9.90 \times 10^{-11}$ M) with neutralizing activity. These results demonstrated that GANP is involved in *V* region alteration generating high-affinity Ab. *The Journal of Immunology,* 2005, 174: 4485–4494.

The Ag-driven B cells expressing high-affinity BCR, which have been selected in secondary lymphoid organs, generate high-affinity Ab. Ag-driven B cells proliferate rapidly by the stimulation with Ag and costimulatory molecules from Th cells surrounding the germinal center (GC)[3] region, in which such B cells undergo affinity maturation of Ig V region and class switching of the C region during the response to T cell-dependent Ag (TD-Ag) in vivo (1, 2). For affinity maturation, introduction of somatic hypermutation (SHM) in the *V* region is probably essential and, in addition to this molecular alteration, the Ag-driven B cells with high-affinity BCR must be selected or further enriched during the maturation of Ag-driven B cells in GCs.

A 210-kDa germinal center-associated DNA primase (GANP) protein, bearing RNA-primase and minichromosome maintenance (MCM)3-binding activities, is up-regulated in GC B cells upon immunization with TD-Ag in vivo and is induced by the stimulation to BCR and CD40 in vitro (3, 4). The mutant mouse with *ganp* gene knockout B cells (B-$ganp^{-/-}$) has a severe defect in mounting high-affinity Ab responses to TD-Ag (5), suggesting that GANP is required for generation of high-affinity Ab in response to TD-Ag in vivo. However, there remained several possibilities to account for the molecular mechanism in generation of high-affinity V regions by the expression of GANP in GC B cells. GANP might augment the induction of SHM in the *V* regions, resulting in the affinity maturation of V regions during the proliferation and differentiation of Ag-driven B cells in GCs. Alternatively, GANP might be involved in the survival of the high-affinity BCR^+ B cells for the positive selection through the interaction of Ags captured on the follicular dendritic cell network. The GCs of the B-$ganp^{-/-}$ mice displayed an increase of apoptotic cells upon immunization with TD-Ag SRBC, which suggested a partial involvement of GANP in the survival of GC B cells. However, the $ganp^{-/-}$ B cells do not show marked abnormalities in the levels of apoptotic and proapoptotic molecules after BCR cross-linkage (5). To study the function of GANP in generation of high-affinity Ab response, it is necessary to examine whether the affinity maturation of BCR on the GC B cell is generated by the genetic alteration in the *V* region gene.

We speculated that it would be possible to generate a high-affinity Ab if we used mice with higher level GANP expression in B cells. We studied whether the transgenic mouse with increased expression of *ganp* gene could generate high-affinity Ab against TD-Ag using a model epitope of 4-hydroxy-3-nitrophenyl acetyl (NP)-hapten in the C57BL/6 background. To demonstrate the increased affinity of the Ab in detail, we established the hybridomas secreting anti-NP mAbs after immunization with NP-chicken gammaglobulin (CG) in $Ganp^{Tg}$ mice. After selecting the high-affinity mAbs against NP-hapten by differential ELISA method and the BIAcore system, we examined the *V* region gene usage of the hybridomas and compared the sequences with those from wild-type C57BL/6 mice. The results suggest that the affinity maturation of BCR on GC B cells is generated by the altered V_H region usage with increased SHM in $Ganp^{Tg}$ mice.

Materials and Methods

$Ganp^{Tg}$ *mouse*

The expression construct of mouse *ganp* cDNA under the mouse Ig promoter and human Ig enhancer (6) was used for establishing the transgenic

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[3] Abbreviations used in this paper: GC, germinal center; GANP, germinal center-associated DNA primase; NP, 4-hydroxy-3-nitrophenyl acetyl; SHM, somatic hypermutation; TD-Ag, T cell-dependent Ag; MCM, minichromosome maintenance; CG, chicken gammaglobulin; KLH, keyhole limpet hemocyanin; TNP, 2,4,6-trinitrophenyl; LTR, long terminal repeat.

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