requirement of homozygosity at the class I locus for resistance to FV was later attributed to the influence of the class I genotypes on the production of cytokines upon FV inoculation [90,91]. Now that heterozygous $H2^{a/b}$ mice have been shown to exhibit resistance to FV-induced disease development even after a high-dose inoculation, provided that the FV complex used is free of LDV contamination, the above requirement for class I homozygosity must be reconsidered. In particular, since LDV induces rapid production of IFN-γ as well as type-I IFN, and also suppresses T-cell responses at an early stage of infection [74–77], the previous data on cytokine production after FV infection must be reexamined.

In the experiments performed with highly susceptible $CB6F_1$ mice and a low dose of FV, we have shown that the mice were protected from the development of splenomegaly, as well as fatal leukemia, by a single immunization with the peptide that harbored an F-MuLV epitope recognized by $CD4^+$ T-cells [72,73]. The reported experiments were performed with an LDV-contaminated stock of FV, but the same protective efficacy was reproduced by using the LDV-free preparation of FV (Fig. 4A). Importantly, cells producing FV became undetectable by 1 month after the challenge infection in the peptide-immunized animals [73]: even when cells from the entire spleen were inoculated as infectious centers, no FV production was detected in the immunized and challenged animals, indicating successful induction of sterilizing immunity (Fig. 5). Interestingly, similarly effective protection against FV infection was induced by immunization with the same $CD4^+$ T-cell epitope in the animals lacking $CD8^+$ T cells [73]. Sterilizing immunity was confirmed in the protected, $CD8^+$ T cell-deficient animals, although some of the immunized animals did develop leukemia in the absence of $CD8^+$ T-cells. On the other hand, mice lacking B cells due to the targeted disruption of the immunoglobulin μ-chain gene were not protected at all (Fig. 5), despite successful priming of $CD4^+$ T-cells with the peptide and the activation of $CD4^+$ as well as $CD8^+$ T-cells upon FV infection [73].

To further strengthen the efficacy of $CD4^+$ T-cell priming for immunity against FV challenge, we performed another set of experiments utilizing recombinant vaccinia virus expressing the *gag* gene as immunogens. Two partially overlapping CTL epitopes have been identified in the leader peptide of the gag precursor, $Pr75^{gag}$ [63,65,69], while a $CD4^+$ T-cell epitope has been identified within the MA protein [68]. The latter epitope was shown to induce protection against a high dose of FV in $H2^{a/b}$ (B6 × A/WySn)F_1 mice, but this previous experiment was performed with the naturally resistant strain and the LDV-contaminated stock of FV. Thus, highly susceptible $CB6F_1$ mice and a low dose of FV were utilized for the reexamination. Mice immunized with the recombinant vaccinia virus that expressed the leader peptide and the entire MA were protected, while those immunized with the control vaccine died (Fig. 6). Interestingly, the recombinant vaccinia virus (ΔBS) that expressed the leader peptide plus a shorter fragment of the MA portion, which lacked the identified $CD4^+$ T-cell epitope, and another virus expressing the CTL epitopes alone, were both unable to protect immunized $CB6F_1$ mice from FV challenge. The results shown in Figs. 5 and 6 indicate that in the presence of efficiently primed $CD4^+$ T-helper cells, retrovirus-infected cells can be eliminated through effector mechanisms other than CTL. B

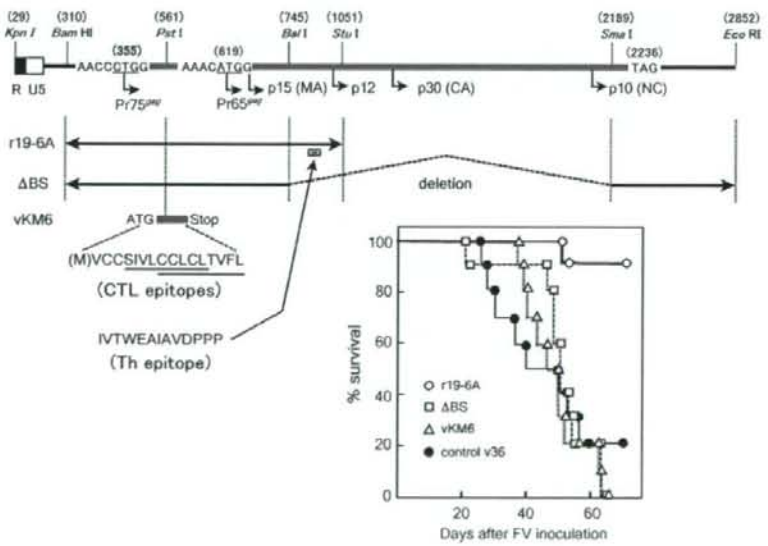

Figure 6 Expression of shorter fragments of the F-MuLV *gag* gene or the overlapping CTL epitopes alone in recombinant vaccinia viruses and protection of highly susceptible $CB6F_1$ mice against FV infection. Immunized $CB6F_1$ mice were challenged with 30 SFFU FV. vKM6 harbored a synthetic oligonucleotide that encoded the indicated epitopes plus an initiation codon (M) added at the N-terminus. The oligonucleotide was kindly provided by Dr. S. Takeshita, Department of Biophysics, Graduate School of Kyoto University, Kyoto, Japan. See [57,65] for the preparation of recombinant vaccinia viruses used, and [68] for the methods of immunization, FV challenge, and disease evaluation.

cells are absolutely required, however, for immune protection against retroviral infections.

Rfv3 gene that influences the production of virus-neutralizing antibodies

An association between genotypes at a non-MHC host gene and the development of FV-induced disease was first described by analyzing the persistence of viremia after FV infection [92]: A/WySn mice remained viremic at >30 days after FV infection, while B10.A mice had cleared viremia by post-inoculation day (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 *Rfv3*[s]. Rfv stands for "recovery from Friend virus" [93], and two other *Rfv* loci are described in Table 1 and were discussed earlier. Thus, B10 mice possess a dominant allele, the *Rfv3*[r], conferring the early clearance of viremia. The *Rfv3* locus was later mapped within chromosome 15 [94,95]. To clarify the relationships between the *Rfv3* genotypes and the production of virus-neutralizing Ab, we performed genetic mapping experiments by using >200 (B10.A × A/WySn) × A/WySn backcross mice [96]. (B10.A × A/WySn)F_1 mice possessed F-MuLV-neutralizing Ab in their sera by PID 14, while A/WySn mice failed to produce detectable levels of virus-neutralizing Ab even at PID 21. 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 (Fig. 7), colocalizing with the *Rfv3* locus previously mapped by measuring viremia [94,95].

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 receptor for B cell-activating factor belonging to the TNF family (BAFF-R) that results in the attenuation of germinal center responses of antigen-simulated B-lymphocytes [97,98]. The structural gene for the BAFF-R is located in chromosome 15, a few megabase pairs (Mbp) telomeric to the D15Mit71 marker (Fig. 7). The autosomal codominant gene defect at the *Baffr* locus in A/WySn mice was described as the B-cell maturation defect (*Bcmd*) locus, and was characterized by a reduced number of peripheral B cells, shorter B-cell half life, and poorer secondary IgG responses in comparison with those in control A/J mice, despite unaffected primary humoral responses to both T-dependent and T-independent antigens [99]. An approximately 4.7-kb insertion in the exon 3 of the *Baffr* gene in A/WySn mice has resulted in the frame-shifting and changes in the amino acid sequence of the cytoplasmic domain of BAFF-R [98]. The observed phenotypes of A/WySn mice are similar to those of the BAFF-deficient mice, both characterized by reduced numbers of surface (s) IgM^{low}, $sIgD^{high}$ spelenic B cells and the reduced sizes and rapid disappearance of germinal centers after an antigenic stimulation [100]. To examine if the expression of the unaffected *Baffr* allele in the A/WySn background restores the above functional defects in peripheral B cells, we infected fertilized eggs from A/WySn mice with a recombinant lentivirus that harbored the *Baffr* cDNA cloned from a B6 mouse and established several lines of transgenic mice. The expression of the *Baffr* was driven with the $Ig\kappa$ 3′ enhancer and a V_H

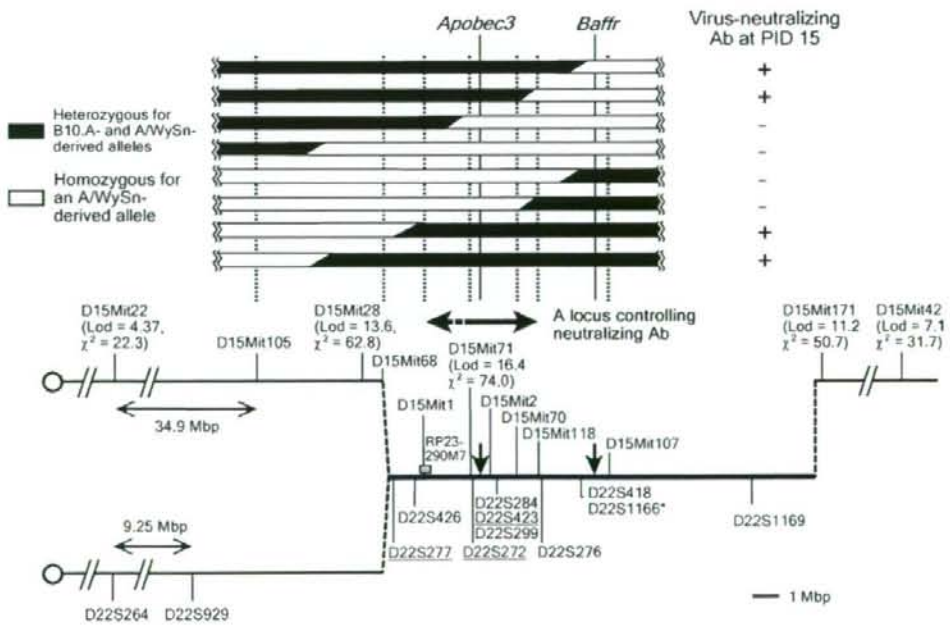

Figure 7 Mapping of the *Rfv3* locus in mouse chromosome 15. See [96] for details. The *Baffr* genotypes were determined by polymerase chain reaction detecting the 4.7-kbp insert found in A/WySn mice [98].

promoter. The expression of the B6 *Baffr* cDNA in the transgenic A/WySn mice was detected, and an increase in the numbers of $sIgM^{low}$, $sIgD^{high}$ splenic B cells was observed (Tsuji-Kawahara S, Kinoshita S, Miyazawa M, unpublished observation). Infection of the progenies from the above *Baffr*-transgenic A/WySn mice with FV is currently underway.

It should be noted, however, that the putative *Rfv3* locus was mapped centromeric to the *Baffr* locus, and two backcross mice that possessed a critical recombination between D15Mit118 and the *Baffr* locus nevertheless produced F-MuLV-neutralizing Ab by PID 15 in the absence of the wild-type *Baffr* allele (Fig. 7). Further, A/WySn mice have been shown to produce high-affinity, class-switched Ab to T-dependent antigens [100], albeit for a shorter duration than A/J mice, a phenotype apparently contradictory to the observed lack of the production of virus-neutralizing Ab in FV-infected A/WySn mice [96]. Thus, it is also possible that the *Rfv3* may not be identical to the *Baffr*, but may rather be explained by the functions of another locus within the same candidate region on chromosome 15. To explore the possible candidate locus for *Rfv3*, we compared at different time-points after FV infection expression levels of all of the open reading frames located within the segment of mouse chromosome 15 that had been narrowed by the backcross studies and found that mouse APOBEC3 was expressed in (B10.A × A/WySn)F_1 mice at higher levels than in A/WySn mice at 5–7 days after FV infection (Fig. 8). As a control, the levels of expression of F-MuLV *env* were not different between the two strains at PID 5, and higher copy numbers of F-MuLV *env* were detected in A/WySn mice at PID 7, indicating more vigorous replication of FV in susceptible A/WySn mice. Importantly, the *Apobec3* locus is located in the middle of the mapped candidate region, between the D15mit71 and D15Mit2 markers (Fig. 7). As has been described in the earlier part of this review, mouse APOBEC3 is not supposed to restrict the replication of ecotropic gammaretroviruses [40,41]. Recent studies have indicated, however, that a certain isoform of mouse APOBEC3 can be preferentially packaged into MuLV virions, and mouse APOBEC3 does restrict the replication of mouse mammary tumor virus [41,42]. Thus, it is possible that the replication of FV might also be restricted, at least partially, in the presence of the resistance-associated isoform of mouse APOBEC3. If this is the case, the resultant less massive expansion of erythroid progenitor cells may allow the preservation of normal splenic architecture, which is required for efficient interactions between B-lymphocytes and follicular dendritic and/or T-helper cells. Thus, the higher expression of mouse APOBEC3 in B6/B10 mice (Fig. 8) may thereby provide the host immune system a window to produce FV-neutralizing Ab before the virus replication becomes uncontrollable. In other words, in A/WySn mice rapid replication of FV in the poor expression of APOBEC3 may result in a rapid expansion in the number of erythroid progenitor cells in the spleen which, in turn, derange the interactions between immune cells before virus-neutralizing Ab can be produced. In this regard, the expression levels of human APOBEC3G were significantly higher in the peripheral blood monocytes from HIV-exposed but uninfected individuals, who possess HIV-1-reactive IgA in the absence of IgG seroconversion, in

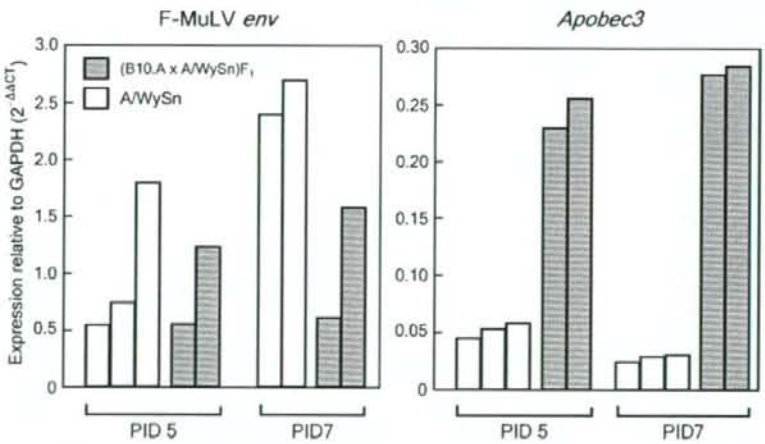

Figure 8 Quantification of the F-MuLV *env* and *Apobec3* mRNA expression by real-time polymerase chain reaction in the spleen of FV-infected A/WySn and (B10.A × A/WySn)F1 mice. The experiments were performed essentially as described in [101]. RNA extraction from dry-ice pressed spleen tissues was performed by using the TRIzol solution (Invitrogen, Japan; K.K., Tokyo, Japan) and poly-A^+ RNA was purified from 1 mg of total RNA for each sample with the use of MicroFast Track 2.0 system (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR reaction was performed by using the Platinum Quantitative RT-PCR ThermoScript One-Step system (Invitrogen) and an ABI Prizm 7700 cycler/detector (Applied Biosystems, Foster City, California), according to the manufacturers' instructions. Sequence-specific primers and probes used are as follows: mouse *Apobec3* forward, CTCTACAACATACGGGACCCAGA; reverse, CCACCTGGGCTCCTTCCT; probe, AACCAGCAGAATCTTTGCAGGCTGGTT; F-MuLV *env* forward, GCTGCGAGACAACCGGTAGA; reverse, GCATACCTGAACAGCCTGGTTA; and probe, TTCTTGGGACTACATCACAGT. TaqMan rodent *GAPDH* control (Applied Biosystems) was added to each reaction mixture to be used as a normalizer, and the levels of expression of each gene tested were expressed by $2^{-\Delta CT}$.

comparison with those from their HIV-infected partners [101].

Immunological characteristics of HIV-1-exposed but uninfected individuals

Susceptibility to HIV infection can vary widely among human individuals. There are individuals who are repeatedly exposed to HIV-1, but exhibit no evidence of productive infection. Such cases are called HIV-1-exposed but uninfected or HIV-1-exposed, seronegative (ESN) individuals (reviewed in [4]). Examples of ESN individuals include seronegative infants born to HIV-1-infected mothers, highly exposed but persistently seronegative blood donors or intravenous drug users, commercial sex workers who have remained uninfected despite years of high-risk exposure, and AIDS widows who have had unprotected sexual contacts with their infected husbands, but remain uninfected. It is noteworthy that a large-scale cohort study of commercial sex workers in Nairobi [102] has indicated that a sizable fraction, estimated to be about 15%, of the at-risk sex workers apparently show a naturally acquired resistance to HIV-1 infection and remain uninfected for years. A critical and extensive review of literature describing correlates of highly HIV-1-exposed but persistently uninfected individuals [4] summarized that natural resistance to HIV-1 acquisition is mediated by multiple mechanisms, including genetic and immunological ones. Cytotoxic T-cell activities specific for HIV-1 antigens have been observed at a high prevalence and strongly associated with ESN status, while genetic factors which can confer cellular resistance to HIV-1 infection, such as *CCR5Δ32* homozygosity, are observed at only low frequencies. In fact, it has been estimated that the cumulated effects of known AIDS-restricting genes can explain only up to 10% of the host determinants that control HIV-1/AIDS [5].

The detection of HIV-1-reactive T-cell responses in the absence of HIV-1 genome or proviruses in the peripheral blood in ESN individuals suggests that such individuals may be protected against HIV-1 infection through prior priming of T cells with HIV-1 antigens and the resultant efficient induction of effector mechanisms upon reentry of the virus [4,103]. Further, the presence of HIV-1-reactive IgA in mucosal secretions or sera in the absence of apparent IgG or IgM seroconversion has been reported in ESN individuals [104–107], and some of these IgA Ab have been shown to neutralize HIV infectivity [105–107]. The production of antiviral IgA in the absence of IgG seroconversion might appear peculiar in view of the conventional understanding of immunoglobulin class-switching. It has been well-documented, however, that mucosal and systemic antibody responses are mediated by quite independent cellular compartments, and pathogen-reactive IgA responses can be induced in the absence of serum IgG production [108]. Further, signaling requirements in T- and B-cell interactions for mucosal and systemic IgA responses are quite different [109], and $CD4^+$ T cell-dependent, virus-neutralizing IgA production can be induced in the absence of MHC class II- and CD40-dependent T–B interactions that are required for IgM and IgG production [110]. Thus, it is possible that ESN individuals may have been exposed to HIV-1 in such a way that selective IgA production is induced in the absence of serum IgG, or that they are genetically predisposed to exert preferential mucosal IgA responses in association with strong T-cell responses.

Genes associated with HIV-1-exposed but uninfected status

Previous studies have shown that none of the known AIDS-restricting alleles are found at a significantly high frequency in cohorts of ESN individuals [4]. In fact, the homozygous *CCR5Δ32* mutation, which results in the lack of the HIV coreceptor, is known to be rare among the ESN individuals in Italy and Thailand [111–113], and a recent analysis of a separate cohort of repeatedly exposed but HIV-1-seronegative individuals in the United States [114] demonstrated the lack

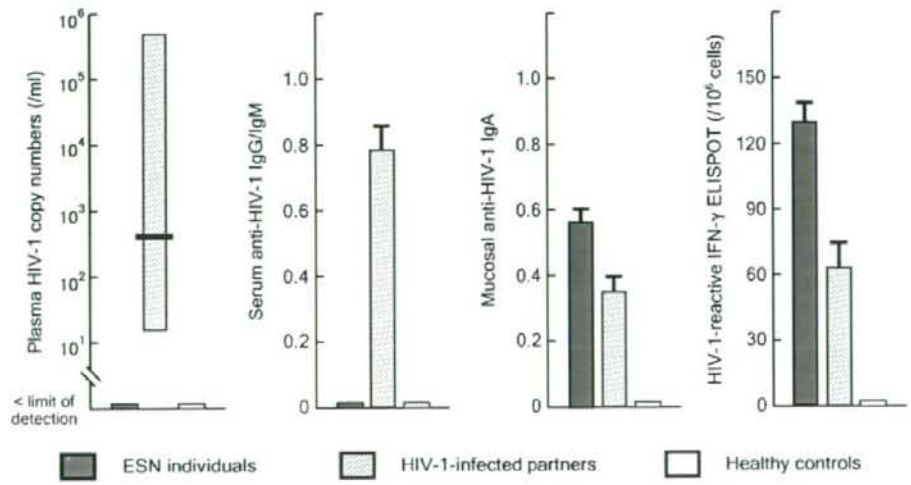

Figure 9 Phenotypes of HIV-1-exposed but uninfected (exposed seronegative: ESN) individuals, their HIV-infected partners, and healthy controls from the same geographic region. See [96] for details.

of association between genotypes at the *CCR2*, *SDF1*, and *RANTES* loci and the uninfected status. To characterize the ENS individuals genetically, we analyzed a cohort of ESN individuals who showed strong HIV-1-reactive T-cell responses and possessed HIV-1-reactive IgA in mucosal secretions, but had remained negative for HIV-1 genome and proviruses in the peripheral blood, despite years of unprotected sexual contacts with their HIV-1-infected partners (Fig. 9). Previous genetic analyses have mapped an ESN-associated gene locus in a segment of human chromosome 22, harbouring the microsatellite markers D22S277, D22S272, and D22S423 [96]. This region is syntenic to the segment of mouse chromosome 15 where the *Rfv3* locus was mapped (Fig. 7). To further narrow down the location of the putative HIV-1 resistance gene, we genotyped additional ESN and HIV-infected individuals enrolled from the same area of Italy at multiple loci of known single nucleotide polymorphism (SNP). Consistent with our previous observations, the number of enrollees who possessed the allele 229 at the D22S423 locus was significantly higher among the ESN than among the HIV-infected individuals. Further, analyses of linkage disequilibrium (LD) revealed a strong linkage ($p < 10^{-5}$) between the rs202642 and rs139562 loci in the HIV-1-infected individuals, but a lack of such linkage among the ESN individuals, consistent with the previously reported disruption of LD at the D22S276 locus (Fig. 10).

These results indicate that a significant fraction of ESN individuals share a similar genetic background that differs from those of their HIV-1-infected partners. In other words, ESN individuals exhibit the resistance-associated phenotypes (Fig. 9) not because of their possible exposures to replication-defective HIV-1 or non-infectious forms of the viral antigens, but they may exert stronger and IgA-oriented immune responses due to the possession of a specific genotype at a polymorphic locus in chromosome 22. Extensive genome sequencing of the candidate regions along with expression analyses utilizing DNA microarrays have already indicated the presence of such functional polymorphism in chromosome 22 (Kanari Y, Irie S, Sakamoto M, Trabattoni D, Biasin M, Piacentini L, et al., submitted for publication).

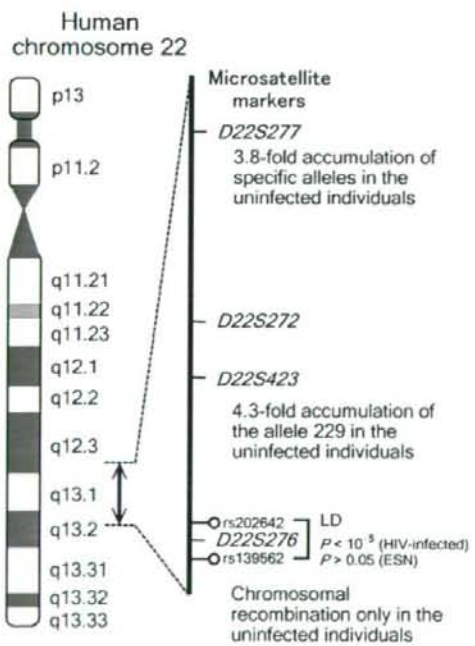

Figure 10 Genetic differences between ESN and HIV-1-infected individuals in a segment of chromosome 22. See [96] for details.

Acknowledgements

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References

[1] UNAIDS/WHO. Report on the global AIDS epidemic; 2006. http://www.unaids.org/en/HIV_data/2006GlobalReport/default.asp.
[2] Shao Y. AIDS epidemic at age 25 and control effort in China. Retrovirology 2006;3:87.
[3] O'Brien SJ, Nelson GW, Winkler CA, Smith MW. Polygenic and multifactorial disease gene association in man: lessons from AIDS. Annu Rev Genet 2000;34:563–91.
[4] Kulkarni PS, Butera ST, Duerr AC. Resistance to HIV-1 infection: lessons learned from studies of highly exposed persistently seronegative (HEPS) individuals. AIDS Rev 2003;5:87–103.
[5] O'Brien SJ, Nelson GW. Human genes that limit AIDS. Nat Genet 2004;36:565–74.
[6] Lilly F. Susceptibility to two strains of Friend leukemia virus in mice. Science 1967;155:461–2.
[7] Kabat D. Molecular biology of Friend viral erythroleukemia. Curr Top Microbiol Immunol 1989;148:1–42.
[8] Chesebro B, Miyazawa M, Britt WJ. Host genetic control of spontaneous and induced immunity to Friend murine retrovirus infection. Annu Rev Immunol 1990;8:477–99.
[9] Hasenkrug KJ, Chesebro B. Immunity to retroviral infection: the Friend virus model. Proc Natl Acad Sci USA 1997;94:7811–6.
[10] Miyazawa M. Host genes that influence immune and non-immune resistance mechanisms against retroviral infections. Recent Res Dev Virol 2004;6:105–18.
[11] Nethe M, Berkhout B, van der Kuyl AC. Retroviral superinfection resistance. Retrovirology 2005;2:52.
[12] Evans LH, Malik FG. Class II polytropic murine leukemia viruses (MuLVs) of AKR/J mice: possible role in the generation of class I oncogenic polytropic MuLVs. J Virol 1987;61:1882–92.
[13] Overbaugh J, Miller AD, Eiden M. Receptors and entry cofactors for retroviruses include single and multiple transmembrane-spanning proteins as well as newly described glycophosphatidylinositol-anchored and secreted proteins. Microbiol Mol Biol Rev 2001;65:371–89.
[14] Albritton LM, Tseng L, Scadden D, Cunningham JM. A putative murine ecotropic retrovirus receptor gene encodes a multi-

ple membrane-spanning protein and confers susceptibility to virus infection. Cell 1989;57:659–66.
[15] Kim JW, Closs EL, Albritton LM, Cunningham JM. Transport of cationic amino acids by the mouse ecotropic retrovirus receptor. Nature 1991;352:725–8.
[16] Wang H, Kavanaugh MP, North RA, Kabat D. Cell-surface receptor for ecotropic murine retroviruses is a basic amino-acid transporter. Nature 1991;352:729–31.
[17] Tailor CS, Nouri A, Lee CG, Kozak C, Kabat D. Cloning and characterization of a cell surface receptor for xenotropic and polytropic murine leukemia viruses. Proc Natl Acad Sci USA 1999;96:927–32.
[18] Battini JL, Rasko JE, Miller AD. A human cell-surface receptor for xenotropic and polytropic murine leukemia viruses: possible role in G protein-coupled signal transduction. Proc Natl Acad Sci USA 1999;96:1385–90.
[19] Yang YL, Guo L, Xu S, Holland CA, Kitamura T, et al. Receptors for polytropic and xenotropic mouse leukaemia viruses encoded by a single gene at *Rmc1*. Nat Genet 1999;21:216–9.
[20] Marin M, Tailor CS, Nouri A, Kozak SL, Kabat D. Polymorphisms of the cell surface receptor control mouse susceptibilities to xenotropic and polytropic leukemia viruses. J Virol 1999;73:9362–8.
[21] Miller DG,R, Edwards H, Miller AD. Cloning of the cellular receptor for amphotropic murine retroviruses reveals homology to that for gibbon ape leukemia virus. Proc Natl Acad Sci USA 1994;91:78–82.
[22] van Zeijl M, Johann SV, Closs E, Cunningham J, Eddy E, Shows TB, et al. A human amphotropic retrovirus receptor is a second member of the gibbon ape leukemia virus receptor family. Proc Natl Acad Sci USA 1994;91:1168–72.
[23] Olah Z, Lehel C, Anderson WB, Eiden MV, Wilson CA. The cellular receptor for gibbon ape leukemia virus is a novel high affinity sodium-dependent phosphate transporter. J Biol Chem 1994;269:25426–31.
[24] Bahrami S, Duch M, Pedersen FS. Change of tropism of SL3-2 murine leukemia virus, using random mutational libraries. J Virol 2004;78:9343–453.
[25] Van Hoeven NS, Miller AD. Use of different but overlapping determinants in a retrovirus receptor accounts for non-reciprocal interference between xenotropic and polytropic murine leukemia viruses. Retrovirology 2005;2: 76.
[26] Miller DG, Miller AD. A family of retroviruses that utilize related phosphate transporter for cell entry. J Virol 1994;68:8270–6.
[27] Ikeda H, Laigret F, Martin MA, Repaske R. Characterization of a molecularly cloned retroviral sequence associated with Fv-4 resistance. J Virol 1985;55:768–77.
[28] Ikeda H, Sugimura H. *Fv-4* resistance gene: a truncated endogenous murine leukemia virus with ecotropic interference properties. J Virol 1989;63:5405–12.
[29] Nisole S, Saïb A. Early steps of retrovirus replication cycle. Retrovirology 2004;1:9.
[30] Suzuki Y, Craigie R. The road to chromatin—nuclear entry of retroviruses. Nat Rev Microbiol 2007;5:187–96.
[31] Lori F, di Marzo Veronese F, de Vico AL, Lusso P, Reitz Jr MS, Gallo RC. Viral DNA carried by human immunodeficiency virus type 1 virions. J Virol 1992;66:5067–74.
[32] Trono D. Partial reverse transcripts in virions from human immunodeficiency and murine leukemia viruses. J Virol 1992;66:4893–900.
[33] Zhu J, Cunningham JM. Minus-strand DNA is present within murine type C ecotropic retroviruses prior to infection. J Virol 1993;67:2385–8.
[34] Harris RS, Bishop KN, Sheehy AM, Craig HM, Petersen-Mahrt SK, Watt IN, et al. DNA deamination mediates innate immunity to retroviral infection. Cell 2003;113:803–9.
[35] Zhang H, Yang B, Pomerantz RJ, Zhang C, Arunachalam SC, Gao L. The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. Nature 2003;424:94–8.
[36] Zheng YH, Irwin D, Kurosu T, Tokunaga K, Sata T, Peterlin BM. Human APOBEC3F is another host factor that blocks human immunodeficiency virus type 1 replication. J Virol 2004;78:6073–6.
[37] Mariani R, Chen D, Schrofelbauer B, Navarro F, Koening R, Bollman B, et al. Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. Cell 2003;114:21–31.
[38] Mehle A, Strack B, Ancute P, Zhang C, McPike M, Gabzda D. Vif overcomes the innate antiviral activity of APOBEC3G by promoting its degradation in the ubiquitin–proteasome pathway. J Biol Chem 2004;279:7792–8.
[39] Yu X, Yu Y, Liu B, Luo K, Kong W, Mao P, et al. Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif-Cul5-SCF complex. Science 2003;302:1056–60.
[40] Doehle BP, Schäfer A, Wiegand HL, Bogerd HP, Cullen BR. Differential sensitivity of murine leukemia virus to APOBEC3-mediated inhibition is governed by virion exclusion. J Virol 2005;79:8201–7.
[41] Abudu A, Takaori-Kondo A, Izumi T, Shirakawa K, Kobayashi M, Sasada A, et al. Murine retrovirus escapes from murine APOBEC3 via two distinct novel mechanisms. Curr Biol 2006; 16:1565–70.
[42] Okeoma CN, Lovsin N, Peterlin BM, Ross SR. APOBEC3 inhibits mouse mammary tumor virus replication in vivo. Nature 2007;445:927–30.
[43] Goff SP. Retrovirus restriction factors. Mol Cell 2004;16: 849–59.
[44] Best S, Le Tissier P, Towers G, Stoye JP. Positional cloning of the mouse retrovirus restriction gene Fv1. Nature 1996;382:826–9.
[45] Bénit L, De Parceval N, Casella J-F, Callebaut I, Cordonnier A, Heidmann T. Cloning of a new murine endogenous retrovirus, MuERV-L, with strong similarity to human HERV-L element and with a *gag* coding sequence closely related to the *Fv1* restriction gene. J Virol 1997;71:5652–7.
[46] Bishop KN, Bock M, Towers G, Stoye JP. Identification of the regions of Fv1 necessary for murine leukemia virus restriction. J Virol 2001;75:5182–8.
[47] Kozak CA, Chakraborti A. Single amino acid changes in the murine leukemia virus capsid protein gene define the target of Fv1 resistance. Virology 1996;225:300–5.
[48] Stevens A, Bock M, Ellis S, Le Tissier P, Bishop KN, Yap MW, et al. Retroviral capsid determinants of *Fv1* NM and NR tropism. J Virol 2004;78:9592–8.
[49] Lassaux A, Sitbon M, Battini J-L. Residues in the murine leukemia virus capsid that differentially govern resistance to mouse *Fv1* and human *Ref1* restrictions. J Virol 2005;79:6560–4.
[50] Robertson MN, Miyazawa M, Mori S, Caughey B, Evans LH, Hayes SF, et al. Production of monoclonal antibodies reactive with a denatured form of the Friend murine leukemia virus gp70 envelope protein: use in a focal infectivity assay, immunohistochemical studies, electron microscopy, and Western blotting. J Virol Methods 1991;34:255–71.
[51] Persons DA, Paulson RF, Loyd MR, Herley MT, Bodner SM, Bernstein A, et al. *Fv2* encodes a truncated form of the Stk receptor tyrosine kinase. Nat Genet 1999;23:159–65.
[52] Nishigaki K, Thompson D, Hanson C, Yugawa T, Ruscetti S. The envelope glycoprotein of Friend spleen focus-forming virus covalently interacts with and constitutively activates a truncated form of the receptor tyrosine kinase Stk. J Virol 2001;75:7893–903.
[53] Lilly F. Fv-2: identification and location of a second gene governing the spleen focus response to Friend leukemia virus in mice. J Natl Cancer Inst 1970;45:163–9.

[54] Munroe DG, Peacock JW, Benchimol S. Inactivation of the cellular p53 gene is a common feature of Friend virus-induced erythroleukemia: relationship of inactivation to dominant transforming alleles. Mol Cell Biol 1990;10:3307–13.
[55] Moreau-Gachelin F, Tavitian A, Tanbourin P. *Spi-1* is a putative oncogene in virally induced murine erythroleukaemias. Nature 1988;331:277–80.
[56] Hasenkrug KJ. Lymphocyte deficiencies increase susceptibility to Friend virus-induced erythroleukemia in *Fv-2* genetically resistant mice. J Virol 1999;73:6468–73.
[57] Miyazawa M, Nishio J, Chesebro B. Genetic control of T cell responsiveness to the Friend murine leukemia virus envelope antigen: Identification of class II loci of the H-2 as immune response genes. J Exp Med 1988;168:1587–605.
[58] Miyazawa M, Nishio J, Wehrly K, Chesebro B. Influence of MHC genes on spontaneous recovery from Friend retrovirus-induced leukemia. J Immunol 1992;148:644–7.
[59] Miyazawa M, Nishio J, Wehrly K, David CS, Chesebro B. Spontaneous recovery from Friend retrovirus-induced leukemia. Mapping of the *Rfv-2* gene in the Q/TL region of mouse MHC. J Immunol 1992;148:1964–7.
[60] Miyazawa M, Nishio J, Wehrly K, Jay G, Melvold RW, Chesebro B. Detailed mapping of the *Rfv-1* gene that influences spontaneous recovery from Friend retrovirus-induced leukemia. Eur J Immunogenet 1992;19:159–64.
[61] Robertson MN, Spangrude GJ, Hasenkrug K, Perry L, Nishio J, Wehrly K, et al. Role and specificity of T-cell subsets in spontaneous recovery from Friend virus-induced leukemia in mice. J Virol 1992;66:3271–7.
[62] Perry LL, Miyazawa M, Hasenkrug K, Wehrly K, David CS, Chesebro B. Contrasting effects from a single major histocompatibility complex class II molecule (H-2E) in recovery from Friend virus leukemia. J Virol 1994;68:4921–6.
[63] Chen W, Qin H, Chesebro B, Cheever MA. Identification of a *gag*-encoded cytotoxic T-lymphocyte epitope from FBL-3 leukemia shared by Friend, Moloney, and Rauscher murine leukemia virus-induced tumors. J Virol 1996;70:7773–82.
[64] Iwashiro M, Kondo T, Shimizu T, Yamagishi H, Takahashi K, Matsubayashi Y, et al. Multiplicity of virus-encoded helper T-cell epitopes expressed on FBL-3 tumor cells. J Virol 1993;67:4533–42.
[65] Kondo T, Uenishi H, Shimizu T, Hirama T, Iwashiro M, Kuribayashi K, et al. A single retroviral gag precursor signal peptide recognized by FBL-3 tumor-specific cytotoxic T lymphocytes. J Virol 1995;69:6735–41.
[66] Ruan K, Lilly F. Identification of an epitope encoded in the *env* gene of Friend murine leukemia virus recognized by anti-Friend virus cytotoxic T lymphocytes. Virology 1991;181:91–100.
[67] Shimizu T, Uenishi H, Teramura Y, Iwashiro M, Kuribayashi K, Tamamura H, et al. Fine structure of a virus-encoded helper T-cell epitope expressed on FBL-3 tumor cells. J Virol 1994;68:7704–8.
[68] Sugahara D, Tsuji-Kawahara S, Miyazawa M. Identification of a protective $CD4^+$ T-cell epitope in $p15^{gag}$ of Friend murine leukemia virus and role of the MA protein targeting to the plasma membrane in immunogenicity. J Virol 2004;78:6322–34.
[69] Uenishi H, Iwanami N, Kuribayashi K, Tamamura H, Fujii N, Nakatani T, et al. Overlapping epitopes of Friend murine leukemia virus *gag*-encoded leader sequence recognized by single cytotoxic T-lymphocyte clones. Immunol Lett 1998;62:33–8.
[70] Uenishi H, Iwanami N, Yamagishi H, Nakatani T, Kawasaki T, Tamamura H, et al. Induction of cross-reactivity in an endogenous viral peptide non-reactive to FBL-3 tumor-specific helper T-cell clones. Microbiol Immunol 1998;42:479–84.
[71] Miyazawa M, Fujisawa R, Ishihara C, Takei YA, Shimizu T, Uenishi H, et al. Immunization with a single T helper cell epitope abrogates Friend virus-induced early erythroid proliferation and prevents late leukemia development. J Immunol 1995;155:748–58.
[72] Iwanami N, Niwa A, Yasutomi Y, Tabata N, Miyazawa M. Role of natural killer cells in resistance against Friend retrovirus-induced leukemia. J Virol 2001;75:3152–63.
[73] Kawabata H, Niwa A, Tsuji-Kawahara S, Uenishi H, Iwanami N, Matsukuma H, et al. Peptide-induced immune protection of $CD8^+$ T cell-deficient mice against Friend retrovirus-induced disease. Int Immunol 2006;18:183–98.
[74] Gerlach N, Schimmer S, Weiss S, Kalinke U, Dittmer U. Author's correction. Effect of type I interferons on Friend retrovirus infection. J Virol 2007;81:6160.
[75] Robertson SJ, Ammann CG, Messer RJ, Carmody AR, Myers L, Dittmer U, et al. Suppression of acute anti-Friend virus $CD8^+$ T cell responses by co-infection with lactate dehydrogenase-elevating virus. J Virol 2008;82:408–18.
[76] Plagemann PGW, Rowland RRR, Even C, Faaberg KS. Lactate dehydrogenase-elevating virus: an ideal persistent virus? Springer Semin Immunopathol 1995;17:167–86.
[77] Markin-Goryaynoff D, Hulhoven X, Cambiaso CL, Montayne P, Briet T, Gonzalez M-D, et al. Natural killer cell activation after infection with lactate dehydrogenase-elevating virus. J General Virol 2002;83:2709–16.
[78] Coutelier J-P, Coulie PG, Wauters P, Heremans H, van der Logt JTM. In vivo polyclonal B-lymphocyte activation elicited by murine viruses. J Virol 1990;64:5383–8.
[79] Steeves RA, Mirand EA, Thomson S, Avita L. Enhancement of spleen focus formation and virus replication in Friend virus-infected mice. Cancer Res 1969;29:1111–6.
[80] Hasenkrug KJ, Brooks DM, Nisho J, Chesebro B. Differing T-cell requirements for recombinant retrovirus vaccines. J Virol 1996;70:368–72.
[81] Hasenkrug KJ, Brooks DM, Robertson MN, Srinivas RV, Chesebro B. Immunoprotective determinants in Friend murine leukemia virus envelope protein. Virology 1998;248:66–73.
[82] McMichael A. HIV vaccines. Annu Rev Immunol 2006;24:227–55.
[83] Carrinton M, Nelson GW, Martin MP, Kissner T, Vlahof D, Goedert JJ, et al. *HLA* and HIV-1: Heterozygote advantage and *B*35-Cw*04* disadvantage. Science 1999;283:1748–52.
[84] Maritin P, Gao X, Lee J-H, Nelson GW, Detals R, Goedert JJ, et al. Epistatic interaction between *KIR3DS1* and *HLA-B* delays the progression to AIDS. Nat Genet 2002;31:429–34.
[85] Fellay J, Shianna KV, Ge D, Colombo S, Ledergerber B, Weale M, et al. A whole-genome association study of major determinants for host control of HIV-1. Science 2007;317:944–7.
[86] Matano T, Kobayashi M, Igarashi H, Takeda A, Nakamura H, Kano M, et al. Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial. J Exp Med 2004;199:1709–18.
[87] Costello RT, Fauriat C, Sinori S, Marcenaro E, Olive D. NK cells: innate immunity against hematopoietic malignancies? Trends Immunol 2004;26:328–33.
[88] Yokoyama WM, Plougastel BFM. Immune functions encoded by the natural killer gene complex. Nat Rev Immunol 2003;3:304–16.
[89] Hasenkrug KJ, Spangrude GJ, Nishio J, Brooks DM, Chesebro B. Recovery from Friend disease in mice with reduced major histocompatibility complex class I expression. J Virol 1994;68:2059–64.
[90] Peterson KE, Iwashio M, Hasenkrug KJ, Chesebro B. Major histocompatibility complex class I gene controls the generation of gamma interferon-producing $CD4^+$ and $CD8^+$ T cells important for recovery from Friend retrovirus-induced leukemia. J Virol 2000;74:5363–7.

[91] Peterson KE, Stromnes I, Messer R, Hasenkrug K, Chesebro B. Novel role of CD8+ T cells and major histocompatibility complex class I genes in the generation of protective CD4+ Th1 responses during retrovirus infection in mice. J Virol 2002;76:7942–8.

[92] Chesebro B, Wherly K. Identification of a non-H-2 gene (*Rfv-3*) influencing recovery from viremia and leukemia induced by Friend virus complex. Proc Natl Acad Sci USA 1979;76:425–9.

[93] Chesebro B, Wehrly K. *Rfv-1* and *Rfv-2*, two *H-2*-asspciated genes that influence recovery from Friend leukemia virus-induced splenomegaly. J Immunol 1978;120:1081–5.

[94] Hasenkrug KJ, Valenzuela A, Letts VA, Nishio J, Chesebro B, Frankel WN. Chromosome mapping of *Rfv3*, a host resistance gene to Friend murine retrovirus. J Virol 1995;69:2617–20.

[95] Super HJ, Hasenkrug KJ, Simmons S, Brooks DM, Konzek R, Sarge KD, et al. Fine mapping of the Friend retrovirus resistance gene, *Rfv3*, on mouse chromosome 15. J Virol 1999;73:7848–52.

[96] Kanari Y, Clerici M, Abe H, Kawabata H, Trabattoni D, Lo Caputo S, et al. Genotypes at chromosome 22q12-13 are associated with HIV-1-exposed but uninfected status in Italians. AIDS 2005;19:1015–24.

[97] Thompson JS, Bixler SA, Qian F, Vora K, Scott ML, cachero TG, et al. BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF. Science 2001;293:2108–11.

[98] Yan M, Brady JR, Chan B, Lee WP, Hsu B, Harless S, et al. Identification of a novel receptor for B lymphocyte stimulator that is mutated in a mouse strain with severe B cell deficiency. Curr Biol 2001;11:1547–52.

[99] Lents VM, Cancro MP, Nashold FE, Hayes CE. *Bcmd* governs recruitment of new B cells into the stable peripheral B cell pool in the A/WySnJ mouse. J Immunol 1996;157:598–606.

[100] Rahman ZSM, Rao SP, Kalled SL, Manser T. Normal induction but attenuated progression of germinal center responses in BAFF and BAFF-R signaling-deficient mice. J Exp Med 2003;198:1157–69.

[101] Biasin M, Piacentini L, Lo Caputo S, Kanari Y, Magri G, Trabattoni D, et al. APOBEC3G: A possible role in resistance of HIV-exposed seronegative individuals. J Infect Dis 2007;195:960–4.

[102] Fowke KR, Nagelkerke NJ, Kimani J, Simonsen JN, Anzala AO, Bwayo JJ, et al. Resistance to HIV-1 infection among persistently seronegative prostitutes in Nairobi, Kenya. Lancet 1996;348:1347–51.

[103] Shearer GM, Clerici M. Protective immunity against HIV infection: has nature done the experiment for us? Immunol Today 1996;17:21–4.

[104] Mazzoli S, Trabattoni D, Lo Caputo S, Piconi S, Blé C, et al. HIV-specific mucosal and cellular immunity in HIV-seronegative partners of HIV-seropositve individuals. Nat Med 1997;3:1250–7.

[105] Lieng Q, Nilsson C, Sourial S, Andersson S, Larsen O, Aaby P, et al. Potent neutralizing serum immunoglobulin A (IgA) in human immunodeficiency virus type 2-exposed IgG-seronegative individuals. J Virol 2004;78:701607022.

[106] Mazzoli S, Lopalco L, Siavi A, Trabattoni D, Lo Caputo S, Semplici F, et al. Human immunodeficiency virus (HIV)-specific IgA and HIV neutralizing activity in the serum of exposed seronegative partners of HIV-seropositive persons. J Infect Dis 1999;180:871–5.

[107] Devito C, Broliden K, Kaul R, Svensson L, Johansen K, Kiama P, et al. Mucosal and plasma IgA from HIV-1-exposed uninfected individuals inhibit HIV-1 transcytosis across human epithelial cells. J Immunol 2000;165:5170–6.

[108] Fagarasan S, Honjo T. T-independent immune response: new aspects of B cell biology. Science 2000;290:89–92 (2000).

[109] Gardby E, Wrammert J, Schön, Ekman L, Leanderson T, Lycke N. Strong differential regulation of serum and mucosal IgA responses as revealed in CD28-deficient mice using cholera toxin adjuvant. J Immunol 2003;170:55–63.

[110] Sangster MY, Riberdy JM, Gonzalez M, Topham DJ, Baumgarth N, Doherty PC. An early CD4+ T cell-dependent immunoglobilin A responses to influenza virus in the absence of key cognate T–B interactions. J Exp Med 2003;198:1011–21.

[111] Mazzoli S, Trabattoni D, Lo Caputo S, Piconi S, Blé C, Meacci F, et al. HIV-specific mucosal and cellular immunity in HIV-seronegative partners of HIV-seropositive individuals. Nat Med 1997;3:1250–7.

[112] Lo Caputo S, Trabattoni D, Vichi F, Piconi S, Lopalco L, Villa ML, et al. Mucosal and systemic HIV-specific immunity in HIV-exposed but uninfected heterosexual males. AIDS 2003;17:531–8.

[113] Beyrer C, Artenstein AW, Rugpao S, Stephens H, Van Cott TC, Robb ML, et al. Epidemiologic and biologic characterization of a cohort of human immunodeficiency virus type 1 highly exposed, persistently seronegative female sex workers in northern Thailand. J Infect Dis 1999;79:59–68.

[114] Liu H, Hwangbo Y, Holte S, Lee J, Wang C, Kaupp N, et al. Analysis of genetic polymorphisms in CCR5, CCR2, stromal cell-derived factor-1, RANTES, and dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin in seronegative individuals repeatedly exposed to HIV-1. J Infect Dis 2004;190:1055–8.

Chapter 20

Chimeric Recombinant Hepatitis E Virus-like Particles Presenting Foreign Epitopes as a Novel Vector of Vaccine by Oral Administration

Yasuhiro Yasutomi[*,†]

Virus-like particles (VLPs) are useful for studies on virion formation, host immune responses to viruses, and vaccines in no practical cell culture systems to allow the growth of virus. Among the various non-replicating molecules, VLP, an empty particle with a structure similar to that of an authentic virus particle, offers the possibility of a new approach for these studies. Hepatitis E virus (HEV) is an unclassified calicivirus-like, positive-strand RNA virus that causes human acute hepatitis by fecal-oral transmission. HEV first infects epithelial cells of the small intestine and then reaches the liver through the portal vein. It has been reported that intact open reading frame 2 (ORF2) of HEV is expressed as a membrane glycoprotein when artificially expressed in mammalian cells *in vitro*, probably because the N-terminal amino acid sequence serves as a signal peptide.[1,2] On the other hand, the intact ORF2 expression in insect cells resulted in various sizes of proteins with cleavages on both N- and C-termini, of which 53 kD polypeptides secreted in the culture supernatant.[3–5] It has also been reported that only after cleavage of C-terminal, which results in molecular weight reduction to

*Laboratory of Immunoregulation and Vaccine Research, Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba, Ibaraki 305-0843, Japan.
†Department of Immunoregulation, Mie University Graduate School of Medicine, Mie 514-8507, Japan. E-mail: yasutomi@nibio.go.jp; yasutomi@doc.medic.mie-u.ac.jp.

54 kD, does ORF2 participate in the VLP formation.[6,7] In this chapter, investigation of HEV-VLP could be carried a molecule for foreign antigenic epitopes and to stimulate mucosal immunity without the need for adjuvant.

Chimerization of VLP

Chimerization of VLP is a unique and useful method for studying morphology, assembly and host recognition of a virus. However, there are not many reports about chimeric VLP in the fields of virology and immunology. Moreover, the induction of immune responses through natural infectious route against not only VLPs but also carrying epitopes is limited.[8–12] Some chimeric plant virus particles carrying foreign epitopes have been reported.[13] These chimeric virus particles are replication-competent and elicit immune responses through mucosal immunization. These particles were derived by foreign epitope insertion in a cDNA of a virus. Chimeric VLPs obtained by the same method have also been reported.[8–11] Successful chimerization is dependent on selection of an appropriate insertion site in VLPs. Another system for chimerization of VLP is co-infection of a couple of baculoviruses in the same cells, which allows VLPs to be obtained as chimeric VLPs.[12] This method is an easy way to obtain the chimeric VLP, although the stability of molecular constructs and the characteristics of morphology to original virus are not promised. Chimeric VLPs obtained by foreign-molecule insertion are suitable for studying morphogenesis of viruses and host recognition to both VLPs and inserted molecules.

HEV-VLP

Hepatitis E is an acute viral hepatitis caused by infection with HEV that was first recognized in India 1955. The HEV has been isolated from various animals, suggesting that hepatitis E is a zoonosis.[14,15] Although an *in vitro* culture system to amplify HEV has not been developed, over-expression of a part of ORF2 in a baculovirus expression system allows this protein to assemble into a VLP.[6] Formation of this VLP occurs only when N-terminal of ORF2 — where potential

signal sequence is encoded — was deleted from the expression construct.[6,16] It has also been reported that additional endogenous cleavage of 52 amino acids at the C-terminal is necessary for the assembly of a VLP.[6,7] HEV-VLP appears as an empty particle of a slightly smaller size than that of a mature HEV particle.[6,7] An HEV-VLP has several advantages for studying virus formation or host recognition. In our experience, large amounts can be easily obtained from standard cultivation protocols compared with amounts of other VLPs obtained. The amount of purified HEV-VLPs collected from culture supernatant of 50 to 100 μg/ml is more than 100 times greater than that of other VLPs. It has recently been found that the VLPs elicit strong immune responses when administrated orally into mice as same to a natural infection route.[17] Moreover, it has been shown that oral inoculation of cynomolgus monkeys with HEV-VLP prevents the infection of native HEV by intravenous injection.[18] These findings indicated that HEV-VLPs conserved original HEV construction to enter the target cells. Conservation of the virus construct in VLPs is very attractive for vaccines inducing the same type of immune responses to virus infection.

Chimeric HEV-VLP Carrying Foreign Epitope

pVL5480/7126, a baculovirus transfer vector that includes a portion of the ORF2 from HEV (dORF2), was described previously.[6] To insert the tag sequence within dORF2, oligonucleotides that encode the tag amino acid sequence were synthesized as shown in Table 1, and described previously.[9] The restriction sites used for insertion sites 1 to 4 were *Hind*III, *Sac*II, *Bss*HII, and *Sac*II sites at nucleotide positions 5679, 6245, 6664, and 6773, respectively. For each site, oligonucleotide pairs of Htg5(0) and Htg3(GA), Htg5(+1) and Htg3(0), Htg5(0) and Htg3(GG), and Htg5(+1) and Htg3(0) were used, respectively. A C-terminal tag was added at a position 52 amino acids upstream from the translational terminal. This site was chosen because the last 52 amino acids at the C-terminal of ORF2 are cleaved off during the formation of VLPs. The nucleotide sequences around the inserted tag are schematically shown in Fig. 1. The plasmid containing the chimeric dORF2 was co-transfected

Table 1. Oligonucleotides Used in this Study

Oligonucleotide	Sequence (5′ to 3′)
HEVBacBg	CGCAGATCTATGGCGGTCGCTCCAGCCC
HEV52Pr	CTGCAGCTATGCTAGCGCAGAGTG
Htg5(0)	CAGCCTGAACTCGCTCCAGAGGA
Htg5(+1)	GCCAGCCTGAACTCGCTCCAGAGGA
Htg3(0)	ATCTTCCGGATCCTCTGGAGCGAG
Htg3(GA)	TCATCTTCCGGATCCTCTGGAGCGAG
Htg3(GG)	CCATCTTCCGGATCCTCTGGAGCGAG
Tag(-52)	CTGCAGCTAATCTTCGGGGTCCTCCGGGGCGAGCT CAGGCTGTGCTAGCGCAGAGTGG
BglTag	AGATCTATGGCGCAGCCTGAACTCGCTCCAGAGGA TCCAGAAGATGCGGTCGCTCCAGCCCATGAC

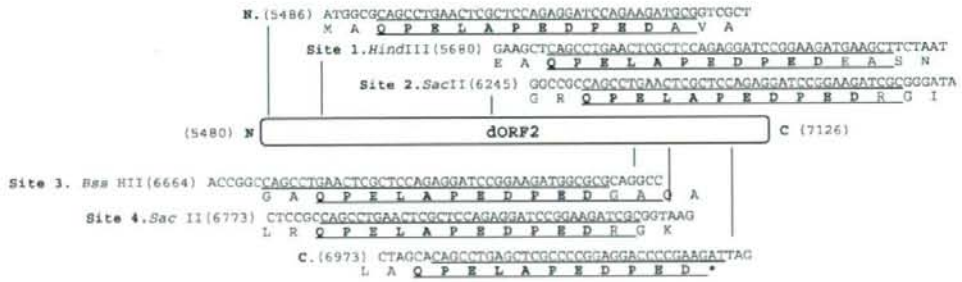


Fig. 1. Schematic diagram and sequences around the tag epitope insertion sites in dORF2. Upper rows show nucleotide sequences and lower rows show the corresponding amino acid sequences. Amino acid numbers relative to the full-length ORF2 are indicated next to the amino acid before the inserted amino acids. Nucleotide numbers referring to HEV genome are in parenthesis. Inserted sequences are underlined. The tag epitope amino acid sequence is in bold face.

with baculovirus DNA, and the recombinant baculovirus was generated as described previously.[6] The production and purification of HEV-VLP was performed as described previously.[6,7]

Utilizing any of the insertion sites, the chimeric dORF2 was expressed at almost equal levels in the cell lysates (Fig. 2A). The antigenicity of the tag epitope was maintained in all cases, as shown by

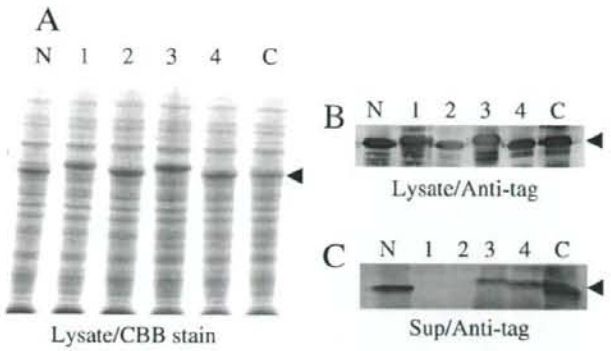


Fig. 2. Expression of chimeric dORF2. A. Expression in the cell lysates was examined by Coomasie brilliant blue staining. B. Antigenicity of the tag epitope in the cell lysates was confirmed by Western blotting with the anti-tag antibody. C. Presence of each chimeric dORF2 in cell supernatant (8 μl) was examined by Western blotting with the anti-tag antibody. The insertion site for each chimera is indicated at the top of the panel. N; N-terminal, 1 to 4; sites 1 to 4, respectively, C; C-terminal. The arrowhead on the right of each panel indicates the position of the chimeric dORF2.

Western blot analysis (Fig. 2B). Even at the C-terminal region, where the native HEV amino acid sequence is cleaved, the tag was not cleaved off from dORF2 (Fig. 2B, lane C). Among these chimeras, only the N- and C-terminal insertions resulted in release of a large amount of chimeric dORF2 into the culture supernatant (Figs. 2C and 2D), although small amounts were released when the insertions were made at either site 3 or 4. These results indicate that internal insertions somehow disturbed the release of dORF2 into the culture supernatant. The precise mechanisms involved in the HEV virion formation are not yet clear. The added tag at 52 amino acids upstream from the C-terminal region, where dORF2 is normally cleaved in insect cells, was not cleaved off in the infected cells during the generation of the chimeric VLPs. This is most likely due to alteration of the amino acid sequence recognized by the proteolytic enzyme involved in the C-terminal modification of HEV-VLP. The successful addition of extra amino acid sequences to the C-terminal of dORF2 suggests that the presence of extra amino acids at the C-terminal is not crucial

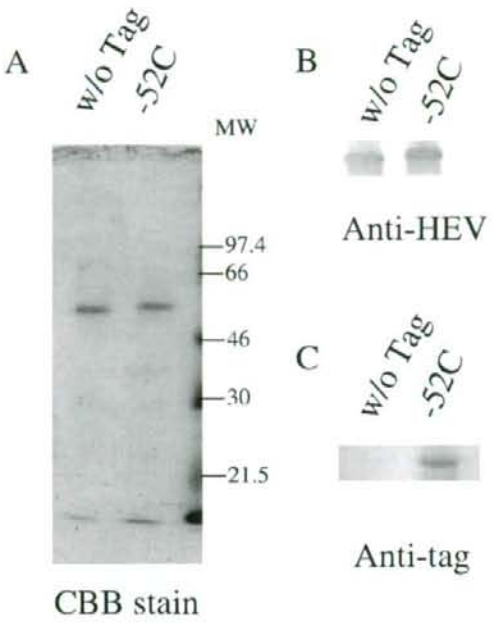
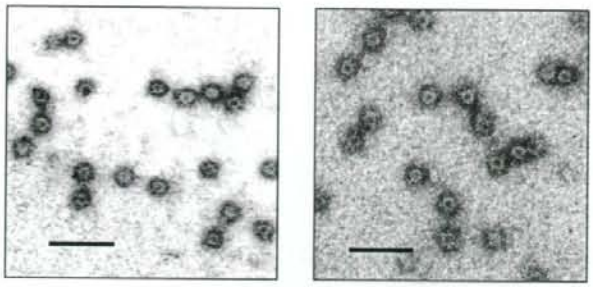


Fig. 3. Purification of the chimeric VLP. Purified VLP-52C was analyzed for its purity (A) and reactivity to anti-HEV (B) and anti-tag antibodies (C). A. Equal amounts (0.3 μg) of purified VLP-52C (–52C) and VLP without tag (w/o Tag) were separated on SDS-PAGE and stained by Coomasie brilliant blue staining. Positions of molecular weight markers are indicated on the right of the panel. B and C. Equal amounts (0.1 μg) of VLP-52C (–52C) and VLP without tag (w/o Tag) were analyzed by Western blotting using anti-HEV (B) and anti-tag (C) antibodies, respectively.

for preventing dORF2 from being incorporated into a VLP form. Rather, the amino acid sequences encoded by the HEV ORF2 genome prevented the formation of VLP.

We attempted to purify chimeric VLPs from the supernatant of Tn5 cells expressing chimeric dORF2 with a tag at either C-termini. The VLP-52C was slightly larger than the HEV-VLP without the tag (Fig. 3A). The purified VLP-52C retained the antigenicity of HEV as well as the intact tag epitope, as shown by the reactivity of specific antibodies (Figs. 3B and 3C).

Electron microscopic observation showed that VLP-52C was approximately 25 nm in diameter and indistinguishable from the



A. -52C B. w/o Tag

Fig. 4. Electron micrograph of VLP-52C. VLP-52C (A) and VLP without tag (B) were observed under electron microscopy after negative staining at a magnification of ×60,000. Inserted bar indicates 100 nm.

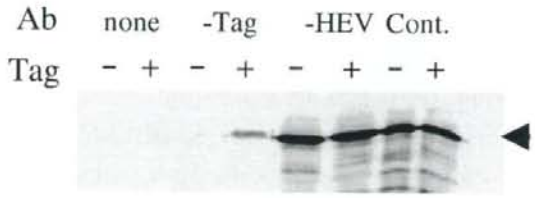


Fig. 5. Surface exposure of the tag epitope on VLP-52C. Surface exposure of the tag epitope on intact VLP-52C was examined by immunoprecipitation with the anti-tag antibody. Antibodies used are indicated at the top of panel. None, negative control without antibody; α-Tag, anti-tag antibody; α-HEV, anti-HEV antibody; cont., purified VLP-52C and VLP without tag were run as controls. The second row indicates either VLP with (+) or without the tag (−).

VLP without the tag (Fig. 4). Using two methods, we confirmed that the inserted epitope tag was exposed on the surface. The intact VLP-52C was immunoprecipitated with the anti-tag antibody, while the anti-HEV antibody immunoprecipitated both VLP-52C and the VLP without the tag (Fig. 5). Furthermore, the anti-tag antibody specifically reacted with the intact VLP-52C in an ELISA (data not shown). The results of immunoprecipitation and ELISA using intact chimeric VLP suggest that the tag epitope is exposed on the surface of the HEV-VLP.

Immune Responses to Chimeric HEV-VLPs through Oral Administration

Since many pathogenic viruses and bacteria establish their initial infections through the mucosal surface, vaccine strategies that can stimulate mucosal immunity have been widely studied (reviewed in Ogra *et al.*).[19] However, there are several difficulties in oral immunization with non-replicating molecules, such as low pH in the stomach, presence of proteolytic enzymes in the digestive tract, and presence of physical as well as biochemical barriers associated with the mucosal surface itself.[19] We previously reported that the HEV-VLP preserved original HEV construction and entered the epithelial cells of the small intestine by oral administration.[20] From these findings, mice were immunized with 50 μg of purified VLP-52C by the oral route four times at two-week intervals, with the mice having the ability to induce mucosal and systemic epitope-specific antibody responses. Specific IgG antibodies to the tag as well as to HEV were detected in intestinal fluids as early as two wpi (Fig. 6A). IgG levels in intestinal fluids continued to increase until the termination of the experiments. Specific IgA to both the tag and HEV also appeared in intestinal fluids from two wpi, paralleling the IgG levels (Fig. 6B). The IgA levels also continued to increase until the termination of experiments. As expected, the control mice immunized with VLP without the tag developed IgG and IgA only to HEV. In sera, levels of a specific IgG antibody to both the tag and HEV showed slightly higher OD values than those in non-immunized controls, but they never reached significant levels, as occurred in the intestinal fluids (Fig. 6C). The levels of specific IgA in sera were also low, although the OD values were also higher than the non-immunized controls (Fig. 6D). The control mice immunized with VLP without the tag showed similarly low OD values to HEV. The specific antibodies in the intestinal fluids were analyzed for their isotypes at 10 wpi. At this point, average OD values and SD for IgG and IgA in the intestinal fluids were 1.02±0.22 and 0.64±0.038, and 0.96±0.086 and 0.66±0.040, respectively, to HEV and the tag in three mice immunized with the chimeric VLP. In the control mice immunized with VLP without the tag, the average OD values and SD for IgG and IgA were 0.88±0.047 and 0.64±0.027, and 0.11±0.024 and 0.084±0.013, respectively, to HEV and the tag. All

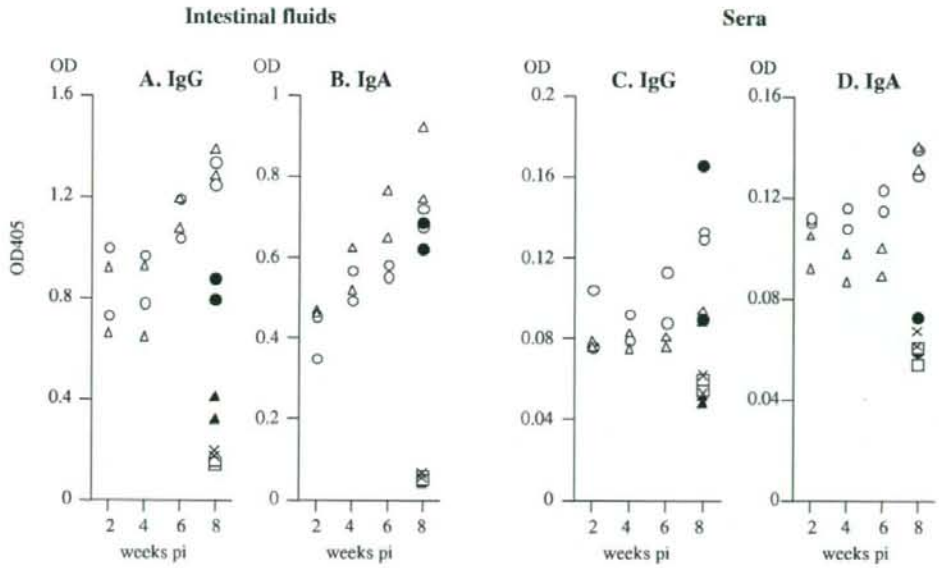


Fig. 6. IgG (A and C) and IgA (B and D) levels in intestinal fluids (A and B) and sera (C and D) of orally immunized mice. Circles and triangles indicate HEV-specific and the tag epitope-specific antibody levels, respectively, in individual mice. Two immunized mice were sacrificed at each time point (two, four, six and eight wpi). Specific antibody levels to HEV and the tag epitope of control mice immunized with VLP without the tag (closed circles and closed triangles, respectively) and background levels to HEV and the tag epitope of non-immunized mice (squares and crosses, respectively) are also shown. Antibody levels are indicated as OD405 in ELISA when sera and intestinal fluids were diluted at 1:100 and 1:2, respectively.

subclasses of IgGs to HEV — except IgG3, IgM, and IgA — were evident in all mice (Fig. 7B). Both to the tag and HEV, all mice failed to develop IgG3 above the detectable level (Figs. 7A and 7B). In the control mice immunized with VLPs without the tag, HEV-specific antibody reactions similar to the those with the chimeric VLPs were shown (Fig. 7B), while no detectable level of any isotype antibody specific to the tag was observed (Fig. 7A), as expected.

Induction of foreign epitope-specific antibody (Ab) responses by chimeric VLP administration is not easy compared with inducing cellular immune responses such as a cytotoxic T lymphocyte (CTL) response.[8,10,11,21,22] Moreover, our results showed Ab responses by oral

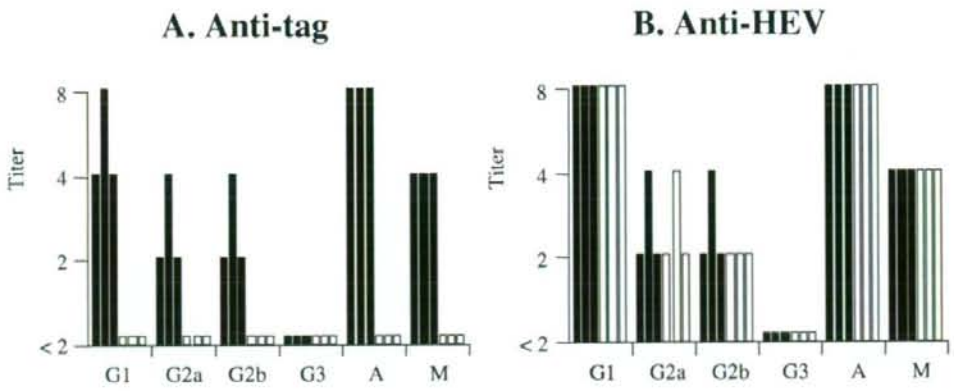


Fig. 7. Isotypes of antibodies specific to the tag epitope (A) and HEV (B) in intestinal fluids in orally immunized mice sacrificed at 10 wpi. Levels of IgA (A), IgM (M), and IgG subclasses (G1, G2a, G2b and G3) were examined by ELISA using isotype-specific secondary antibodies and are shown as end-point titers. Solid and open bars indicate antibody levels of each mouse immunized with the chimeric VLP and VLP without the tag epitope insertion, respectively.

administration to overcome the difficulties of a severe environment through the digestive tract. It is plausible that HEV-VLPs, which are derived from an orally transmissible virus, were incorporated into HEV-permissive epithelial cells in the small intestine because they retained structures and properties similar to those of HEV particles, producing an infection similar to that induced naturally.[17] It has been shown that the VLP structure should provide resistance to severe environments in the digestive tracts and enable specific binding to the mucosal surface if an appropriate VLP is chosen.[23] The delivery of a vaccine antigen (Ag) for induction of mucosal immune responses is usually achieved through the upper nasopharynx-associated lymphoid tissue (NALT), upper airway, salivary glands and tonsils.[24,25] Despite its obvious convenience, oral administration is rarely successful since it is quite difficult to protect vaccine Ag from the environment in the digestive tract.

The results of immunoprecipitation and ELISA using intact chimeric VLPs suggest that the tag epitope is exposed on the surface of the HEV-VLP. The successful induction of antibodies to the tag

also supports the hypothesis that the tag is exposed on the surface, since an internally localized B cell epitope in chimeric parvovirus-VLP failed to induce a specific antibody response.[11] Furthermore, this hypothesis is consistent with the results of three-dimensional analysis of the Norwalk virus-VLP particle, in which the C-terminal is exposed to the VLP surface.[26] Considering that B cell epitopes are generally hydrophilic and most likely exposed to the VLP surface, B cell epitope regions may not be directly involved in the protein-protein interactions to form a VLP. Our unsuccessful insertions into internal sites suggest that the integrity of internal regions must be maintained for proper protein folding and VLP formation. To find potential internal insertion sites, a precise three-dimensional structural map of the HEV-VLP may be necessary.

Oral vaccination has obvious advantages for a field trial in a large-scale public health vaccination program.[27] From a practical standpoint, oral administration is less stressful for vaccine recipients and does not require professional skill for administration. Moreover, delivery of vaccines via the intestinal tract is considered to be inherently safer than systemic injection. Encouraging results of phase I trials using Norwalk virus VLPs have recently been reported.[28] We also confirmed that chimeric HEV-VLPs carrying foreign CTL and B cell epitope at C-termini can elicit mucosal and systemic cellular immune responses as well as humoral immune responses by oral administration (submitted). It has become apparent that mucosal immune responses on different mucosal surfaces were achieved simultaneously, despite the initial stimulation of a single mucosal site.[29,30] Therefore, it is probable that oral administration of chimeric HEV-VLPs stimulates immune responses simultaneously on distant mucosal surfaces as well. This phenomenon significantly extends the potential use of chimeric HEV-VLPs as an oral vaccine vehicle.

A chimeric HEV-VLP has several advantages as an oral vaccine vector. First, large amounts can be easily obtained from standard cultivation protocols compared with amounts of other VLPs obtained. Second, the outcome of delivery of vaccine Ag in humans can be predicted using conventional laboratory animals, since HEV naturally infects various animals as well as humans through the same infectious

route and target cells.[6,31] Third, HEV-VLPs are stable at room temperature. Fourth, anti-HEV immune responses had no effect on boosting administration in the present study. Thus, HEV-VLPs are an attractive vaccine vector in developing countries because these VLPs can be preserved without the requirement of any particular equipment. These findings suggest that chimeric VLPs derived from orally transmissible viruses can be used as vaccine vectors to mucosal tissue by oral administration for the purpose of vaccination.

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References

1. Jameel S, Zafrullah M, Ozdener MH, Panda SK. (1996) Expression in animal cells and characterization of the hepatitis E virus structural proteins. *J Virol* **70**: 207–216.
2. Zafrullah M, Ozdener MH, Kumar R, *et al.* (1999) Mutational analysis of glycosylation, membrane translocation, and cell surface expression of the hepatitis E virus ORF2 protein. *J Virol* **73**: 4074–4082.
3. McAtee C, Zhang Y, Yarbrough PO, *et al.* (1996) Purification of a soluble hepatitis E open reading frame 2-derived protein with unique antigenic properties. *Protein Expr Purif* **8**: 262–270.
4. McAtee CP, Zhang Y, Yarbrough PO, *et al.* (1996) Purification and characterization of a recombinant hepatitis E protein vaccine candidate by liquid chromatography-mass spectrometry. *J Chromatogr B Biomed Appl* **685**: 91–104.
5. Robinson RA, Burgess WH, Emerson SU, *et al.* (1998) Structural characterization of recombinant hepatitis E virus ORF2 proteins in baculovirus-infected insect cells. *Protein Expr Purif* **12**: 75–84.