34. **Musey, L., J. Hughes, T. Schacker, T. Shea, L. Corey, and M. J. McElrath.** 1997. Cytotoxic-T-cell responses, viral load, and disease progression in early human immunodeficiency virus type 1 infection. N. Engl. J. Med. **337**:1267–1274.
35. **Nabel, G., W. Makgoba, and J. Esparza.** 2002. HIV-1 diversity and vaccine development. Science **296**:2335.
36. **Nabel, G. J.** 2002. HIV vaccine strategies. Vaccine **20**:1945–1947.
37. **Nishimura, Y., T. Igarashi, N. Haigwood, R. Sadjadpour, R. J. Plishka, A. Buckler-White, R. Shibata, and M. A. Martin.** 2002. Determination of a statistically valid neutralization titer in plasma that confers protection against simian-human immunodeficiency virus challenge following passive transfer of high-titered neutralizing antibodies. J. Virol. **76**:2123–2130.
38. **Ogg, G. S., X. Jin, S. Bonhoeffer, P. R. Dunbar, M. A. Nowak, S. Monard, J. P. Segal, Y. Cao, S. L. Rowland-Jones, V. Cerundolo, A. Hurley, M. Markowitz, D. D. Ho, D. F. Nixon, and A. J. McMichael.** 1998. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. Science **279**:2103–2106.
39. **Okamoto, Y., Y. Eda, A. Ogura, S. Shibata, T. Amagai, Y. Katsura, T. Asano, K. Kimachi, K. Makizumi, and M. Honda.** 1998. In SCID-hu mice, passive transfer of a humanized antibody prevents infection and atrophic change of medulla in human thymic implant due to intravenous inoculation of primary HIV-1 isolate. J. Immunol. **160**:69–76.
40. **Ota, M. O., J. Vekemans, S. E. Schlegel-Haueter, K. Fielding, M. Sanneh, M. Kidd, M. J. Newport, P. Aaby, H. Whittle, P. H. Lambert, K. P. McAdam, C. A. Siegrist, and A. Marchant.** 2002. Influence of *Mycobacterium bovis* bacillus Calmette-Guerin on antibody and cytokine responses to human neonatal vaccination. J. Immunol. **168**:919–925.
41. **Reimann, K. A., J. T. Li, R. Veazey, M. Halloran, I. W. Park, G. B. Karlsson, J. Sodroski, and N. L. Letvin.** 1996. A chimeric simian/human immunodeficiency virus expressing a primary patient human immunodeficiency virus type 1 isolate *env* causes an AIDS-like disease after in vivo passage in rhesus monkeys. J. Virol. **70**:6922–6928.
42. **Robinson, H. L.** 2002. New hope for an AIDS vaccine. Nat. Rev. Immunol. **2**:239–250.
43. **Roche, P. W., P. W. Peake, H. Billman-Jacobe, T. Doran, and W. J. Britton.** 1994. T-cell determinants and antibody binding sites on the major mycobacterial secretory protein MPB59 of *Mycobacterium bovis*. Infect. Immun. **62**: 5319–5326.
44. **Sasaki, Y., Y. Ami, T. Nakasone, K. Shinohara, E. Takahashi, S. Ando, K. Someya, Y. Suzaki, and M. Honda.** 2000. Induction of CD95 ligand expression on T lymphocytes and B lymphocytes and its contribution to apoptosis of CD95-up-regulated $CD4^+$ T lymphocytes in macaques by infection with a pathogenic simian/human immunodeficiency virus. Clin. Exp. Immunol. **122**: 381–389.
45. **Schmitz, J. E., M. J. Kuroda, S. Santra, V. G. Sasseville, M. A. Simon, M. A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B. J. Scallon, J. Ghrayeb, M. A. Forman, D. C. Montefiori, E. P. Rieber, N. L. Letvin, and K. A. Reimann.** 1999. Control of viremia in simian immunodeficiency virus infection by $CD8^+$ lymphocytes. Science **283**:857–860.
46. **Shibata, R., T. Igarashi, N. Haigwood, A. Buckler-White, R. Ogert, W. Ross, R. Willey, M. W. Cho, and M. A. Martin.** 1999. Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys. Nat. Med. **5**:204–210.
47. **Shinohara, K., K. Sakai, S. Ando, Y. Ami, N. Yoshino, E. Takahashi, K. Someya, Y. Suzaki, T. Nakasone, Y. Sasaki, M. Kaizu, Y. Lu, and M. Honda.** 1999. A highly pathogenic simian/human immunodeficiency virus with genetic changes in cynomolgus monkey. J. Gen. Virol. **80**:1231–1240.
48. **Someya, K., K. Q. Xin, K. Matsuo, K. Okuda, N. Yamamoto, and M. Honda.** 2004. A consecutive prime-boost vaccination of mice with simian immunodeficiency virus (SIV) *gag/pol* DNA and recombinant vaccinia virus strain DIs elicits effective anti-SIV immunity. J. Virol. **78**:8942–8953.
49. **Stover, C. K., V. F. de la Cruz, T. R. Fuerst, J. E. Burlein, L. A. Benson, L. T. Bennett, G. P. Bansal, J. F. Young, M. H. Lee, G. F. Hatfull, S. B. Snapper, R. G. Barletta, W. R. Jacobs, Jr., and B. R. Bloom.** 1991. New use of BCG for recombinant vaccines. Nature **351**:456–460.
50. **Weiss, R. A.** 2002. HIV receptors and cellular tropism. IUBMB Life **53**:201–205.
51. **Wu, S. C., J. L. Spouge, S. R. Conley, W. P. Tsai, M. J. Merges, and P. L. Nara.** 1995. Human plasma enhances the infectivity of primary human immunodeficiency virus type 1 isolates in peripheral blood mononuclear cells and monocyte-derived macrophages. J. Virol. **69**:6054–6062.
52. **Yamanaka, T., Y. Fujimura, S. Ishimoto, A. Yoshioka, M. Konishi, N. Narita, J. Mimaya, T. Meguro, T. Nakasone, Y. Okamoto, H. Yoshizaki, K. Yamada, and M. Honda.** 1997. Correlation of titer of antibody to principal neutralizing domain of HIVMN strain with disease progression in Japanese hemophiliacs seropositive for HIV type 1. AIDS Res. Hum. Retrovir. **13**: 317–326.
53. **Ye, Y., Z. H. Si, J. P. Moore, and J. Sodroski.** 2000. Association of structural changes in the V2 and V3 loops of the gp120 envelope glycoprotein with acquisition of neutralization resistance in a simian-human immunodeficiency virus passaged in vivo. J. Virol. **74**:11955–11962.
54. **Zhang, Y., B. Lou, R. B. Lal, A. Gettie, P. A. Marx, and J. P. Moore.** 2000. Use of inhibitors to evaluate coreceptor usage by simian and simian/human immunodeficiency viruses and human immunodeficiency virus type 2 in primary cells. J. Virol. **74**:6893–6910.

Transworld Research Network
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

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

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

Masaaki Miyazawa
Department of Immunology, Kinki University School of Medicine
377-2 Ohno-higashi, Osaka-Sayama, Osaka 589-0023, Japan

Abstract

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

Correspondence/Reprint request: Dr. Masaaki Miyazawa, Department of Immunology, Kinki University School of Medicine, 377-2 Ohno-higashi, Osaka-Sayama, Osaka 589-0023, Japan. E-mail: Masaaki@med.kindai.ac.jp

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

Introduction

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

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

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

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

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

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

Cellular genes that directly influence the replication of FV

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

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

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

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

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

Molecular mechanisms of Friend virus-induced disease development

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

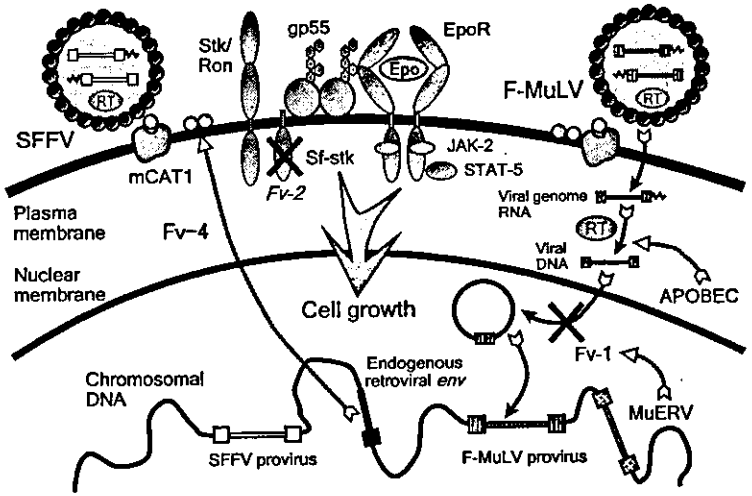

Figure 1. Schematic summary of the mechanisms of FV-induced erythroid cell proliferation and cellular factors that interfere with FV infection.

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

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

Host genes that control immune responses to FV infection

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

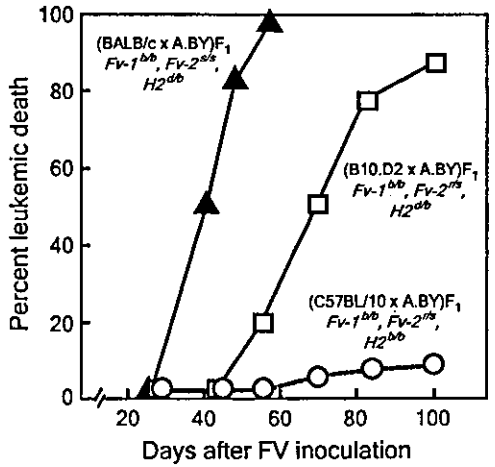

Figure 2. Comparison of tempos of leukemic death after FV infection in three different strains of mice.

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

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

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

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

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

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

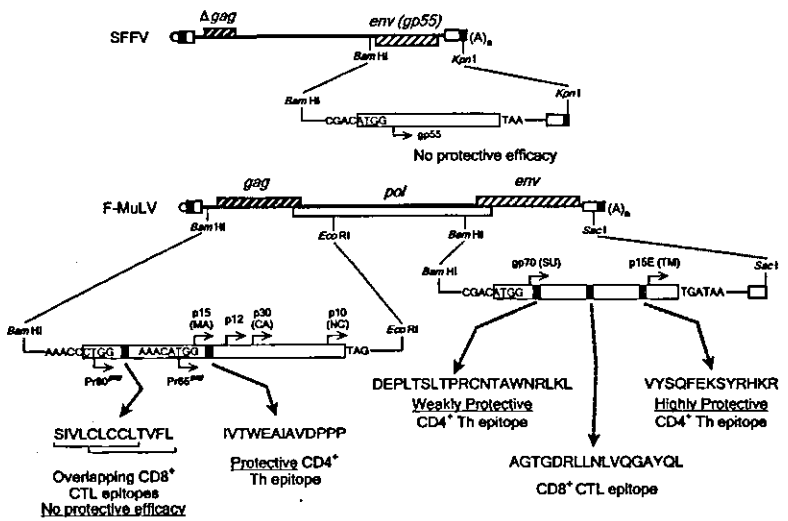

Figure 3. Amino acid sequence and location of the epitopes recognized by T cells in FV gene products.

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

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

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

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

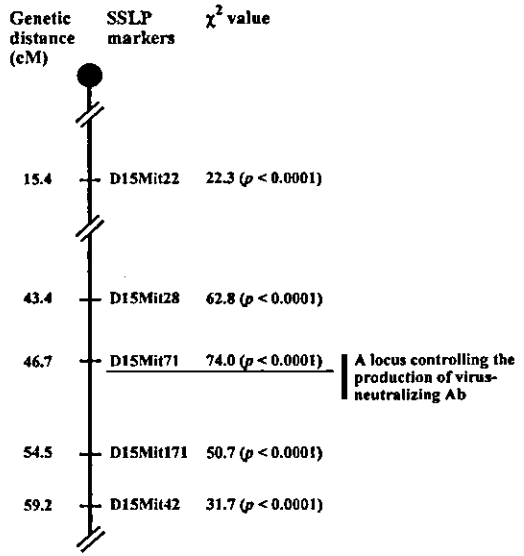

Figure 4. Genetic mapping of the host gene that controls serum titers of virus-neutralizing Ab in FV-infected mice.

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

Acknowledgements

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

References

1. Zinkernagel, R. M., and Hengartner, H. 1994, Immunol. Today, 15, 262.
2. Gonzaléz-Scarano, F., Nathanson, N., and Wong, K. Y. 1995, The Retroviridae vol. 4, J. A. Levy (Ed.), Plenum Press, New York, 409.
3. Garry, R. F., Krieg, A. M., Cheevers, W. S., Monteralo, R. C., Golding, H., Fermin, C. D., and Gallaher, W. R. 1995. The Retroviridae vol. 4, J. A. Levy (Ed.), Plenum Press, New York, 491.

4. Fan, H., Chen, I., Rosenberg, H., and Sugden, B. 1991, H. Fan, I. S. Y. Chen, N. Rosenberg, and W. Sugden (Eds.) Viruses That Affect the Immune System, American Society for Microbiology, Washington, D. C., 1.
5. Kabat, D. 1989, Molecular biology of Friend viral erythroleukemia. Curr. Top. Microbiol. Immunol., 148, 1.
6. Chesebro, B., Miyazawa, M., and Britt, W. J. 1990, Annu. Rev. Immunol., 8, 477.
7. Evans, L. H., and Malik, F. G. 1987, J. Virol., 61, 1882.
8. Albritton, L. M., Tseng, L., Scadden, D., and Cunningham, J. M. 1989, Cell 57, 659.
9. Kim, J. W., Closs, E. L., Albritton, L. M., and Cunningham, J. M. 1991, Nature 352, 725.
10. Wang, H., Kavanaugh, M. P., North, R. A., and Kabat, D. 1991, Nature 352, 729.
11. Tailor, C. S., Nouri, A., Lee, C. G., Kozak, C., and Kabat, D. 1999, Proc. Natl. Acad. Sci. USA., 96, 927.
12. Battini, J. L., Rasko, J. E., and Miller, A. D. 1999, Proc. Natl. Acad. Sci. USA., 96, 1385.
13. Yang, Y. L., Guo, L., Xu, S., Holland, C. A., Kitamura, T., Hunter, K., and Cunningham, J. M. 1999, Nat. Genet., 21, 216.
14. Marin, M., Tailor, C. S., Nouri, A., Kozak, S. L., and Kabat, D. 1999, J. Virol., 73, 9362.
15. Miller, D. G., R. Edwards, H., and Miller, A. D. 1994, Proc. Natl. Acad. Sci. USA., 91, 78.
16. van Zeijl, M., Johann, S. V., Closs, E., Cunningham, J., Eddy, E., Shows, T. B., and O'Hara, B. 1994, Proc. Natl. Acad. Sci. USA., 91, 1168.
17. Olah, Z., Lehel, C., Anderson, W. B., Eiden, M. V., and Wilson, C. A. 1994, J. Biol. Chem., 269, 25426.
18. Ikeda, H., Laigret, F., Martin, M. A., and Repaske, R. 1985, J. Virol. 55, 768.
19. Ikeda, H., and Sugimura, H. 1989, J. Virol., 63, 5405.
20. Harris, R. S., Bishop, K. N., Sheehy, A. M., Craig, H. M., Petersen-Mahrt, S. K., Watt, I. N., Neuberger, M. S., and Malim, M. H. 2003, Cell, 113, 803.
21. Zhang, H., Yang, B., Pomerantz, R. J., Zhang, C., Arunachalam, S. C., and Gao, L. 2003, Nature, 424, 94.
22. Vartanian, J. P., Sommer, P., and Wain-Hobson, S. 2003, Trends Mol. Med., 9, 409.
23. Zheng, Y. H., Irwin, D., Kurosu, T., Tokunaga, K., Sata, T., and Peterlin, B. M. 2004, J. Virol., 78, 6073.
24. Yu, X., Yu, Y., Liu, B., Luo, K., Kong, W., Mao, P., and Yu, X. F. 2003, Science, 302, 1056.
25. Best, S., Le Tissier, P., Towers, G., and Stoye, J. P. 1996, Nature, 382, 826.
26. Towers, G., Bock, M., Martin, S., Takeuchi, Y., P. Stoye, J. P., and Danos, O. 2000, Proc. Natl. Acad. Sci. USA., 97, 12295.
27. Robertson, M. N., Miyazawa, M., Mori, S., Caughey, B., Evans, L. H., Hayes, S. F. and Chesebro, B. 1991, J. Virol. Methods, 34, 255.
28. Persons, D. A., Paulson, R. F., Loyd, M. R., Herley, M. T., Bodner, S. M., Bernstein, A., Correll, P. H., and Ney, P. A. 1999, Nat. Genet., 23, 159.
29. Nishigaki, K., Thompson, D., Hanson, C., Yugawa, T., and Ruscetti, S. 2001, J. Virol., 75, 7893.
30. Munroe, D. G., Peacock, J. W., and Benchimol, S. 1990, Mol. Cell Biol., 10, 3307.

31. Moreau-Gachelin, F., Tavitian, A., and Tanbourin, P. 1988, Nature, 331, 277.
32. Hasenkrug, K. J. 1999, J. Virol., 73, 6468.
33. Miyazawa, M., Nishio, J., and Chesebro, B. 1988, J. Exp. Med., 168, 1587.
34. Miyazawa, M., Nishio, J., Wehrly, K., and Chesebro, B. 1992, J. Immunol., 148, 644.
35. Miyazawa, M., Nishio, J., Wehrly, K., David, C. S., and Chesebro, B. 1992, J. Immunol. 148, 1964.
36. Miyazawa, M., Nishio, J., Wehrly, K., Jay, G., Melvold, R. W., and Chesebro, B. 1992, Eur. J. Immunogenet., 19, 159.
37. Robertson, M. N., Spangrude, G. J., Hasenkrug, K., Perry, L., Nishio, J., Wehrly, K., and Chesebro, B. 1992, J. Virol., 66, 3271.
38. Perry, L. L. Miyazawa, M., Hasenkrug, K., wehrly, K., David, C. S., and Chesebro, B. 1994, J. Virol., 68, 4921.
39. Chen, W., Qin, H., Chesebro, B., and Cheever, M. A. 1996, J. Virol., 70, 7773.
40. Iwashiro, M., Kondo, T., Shimizu, T., Yamagishi, H., Takahashi, K., Matsubayashi, Y., Masuda, T., Otaka, A., Fujii, N., Ishimoto, A., Miyazawa, M., Robertson, M. N., Chesebro, B., and Kuribayshi, K. 1993, J. Virol., 67, 4533.
41. Kondo, T., Uenishi, H., Shimizu, T., Hirama, T., Iwashiro, M., Kuribayashi, K., Tamamura, H., Fujii, N., Fujisawa, R., Miyazawa, M., and Yamagishi, H. 1995, J. Virol., 69, 6735.
42. Ruan, K., and Lilly, F. 1991, Virology, 181, 91.
43. Shimizu, T., Uenishi, H., Teramura, Y., Iwashiro, M., Kuribayashi, K., Tamamura, H., Fujii, N., and Yamagishi, H. 1994, J. Virol., 68, 7704.
44. Sugahara, D., Tsuji-Kawahara, S., and Miyazawa, M. 2004, J. Virol., 78, 6322.
45. Uenishi, H., Iwanami, N., Kuribayashi, K., Tamamura, H., Fujii, N., Nakatani, T., Kawasaki, T., and Yamagishi, H. 1998, Immunol. Lett., 62, 33.
46. Uenishi, H., Iwanami, N., Yamagishi, H., Nakatani, T., Kawasaki, T., Tamamura, H., Fujii, H., and Kuribayashi, K. 1998, Microbiol. Immunol., 42, 479.
47. Miyazawa, M., Fujisawa, R., Ishihara, C., Takei, Y. A., Shimizu, T., Uenishi, H., Yamagishi, H., and Kuribayashi, K. 1995, J. Immunol., 155, 748.
48. Iwanami, N., Niwa, A., Yasutomi, Y., Tabata, N., and Miyazawa, M. 2001, J. Virol., 75, 3152.
49. Miyazawa, M., Nishio, J., Kyogoku, M., and Chesebro, M. 1992. Molecular Approaches to the Study and Treatment of Human Diseases, T. O. Yoshida, and J. M. Wilson (Eds.), Excerpta Medica, Amsterdam, 177.
50. Cerwenka, A., and Lanier, L. L. 2001, Immunol. Rev., 181, 158.
51. Chesebro, B., and Wherly, K. 1979, Proc. Natl. Acad. Sci. USA. 76, 5784.
52. Hasenkrug, K. J., Valenzuela, A., Letts, V. A., Nishio, J., Chesebro, B., and Frankel, W. N. 1995, J. Virol., 69, 2617.
53. Super, H. J., Hasenkrug, K. J, Simmons, S., Brooks, D. M., Konzek, R., Sarge, K. D., Morimoto, R. I., Jenkins, N. A., Gilbert, D. J., Copeland, N. G., Frankel, W., and Chesebro, B. 1999, J. Virol., 73, 7848.
54. Rahman, Z. S. M., Rao, S. P., Kalled, S. L., and Manser T. 2003, J. Exp. Med. 198, 1157.

K. Conrad, M. P. Bachmann, E. K. L. Chan,
M. J. Fritzler, R. L. Humbel, U. Sack, Y. Shoenfeld
(Eds.)

From Animal Models to Human Genetics: Research on the Induction and Pathogenicity of Autoantibodies

Report on the 7th Dresden Symposium on Autoantibodies held in Dresden on September 1-4, 2004

PABST SCIENCE PUBLISHERS
Lengerich, Berlin, Bremen, Miami,
Riga, Viernheim, Wien, Zagreb

Pathogenicity of autoantibodies reactive with the endogenous retroviral envelope glycoprotein gp70

M. Miyazawa[1], E. Kajiwara[1], N. Tabata[1,2], T. Ogawa[1,3], T. Yuasa[1], H. Matsumura[1]

[1]Departments of Immunology, [2]Pediatrics, and [3]Cardiovascular Surgery, Kinki University School of Medicine, Osaka-Sayama, Osaka, Japan

masaaki@med.kindai.ac.jp

Abstract

MRL/MpJ-lpr/lpr (MRL/lpr) mice spontaneously develop immune complex-mediated glomerulonephritis, granulomatous arteritis, chronic destructive arthritis, and thrombocytopenia. Recent genetic analyses in a variety of lupus-prone strains of mice have pointed out a close correlation between autoantibodies reactive with the endogenous retroviral env gene product, gp70, and the development and severity of glomerulonephritis. However, the suggested pathogenicity of anti-gp70 autoantibodies had not been directly demonstrated. To examine if anti-retroviral gp70 autoantibodies induce glomerular and vascular pathology, we established from unmanipulated MRL/lpr mice hybridoma clones that secrete monoclonal antibodies reactive with the endogenous xenotropic viral env gene product. This gp70 is known to be expressed in the liver and secreted as a normal constituent of mouse serum. A high proportion of these anti-gp70 antibody-producing hybridoma clones induced proliferative or wire loop-like glomerular lesions with massive depositions of gp70, IgG, and C3 in affected glomeruli when transplanted into syngeneic non-autoimmune or severe combined immunodeficiency mice. Furthermore, we have successfully demonstrated that repeated intravenous injections of purified monoclonal anti-gp70 autoantibodies induce glomerular pathology associated with gp70 deposition. The development of the glomerular pathology after injection of purified anti-gp70 autoantibodies was dependent on the amounts of serum gp70 expressed in the injected mice, and the development of granulomatous arteritis was also observed after repeated injections of one of the pathogenic clones of anti-gp70 autoantibodies. These results directly prove the long-debated pathogenicity of anti-retroviral autoantibodies in the mouse lupus models.

By the late 1970s a mistaken consensus emerged that viruses did not cause human cancer and that human retroviruses did not exist.

Robert C. Gallo [1]

Introduction

Nearly half century has passed since the first description of DNA-binding antibodies in the sera of patients with systemic lupus erythematosus (SLE), yet correlation between levels of anti-DNA autoantibodies and disease activity in SLE still remains controversial [2,3]. In recent cross-sectional studies, a correlation between the presence of histologically proven lupus nephritis and levels of autoantibodies reactive to double-stranded (ds) DNA has been observed [2,4]. However, most anti-DNA antibodies are actually anti-nucleosome antibodies, binding to DNA only if it is complexed to nucleosomes [3,5]. The observed nephritogenicity of experimentally established and patient-derived anti-dsDNA antibodies seems to depend on the binding of nucleosomes to the major component of glomerular basement membrane, heparan sulfate.

Pathogenicity of anti-DNA autoantibodies has been experimentally analyzed in details by using monoclonal antibodies established from mouse strains that spontaneously develop an SLE-like syndrome. These include New Zealand, MRL, and BXSB strains and their crosses, and they have provided useful tools for the immunologic and genetic dissection of pathogenetic and predisposing factors that contribute to the development of lupus-like lesions. In particular, the immunologic factors that have long been associated with the development of fatal glomerulonephritis in the above lupus-prone mice are immune complexes, anti-DNA antibodies, and antibodies reactive with the env gene product of an endogenous retrovirus, gp70 [6,7].

It has long been postulated that endogenous retroviruses might be involved in the development of human autoimmune diseases [8-10]. They may encode a part of target molecules bound by autoantibodies, or may derange and modulate immune responses through the production of superantigens, modification of cytokine gene expression, and stimulation of anti-idiotypic antibody responses. Infections with exogenous human retroviruses, HTLV-I and HIV-1, as well as exogenous retroviruses of some other species, have been associated with the development of chronic inflammatory diseases involving the central nervous system, joints, lungs, salivary glands, and blood vessels (reviewed in [9]). However, the possible involvement of endogenous retroviruses in the development of human autoimmune diseases is still controversial and largely unproven [11,12]. In contrast, there is compelling evidence indicating the involvement of endogenous retroviruses in the development of glomerulonephritis and chronic vasculitis in mouse models of systemic autoimmune diseases [8,9,13,14]. The involvement of the envelope glycoprotein gp70 of an endogenous retrovirus in the formation of circulating immune complexes, and their deposition in the glomerular and vascular lesions have been

demonstrated [7,8,13,14]. Importantly, recent genetic linkage analyses have demonstrated the strongest association of serum levels of gp70-anti-gp70 immune complexes, rather than the levels of anti-dsDNA autoantibodies, with the development and severity of glomerulonephritis [15-18], suggesting a major pathogenic role of anti-gp70 autoantibodies in the lupus-prone mice. However, suggested pathogenicity of anti-gp70 autoantibodies had not been directly tested until recently. To examine if anti-gp70 autoantibodies induce glomerular and vascular pathology, we have established from unmanipulated MRL/lpr mice hybridoma clones that secrete monoclonal antibodies reactive with endogenous xenotropic viral env gene products [19,20]. A high proportion of these monoclonal anti-gp70 autoantibodies induced in syngeneic non-autoimmune (BALB/c x MRL/Mp-+/+)F1 and severe combined immunodeficiency (SCID) mice proliferative or wire loop-like glomerular lesions associated with massive gp70 deposition [20]. Furthermore, we found an IgG2a-producing anti-gp70 hybridoma clone that induced acute hemorrhagic death upon transplantation into syngeneic non-autoimmune mice. Subsequent analyses have demonstrated the binding of the anti-gp70 autoantibody onto mouse platelets, and induction of diffuse intraluminal platelet aggregation in mice transferred with this autoantibody [19].

In the present paper, we summarize our observations on the pathogenicity and molecular characteristics of the anti-gp70 autoantibodies, and report the development of vascular lesions in non-autoimmune mice by intravenous injection of a purified anti-gp70 autoantibody.

Materials and methods

Mice

Breeding pairs of MRL/MpJ-+/+ (MRL), MRL/lpr, and BALB/cCrSlc mice were purchased from Japan SLC, Inc., Hamamatsu, Japan, and (BALB/c x MRL)F_1 hybrid mice were bred in our animal facilities. NZW/NSlc and C57BL/6CrSlc (B6) mice were also purchased from Japan SLC, Inc. All the animal experiments described in this report were approved by and performed under the relevant guidelines of Kinki University.

Production and screening of hybridoma cells

For the screening of anti-gp70 autoantibodies, endogenous retroviral env genes and their chimeras were expressed by using recombinant vaccinia viruses as described [20]. A recombinant vaccinia virus expressing the influenza virus hemagglutinin (HA) gene [21] was used as a negative control throughout the experiment. Spleen and lymph node cells were prepared from

unmanipulated MRL/lpr mice and P3/NSI/1-Ag4-1 myeloma cells were used as fusion partner cells. Hybridoma cell fusion, hypoxanthine-aminopterin-thymidine selection, and cloning by colony formation in fibrin gels were performed as described previously [22,23]. For immunofluorescence detection of the reactivities of hybridoma-derived antibodies to expressed env gene products, monkey CV-1 cells were grown in wells of 96-well tissue culture plates, infected with a recombinant vaccinia virus at 100-200 plaque-forming units per well for 20-36 hours, and incubated at 4 °C, overnight with a hybridoma culture supernatant. After incubation, the cells in each well were washed with phosphate-buffered balanced salt solution (PBBS), fixed with methanol, blocked with 10 % skim milk, and were stained with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig antibody. Control hybridoma cell lines, N-S.7 producing anti-sheep red blood cell (SRBC) IgG3 and 2B12-2 producing anti-TNP IgM antibodies were used as negative controls.

Transfer of hybridoma cells or purified antibodies into non-autoimmune mice and pathological analyses in the recipients

(BALB/c x MRL)F_1 and SCID mice were transplanted intraperitoneally (i. p.) with 1-2 x 10^7 hybridoma cells after a pretreatment with a 0.5 ml/mouse i. p. dose of 2, 6, 10, 14-tetramethylpentadecane (pristane) given 1-3 weeks prior to hybridoma transplantation. For purification of a clonal anti-gp70 IgG, hybridoma cells were grown in a serum-free medium (Hybridoma SFM; Gibco BRL, Rockville, Maryland, U.S.A.) using 20-l spinner flasks, and culture supernates were concentrated by using a tangential flow ultrafiltration system. IgG was purified by Protein A-Sepharose affinity chromatography. Special attention was paid to perform the purification aseptically at room temperature, and to elute each antibody from the affinity column at pH = 4.4. Purified antibodies dissolved in PBBS at 0.5-1.0 mg/ml were injected into the tail vein after removing possibly contaminating Ig aggregates by centrifugation at 10,000 x g for 15 min. The methods for preparation and staining of formalin-fixed, paraffin-embedded tissue sections, preparation of specimens for electron microscopy, and immunohistochemical staining of frozen sections have been described [13,19,20,22,23].

Cloning and sequencing of Ig heavy and light chain cDNA

Total RNA was prepared from each clone of the hybridoma cells using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, California, U.S.A.) and mRNA was purified by using the poly(A)+ isolation kit (Nippon Gene, Tokyo, Japan). 5' RACE reaction was performed by using the SMART RACE cDNA Amplification kit (Clontech, Palo Alto, California, U.S.A.) with the following primers:

5'-GGGGAGGGCACTGACCACCCGGAGA-3' for Cγ2a,
5'-TTGGGGGGAAGATGAAGACGGATGG-3' for Cγ3, and
5'-TTGGTCAACGTGAGGGTGCTGCTCATGC-3' for Cκ.

The amplified full-length cDNA was purified by cutting the band out from agarose gel, and cloned into pGEM-T Easy vector (Promega Corporation, Madison, Wisconsin, U.S.A.), and resultant clones were subjected to DNA sequencing of each insert by using BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, California, U.S.A.). Sequencing data were considered valid when > 8 independent plasmid clones with an identical sequence were observed.

Detection of serum gp70

For the detection of serum gp70, sera from NZW, (BALB/c × MRL)F1 and B6 mice were mixed at 1:20 with the SDS sample buffer containing no reducing agent, and serum proteins were separated through 7.5 % polyacrylamide gel and blotted onto polyvinylidenedifluoride membrane. After being blocked with 10 % skim milk, serum gp70 molecules were detected with a biotin-conjugate of anti-gp70 mAb 24-6 [24] by chemiluminescence reaction using horse radish peroxidase-conjugated streptavidin and ECL+ reagent (Amershan Pharmacia Biotech, Uppsala, Sweden) according to the manufacturers' instructions.

Results

Developement of glomerulonephritis in non-autoimmune mice transferred with monoclonal anti-gp70 autoantibodies. Sixteen independent hybridoma clones that produce antibodies reactive with the endogenous xenotropic viral gp70 were established by the fusion of B cells from unmanipulated MRL/lpr mice with the myeloma cells. These antibodies reacted with the cells infected with the recombinant vaccinia virus that expressed the endogenous retroviral env gene, but not with the cells infected with the vaccinia virus-influenza virus HA recombinant. Further analyses performed by using recombinant vaccinia viruses expressing reciprocal chimeras between the NZB xenotropic viral and polytropic Friend spleen focus-forming viral (SFFV) env genes indicated that 8 of these hybridoma antibodies are reactive with an epitope localized within the N-terminal one third of the xenotropic viral gp70, while two others require the presence of the C-terminal 2/3 of the xenotropic viral gp70 for their binding to the expressed antigens. Six other clones were cross-reactive to both the xenotropic viral and polytropic SFFV env gene products.

All these hybridoma clones were transplanted into syngenic, non-autoimmune (BALB/c x MRL)F1 mice, and 10 of them induced glomerular pathology of varying severity and morphology. Among them, two representative IgG3-producing clones, 12H5.1 and 37C4.1, induced the most severe glomerular pathology with the highest frequency but with distinct

morphological features. The lesions induced by the transplantation of clone 12H5.1 were characterized by the accumulation of inflammatory cells within the capillary lumina with numerous droplet-like granules within the cytoplasm of the infiltrated cells (Fig. 1). Deposits of an electron-dense material between the basement membrane and endothelial cells were also observed. Immunohistochemical analyses showed granular depositions of IgG, C3, and gp70, indicating the above glomerular deposits to be gp70-anti-gp70 immune complexes. On the other hand, the lesions induced by the transplantation of clone 37C4.1 were characterized by the massive and homogeneous subendothelial deposition of an electron-dense material, which resembled wire-loop lesions under light microscopy. The depositions of IgG, C3, fibrin, and gp70 were also observed. Four other clones, 37C6.1 (IgG2a), 51D1.1 (IgG3), 58C5.1 (IgM), and 60A5.1 (IgG3) also induced glomerular pathology with proliferative and/or sclerosing changes when transplanted into the syngeneic non-autoimmune mice with lower frequencies. Interestingly, clone 36D1.1, which did not induce glomerular pathology, instead induced hemorrhagic thrombocytopenia morphologically resembling the microvascular changes of human immune thrombocytopenic purpura. This antibody has been shown to react with a gp70-related membrane protein expressed on the surface of mouse platelets.

To exclude the possibility that hybridoma-derived cytokines and other cellular products were involved in the formation of glomerular pathology in the above transplanted animals, we next purified IgG from the cultures of representative anti-gp70 antibody-producing cells, and injected purified antibodies into non-autoimmune mice. Purified anti-gp70 IgG3 12H5.1, 37C4.1, and 51D1.1 induced glomerular pathology associated with thickening of capillary walls and increased cellularity. Depositions of gp70 were also demonstrated by immunohistochemistry in glomeruli of the mice injected with the purified anti-gp70 IgG. Severer glomerular pathology was induced when two anti-gp70 antibodies of separate epitope specificities, 12H5.1 and 51D1.1 were mixed and injected together.

Since mouse IgG3 lack allotypes, we next injected purified anti-gp70 IgG3 into three different strains of mice that differ in their amounts of constitutively expressed serum gp70. The same amount (3-4 mg/mouse) of purified anti-gp70 antibody 12H5.1 was injected into NZW, (BALB/c x MRL)F1, and B6 strains of mice, which express high, medium, and very low amounts of serum gp70, respectively. Glomerular pathologies with gp70 depositions were observed in antibody-injected NZW and the F1 mice, while no significant pathology was induced in the injected B6 mice (Fig. 1). These data indicated that the formation of gp70 immune complexes was involved in the development of glomerular pathology in anti-gp70 antibody-injected mice. By absorbing plasma immune complexes to Protein A-Sepharose and performing Western blot analyses of the Protein A-bound materials with labeled anti-gp70 monoclonal antibody, we actually detected the presence of gp70-containing immune complexes in mice injected with the purified anti-gp70 autoantibody (data not shown).

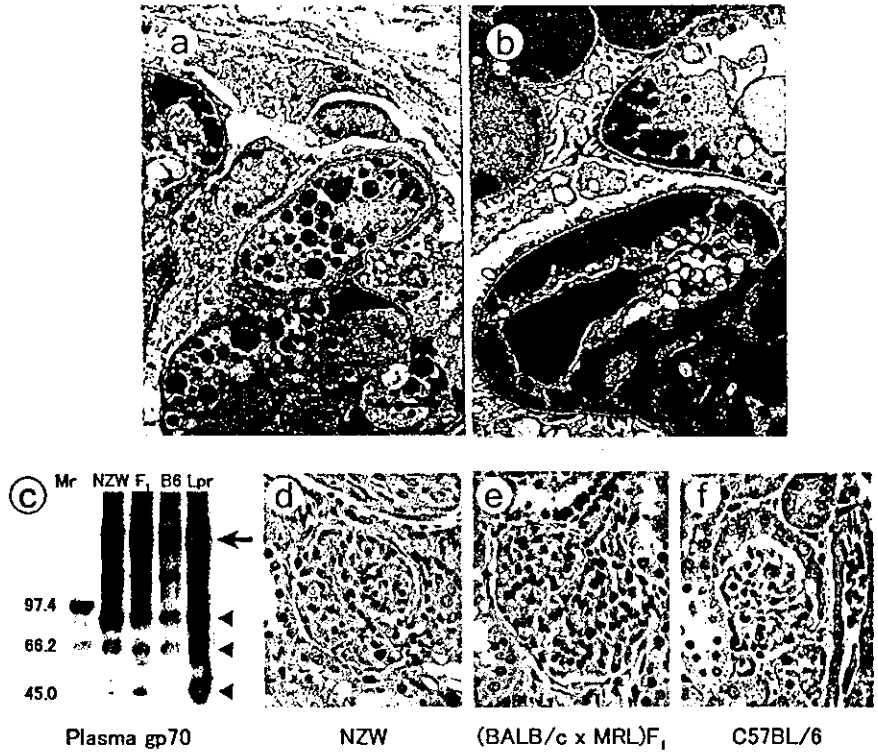

Fig. 1: Pathogenicity of monoclonal anti-gp70 autoantibodies established from MRL/lpr lupus mice shown by passive transfer into non-autoimmune mice. a, An electron micrograph showing cellular proliferation filling the glomerular capillaries and accumulation of electron-dense droplets in the cytoplasm of glomerular cells in (BALB/c x MRL)F1 mice transplanted with anti-gp70 antibody-producing hybridoma cell clone, 12H5.1. Bar = 5 mm. b, Massive deposition of electron-dense materials between the basement membrane and the endothelial cells in the glomeruli of a representative (BALB/c x MRL)F1 mouse transplanted with another anti-gp70 autoantibody-producing hybridoma cell line, 37C4.1. Bar = 2 mm. c, A representative western blot analysis showing differences in serum gp70 expression in NZW, (BALB/c x MRL)F1, and B6 mice. Sera were diluted 1:20 into the SDS-sample buffer without a reducing reagent, boiled for 5 min., and 10 ml of each boiled mixture was loaded into a well of 7.5 % polyacrylamide gel. Plasma from a 4 month-old female MRL/lpr mouse (Lpr) that should contain a large amount of gp70-anti-gp70 immune complexes, was used as a positive control. NZW mice expressed a high level of serum gp85 (gp70 + p15E), gp70, and a degradation product gp45 (arrowheads), while their expression in B6 mice was low. (BALB/c x MRL)F1 mice (F1) expressed an intermediate level of serum gp70. Mr, biotinylated markers with numbers on the left showing relative molecular mass x 10–3. The viral envelope gp70 is known to form trimers, and the corresponding band of apparent molecular mass > 220 kDa was observed (arrow). d-f, Representative photomicrographs taken from kidney sections of NZW (d), (BALB/c x MRL)F1 (e), and B6 (f) mice injected with purified anti-gp70 IgG3, 12H5.1.

Hematoxylin and eosin staining, x 300. Note apparent thickening of the capillary walls and inflammatory cell infiltration in d, and the marked increase in glomerular cellularity and evident neutrophilic infiltration in e.

Immunoglobulin variable region gene usage and amino acid sequences of the pathogenic anti-gp70 autoantibodies

Immunoglobulin heavy and light chain V gene usages and amino acid sequences of the above pathogenic anti-gp70 autoantibodies established from MRL/lpr lupus mice were analyzed by determining nucleic acid sequences of the cDNAs (Tab. 1). Four of the 5 examined clones were using the germ line VH558 family gene which is known to be preferentially used by many anti-DNA antibody clones, including those established from an MRL/lpr mouse [25,26]. VH7183 family gene that is used by the anti-gp70 clone 51D1.1 is also known to be used preferentially by anti-DNA antibodies in (NZB x NZW)F1 mice [26]. Accordingly, the heavy chain CDR1 and CDR2 amino acid sequences of the examined pathogenic anti-gp70 autoantibodies are similar to those reported for the anti-DNA antibody clones established from the MRL/lpr mouse. However, the observed amino acid sequences of heavy chain CDR3 of the pathogenic anti-gp70 antibodies were quite dissimilar to those reported for anti-DNA antibodies, suggesting that CDR3 is mainly involved in the determination of anti-gp70 specificity of these antibodies. In addition, a high frequency of Arg or Lys residues in CDR3 that is know to favor DNA binding of autoantibodies [26,27] was not a common feature of the examined anti-gp70 antibodies, and accumulation of these basic residues in CDR3 was observed in only two of the 5 examined clones. Interestingly, these 2 clones, 36D1.1 and 37C4.1, established from a single fusion, shared the identical heavy chain gene sequence and hence the CDR amino acid sequences, but differed in their light chain gene usages. These results suggest that the two clones have probably developed from a common pre-B cell. Further, since observed pathogenicities of the two clones were different, pathogenic potentials in these two clones of anti-gp70 autoantibodies might be determined by their light chain amino acid sequences. Further analyses are required to experimentally prove this possibility.

Induction of granulomatous arteritis by injection of purified anti-gp70 monoclonal antibody into non-autoimmune mice

During the process of examining glomerular pathology in anti-gp70 antibody-injected animals, we noted the presence of granulomatous arteritis in the lungs of some injected mice. The lesions were characterized by dense accumulation of neutrophyls and macrophage-like cells in the adventitia with focal destruction of the muscular media. Subendothelial accumulation of neutrophils in the intima was also observed, but less frequently. Small arterioles were mainly involved, but larger branches of pulmonary arteries were also affected at a low frequency. To systemically analyze the development of this arterial pathology, we purified large amounts of the anti-gp70 and control anti-SRBC IgG3 antibodies, and injected them into (BALB/c x MRL)F1, (B6 x MRL)F1, and NZW mice. A total of 3-6 mg/mouse of a purified antibody was injected in 6 to 10 split doses of 0.7 ml twice a day for 3 to 5 days, and injected animals were examined 2 days after the final injection based on the results of preliminary experiments in which pathological analyses were done 1, 2, 4, and 7 days after the final injection. The granulomatous arterial lesions were observed only in mice injected with anti-gp70 clone 12H5.1, but not in those injected with 51D1.1 or control anti-SRBC IgG3, N-S.7. The frequency of the development of the arteritis was higher in NZB mice than in (BALB/c x MRL)F1, suggesting possible involvement of gp70-anti-gp70 immune complexes. More detailed analyses on the pathogenesis of this granulomatous arteritis are currently under way, and will be reported separately.

Tab. 1. Immunoglobulin variable region gene usage and deduced amino acid sequence of pathogenic anti-gp70 autoantibodies.

Clone	Isotype	V_H	J_H	$V\kappa_K$	$J\kappa_K$
12H5.1	IgG_3, κ	558	4	21	2
36D1.1	IgG_{2a}, κ	558	3	19-20	2
37C4.1	IgG_3, κ	558	3	8-21	4
37C6.1	IgG_{2a}, κ	558	3	10	2
51D1.1	IgG_3, κ	7183	4	21B	1

Clone	V_H		
	CDR1	CDR2	CDR3*
12H5.1	RYWMH	AIYPGNSDTSYNQKFKGK	EGISIYDGLYFAMDY
36D1.1	DYSMD	YIYPNNGYTGYNQKFKSK	KLGRREAYFDV
37C4.1	DYYMD	YIYPNNDGTNYNQKFKGK	GGLAGYYLYYAMDY
37C6.1	DYSMD	YIYPNNGYTGYNQKFKSK	KLGRREAYFDV
51D1.1	DYYMA	NINYDGSSTYYLDSLKSR	TPTGYYAMDY

Clone	V_L		
	CDR1	CDR2	CDR3
12H5.1	RASKSVSTSSYSYMH	YASYLES	QHSREFPYT
36D1.1	KASENVVTYVS	GASNRYT	GQGYSYPYT
37C4.1	KSTQSLFNSRTRKNYLA	WASTRES	TQSYYLH
37C6.1	RASQDISNYLN	YTSRLHS	QQYSKLPYT
51D1.1	RASKSVDRYGNSFMH	RTSNLES	QQNNEDPWT

*Basic amino acid residues within CDR3 are shown in boldface.

Discussion

Since the discovery of spontaneous mouse models of SLE, it has long been debated if anti-DNA antibodies are mainly responsible for the development of fatal nephritis, or anti-gp70 antibodies reactive to the endogenous retroviral env gene product play more important roles in the formation of the glomerular injury. Pathogenic potentials of anti-DNA antibodies have been shown in various experimental settings, but recent genetic analyses have indicated a closer correlation between the production of gp70-anti-gp70 immune complexes with the development of glomerulonephritis than that between the anti-DNA antibody production and fatal nephritis. We have shown here that monoclonal anti-gp70 autoantibodies established from MRL/lpr lupus mice are directly pathogenic when transferred into non-autoimmune mice. The deposition of gp70 in affected glomeruli along with IgG and C3, and the development of severer lesions in NZW mice that express higher concentrations of serum gp70 than B6 mice that developed no significant pathology indicate that gp70-anti-gp70 immune complexes are involved in the development of the glomerular pathology.

The pathogenic anti-gp70 autoantibodies used the same VH gene family as known anti-DNA antibody clones, but possessed unique CDR3 sequences that are dissimilar to those of anti-DNA antibodies. Further studies are required to conclude if antigen-driven somatic mutations are accumulated in the VH and VL genes of the anti-gp70 clones as has been reported for anti-DNA antibodies [25,27].

Vascular lesions of necrotizing and/or granulomatous types are frequently observed in mice predisposed to develop systemic autoimmune diseases [6-8,14]. However, pathogenesis of spontaneous vascular injury has been largely unknown. In a previous study, we have shown a close correlation between the presence of anti-gp70 autoantibodies and the development of necrotizing arteritis in SL/Ni strain of mice [14]. Vascular lesions were also induced in mice transplanted with the pathogenic anti-gp70 antibody-producing hybridoma cells as we reported previously [28] and in the present study. The development of granulomatous arteritis by the injection of purified anti-gp70 autoantibody is a unique phenomenon, and this model promises to be useful for the analyses of pathogenetic mechanisms of and the development of therapeutic means for human inflammatory vascular diseases.

Our model of vascular injury induced by the injection of anti-retroviral autoantibodies also suggests that immune responses to human retroviruses might be involved in the development of human inflammatory vascular diseases. This is particularly of interest because infections with exogenous human retroviruses, HTLV-I and HIV-1, are known to be associated with vasculitis [8,9]. Although sceptical views are common among retrovirologists on the possible involvement of human endogenous retroviruses in the pathogenesis of autoimmune diseases [11,12], it might be worth recalling that most retrovirologists had been sceptical to the point of nearly denying the possibility of retroviruses playing a role in human cancers until HTLV-I was finally discovered [1].

References

1. Gallo RC. The early years of HIV/AIDS. Science 2002; 298: 1728-31.
2. Rahman A, Hiepe F. Anti-DNA antibodies – overview of assays and clinical correlations. Lupus 2002; 11: 770-773.
3. Smeenk RJT. Antinuclear antibodies: cause of disease or caused by disease? Rheumatology 2000; 39: 581-584.
4. Ravirajian CT, Rowse L, MacGowan JR, Isenberg DA. An analysis of clinical disease activity and nephritis-associated serum autoantibody profiles in patients with systemic lupus erythematosus: a cross-sectional study. Rheumatology 2001; 40: 1405-12.
5. Amoura Z, Piette J-C, Bach J-F, Koutouzov S. The key role of nucleosomes in lupus. Arthritis Rheum. 1999; 42: 833-843.
6. Andrews BS, Eisenberg RA, Theofilopoulos AN, et al. Spontaneous murine lupus-like syndromes. Clinical and immunopathological manifestations in several strains. J. Exp. Med. 1978; 148:1198-1215.
7. Theofilopoulos AN, Dixon FJ. Murine models of systemic lupus erythematosus. Adv. Immunol. 1985; 37:269-390.
8. Krieg AM, Steinberg AD. Retroviruses and autoimmunity. J. Autoimmun. 1990; 3:137-166.
9. Garry RF, Krieg AM, Cheevers WP et al. Retroviruses and their roles in chronic inflammatory diseases and autoimmunity. In: The Retroviridae. vol 4. New York: Plenum Press, 1995; 491-603.
10. Conrad B, Weissmahr RN, Boni J, et al. A human endogenous retroviral superantigens as candidate autoimmune gene in type I diabetes. Cell 1997; 90:303-313.
11. Stoye JP. The pathogenic potential of endogenous retroviruses: a skeptical view. Trends Microbiol. 1999; 7: 430.
12. Portis JL. Perspectives on the role of human endogenous retroviruses in autoimmune diseases. Virology 2002; 296: 1-5.
13. Izui S, Elder JH, McConahey PJ, Dixon FJ. Identification of retroviral gp70 and anti-gp70 antibodies involved in circulating immune complexes in NZB × NZW mice. J. Exp. Med. 1981; 153:1151-60.
14. Miyazawa M, Nose M, Kawashima M, Kyogoku M. Pathogenesis of arteritis of SL/Ni mice. Possible lytic effect of anti-gp70 antibodies on vascular smooth muscle cells. J. Exp. Med. 1987; 166: 890-908.
15. Vyse TJ, Drake CG, Rozzo SJ, et al. Genetic linkage of IgG autoantibody production in relation to lupus nephritis in New Zealand hybrid mice. J. Clin. Invest. 1996; 98:1762-1772.
16. Tucker RM, Vyse TJ, Rozzo S, et al. Genetic control of glycoprotein 70 autoantigen production and its influence on immune complex levels and nephritis in murine lupus. J. Immunol. 2000; 165: 1665-72.
17. Haywood ME, Vyse TJ, McDermott A, et al. Autoantigen glycoprotein 70 expression is regulated by a single locus, which acts as a checkpoint for pathogenic anti-glycoprotein 70 autoantibody production and, hence for the corresponding development of severe nephritis, in lupus-prone BXSB mice. J. Immunol. 2001; 167: 1728-33.

18. Rigby RJ, Rozzo SJ, Gill H, et al. A novel locus regulates both retroviral glycoprotein 70 and anti-glycoprotein 70 antibody production in New Zealand mice when crossed with BALB/c. J. Immunol. 2004; 172: 5078-85.
19. Hashimoto K, Tabata N, Fujisawa R, Matsumura H, Miyazawa M. Induction of microthrombotic thrombocytopenia in normal mice by transferring a platelet-reactive, monoclonal anti-gp70 autoantibody established from MRL/lpr mice: an autoimmune model of thrombotic thrombocytopenic purpura. Clin. Exp. Immunol. 1999; 119: 47-56.
20. Tabata N, Miyazawa M, Fujisawa R, et al. Establishment of monoclonal anti-retroviral gp70 autoantibodies from MRL/lpr lupus mice and induction of glomerular gp70 deposition and pathology by transfer into non-autoimmune mice. J. Virol. 2000; 74: 4116-26.
21. Smith GL, Murphy BR, Moss B. Construction and characterization of an infectious vaccinia virus recombinant that expresses the influenza hemagglutinin gene and induces resistance to influenza virus infection in hamsters. Proc. Natl. Acad. Sci. USA. 1983; 80:7155-7159.
22. Robertson MN, Miyazawa M, Mori S, et al. Production of monoclonal antibodies reacting 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; 34:255-271.
23. Miyazawa M, Mori S, Spangrude GJ, et al. Production and characterization of new monoclonal antibodies that distinguish subsets of mink lymphoid cells. Hybridoma 1994; 13:107-114.
24. Portis JL, McAtee FJ, Cloyd MW. Monoclonal antibodies to xenotropic and MCF murine leukemia viruses derived during the graft-versus-host reaction. Virology 1982; 118: 181-190.
25. Swanson PC, Yung RL, Blatt NB, et al. Ligand recognition by murine anti-DNA autoantibodies. II. Genetic analysis and pathogenicity. J. Clin. Invest. 1996; 97: 1748-60.
26. Marion TN, Tillman DM, Krishnan MK, et al. Immunoglobulin variable-region structures in immunity and autoimmunity to DNA. Tohoku J. Exp. Med. 1994; 173: 43-64.
27. Jang YI, Stollar BD. Anti-DNA antibodies: aspects of structure and pathogenicity. Cell. Mol. Life Sci. 2003; 60: 309-320.
28. Miyazawa M, Tabata N, Fujisawa R, et al. Roles of endogenous retroviruses and platelets in the development of vascular injury in spontaneous mouse models of autoimmune diseases. Int. J. Cardiol. 2000; 75: S65-S73.

Acknowledgements

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. We thank Mr. M. Patrick Gorman for critically reading and correcting the manuscript.

Rheumatic Fever: an animal model for a human disease

F.F. Alcantara, E. Postol, E.R. Alencar, J. Kalil, L. Guilherme

Immunology Laboratory Heart Institute (InCor), University of Sao Paulo Medical School and Department of Allergy and Clinical Immunology, University of Sao Paulo - School of Medicine, Brazil

flaviofalcantara@yahoo.com

Rheumatic fever is an inflammatory autoimmune condition following 3 % of non treated group A streptococci pharyngitis of certain M serotype strains. In addition to a cellular heart valve attack, anti-basal ganglia autoantibodies arise later in the autoimmune process targeting caudate and subthalamic nuclei antigens, being responsible for the neurologic symptoms of Sydenham's chorea. Mammalian lysoganglioside and N-acetyl-beta-D-glucosamine (GlcNAc), the dominant epitope of the group A streptococcal (GAS) carbohydrate are the probable molecules involved in neurologic damage. Antibodies targeting both heart antigens and M proteins were found in blood of affected patients. Antigenic mimicry between streptococcal M protein epitopes and heart components have been proposed as the triggering factor leading to the heart autoimmune attack. The understanding of the disease process and therapeutic advances has been hampered by the lack of an adequate animal model for the disease. We have injected Lewis rats with streptococcus recombinant M1 protein 500 µg on day 0 followed by 500 µg boost on day 7 and sacrifice on day 21, in order to reproduce a recently described animal model of rheumatic fever [Quinn, A. et al, 2001, Infect.Immun. 69(6):4072-78]. Rat hearts were subjected to histopathological analyzes. Spleen and lymph node lymphocyte cells, as well as sera, were harvested and probed against ABC domains, AB domains (N-terminus), or C domain (C-terminus) of the M1 complete protein and myosin or control proteins. We have obtained specific lymphoproliferative responses against selected M protein fragments and specific cardiac proteins, as seen in our previous results with patient samples [Guilherme et. al, 1995, Circulation 92:415-20]. We are currently using the rat-immunized cells for FACS analysis to study their phenotypic profile and cytokine production. The aim of our studies is to map the minimal M protein epitope(s) responsible for rheumatic fever after immunization with different recombinant M protein fragments.

JOURNAL OF VIROLOGY, June 2004, p. 6322–6334 Vol. 78, No. 12
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.12.6322–6334.2004

Identification of a Protective CD4$^+$ T-Cell Epitope in p15^{gag} of Friend Murine Leukemia Virus and Role of the MA Protein Targeting the Plasma Membrane in Immunogenicity

Daisuke Sugahara, Sachiyo Tsuji-Kawahara,* and Masaaki Miyazawa

Department of Immunology, Kinki University School of Medicine, Osaka-Sayama, Osaka 589-8511, Japan

Received 15 October 2003/Accepted 19 February 2004

Recent studies have demonstrated an essential role of Gag-specific CD4$^+$ T-cell responses for viral control in individuals infected with human immunodeficiency virus type 1. However, little is known about epitope specificities and functional roles of the Gag-specific helper T-cell responses in terms of vaccine-induced protection against a pathogenic retroviral challenge. We have previously demonstrated that immunization with Friend murine leukemia virus (F-MuLV) Gag proteins protects mice against the fatal Friend retrovirus (FV) infection. We report here the structure of a protective T helper cell (Th) epitope, (I)VTWEAIAVDPPP, identified in the p15 (MA) region of F-MuLV Gag. In mice immunized with the Th epitope-harboring peptide or a vaccinia virus-expressed native full-length MA protein, FV-induced early splenomegaly regressed rapidly. In these mice, FV-infected cells were eliminated within 4 weeks and the production of virus-neutralizing antibodies was induced rapidly after FV challenge, resulting in strong protection against the virus infection. Interestingly, mice immunized with the whole MA mounted strong CD4$^+$ T-cell responses to the identified Th epitope, whereas mice immunized with mutant MA proteins that were not bound to the plasma membrane failed to mount efficient CD4$^+$ T-cell responses, despite the presence of the Th epitope. These mutant MA proteins also failed to induce strong protection against FV challenge. These data indicate the importance of the properly processible MA molecule for CD4$^+$ T-cell priming and for the resultant induction of an effective immune response against retrovirus infections.

Defining the immune mechanisms that facilitate resistance to viral infections is vital for the rational development of preventative and therapeutic modalities against virus-induced diseases. Substantial evidence indicates that virus-specific CD4$^+$ T helper (Th) cells play a key role in the control of many different viral infections (reviewed in references 14 and 36). In mouse models, maintenance of CD8$^+$ cytotoxic T-cell (CTL) responses and control of viremia have been demonstrated to depend on virus-specific CD4$^+$ T cells during chronic viral infections (1, 28, 57, 62). In addition, cooperation between antigen-specific CD4$^+$ T cells and neutralizing antibody (Ab)-producing B cells is required for long-term virus control in lymphocytic choriomeningitis virus infections (43, 53). With regard to immunosuppressive retrovirus infections, activation of virus-specific CTL responses alone is largely ineffective in inducing protection against simian immunodeficiency virus (SIV) infection (12, 49, 60). In contrast, adoptive transfer of autologous CD4$^+$ T cells results both in the induction of virus-specific CTL responses and in the production of neutralizing Abs, with long-term anti-SIV control (56). Thus, the development and maintenance of functional CTL and B-cell responses that are aided by the activation of virus-specific CD4$^+$ T cells might be required for effective protection against chronic virus infections. However, the precise nature of the virus-specific CD4$^+$ T cells that contribute to effective antiviral immunity remains unclear. More recently, an inverse association between human immunodeficiency virus type 1 (HIV-1)-specific CD4$^+$ T-cell responses and plasma viral load has been demonstrated in long-term nonprogressors and individuals treated with highly active antiretroviral therapy (22, 26, 42, 46, 47). Intriguingly, in such HIV-1-infected individuals, strong Gag-reactive CD4$^+$ T-cell responses were detected in association with a high level of HIV-1-specific CTL responses.

The Gag protein of retroviruses is a major viral component and is relatively conserved in its structure among various isolates and between retroviruses of different host species in comparison with the Env protein. Broadly cross-reactive Th epitopes, as well as CTL epitopes, have been identified in conserved regions of retroviral Gag proteins (11, 29, 48, 58). Finally, by use of a mouse model of Friend retrovirus (FV) infection, it has been found that immunization with *gag* gene products induces CD4$^+$ T-cell-mediated protective immunity (32), although the precise epitopes involved have not been identified. Given these observations, there is compelling evidence indicating that Gag-specific CD4$^+$ T cells are effective in controlling retrovirus infections, and therefore they may be potential targets for the development of effective antiretrovirus vaccines.

FV is an immunosuppressive retrovirus complex that induces fatal erythroleukemia in adult immunocompetent mice. Since the cell surface receptors, intracellular signaling, and host factors controlling virus replication and host immune responses have been well characterized, infection with this retrovirus represents a useful model in which to study both acute and persistent viral infections, as well as virus-host interactions

* Corresponding author. Mailing address: Department of Immunology, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan. Phone and fax: 81-72-367-7660. E-mail: skawa@immunol.med.kindai.ac.jp.

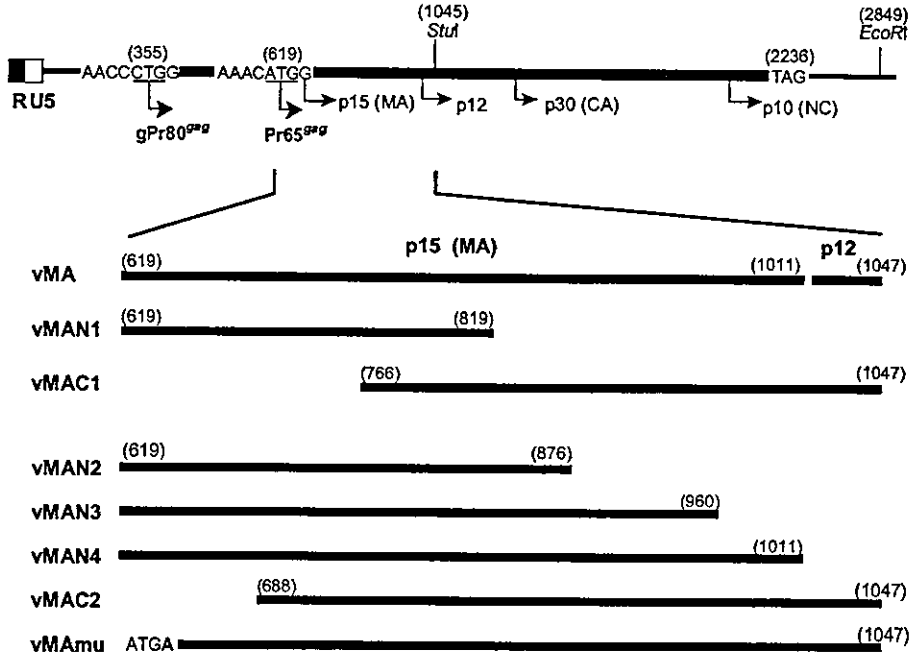

FIG. 1. Schematic representation of the F-MuLV *gag* gene and strategies for construction of the rVVs expressing portions of the MA protein. Base numbers of the *gag* gene in parentheses are given according to the published sequence of F-MuLV FB29 (39).

(reviewed in references 8 and 13). The replication-competent helper component of FV, Friend murine leukemia virus (F-MuLV), contains the immunological determinants necessary for anti-FV immune responses, while the replication-defective spleen focus-forming virus (SFFV) is required for the pathogenicity of FV complex in adult mice (21, 34). FV induces rapid splenomegaly because the SFFV envelope protein binds to the erythropoietin receptor on erythroid precursor cells, causing false proliferation signals. Susceptible animals develop acute and severe splenomegaly after FV inoculation, and unresolved infection leads to leukemic death within several weeks after challenge.

In order to understand and characterize the role of Gag-specific $CD4^+$ T cells in protective immunity against retrovirus infections, we attempted here to identify a Th epitope in the MA protein of F-MuLV Gag and investigated the possible association of Gag-primed $CD4^+$ T-cell responses with host protection. Furthermore, we examined structural features of the MA required for the induction of efficient cellular and humoral immune responses in vivo. The results provide new insights into different accessibilities for antigen presentation of the membrane-bound and unbound MA proteins and underscore their importance in vaccine development for retrovirus infections.

MATERIALS AND METHODS

Mice and virus. Female C57BL/6 (B6) and BALB/c mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). A/WySnJ mice were originally purchased from The Jackson Laboratory (Bar Harbor, Maine). (B6 × A)F_1 mice were bred and maintained at the animal facility, Kinki University School of Medicine, and these mice, aged 8 to 16 weeks at the time of immunization, were used for the experiments described below. A stock of B-tropic FV was originally given by Bruce Chesebro, Laboratory of Persistent Viral Diseases, National Institute of Allergy and Infectious Diseases (Hamilton, Mont.), and the stock used in the present study was prepared from infected BALB/c mice as a 20% spleen homogenate as described previously (31). For virus challenge, mice were injected in the tail vein with 1,500 spleen focus-forming units (SFFU) of FV complex in 0.5 ml of phosphate-buffered balanced salt solution (PBBS) containing 2% fetal bovine serum. After virus challenge, mice were observed daily, and the number of surviving mice was counted. The development of splenomegaly was monitored by palpation as described elsewhere (32). In some experiments, moribund mice were killed by cervical dislocation and spleen weights were measured to compare the results of palpation to actual spleen weights. Spleens weighing >0.5 g were consistently marked as palpable splenomegaly. All the animal experiments were approved and performed under relevant guidelines of the Japanese government and of Kinki University.

Construction of rVVs expressing the F-MuLV *gag* genes. Recombinant vaccinia viruses (rVVs) were constructed by the standard homologous recombination method using transfer plasmids based on pSC11 (6). Fragments of a *gag* gene from an infectious molecular clone of F-MuLV, FB29 (39) (GenBank accession no. Z11128), that were cloned into rVV are shown in Fig. 1. An rVV, r9-28B, expressing the entire $gPr80^{gag}$ and $Pr65^{gag}$ proteins has been described previously (32). All fragments of MA were fused with a polyhistidine metal-binding peptide (His tag) at their C termini so that their expression could be visualized with an anti-His tag Ab. For construction of rVVs expressing the His tag-conjugated proteins, a derivative of pSC11 (pSC11-His) was newly generated by inserting a His tag sequence and the multiple cloning site from the pcDNA3.1/V5-His vector (Invitrogen Corp., Carlsbad, Calif.) into a StuI site of pSC11-SS as described elsewhere (17). All DNA fragments encoding portions of F-MuLV MA (Fig. 1) were synthesized by PCR using pairs of oligonucleotide primers with additional sequences to generate the restriction enzyme site at their 5′ ends. After digestion with the corresponding restriction enzymes, PCR-amplified fragments were inserted in frame into the multiple cloning site of pSC11-His, which allowed fusion of the MA gene fragments to the N-terminal end of the His tag. The resultant plasmids were used to generate rVV vMAs. An unmyristylated form of the MA in which the N-terminal glycine required for protein myristylation (44) was replaced with an alanine was created by site-directed mutagenesis. To introduce a glycine-to-alanine point mutation, the following oligonucleotide was used as the sense primer: 5′-CCCCGTCGACCATGGCCCAGGCTGTT-3′. The PCR product amplified with the mutagenic primer pair and with the plasmid harboring the whole F-MuLV *gag* gene (32) as the template was inserted into pSC11-His as described above to generate an rVV vMAmu. Nucleotide sequences of all the cloned DNA fragments were confirmed, and the protein expression from the newly constructed rVV was detected by Western blotting and/or immunofluo-

rescent staining with an anti-His Ab (Santa Cruz Biotechnology, Santa Cruz, Calif.). A control rVV expressing the influenza virus hemagglutinin gene (vHA) has been described previously (51). As another control, an rVV, vHS1, expressing His tag-conjugated HS1, a hematopoietic-cell-specific intracellular molecule, was made by inserting a cDNA fragment encoding the N-terminal part (residues 1 to 204) of human HS1 (24) (GenBank accession no. H16663) into pSC11-His. Mice were inoculated with 10^7 PFU of an rVV via tail scratch (32), followed by an intravenous injection with the same amount of the identical virus 2 weeks later as a booster. Four weeks after the booster immunization, the mice were challenged with FV complex by intravenous inoculation.

Synthetic peptides and immunization. Locations within MA and sequences of the peptides used in this study are shown in Fig. 6A and 7C. Overlapping 30-mer or 9- to 17-mer peptides covering the C-terminal half of the F-MuLV MA were ordered from QIAGEN K. K. (Tokyo, Japan). Lyophilized powder of each purified peptide was dissolved in Dulbecco's phosphate-buffered saline and emulsified with an equal volume of complete Freund's adjuvant (CFA; Difco, Detroit, Mich.). Mice were immunized once subcutaneously in the abdominal wall with multiple split doses for a total of 100 μl of emulsion containing 50 μg of a peptide; 4 weeks later, they were challenged with FV complex. Control mice were given the same amount of CFA emulsified with phosphate-buffered saline without any peptide.

Flow cytometry. Spleen tissue was dissociated in PBBS containing 2% fetal bovine serum, and a single-cell suspension was prepared as described elsewhere (16). Cells were incubated with 10 μg of anti-mouse CD16/CD32 (BD Biosciences PharMingen, San Diego, Calif.)/ml to prevent test Abs from binding to Fc receptors. For detection of erythroblasts infected with F-MuLV, spleen cells were incubated with R-phycoerythrin-conjugated TER-119 (BD Biosciences PharMingen) and biotinylated monoclonal Ab (MAb) 720 followed by fluorescein isothiocyanate-conjugated streptavidin (BD Biosciences PharMingen). TER-119 is specific for late erythroblasts and mature erythrocytes (23), and MAb 720 reacts specifically to F-MuLV gp70 but not to any other mouse retrovirus (45). Cells were also stained with isotype-matched control Abs. Dead cells were excluded from analyses by staining with 7-aminoactinomycin D (Beckman Coulter, Marseille, France), and viable cells were analyzed for specific staining with a FACScalibur (Becton Dickinson Immunocytometry Systems, Franklin Lakes, N.J.).

Infectious center assays. Infectious center assays were performed as described previously (31). Briefly, spleen cell suspensions prepared from mice challenged with FV complex were serially diluted, plated in triplicate onto monolayers of *Mus dunni* cells, and then cocultured for 2 days. After fixation with methanol, F-MuLV-infected cell foci were stained with MAb 720, visualized by using the avidin-biotinylated peroxidase complex (ABC; Vector Laboratories, Burlingame, Calif.), and counted under a magnifier.

Assays for virus-neutralizing Abs. Mice were bled of 100 μl from the retro-orbital sinuses under ether anesthesia before immunization, at 2 weeks after the last immunization, and once a week after FV inoculation. The details of the assay for F-MuLV-neutralizing Abs have been described previously (31, 32).

Assays for T-cell proliferative responses. Proliferative responses of T cells against MA peptides were analyzed at 3 weeks after immunization with each rVV. The assay method has been described elsewhere (15, 32). Briefly, nylon wool-passed T cells were prepared from the spleen, and irradiated (4,000 rads) syngeneic or parental spleen cells were used as antigen-presenting cells (APC). The T cells (5×10^5) were incubated with the APC (5×10^5) and each synthesized peptide (20 μM) in a total volume of 200 μl. Three days later, the cells were pulsed with [^{3}H]thymidine (Amersham Biosciences, Piscataway, N.J.) added at 1.0 μCi per well; its uptake was measured 18 h later with a scintillation counter (Perkin-Elmer Applied Biosystems, Foster City, Calif.). All data are expressed as the mean difference in counts per minute (Δcpm, calculated as the average incorporation of [^{3}H]thymidine by cultures stimulated with a peptide minus that by unstimulated cultures). In some experiments, proliferative responses of T-cell subsets were analyzed. Nylon wool-passed spleen T cells were incubated with an anti-CD4 or anti-CD8 MAb conjugated with magnetic microbeads and were passed through a separation column placed in a magnetic sorter I (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The purity of the cell fractions was determined by flow cytometry after staining with appropriate fluorescence-labeled MAbs as described under "Flow cytometry" above. Each preparation contained less than 0.5% of the depleted cell type.

Western blotting. For some mice immunized with an rVV, production of anti-MA Abs in sera was analyzed by immunoblotting. F-MuLV particles were purified from the culture supernatant of *M. dunni* cells chronically infected with FB29 as described previously (15, 33). The purified virus particles (8 μg/lane) were subjected to sodium dodecyl sulfate-polyacrylamide gradient gel (PAGEL AE6000; ATTO Corp., Tokyo, Japan) electrophoresis and transferred to a polyvinylidene difluoride membrane. After blocking with 10% skim milk, the membranes were incubated with a 1:10 dilution of each serum sample, followed by another incubation with a horseradish peroxidase-conjugated anti-mouse immunoglobulin (Ig) Ab (Zymed, South San Francisco, Calif.). MA proteins were visualized by using an enhanced chemiluminescence detection system (Amersham Biosciences) as described elsewhere (52). MAb 690, directed against F-MuLV MA (30), was used as a positive control for detection of blotted MA proteins.

Analysis of intracellular localization of His tag-conjugated MA proteins by confocal microscopy. CV-1 cells were infected with a low titer of rVV and incubated overnight so that isolated infectious plaques were visible. The cells were fixed for 10 min in 3.7% formaldehyde, permeabilized by 0.4% Triton X-100, and blocked with 5% goat serum. His tag-conjugated MA proteins were stained with the anti-His Ab, followed by incubation with a fluorescein isothiocyanate-conjugated anti-rabbit Ig Ab (Southern Biotechnology, Birmingham, Ala.). The stained samples were scanned with an LSM 5 PASCAL laser confocal microscope (Carl Zeiss, Berlin, Germany).

Statistical analyses. Survival data were expressed by the Kaplan-Meier method, and the Mantel-Haenszel log rank test was employed for comparison of survival curves using GraphPad Prism (GraphPad Software, Inc., San Diego, Calif.). Student's *t* test was used for comparison of data for T-cell proliferative responses and frequencies of spleen infectious centers between experimental groups.

RESULTS

Early protection against FV infection in mice immunized with F-MuLV MA. The F-MuLV *gag* gene codes for two alternatively translated polyproteins, $Pr65^{gag}$ and $gPr80^{gag}$ (9). $Pr65^{gag}$ is the precursor to virion core structural proteins and is myristylated on the N-terminal glycine and proteolytically cleaved into four proteins (p15, p12, p30, and p10) during virion maturation. The glycosylated cell surface Gag protein, $gPr80^{gag}$, contains the entire amino acid sequence of $Pr65^{gag}$ plus a leader sequence (Fig. 1). We previously showed that protective immune responses against FV infection mediated by $CD4^+$ T cells were induced by immunization with the full-length *gag* gene products. Moreover, immunization with an rVV expressing full-length $Pr65^{gag}$ (positions 619 to 2849) or with an rVV expressing an N-terminal portion of Gag (positions 355 to 1047) representing the 5′ leader sequence, the entire MA, and a short N-terminal fragment of p12, elicited similarly efficient protection, suggesting that the major protective epitope might be located within a *gag* gene product encoded by the segment corresponding to positions 619 to 1047 (32). However, since it has been shown that two overlapping CTL epitopes are located within the leader peptide (7, 25, 54), it is not certain whether the MA protein alone can be protective.

To further narrow the region containing the protective Th epitope, rVVs expressing full-length MA (vMA; positions 619 to 1047), the N-terminal half (vMAN1; positions 619 to 819), or the C-terminal half (vMAC1; positions 766 to 1047) were constructed (Fig. 1), and (B6 × A)F_1 mice, which are susceptible to FV infection, were immunized twice with one of these rVVs before FV infection. As shown in Fig. 2, when immunized with the rVV expressing the entire $Pr65^{gag}$/$gPr80^{gag}$ (r9-28B), more than 80% of the (B6 × A)F_1 mice recovered from the initial development of splenomegaly by 6 weeks and survived longer than 12 weeks after inoculation with 1,500 SFFU of FV. On the other hand, all the control mice given the rVV expressing influenza virus HA or His tag-conjugated HS1 showed enlargement of the spleen shortly after the FV challenge, and about half of them died by 12 weeks postchallenge,

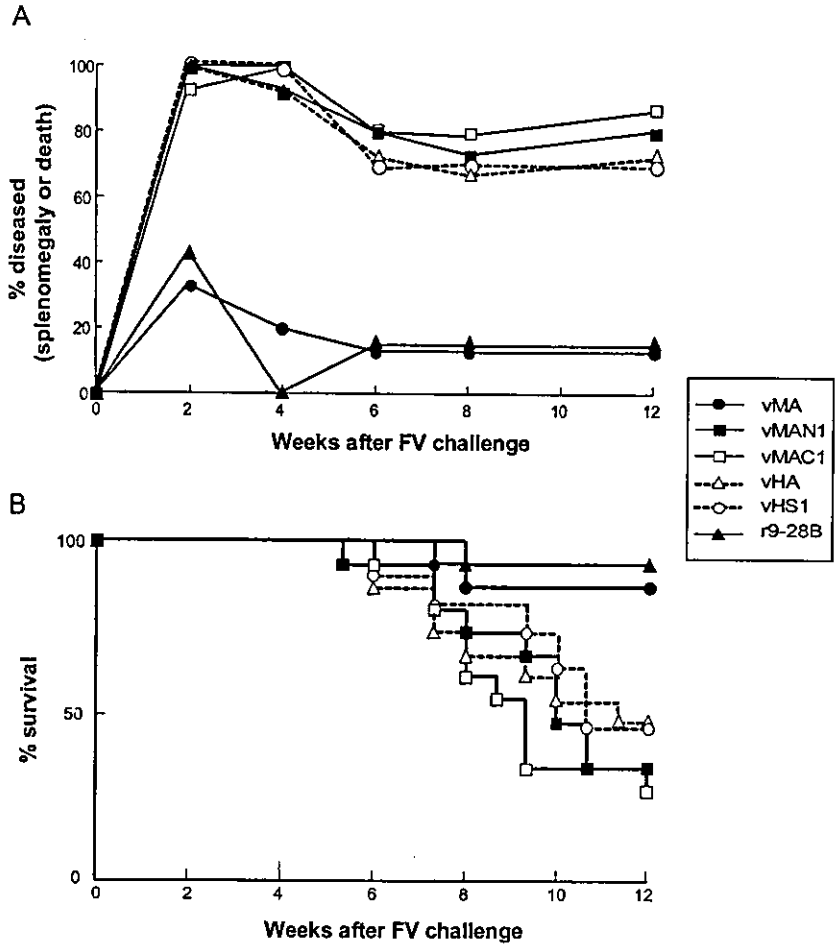

FIG. 2. Induction of protective immunity against FV infection with the MA region of F-MuLV Gag. Each group of (B6 × A)F_1 mice (10 to 15 per group) was immunized twice with one of the rVVs shown. Four weeks after the second immunization, mice were injected intravenously with 1,500 SFFU of FV and then monitored for the development of splenomegaly and death. (A) The incidence of disease at each time point was calculated by adding the numbers of mice that had splenomegaly (>0.5 g) and mice that had died. (B) Survival curves of the groups of mice examined. Statistically significant differences ($P < 0.05$) between the upper two (▲, ●) and the lower four survival curves were confirmed by the Mantel-Haenszel log rank test.

results comparable to those observed for nonimmunized mice of the same strain. As expected, significant protection was observed in mice immunized with vMA, expressing the entire MA plus the short fragment of p12 but not the leader peptide, confirming the predicted existence of a protective epitope(s) in the MA region. Surprisingly, however, neither the N-terminal (vMAN1) nor the C-terminal (vMAC1) half of MA induced significant protection against FV infection. At 12 weeks postchallenge, the spleens of the surviving mice were weighed, and the results corresponded well with those of palpation, indicating that the individuals shown as diseased in Fig. 2A were indeed leukemic. The incidences of splenomegaly at 6 weeks after challenge were well correlated with the incidence of leukemic death or splenomegaly at 12 weeks after challenge. In addition, in the previous experiments performed with the same or similar mouse strains and ≥1,500 SFFU of FV, recovery from splenomegaly present at 6 to 7 weeks postchallenge has rarely been observed (31, 32). Therefore, in subsequent experiments, splenomegaly was monitored, as an indicator of the FV-induced disease, over a period of 6 weeks postchallenge. Furthermore, since mice immunized with vHA or vHS1 showed similar incidences of disease development, the rVV-expressing His tag-conjugated HS1 was subsequently used as the negative control.

Kinetics of the development of early protective immunity in mice immunized with the MA proteins. To elucidate how rapidly antiviral protection developed as a result of immunization with full-length MA, we prepared spleen cells from vMA-immunized mice at early time points after FV challenge and examined the numbers of virus-producing cells by infectious center assays (Fig. 3A). In both groups of mice, those immunized with vMA and those immunized with vHS1 (negative control), cells producing infectious F-MuLV particles were detected in the spleen at 1 week after FV challenge, but the average number of spleen infectious centers was significantly lower in vMA- than in vHS1-immunized mice (3.3×10^1 versus 2.7×10^2 per 10^5 nucleated cells, respectively). In control mice, spleens were dramatically enlarged, reaching a peak average weight of 1.96 g at 3 weeks postchallenge, and average numbers of spleen infectious centers increased and remained high