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MN      CTRPNYNKRKRRIHL  GPGRAFYVTKNIIGTRQAHC
BZ167  -----NKA-R-R-  ---T---G- -V-D---Y-
SF2    ---LSN-T-C-PL  ---V-A-DI- -D-----
CI2    -----SN-T-R-  ---RQ-R-D-----
MNP    -----N-R-T-  -----

89.6P  -----N-T-E-LS-  -----ARR----D-----
SF33   -----N-R-R--TS  ---KVL---GE---D--K-Y-
VI131  -----N-T-QSV--  ---Q---A-GDV--D-----
IIIB   -----N-T---KS-QR-----V-IGK- -NM-----
    
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FIG. 5. Alignment of the amino acid sequences of HIV-1 Env V3 from primary and laboratory isolates. The spaces indicate amino acid deletions; dashes indicate homology. The V3 motif of a neutralization-sensitive HIV-1 strain is enclosed in the shaded rectangle (37).

crease in viral load following challenge, and the levels remained high until the animal was sacrificed. These results demonstrate that vaccination with rBCG Env V3 can induce protective immunity in rhesus macaques against a low-dose challenge with SHIV-MN.

**Challenge with high-dose SHIV-MN.** The second group of eight macaques (R-09 through R-16) was similarly challenged with a higher dose (200 TCID<sub>50</sub>) of SHIV-MN by intravenous inoculation at 24 weeks p.i. (Fig. 6). Measurements of the viral loads in PBMC and plasma indicated that all the macaques were infected by the high-dose SHIV-MN challenge. However, the level of viremia during the acute phase of viral infection

was reduced by 1 to 2 log units in macaques immunized with rBCG Env V3 compared with controls (from 10<sup>6</sup> to 10<sup>7</sup>, to <10<sup>5</sup> to 10<sup>4</sup> RNA copies/ml) (Fig. 6A). The control macaques developed a transient decrease in CD4<sup>+</sup>-T-cell counts that rebounded to normal levels ~3 weeks postchallenge (Fig. 6B). In contrast, macaques vaccinated with rBCG Env V3 had little or no change in CD4<sup>+</sup>-T-cell numbers.

Despite the low levels of V3 peptide-specific IFN-γ ELISPOT activities noted for animals R-09 and R-10 above (Table 2), these animals exhibited a plasma viral load and a rate of CD4<sup>+</sup>-cell loss after SHIV challenge that was comparable to those seen in the immunized animals designated R-11, -12, and -13. Thus, immunization with rBCG Env V3 generated even low levels of T-cell responses in only 2 animals out of 5 in this group and out of a total of 15 immunized animals. No evidence of higher virus-specific IFN-γ ELISPOT activity was demonstrated in samples obtained 0, 4, or 6 and 24 weeks after vaccination (Table 2), suggesting that few significant cellular anti-SHIV responses were generated and that those few did not affect virus control in this macaque population.

**Challenge with pathogenic SHIV-89.6PD.** The third group of macaques (R-17 through R-24) was challenged with pathogenic SHIV-89.6PD (20 TCID<sub>50</sub>) 24 weeks postinoculation. The effects of vaccination with rBCG Env V3 on immune induction against the pathogenic virus were followed for 32 weeks, and the macaques were then autopsied. As shown in

TABLE 2. SHIV-MN-specific serum IgG neutralization titers and Env V3-specific ELISPOT responses<sup>a</sup>

Monkey no.	Immunogen	IC <sub>50</sub> of neutralization serum IgG (μg/ml) <sup>b</sup>			V3-specific IFN-γ SFCs/10 <sup>6</sup> cells <sup>c</sup>		
		0 week	4 or 6 weeks <sup>d</sup>	24 weeks <sup>e</sup>	0 week	4 or 6 weeks	24 weeks
R-01	rBCG Env V3	>50	0.5	0.6	<20	30	20
R-02	rBCG Env V3	>50	0.3	0.4	<20	40	40
R-03	rBCG Env V3	>50	0.5	0.6	<20	40	30
R-04	rBCG-Env V3	>50	0.2	0.3	<20	20	40
R-05	rBCG-Env V3	>50	0.08	0.3	<20	30	80
R-06	rBCG-α	>50	>50	>50	<20	<20	<20
R-07	rBCG-α	>50	>50	>50	<20	<20	<20
R-08	rBCG-α	>50	>50	>50	<20	<20	<20
R-09	rBCG-Env V3	>50	0.04	0.3	<20	180	120
R-10	rBCG-Env V3	>50	0.1	0.2	<20	160	110
R-11	rBCG-Env V3	>50	0.05	0.2	<20	20	30
R-12	rBCG-Env V3	>50	0.03	0.4	<20	60	20
R-13	rBCG-Env V3	>50	0.02	0.4	<20	30	30
R-14	rBCG-α	>50	>50	>50	<20	<20	<20
R-15	rBCG-α	>50	>50	>50	<20	<20	<20
R-16	rBCG-α	>50	>50	>50	<20	<20	<20
R-17	rBCG-Env V3	>50	0.2	0.6	<20	40	90
R-18	rBCG-Env V3	>50	0.3	0.3	<20	50	60
R-19	rBCG-Env V3	>50	0.3	0.4	<20	40	30
R-20	rBCG-Env V3	>50	0.5	0.7	<20	20	50
R-21	rBCG-Env V3	>50	0.4	0.5	<20	20	40
R-22	rBCG-α	>50	>50	>50	<20	<20	<20
R-23	rBCG-α	>50	>50	>50	<20	<20	<20
R-24	rBCG-α	>50	>50	>50	<20	<20	<20

<sup>a</sup> Animals were inoculated with either rBCG Env V3 or the vector control. Blood samples were obtained at 0, 4, or 6 and 24 weeks p.i., and antibody inhibitory concentration and the V3-specific IFN-γ ELISPOT activity were compared.

<sup>b</sup> The IC<sub>50</sub> was derived from the data in Fig. 2 based on neutralization dose-response curves similarly obtained from Fig. 3.

<sup>c</sup> Freshly isolated PBMC were assessed for their ability to produce IFN-γ in response to HIV-1<sub>MN</sub> Env V3 peptide.

<sup>d</sup> Mean IC<sub>50</sub>s: R-01 to R-05, 0.32; R-09 to R-13, 0.05; R-17 to R-21, 0.35.

<sup>e</sup> Mean IC<sub>50</sub>s: R-01 to R-05, 0.44; R-09 to R-13, 0.30; R-17 to R-21, 0.50.

TABLE 3. Comparison of low-dose SHIV-MN infections in macaques vaccinated with either rBCG Env V3 or rBCG- $\alpha$  (control)

Monkey	Immunogen (10 mg)	Efficacy analysis	Results <sup>a</sup>							
			0 <sup>b</sup>	2	4	6	8	10	12	16
R-01	rBCG Env V3	Virus isolation	<1	<1	2	<1	<1	<1	<1	<1
		Provirus by PCR	<500	<500	>500	<500	<500	<500	<500	<500
		Plasma viral load	<500	<500	20,000	<500	<500	<500	<500	<500
R-02	rBCG Env V3	Virus isolation	<1	<1	<1	<1	<1	<1	<1	<1
		Provirus by PCR	<500	<500	<500	<500	<500	<500	<500	<500
		Plasma viral load	<500	<500	<500	<500	<500	<500	<500	<500
R-03	rBCG Env V3	Virus isolation	<1	32	<1	<1	2	<1	ND	ND
		Provirus by PCR	<500	>500	<500	<500	>500	<500	ND	ND
		Plasma viral load	<500	310,000	<500	<500	20,000	<500	ND	<500
R-04	rBCG Env V3	Virus isolation	<1	<1	<1	<1	<1	<1	ND	ND
		Provirus by PCR	<500	<500	<500	<500	<500	<500	ND	ND
		Plasma viral load	<500	<500	<500	<500	<500	<500	<500	<500
R-05	rBCG Env V3	Virus isolation	<1	<1	<1	<1	<1	<1	<1	ND
		Provirus by PCR	<500	<500	<500	<500	<500	<500	<500	ND
		Plasma viral load	<500	<500	<500	<500	<500	<500	<500	<500
R-06	rBCG- $\alpha$	Virus isolation	<1	32	16	<1	2	2	<1	<1
		Provirus by PCR	<500	>500	>500	>500	>500	>500	>500	500
		Plasma viral load	<500	300,000	50,000	20,000	20,000	20,000	20,000	20,000
R-07	rBCG- $\alpha$	Virus isolation	<1	2	32	<1	<1	<1	<1	ND
		Provirus by PCR	<500	>500	>500	>500	>500	<500	<500	ND
		Plasma viral load	<500	27,000	310,000	350,000	25,000	<500	<500	<500
R-08	rBCG- $\alpha$	Virus isolation	<1	32	16	<1	2	2	ND	ND
		Provirus by PCR	<500	>500	>500	>500	>500	>500	ND	ND
		Plasma viral load	<500	300,000	50,000	<500	20,000	20,000	ND	<500

<sup>a</sup> Viral loads were determined by either limiting dilution of PBMC or competitive PCR for HIV-1 Env V3 genes, and the results are expressed as the number of infected cells per million PBMC and virus copies per milliliter of blood. Nested PCR for HIV-MN Env V3 was used in all macaques to detect the provirus genome. Naïve macaques were injected intravenously with 20 TCID<sub>50</sub> of SHIV-MN and used as controls for SHIV infection. The results are expressed as the mean of three different assays; <1, <500, and <500 were the detection limits of virus isolation, proviral copy number, and plasma viral load, respectively. ND, not determined.

<sup>b</sup> Weeks after challenge.

Fig. 7, high levels of plasma viremia were detected in the control macaques, with a viral set point of  $\sim 10^6$  RNA copies/ml, accompanied by an abrupt decline in CD4<sup>+</sup>-T-cell counts. Prior vaccination with rBCG Env V3 appeared to have no positive effect on the viral load and CD4<sup>+</sup>-T-cell counts compared with the control animals.

**Association of in vitro neutralization antibody responses following rBCG Env V3 immunization with control of viremia after SHIV challenge.** Of the macaques challenged with low doses of homologous SHIV-MN (group 1), the three virus-controlled macaques R-02, -04, and -05 (Table 1) had higher IC<sub>50</sub>s of SHIV-MN-specific neutralizing antibodies as measured in M8166 cells at 24 weeks p.i. or on the day of challenge, with serum IgG concentrations of 0.4, 0.3, and 0.3  $\mu$ g/ml, respectively (Table 2). The IC<sub>50</sub>s of the uncontrolled macaques R-01 and -03 (Table 1) were both 0.6  $\mu$ g/ml (Table 2).

When the challenge dose was increased 10-fold (Fig. 1), all five animals in group 2 had high neutralizing antibody titers with a mean IC<sub>50</sub> of 0.30  $\mu$ g/ml on the day of challenge (Table 2). These animals in group 2 showed partial protection against the same homologous virus challenge (Fig. 6). In contrast, no animals similarly immunized with rBCG elicited any in vivo protection against a low-dose, heterologous viral challenge with SHIV-89.6PD (Table 2 and Fig. 7).

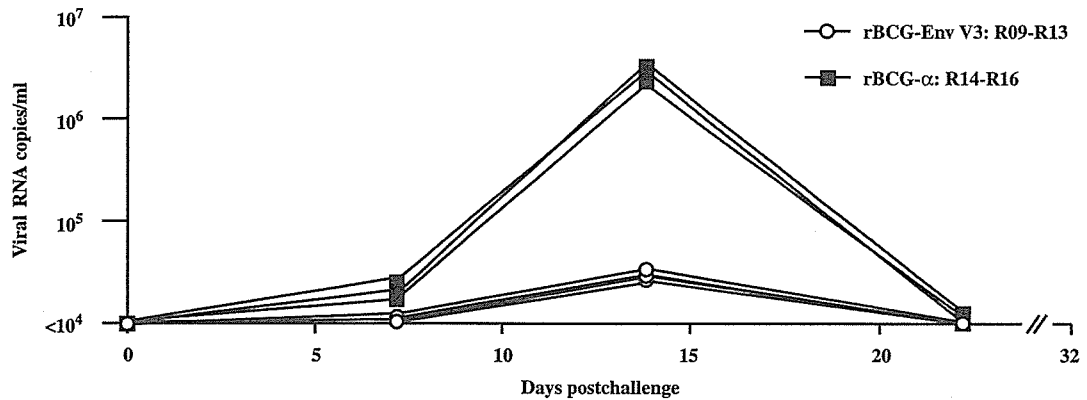
In summary, the rBCG Env V3-elicited NAb response afforded some degree of protection against a homologous viral challenge. However, infection by the heterologous virus SHIV-89.6PD was not controlled by heterologous virus SHIV-MN- or HIV-1<sub>MN</sub>-specific NAb generated by the recombinant HIV-1<sub>MN</sub> Env V3-expressed BCG immunization.

## DISCUSSION

First, our study demonstrates the potential of anti-Env V3 NAb induced by immunization of rhesus macaques with rBCG Env V3 to afford protection against homologous challenge with SHIV-MN but not against the heterologous SHIV-89.6PD. With the low-dose homologous SHIV-MN challenge (20 TCID<sub>50</sub>), sterile protection was achieved in three of five immunized animals. These findings correlate well with our in vitro neutralization data for these animals. Protected animals showed higher levels of potent neutralization antibodies than did unprotected animals. Macaques serving as vector and naïve controls experienced high levels of replication of the SHIV-MN challenge virus. With a high-dose challenge, rBCG Env V3 vaccination was effective at reducing viremia during acute infection by  $\sim 100$ -fold. The vaccine consisted of an rBCG vector that expresses a chimeric HIV-1 Env V3 region peptide and the  $\alpha$ -antigen of *M. bovis*. The kinetics and magnitude of the HIV-1 Env V3-specific antibody responses elicited in macaques were comparable to those observed in our previous studies using guinea pigs vaccinated with rBCG Env V3 (9, 16).

Secondly, the levels of neutralizing antibodies generated after injection with a recombinant BCG vector-based vaccine expressing a chimeric protein of HIV-1 Env V3 peptide and  $\alpha$ -antigen protein were maintained for at least 24 weeks p.i. with no diminishment in titer. A plausible explanation for the longevity of the neutralizing antibody titers after rBCG immunization is that the carrier protein,  $\alpha$ -antigen (also known as MPT59 or antigen 85B), is derived from mycobacteria and has

A



B

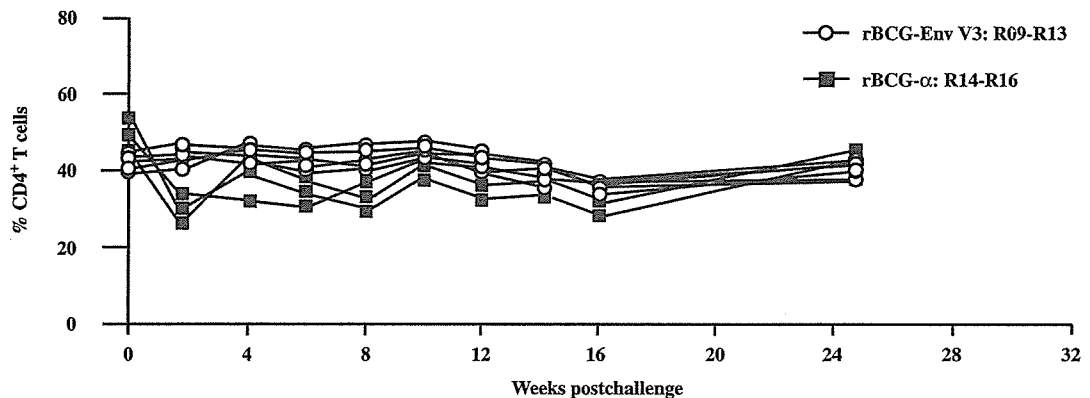


FIG. 6. Comparison of infection kinetics following high-dose (200 TCID<sub>50</sub>) inoculation of SHIV-MN in macaques vaccinated with either rBCG Env V3 or rBCG vector control. (A) Viral RNA copy number per milliliter of serum. (B) CD4<sup>+</sup>-T-cell count as a percentage of total lymphocytes. The results in individual animals are expressed.

the ability to elicit potent Th1-type immune responses (24, 43). Our result is consistent with those of other groups, which have shown that BCG immunity is maintained for at least a few years and that the BCG bacillus is effective at increasing NAb responses (40). These characteristics might help to explain the long-lasting enhanced levels of NAb elicited by vaccination with rBCG Env V3.

The concentration of purified macaque IgG in serum was determined to be  $\sim 10$  mg/ml: By this estimation, 0.5 mg corresponds to a serum dilution of 1:1 in virus neutralization assays. The IC<sub>50</sub> and IC<sub>90</sub> values for neutralization of SHIV-MN were 10<sup>3</sup> to 10<sup>4</sup> and 166, respectively (similar values were obtained for neutralization of HIV-1<sub>MN</sub>). These neutralization titers suggest that antibody responses generated de novo may contribute to a degree of protection against SHIV-MN. The observed relationship of the NAb titer and viral protection is consistent with results obtained by repeated immunization with SHIV-89.6 C4-V3 peptides in guinea pigs and rhesus macaques (6, 27). In this case, NAb titers to homologous SHIV-89.6 were  $\sim 10^3$  greater than those against heterologous HIV-1<sub>MN</sub>, while responses to HIV-1 R5 viruses were weak or absent. This suggests that the protection mediated by a C4-V3 peptide vaccine against SHIV-89.6 may be type (or strain) specific. Thus, we assume that the NAb generated by

SHIV-89.6 C4-V3 peptide immunization (6) would not mediate protection against a heterologous SHIV-MN challenge.

The present study suggests that the vaccine-elicited antibodies directed against the HIV-1 Env V3 peptide can in some cases confer a degree of neutralization against primary isolates of HIV-1 (26). Following vaccination of rhesus macaques with rBCG Env V3, both binding and NAb responses against this novel construct were clearly evident. At the time of SHIV challenge, immune sera from the vaccinated macaques efficiently neutralized a homologous, type-specific TCLA HIV-1 strain (HIV-1<sub>MN</sub>) and a related SHIV strain (SHIV-MN) with IC<sub>90</sub> values of  $< 5$   $\mu$ g/ml. Controls, including preimmune sera and sera from macaques vaccinated with rBCG vector alone, had no neutralizing activity in assays using GHOST cells expressing either CCR5 or CXCR4 or in M8166 cells. Immune sera from macaques vaccinated with rBCG Env V3 were able to neutralize several primary HIV-1 X4 isolates (HIV-1<sub>BZ167</sub>, HIV-1<sub>SF2</sub>, and HIV-1<sub>CI2</sub>); however, neutralization of an X4-R5 dual-tropic strain (HIV-1<sub>MNP</sub>) was weak. No neutralization of HIV-1 R5 isolates and primary HIV-1 isolates from different clades was observed. These findings were confirmed in an independent international neutralization trial (conducted by Simon Beddows and Jonathan Weber, Imperial College School of Medicine, Medical Research Council, London, En-

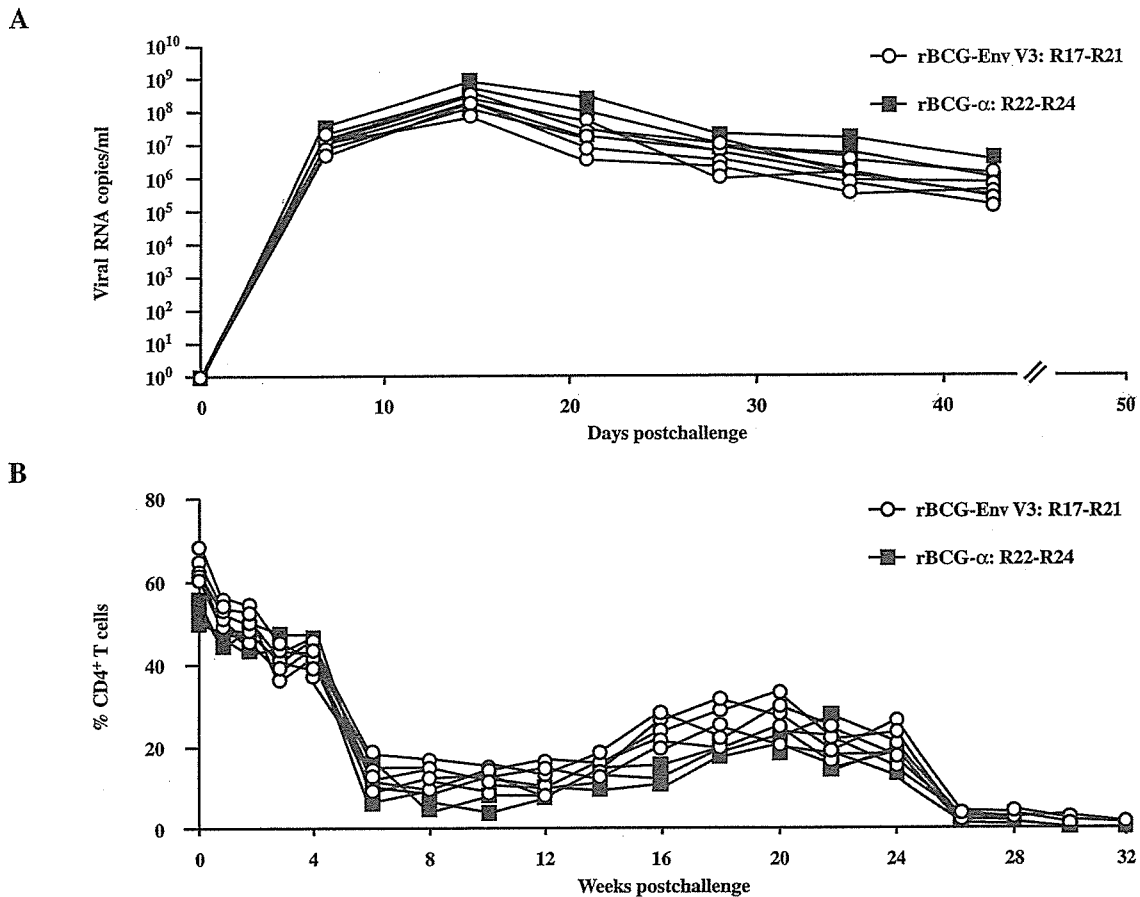


FIG. 7. Comparison of infection kinetics following challenge with pathogenic SHIV-89.6PD in macaques vaccinated with either rBCG Env V3 or rBCG vector control. (A) Plasma viral-RNA copy numbers per milliliter. (B) CD4<sup>+</sup>-T-cell count as a percentage of total lymphocytes. The results in individual animals are expressed.

gland, and Pia Scott and Eva-Maria Fenyo at Microbiology and Tumorbiology Center, Karolinska Institute, Stockholm, Sweden). Preliminary results from this study have had been summarized and reported (11). Despite similarities in the V3 sequence motif, neutralization of the TCLA strain HIV-1<sub>MN</sub> was found to be 10- to 50-fold more sensitive than neutralization of primary HIV-1 isolates, such as HIV-1<sub>CT2</sub>, HIV-1<sub>MNP</sub>, or HIV-1<sub>JR-CSF</sub> (11). A reasonable explanation for the relative insensitivity of primary HIV-1 isolates—particularly primary HIV-1 R5 isolates—to neutralization is the presence of cryptic or occluded sites within the virus-associated V3 region (13, 53).

In the Japanese consensus HIV-1 Env V3 expressed in the rBCG construct, the core V3 motif of the neutralization epitope is IHIGPGRAF (39). Although the consensus sequence of the V3 loop differs from the MN-V3 sequence in five amino acid positions, the neutralization epitope of the tip V3 region in the Japanese consensus is identical to that of MN-V3. Some substitutions of amino acids at certain positions within this motif (for example, H to R and A to T in the core motif in BZ167) are tolerated, suggesting that NAbs generated by immunization with rBCG Env V3 are not strictly type specific. Immune sera from macaques vaccinated with rBCG Env V3 were able to neutralize primary HIV-1 X4 and some HIV-1 X4-R5 dual-tropic isolates, suggesting that the antigenic struc-

ture of the chimeric V3 peptide mimics to some extent that of the virus-associated V3 region. Indeed, the chimeric V3- $\alpha$ -antigen protein is estimated to be 38 kDa and contains four cysteine residues, suggesting the possible formation of a new loop structure in the V3 portion of the protein. With regard to the heterologous SHIV-89.6PD challenge in macaques vaccinated with rBCG Env V3, NAbs specific for SHIV-89.6PD were not generated efficiently ( $IC_{50}$ , >50  $\mu$ g of immune serum IgG/ml) and did not provide any protection against the SHIV-89.6PD challenge. The V3 neutralization site of SHIV-89.6PD may differ in sequence or structure or both from that of SHIV-MN or other viral strains, including some of the HIV-1 isolates, making it unrecognizable to antibodies. Such a difference could account for the poor cross-neutralization activity against SHIV-89.6PD.

Thus, our data from the SHIV-macaque models show that the *in vitro* neutralization titers generated in rBCG-immunized animals correlate with protection. Although a present goal of HIV-1 vaccine development is to reduce the viral set point by eliciting high levels of virus-specific cellular immune responses, induction of cross-reactive NAbs may also contribute to control virus replication in the course of HIV-1 infection and may therefore be useful in the context of a preventive vaccine. Furthermore, although the choice of HIV Env V3 and the

autologous challenge virus SHIV-MN are unlikely to provide information that predicts efficacy in humans, the results presented here demonstrate that recombinant BCG vectors have the potential to deliver a more appropriate immunogen for desirable immune elicitation.

#### ACKNOWLEDGMENTS

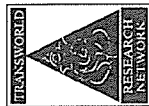
We thank L. Yichen, Harvard AIDS Institute, Harvard University, and A. Schultz, NIAID, National Institutes of Health, for providing the SHIV strains and for their helpful discussions. We also thank J. Esparza and S. Osmanov, UNAIDS, Geneva, Switzerland; S. Beddows and J. Weber, Medical Research Council, London, United Kingdom; and Eva-Maria Fenyo, Microbiology and Tumorigenesis Center Karolinska, Stockholm, Sweden, for their helpful discussions.

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

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### Abstract

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

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*enzymes mutate reverse-transcribed viral DNA, and the Fv-1 gene product interferes with the proviral integration. Growth induction of infected erythroid progenitor cells is affected by the Fv-2 mutation of a hematopoietic cell growth factor receptor, STK. Several genes located within the major histocompatibility complex (MHC) and a separate gene not linked to MHC control the host immune responses. The latter, Rfv-3, controls the production of virus-neutralizing antibodies, and thus affects the duration of viremia. Both MHC class I-restricted CD8<sup>+</sup> and class II-restricted CD4<sup>+</sup> 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<sup>+</sup> T cells are effective in inducing protective immunity against FV, and effector mechanisms including NK cells, CD4<sup>+</sup> and CD8<sup>+</sup> 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 ecotropic 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<sup>++</sup> or Mn<sup>++</sup> 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)<sub>F1</sub> mice are resistant to both N-tropic and B-tropic viruses. Some tissue culture-adapted viruses can infect both N-type and B-type cells with comparable efficiencies, and are called NB-tropic. The exact molecular mechanisms of the action of *Fv-1* gene product are still unknown; interestingly, however, the same mechanism seems to work in human cells restricting the integration of mouse retroviruses [26].

### Molecular mechanisms of Friend virus-induced disease development

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

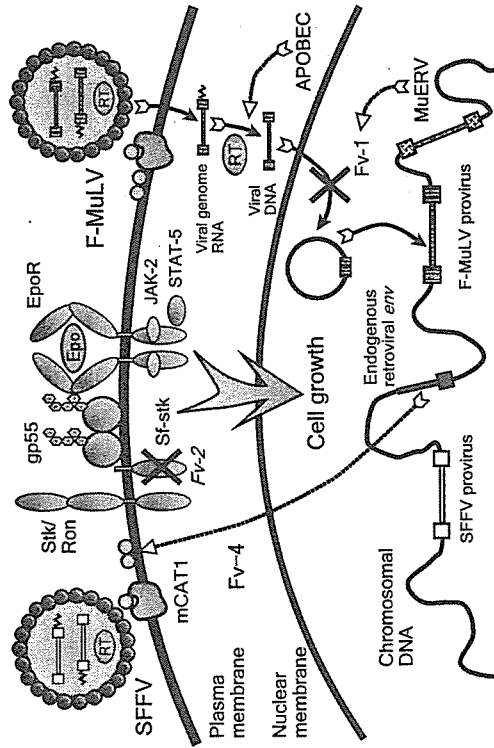


Figure 1. Schematic summary of the mechanisms of FV-induced erythroid cell proliferation and cellular factors that interfere with FV infection.

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

It has been widely accepted that the SFFV-induced erythroid cell proliferation is crucial for the development of FV-induced erythroleukemia. That scheme of FV-induced leukemogenesis is as follows: upon infection of susceptible strains of mice with FV, SFFV gp55 induces rapid proliferation of erythroid progenitor cells. This leads to the development of splenomegaly and polycythemia readily detectable at as early as two weeks after FV inoculation. However, since the stimulation of erythroid progenitor cells through EpoR induces not only their growth but terminal differentiation, the process of erythroid cell expansion is self-limited, and progenies of SFFV-infected erythroid cells ultimately stop dividing and mature into red blood cells. Thus, even continuous infection of erythroid progenitor cells cannot result in the development of leukemia. For the development of malignant transformation, changes in the expression of host genes due to proviral integration into specific chromosomal locations are involved. Rapid expansion of erythroid progenitor cells is believed to provide target cells for repeated integration of SFFV and F-MuLV proviruses, and resultantly increases the chances for the proviruses to "hit" cellular genes by the promoter insertion adjacent to an oncogene or by disruption and silencing of a tumor suppressor gene [30, 31]. In fact, activation of several oncogenes and disruption of a few tumor suppressor genes have been documented in FV-induced leukemia cells. However, the proposed relationships between SFFV-induced early expansion of erythroid progenitor cells and malignant transformation due to proviral integration still remain circumstantial: for instance, it has not been proven if the SFFV-induced proliferation of erythroid cells is a prerequisite to allow repeated proviral integration resulting in a higher frequency of disease-inducing "hits." In this regard, it has been shown that even *Fv-2<sup>+/+</sup>* 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 immunoregulatory genes. (B10 × A.BY)F<sub>1</sub> and (B10.D2 × A.BY)F<sub>1</sub> mice share the same (B10 × A)F<sub>1</sub> background, and thus share the *Fv-1<sup>bb</sup>*, *Fv-2<sup>ss</sup>*, and *Fv-4<sup>ss</sup>* genotypes regarding FV infectivity. Nevertheless, (B10 × A.BY)F<sub>1</sub> mice are highly resistant to FV infection, and only <10% die within 100 days

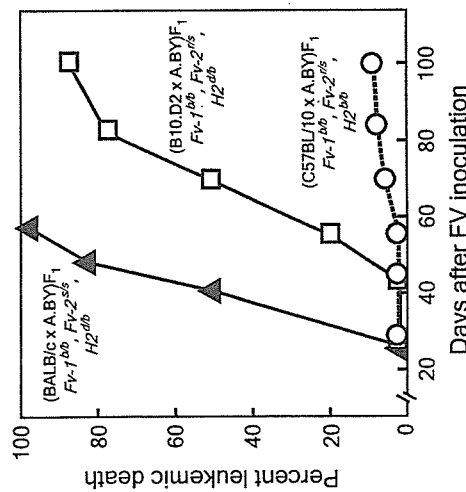


Figure 2. Comparison of tempos of leukemic death after FV infection in three different strains of mice.

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<sub>1</sub> possessing the homozygous *H2<sup>b/b</sup>* haplotype, and (B10.D2 × A.BY)F<sub>1</sub> being *H2<sup>d/b</sup>*. 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<sup>+</sup> and class II-restricted CD4<sup>+</sup> 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
<i>Rfv-3</i>	15	<i>Rfv-3<sup>r</sup></i> (C57BL)	<i>Rfv-3<sup>s</sup></i> (A/WySn)	Recovery from viremia, Kinetics of neutralizing Ab production
<i>Rfv-1</i>	17, <i>H2D</i>	<i>D<sup>b</sup></i>	<i>D<sup>d</sup></i> , <i>D<sup>k</sup></i> , <i>D<sup>q</sup></i> , <i>D<sup>am1.4</sup></i>	Cytokine production from CD8 <sup>+</sup> and CD4 <sup>+</sup> T cells
<i>Rfv-2</i>	17, <i>Q/TL</i>	<i>Qa-1<sup>a</sup></i>	<i>Qa-1<sup>b</sup></i>	NK killing?
<i>H2A</i>	17	<i>A<sup>b</sup></i>	<i>A<sup>c</sup></i> , <i>A<sup>k</sup></i> , <i>A<sup>bm12</sup></i>	T helper cell responses to viral antigens
<i>H2E</i>	17	<i>E<sup>b</sup></i> (hybrid)	<i>E<sup>k</sup></i> , <i>E<sup>d</sup></i>	T helper cell responses to viral antigens

development. The requirement of both CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets for the spontaneous immune resistance of *H2<sup>b/b</sup>* mice was further substantiated by the results of antibody (Ab)-mediated T cell depletions [37]: the otherwise resistant mice depleted of CD8<sup>+</sup> T cells developed rapid splenomegaly, while the same strain of mice depleted of CD4<sup>+</sup> 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<sup>+</sup> T cells nevertheless showed spontaneous recovery from the initial development of splenomegaly. These results suggest that functions of CD8<sup>+</sup> T cells are required in the earlier, and those associated with CD4<sup>+</sup> T cells in the later stages of the infection, together exerting immune resistance against FV infection.

Epitopes recognized by CD4<sup>+</sup> and CD8<sup>+</sup> 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<sup>bd</sup>-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<sup>ab</sup>* or *H2<sup>db</sup>* 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<sup>+</sup> T cell-priming vaccines in facilitating virus-reactive Ab production. Effector cells including CD8<sup>+</sup> and CD4<sup>+</sup> cytotoxic T lymphocytes (CTL) and natural

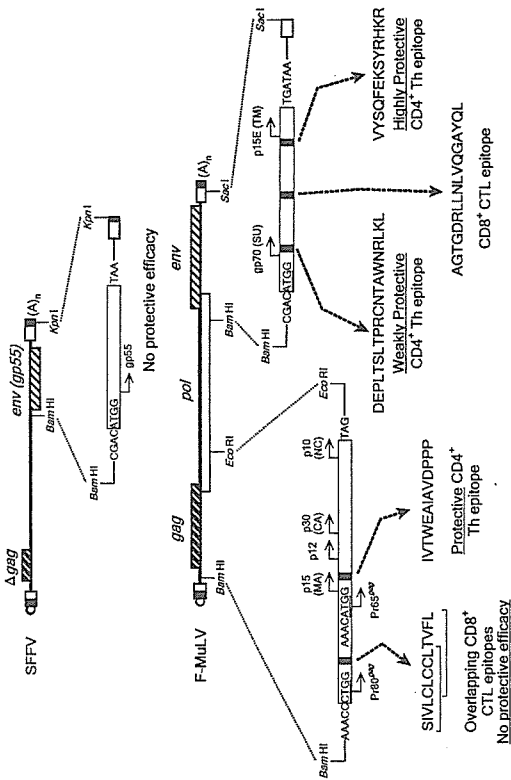


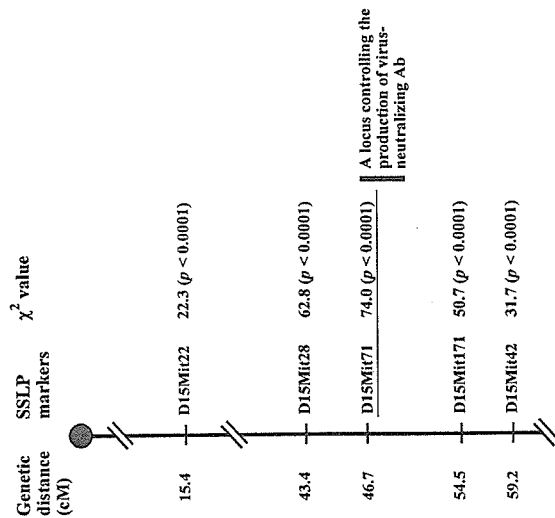
Figure 3. Amino acid sequence and location of the epitopes recognized by T cells in FV gene products.

killer (NK) cells are activated in mice immunized with the *env*-encode peptide within a few days after FV inoculation [48]. The CD4<sup>+</sup> CTL was only detectable in the peptide-immunized mice, but CD8<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cell epitope induces protection against FV infection. Further, immunization of mice with a recombinant vaccinia virus that expresses the SFFV *env* gene did not induce protective immunity [49], suggesting the lack of immunoprotective epitopes within the SFFV *env* gene products.

Another MHC-associated host gene that influences the development of FV-induced splenomegaly has been mapped in the class Ib Q/TL loci [35]. The exact molecular nature of this resistance gene, *Rfv-2*, has not been identified, but given the involvement of a Q-locus gene product, Qa-1<sup>b</sup>, 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<sub>1</sub> and (BALB/c × A.BY)F<sub>1</sub> strains of mice share the same *H2<sup>d/b</sup>* haplotype, they differ completely in their tempo of leukemia development after FV infection. In fact, (BALB/c × A.BY)F<sub>1</sub> mice die within 2 months after FV infection, while nearly half of (B10.D2 × A.BY)F<sub>1</sub> 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<sub>1</sub> 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-3<sup>s</sup>*. Thus, B10 mice possess a dominant allele, *Rfv-3<sup>r</sup>*, 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.

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(Eds.)*

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

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

## **Pathogenicity of autoantibodies reactive with the endogenous retroviral envelope glycoprotein gp70**

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### **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.



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*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 pathogenic 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<sub>1</sub> hybrid mice were bred in our animal facilities. NZW/NSic 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<sub>1</sub> and SCID mice were transplanted intraperitoneally (i. p.) with 1-2 x 10<sup>7</sup> 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)<sup>+</sup> 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'-GGGGAGGGCACTGACCACCCGGAGGA-3' for Cy2a,  
5'-TTGGGGGAAGATGAAGACGGATGG-3' for Cy3, and  
5'-TTGGTCAACGTTGAGGGTGTCTCATGC-3' for Cx.

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 x MRL)F<sub>1</sub> 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<sup>+</sup> reagent (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturers' instructions.

## **Results**

Development 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 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)F<sub>1</sub> 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. Severe 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).

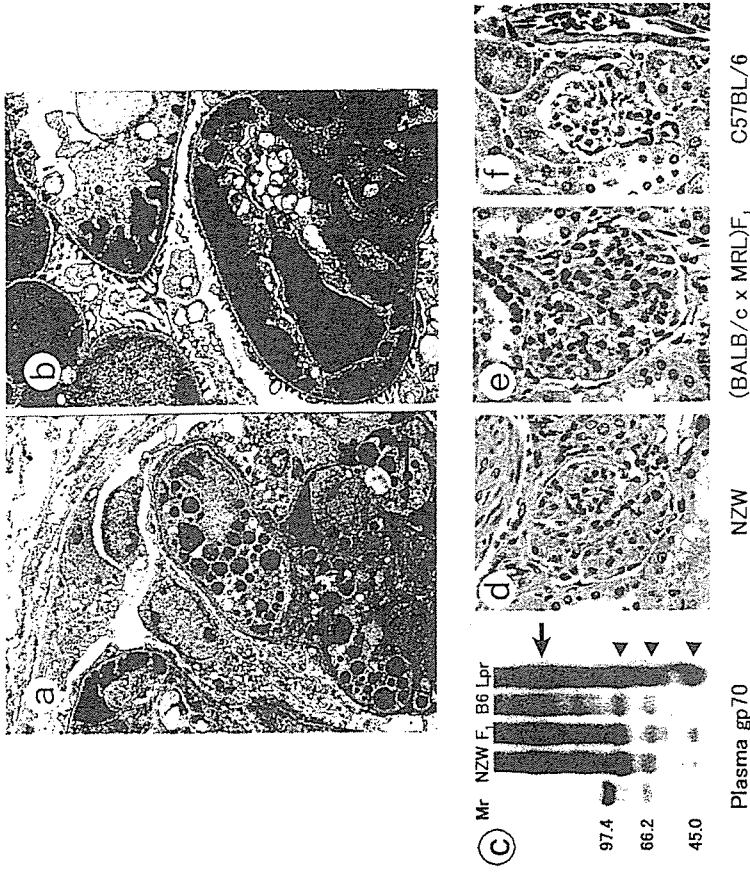


Fig. 1: Pathogenicity of monoclonal anti-gp70 autoantibodies established from MRL/lpr lupus mice shown by passive transfer into non-autoimmune mice. a, An electron micrograph showing cellular proliferation filling the glomerular capillaries and accumulation of electron-dense droplets in the cytoplasm of glomerular cells in (BALB/c x MRL)F1 mice transplanted with anti-gp70 antibody-producing hybridoma cell clone, 12H5.1. Bar = 5  $\mu$ m. 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  $\mu$ m. 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  $\mu$ l 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<sup>-3</sup>. 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 known 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 neutrophils 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 <sub>H</sub>	J <sub>H</sub>	V <sub>K</sub>	J <sub>K</sub>
12H5.1	IgG <sub>3</sub> , κ	558	4	21	2
36D1.1	IgG <sub>2a</sub> , κ	558	3	19-20	2
37C4.1	IgG <sub>3</sub> , κ	558	3	8-21	4
37C6.1	IgG <sub>2a</sub> , κ	558	3	10	2
51D1.1	IgG <sub>3</sub> , κ	7183	4	21B	1

Clone	V <sub>H</sub>		CDR3*
	CDR1	CDR2	
12H5.1	RVMMH	AIYPGNSDTSYNQKFKGK	EGLSIYDGLYFAMDY
36D1.1	DYSMD	YIYPNNGYTGYNQKFKSK	KLGRREAYFDV
37C4.1	DYMD	YIYPNNDGTNNQKFKGK	GGLAGYLYYYAMDY
37C6.1	DYSMD	YIYPNNGYTGYNQKFKSK	KLGRREAYFDV
51D1.1	DYMA	NINYDGSSTYYLDSLKSR	TPTGYAMDY

Clone	V <sub>L</sub>		
	CDR1	CDR2	CDR3
12H5.1	RASKSVSTSSYSYMH	YASYLES	QHSREFFPYT
36D1.1	KASENVVTVYS	GASNRYT	GQGYSPYPT
37C4.1	KSTQSLFNSRTRKNYLA	WASTRES	TQSYLHL
37C6.1	RASQDISNYLN	YTSRLHS	QQYSKLPYT
51D1.1	RASKSVDRYGNFSFMH	RTSNLES	QQNEDPWT

\*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 [1,12], 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].

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