Cell-Free Transmission of Fv-4 Resistance Gene Product Controlling Friend Leukemia Virus-Induced Leukemogenesis: A Unique Mechanism for Interference With Viral Infection

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Fv-4 is a mouse gene that dominantly confers resistance to infection by ecotropic murine leukemia virus (MuLV). We previously demonstrated that mixed radiation bone marrow chimeras containing Fv-4'-bearing BALB/c-Fv-4W' (C4W) bone marrow and Fv-4'-bearing C3H/He (C3H) bone marrow grafted into C3H recipient mice (C4W + C3H→ C3H) were resistant to Friend leukemia virus (FLV)-induced leukemogenesis, even when they contained as high as 70% C3H-derived cells. This indicates that FLV-sensitive C3H-derived cells are rendered refractory to infection and/or transformation with FLV when they coexist in mice with Fv-4'-bearing cells. To investigate the mechanism of Fv-4 resistance to FLV-induced leukemogenesis, we first examined the expression of Fv-4' env antigen in the peripheral blood mononuclear cells (PBMC) of these chimeras. The Fv-4' env antigen was present not only on C4W-derived cells, but also on Fv-4'-bearing C3H-derived cells in C4W + C3H→ C3H mixed bone marrow chimeras. The Fv-4' env antigen that binds to the cell surface of C3H cells was found in sera from normal C4W mice, C4W→ C3H chimeras, and C4W + C3H→ C3H mixed chimeras. The serum Fv-4' env antigen binds to ecotropic MuLV receptors, shown by specific binding to transfected mink cells expressing ecotropic MuLV receptor, but not to parental mink cells. To determine whether the binding of Fv-4' env antigen to the putative MuLV receptors would block FLV infection, C3H thymocytes or spleen cells that had been preincubated with C4W serum were mixed with FLV and the subsequent production of MuLV-specific antigens was examined. C3H thymocytes or spleen cells treated with C4W serum became refractory to binding by FLV. These results provide evidence that the Fv-4' env antigen is released from C4W-derived cells in vivo and binds to cells expressing surface receptors for ecotropic MuLV, thereby protecting them from infection with FLV. The implication of these findings for gene therapy of retrovirus-induced disease such as acquired immune deficiency syndrome (AIDS) is discussed.

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FRIEND LEUKEMIA virus (FLV)-induced leukemogenesis is controlled by many host genes including Fv-1', Fv-2', Fv-3', Fv-4', Fv-5', RfV-1', RfV-2', RfV-3', some of which are closely linked to the mouse major histocompatibility complex (MHC), H-2. Among them, the resistance allele at the Fv-4 locus (Fv-4'), located on mouse chromosome 12, is dominant over the susceptibility allele (Fv-4') and confers strong resistance to infection with ecotropic retroviruses, as well as to retrovirus-induced tumors.3,4 A unique cell surface protein immunologically related to ecotropic murine leukemia virus (MuLV) envelope glycoprotein gp70, has been identified in normal Fv-4' mice.5,6 Further, the Fv-4'-associated, MuLV-related sequence has been cloned and its structure has been shown to be identical to that of the 3' portion of a provirus genome extending from the end of the pol gene through a complete env gene to a 3' long terminal repeat.7,8 Mouse cells transduced with this putative Fv-4' gene expressed a circa 3-kb Fv-4 env RNA, which is comparable to the size of a message expressed in mice carrying the Fv-4' trait. NIH3T3 cell lines transfected with the cloned Fv-4 env gene expressed a cell surface Fv-4'-specific MuLV envelope protein and were variably resistant to superinfection with ecotropic MuLVs.9 More recently, transgenic mouse strains carrying the Fv-4 env gene, which was placed under control of its presumed natural promoter, were shown to be completely or partially resistant to infection with Friend MuLV. The resistance correlated with the level of expression of transgenic RNA and its translated protein.10 A viral interference model has been proposed for the mechanism of Fv-4'-related restriction of MuLV infection.5,22 Viral interference refers to a phenomenon in which cells chronically infected with one virus are resistant to superinfection with other viruses bearing the same virus envelope specificity.11 Interference is probably due to blockage of virus receptors by the envelope protein of the initially infecting virus.9 Thus, the interference model suggests that the Fv-4' env antigen competes with ecotropic MuLV for cell surface receptors. However, the exact mechanism for the virus interference controlled by Fv-4' is still undetermined.

We have been developing a model to test the potential of bone marrow transplantation to cure retrovirus-induced disease. Bone marrow cells from BALB/c-Fv-4W' (C4W) mice were mixed with FLV-susceptible C3H/He (C3H, Fv-4')-derived bone marrow cells in various proportions and were transplanted into supralethally irradiated C3H hosts (C4W + C3H→ C3H). We found that C4W + C3H→ C3H mixed chimeras that contained up to 70% C3H-derived cells, were refractory to FLV-induced leukemogenesis.12 Here we show that in the mixed chimeras the Fv-4' env antigen was released from C4W-derived cells into the serum and bound to C3H-derived cells, presumably via ecotropic MuLV receptors. When the amount of Fv-4' env antigen was sufficient to saturate virus receptors, the mixed bone marrow chimeras were protected from infection with exogenous FLV.

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**MATERIALS AND METHODS**

**Mice.** Specific-pathogen-free (SPF) C3H/HeNcsNs (C3H, Fv-4', H-2) male mice were bred from our colonies at the Animal Production Facility of this institute and were maintained within the clean conventional animal facility in which all animals are maintained SPF by our criteria. BALB/c-Fv-4'W (C4W, Fv-4', H-2') mice, which are Fv-4' congenic with BALB/c mice (Fv-4'), were originally provided by Dr T. Odaka (Professor Emeritus, Tokyo University, Tokyo, Japan) and were maintained in our laboratory.

Preparation of mixed radiation bone marrow chimeras and virus infection. Ten- to 12-week-old C3H mice were irradiated with 12.5 Gy of $^{137}$Cs gamma-rays from GAMMA-CELL-40 (Atomic Energy of Canada, Ltd, Kanata, Canada) at a dose rate of 0.60 Gy/min. Mixed radiation bone marrow chimeras (C4W + C3H → C3H) were constructed by grafting a mixture of bone marrow cells from C4W and C3H donors as described earlier. Four groups of mixed radiation bone marrow chimeras were produced: C3H mice that were supralethally irradiated and reconstructed with a mixture of (1) $1 \times 10^7$ bone marrow cells from C4W and $2 \times 10^6$ cells from C3H mice [C4W(10) + C3H(2) → C3H], (2) $5 \times 10^6$ each of bone marrow cells from C4W and C3H donors [C4W(5) + C3H(5) → C3H], (3) $2 \times 10^6$ bone marrow cells from C4W mice and $1 \times 10^6$ cells from C3H [C4W(2) + C3H(1) → C3H], and (4) $1 \times 10^6$ bone marrow cells from C4W donors [C4W(1) → C3H]. In addition, similarly irradiated C3H mice were transplanted with $1 \times 10^6$ bone marrow cells from C3H donors [C4W(1) → C3H] or with $2 \times 10^7$ bone marrow cells from C3H mice (C3H → C3H). The resulting radiation bone marrow chimeras contained various proportions of C4W- and C3H-derived cells. Three to 4 months after their construction, chimerism was determined by assessing the relative proportion of cells expressing H-2K' or H-2D' antigens on the surface of peripheral blood mononuclear cells (PBMC) using flow cytometry as described previously. They were then inoculated intraperitoneally (IP) with NB-tropic FLV solution. For personal use only.on October 3, 2017. by guest. From www.bloodjournal.org by guest on October 3, 2017. For personal use only.
Presence of Fv-4' env antigen on C3H-derived cells of mixed radiation bone marrow chimeras. To obtain information concerning Fv-4' expression on the surface of lymphohemopoietic cells in mixed radiation bone marrow chimeras, chimeras with different ratios of C3H and C4W cells were constructed and the cell surface expression of H-2 antigen and Fv-4' env antigen on PBMC were analyzed. H-2 typing by FACS analysis 3 months after bone marrow transplantation indicated that PBMC of C4W(10) + C3H(2) → C3H chimeras contained 44% to 74% C4W-derived (H-2'-positive) cells, while the PBMC of C4W(5) + C3H(5) → C3H chimeras contained 30% to 43% and the PBMC of C4W(2) + C3H(10) → C3H chimeras contained 5% to 9% C4W-derived cells. The PBMC from C3H → C3H mice and C4W → C3H chimeras contained more than 95% H-2'-positive and H-2'-positive cells, respectively.

Two-color flow cytometric analysis showed, as expected, that PBMC of C3H → C3H mice were H-2'-positive, but negative for Fv-4' env antigen (Fig 2A). Also, as expected, C4W-derived (H-2'-positive) cells of mixed chimeras were positive for Fv-4' env antigen. Unexpectedly, the majority of C3H-derived (H-2'-positive) PBMC of C4W(10) + C3H(2) → C3H mixed chimeras were also positive for Fv-4' env antigen (Fig 2B). The C3H-derived (H-2'-positive) PBMC of C4W(10) + C3H(2) → C3H and C4W(2) + C3H(10) → C3H mixed chimeras had lower levels of Fv-4' env antigen than C4W → C3H chimeric mice (positive control) (Fig 3A). The H-2'-positive PBMC from C4W(2) + C3H(10) → C3H chimeras appeared to have a lesser amount of Fv-4' env antigen than those from C4W(10) + C3H(2) → C3H chimeras. The amount of Fv-4' env antigen on H-2'-positive PBMC of C4W(5) + C3H(5) → C3H mixed chimeras was intermediate between that of C4W(10) + C3H(2) → C3H and C4W(2) + C3H(10) → C3H mixed chimeras (data not shown). Thus, the intensity of the expression of Fv-4' env antigen correlated with the proportion of C4W-derived cells in these mixed chimeras. In contrast, the intensity of Fv-4' env antigen on C4W-derived (H-2'-positive) cells was not affected by the proportion of coexisting C3H-derived cells.

About 4 months after bone marrow transplantation, all mice were inoculated with FLV. After inoculation, the proportion of Fv-4'-negative cells among H-2'-positive cells increased in the peripheral blood of C4W(10) + C3H(2) → C3H and C4W(2) + C3H(10) → C3H chimeras (Fig 3B).

Chronological changes in the mean intensity of Fv-4' env antigen on C3H-derived PBMC of mixed chimeras after bone marrow transplantation and after inoculation with FLV are shown in Fig 4. The amount of Fv-4' env antigen was high 1 month after bone marrow transplantation and decreased slightly 2 to 3 months after bone marrow transplantation.
tion. After inoculation with FLV, mean expression of the Fv-4' env antigen on the H-2 Ku-positive PBMC decreased further, especially in the C4W(10) + C3H(2) → C3H mixed chimeras, largely due to an increase in the number of Fv-4'-negative cells. The overall mean intensity of Fv-4' expression by H-2 Ku-positive population of PBMC from C4W(10) + C3H(2) → C3H mixed chimeras, (B) that of C4W(5) + C3H(5) → C3H chimeras, and (C) that of C4W(2) + C3H(10) → C3H chimeras. Each group consisted of four or five mice and bars indicate standard deviation of each group.

Detection of Fv-4' env antigen in sera from Fv-4' C3H mice and mixed chimeras. The strong resistance to FLV-induced leukemogenesis together with the presence of Fv-4' env antigen on the H-2 Ku-positive PBMC suggested to us that Fv-4' env antigen might have been transferred from C4W-derived cells to protect C3H-derived cells from infection with FLV. To investigate this possibility, we examined serum from mice containing C4W-derived cells for presence of Fv-4' env antigen and ability of this antigen to bind to the surface of C3H-derived cells in vitro. Thymus or spleen cells from C3H mice were incubated with serum taken from C4W or C4W → C3H mice 1 month after bone marrow transplantation and examined for cell surface Fv-4' env antigen by membrane immunofluorescence. While the incubation of C3H-derived cells with serum from C3H mice did not result in detectable Fv-4' env antigen, C3H thymus cells incubated with serum from C4W(5) + C3H(5) → C3H, C4W → C3H, or C4W mice became positive for cell surface Fv-4' env antigen (Table 1). The same type of experiments using C3H spleen cells showed similar results. These data demonstrate that C4W-derived serum contains a detectable amount of Fv-4' env antigen that can bind to the surface of C3H-derived cells.

Table 1. Relative Intensity of Cell Surface Fv-4' env Antigens on C3H Cells After Incubation With Various Mouse Sera

<table>
<thead>
<tr>
<th>Mouse Sera*</th>
<th>Thymus</th>
<th>Spleen</th>
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<tbody>
<tr>
<td>C3H</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>C4W(5) + C3H(5) → C3H</td>
<td>14.3</td>
<td>92.9</td>
</tr>
<tr>
<td>C4W → C3H</td>
<td>22.6</td>
<td>66.1</td>
</tr>
<tr>
<td>C4W</td>
<td>11.6</td>
<td>37.6</td>
</tr>
</tbody>
</table>

* Thymocytes and spleen cells of Fv-4' C3H mice were incubated with mouse sera from C3H, C4W(5) + C3H(5) → C3H, C4W → C3H, and C4W mice. The cell surface Fv-4' env antigen was measured by flow cytometry using FACSscan. Relative intensity of Fv-4' env antigen fluorescence was calculated by the formula:

\[
\text{Relative Intensity} = \frac{(\text{Sample Intensity}) - (\text{Negative Control})}{(\text{Positive Control}) - (\text{Negative Control})} \times 100
\]

Sample intensity, mean fluorescence intensity of cell surface Fv-4' env antigen by Fv-4' C3H cells after incubation with mouse sera; positive control, mean intensity of C4W thymic (111.4) or spleen cells (40.6); negative control, mean intensity of C3H thymic (1.95), or spleen cells (7.04) before incubation with mouse sera.

Binding of serum Fv-4' env antigen to ecotropic MuLV receptor. To further analyze the binding specificity of Fv-4' env antigen in C4W serum, we made use of a mink cell line transfected with DNA encoding the ecotropic MuLV receptor and parental mink cells that did not express ecotropic MuLV receptors. Fv-4' env antigen present in C4W serum bound to the transfected cells (Fig 5, curve a), but not to the parental cells (Fig 5, curve b), as would be expected if the Fv-4' env antigen was binding to the ecotropic virus receptor.

Fig 5. Specific binding of Fv-4' env antigen to the ecotropic MuLV receptor. Profile of FACS analysis of Fv-4' env binding by (a) Mink fibroblastic cell line transfected with murine ecotropic virus receptor (ecofi) gene and (b) parent mink fibroblastic cell line. Note the specific binding of Fv-4' env antigen to the ecoR transfected cells.
CELL-FREE TRANSMISSION OF Fv-4 Env Product

**Form of Fv-4 Env antigen in C4W serum.** The possibility that the Fv-4 Env antigen in serum was associated with virus particles was investigated by centrifugation to pellet virus.22 Fv-4 Env antigen from C4W serum was found in the supernatant fraction, but not in the sediment fraction, when assessed by binding to C3H thymocytes (Fig 6A). In contrast, most FLV antigen in an FLV solution was pelletable, although a small amount of FLV antigen was also found in the supernatant fraction (Fig 6B). FLV antigen in serum from FLV-infected C3H mice gave similar results (data not shown). These results imply that the Fv-4 Env antigen in the serum is not associated with virions.

Serum Fv-4 Env antigen can block binding by FLV. We previously suggested that Fv-4 resistance may be mediated via receptor interference.5,7,9 To test whether the binding of Fv-4 Env antigen from serum interferes with FLV adsorption, C3H thymocytes or spleen cells were preincubated with C4W serum, followed by incubation with FLV suspension. A large amount of FLV antigen was bound to the cell surface of C3H thymocytes or spleen cells if the cells were preincubated with C3H serum (Fig 7, curve a), whereas preincubation with C4W serum strongly reduced the binding of FLV (Fig 7, curve b). Negative controls included C4W cells incubated with FLV (Fig 7, curve c) and C3H cells not incubated with FLV (Fig 7, curve d).

**DISCUSSION**

MuLV infection is initiated by binding of virus envelope to membrane receptors expressed on susceptible cells. Binding is required for fusion of the virus envelope to the target cell, an event that may occur at the cell surface or within an acidified endosome after receptor-mediated endocytosis that results in entry of the virion core into the cytoplasm and initiation of replication.18,20 The mechanism of Fv-4 resistance is presumed to be mediated via receptor interference.5,7,9 This virus interference model is related to the phenomenon of viral interference in vitro. When tissue culture cells are chronically infected with one retrovirus, they are resistant to superinfection with the same or related retroviruses.31 Mouse cells have three types of virus receptors, Rec-1 for ecotropic MuLVs,21,22 Ram-1 for amphotropic MuLVs,23 and Rmcf-1 for dualtropic MuLVs.24 The observation that mouse cells transfected with the cloned Fv-4 gene became resistant specifically to ecotropic MuLV infection suggested a possible interference between the Fv-4 Env and the ecotropic MuLV receptor. The results presented in this paper clearly demonstrate that the soluble Fv-4 Env antigen (molecular weight of about 80 kD, unpublished data) specifically binds to the ecotropic MuLV receptors (Fig 5) and, further, that binding of Fv-4 Env antigen to the cell surface receptors interferes with the subsequent binding and infection by FLV (Fig 7).

The interference appears to be a consequence of the modification of virus receptor via interaction between receptor and viral envelope glycoprotein.25,26 Experiments demonstrating interference have usually been done in persistently infected cells. Recent findings suggested that, in those cells, intracellular binding of receptor by env glycoprotein induces downregulation of cell surface receptor molecules. A study with reticuloendotheliosis virus shows that envelope mutants that are retained predominantly in the endoplasmic reticulum (ER) can still cause superinfection interference.27 Related studies using the mutant env gene of a newly cloned MuLV showed that the mutant env glycoprotein that remained in the ER caused superinfection resistance. Transfection of the mutant env gene into cells persistently infected with wild-type virus of the same interference group led to trapping of the wild-type Env protein in the ER and suppressed expression of wild-type virus.28 The interference demonstrated in this paper is different from the interference shown in persistently infected cells in that the binding of Fv-4 Env antigen from outside uninfected cells blocked the attachment and replication of infecting virus. Such a phenomenon has not been reported in other retroviruses. Human immunodeficiency virus (HIV) binding to susceptible cells failed to induce receptor (CD4) endocytosis, whereas anti-CD4 antibodies and phorbol esters did.29 Binding inhibition for superinfection was tested in the HIV experiment.

**Fig 6.** Virion-free binding of Fv-4 Env antigen to C3H thymocyte. Binding assay of C4W serum and FLV solution to the C3H thymocytes after separation with centrifuge. (A) Expression of Fv-4 Env antigen by C3H thymocytes after incubation with the (a) original, (b) supernatant, (c) sediment fraction of C4W serum, and (d) C3H serum as a negative control. (B) Expression of MuLV antigen by C3H thymocytes after incubation with the (a) original, (b) supernatant, (c) sediment fraction of FLV solution, and (d) C3H serum. Fv-4 Env antigen was identified in the supernatant fraction of C4W serum, while viral antigen was demonstrated both in the sediment and supernatant fraction of FLV solution.

**Fig 7.** Blocking of FLV binding to C3H cells by preincubation with Fv-4 Env antigen. The expression of MuLV antigen was determined in (A) thymocytes and (B) spleen cells. (a) C3H cells preincubated with C3H serum and then mixed with FLV, (b) C3H cells preincubated with normal C4W serum and then mixed with FLV, (c) C3H cells without incubation with FLV, and (d) C4W cells incubated with FLV.
Although AKR mice are viremic and express the env glycoprotein of an endogenous ecotropic MuLV (AKV virus) on the cell surface,29 they are not as strongly resistant to exogenous infection by ecotropic MuLVs as are Fv-4' mice. Most of the AKV proviral loci of AKR mice appear to be structurally competent for full expression of infectious virus,29 while the Fv-4' gene contains only a truncated provirus genome. Differences between ecotropic AKV proviral loci and the Fv-4' gene in the env region and in structures outside of the env region may account for the different resistance activities.9

The resistance of C4W + C3H → C3H mixed chimeras to FLV depended on the amount of Fv-4' env antigen on the surface of H-2k-positive (C3H-derived) cells. The amount of Fv-4' env antigen on the H-2k-positive PBMC of mixed chimeras was roughly proportional to the ratio of C4W- to C3H-derived bone marrow cells. The C4W(5) + C3H(5) → C3H chimeras were FLV-resistant, while C4W(2) + C3H(10) → C3H chimeras were susceptible to FLV-induced leukemia. The overall average amount of Fv-4' env antigen on C3H-derived PBMC of C4W(2) + C3H(10) → C3H chimeras was below 20% of the normal expression by C4W PBMC. Thus, surface Fv-4' env antigen greater than 20% of that of C4W-derived cells appeared necessary for C3H-derived cells to resist FLV-induced leukemogenesis. Limjoco et al10 have constructed two strains of transgenic Fv-4' mice expressing Fv-4'-related antigen in the thymus and spleen cells. One of the two transgenic strains expressed three to nine times more transgene RNA and protein than the other strain. The former strain of mice were completely resistant to FLV-induced leukemia, while the latter mice were only partially resistant. This finding of Fv-4' gene expression-dependent FLV-resistance in transgenic Fv-4' mice is consistent with our observation in the present study using mixed bone marrow chimeras. Further study may be necessary to clarify the precise mechanism whereby FLV resistance depends on the amount of Fv-4' env antigen on the cell surface.

In the mixed chimeras examined in the present study, the amount of Fv-4' env antigen on PBMC lacking the Fv-4' gene (C3H-derived) appeared highest at 1 month after bone marrow transplantation and then gradually decreased. As shown by the binding assay, serum of C4W → C3H chimeras contained more Fv-4' env antigen than the C4W serum. This finding may be due to increased proliferation or metabolic activity of bone marrow cells after bone marrow transplantation following supralethal irradiation.

Bone marrow transplantation has offered therapeutic prospects for the treatment of otherwise fatal hematologic malignancies.22,23 Severe combined immunodeficiency,24 as well as aplastic anaemia.25 However, the indication of bone marrow transplantation for retroviral infection is still controversial.26 The fact that the soluble or secreted form of viral env appeared to be effective in conferring resistance is auspicious for attempts to establish receptor interference therapy of retrovirus diseases by bone marrow transplantation12,13 or other treatments, because (1) the transfected env gene might not need to be expressed in all marrow-derived cells, and (2) soluble forms of genetically engineered env proteins could be applicable for the therapy. Several approaches to block infection by soluble env or receptor proteins at the level of viral attachment to the cellular receptor have been reported. With HIV in vitro, soluble, secreted forms of the CD4 antigen26 and synthetic peptide segments of the CD4 molecule27 have been shown to block HIV infection of CD4-positive cells. Moreover, anti-CD4 antibody28 and phorbol esters29 induce internalization of the CD4 molecule resulting in a block to HIV binding. HIV-like particles containing modified envelope glycoproteins were used to develop a cross-protective AIDS vaccine in vivo.30 However, the efficacy of soluble CD4 or anti-CD4 antibody therapy, as well as vaccine therapy of AIDS, is still controversial. CD4 epitope masking by envelope protein could also downregulate CD4-positive cell function.31 Nevertheless, the unique interference mechanism of an env gene product shown in this study could contribute to establishing a novel basis for therapeutic intervention in AIDS.

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