ANTI-FRIEND VIRUS ANTIBODY IS ASSOCIATED WITH RECOVERY FROM VIREMIA AND LOSS OF VIRAL LEUKEMIA CELL-SURFACE ANTIGENS IN LEUKEMIC MICE

Identification of Rfv-3 as a Gene

Locus Influencing Antibody Production

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The course and outcome of Friend virus complex (FV)[1]-induced erythroleukemia is influenced by several mouse genes (1, 2). Spontaneous recovery from leukemia occurs in certain strains of mice (3, 4) and appears to be a complex phenomenon requiring the interaction of several mechanisms controlled by different genes (5, 6). For example, genes associated with the mouse major histocompatibility complex (H-2) interact in a complementary manner with at least one non-H-2 gene (Rfv-3) to produce a high incidence of recovery from leukemia (7, 8). In the absence of the appropriate high recovery H-2 genotype, the Rfv-3 gene influences recovery from viremia in spite of progressive fatal leukemia (8).

The present work is an investigation of the mechanism of action of the Rfv-3 gene. A.BY and A mice have the Rfv-$3^{s/s}$ genotype and do not recover from FV viremia, whereas (B10.A $\times$ A.BY)F_1 and (B10.A $\times$ A)F_1 mice have the Rfv-$3^{r/s}$ genotype which is associated with recovery from FV viremia (8). In previous experiments we observed that 30–90 d after FV inoculation, the leukemic spleen cells of these F_1 mice were no longer susceptible to cytolysis by anti-FV antibody plus complement (9). Furthermore, these spleen cells were found to have lost 80% of their FV-induced cell-surface antigens (9). Our present data indicate that in addition to recovery from FV viremia (8), the Rfv-3 gene also influences loss of FV-induced cell-surface antigens and generation of cytotoxic anti-leukemia cell antibody. Evidence is presented which indicates that anti-leukemia cell antibody is responsible for FV antigen loss and recovery from FV viremia.

Materials and Methods

Mouse Strains. C57BL/10Sn (B10), B10.A, A.BY, and A/WySn (A) mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. (B10.A $\times$ A)F_1, (B10.A $\times$ A.BY)F_1, and (B10.A $\times$ A)F_1 $\times$ A backcross mice were bred at the Rocky Mountain Laboratory, Hamilton, Mont.

Virus. The B-tropic strain of FV was used (10), and virus stocks were prepared as previously described (4). 1,500 spleen focus-forming units (FFU) were injected intravenously (i.v.) to induce leukemia.

Virus Assay. The S^+L^- assay of Bassin et al. (11) for the Friend murine leukemia helper virus (F-MuLV) was previously described (12). Results were expressed as plaque-forming units (PFU).

Cytotoxicity Assay. A two-step antibody plus rabbit complement ^{51}Cr-release cytotoxicity assay was employed (9). Sera were tested for anti-FV cytotoxic activity using a (B10.A × A)F$_1$ FV-induced leukemia cell line, AA41, as target cells (13). The titer was defined as the last dilution which gave 10% or greater specific cytotoxicity.

Immunofluorescence. Virus-specific membrane and intracellular immunofluorescence was studied using fluorescein-conjugated goat anti-Moloney leukemia virus (anti-MLV) from the National Cancer Institute Viral Oncology Program as previously described (9).

Immunization. Mice which had recovered from leukemia were inoculated two to five times i.v., every 2–4 wk with 2.5 × 10^7 syngeneic high antigen 8–9 d leukemic spleen cells to produce hyperimmune mice, which then had high titers of circulating anti-FV cytotoxic antibodies.

Results

Recovery from F-MuLV Viremia, Loss of FV Antigen, and Presence of Anti-FV Antibody Are Associated in (B10.A × A)F$_1$ Mice. 35–37 d after inoculation of (B10.A × A)F$_1$ mice with FV, plasma F-MuLV was high (>10^3 PFU/ml) in only 3 of 39 mice (Table I) and leukemic spleen cells from only 1 mouse had high levels of membrane immunofluorescence (>50% positive) (Table I). In addition, most mice (34/43) had cytotoxic anti-FV leukemia cell antibodies, which were capable of lysing early (8–9 d), but not late (30–90 d), leukemic spleen cells (Table I). Similar results for viremia, loss of FV antigen, and presence of anti-FV antibody were seen with (B10.A × A.BY)F$_1$ mice (data not shown).

Viremia, FV Antigen Retention, and Absence of Anti-FV Antibody in A.BY and A/WySn Mice. In contrast to the F$_1$ mice, A.BY and A mice did not recover from viremia. 35–37 d after FV inoculation, 18 of 21 mice still had >10^3 PFU/ml plasma F-MuLV (Table I). In addition, most A.BY and A mice (19/21) did not exhibit a loss of FV-induced cell-surface antigens in late stages of the leukemia (Table I). Finally, most A.BY and A mice failed to develop demonstrable cytotoxic anti-FV antibodies during late stages of the disease (only 2 of 23 had titers ≥4) (Table I). The difference in recovery from viremia between (B10.A × A)F$_1$ and A strains was previously determined to be influenced by a non-H-2 gene, Rfv-3 (8). To determine whether the loss of FV-induced cell-surface antigens and production of cytotoxic anti-FV antibodies were under similar genetic control, individual (B10.A × A)F$_1$ × A backcross mice were studied.

Comparison of F-MuLV Viremia, FV-Induced Cell-Surface Antigens, and Anti-FV Antibody in (B10.A × A)F$_1$ × A Backcross Mice. Plasma and spleens were collected from backcross mice 35–37 days after FV inoculation. Plasma F-MuLV levels in backcross mice segregated nearly equally between high (>10^3 PFU/ml) (24 mice) and low (<10^3 PFU/ml) (27 mice) (Table I). A similar segregation was seen for FV cell-surface antigen loss; 27 mice had low numbers of cells (<50%) with FV-induced cell-surface antigens and 24 mice had high (>50% positive) levels of membrane immunofluorescence (Table I). Fig. 1 (left column) shows the distribution of membrane immunofluorescence values for the backcross mice and demonstrates the existence of two groups corresponding to the F$_1$ and A parents. Cytotoxic anti-FV antibody also segregated

TABLE I

*Comparison of F-MuLV Viremia, FV-Induced Cell-Surface Antigens, and Anti-FV Antibody in (B10.A × A)F₁, A.BY, A, and (B10.A × A)F₁ × A Mice**

Mouse strain	Rfv-3 geno-type	F-MuLV viremia‡	FV-induced cell-surface antigens§	Anti-FV anti-body ‖
		(%)	(%)	(%)
(B10.A × A)F₁	r/s	3/39 (8)	1/39 (3)	34/43 (79)
A.BY and A¶	s/s	18/21 (86)	19/21 (90)	2/23 (9)
(B10.A × A)F₁ × A	50% s/s			
	50% r/s	24/51 (47)	24/51 (47)	28/50 (57)

* 35–37 d after infection with FV, mice were bled and the spleens were harvested.
‡ F-MuLV viremia was assayed by the S^+L^- assay. Values shown are number of mice with $>10^3$ PFU/ml plasma per total mice tested.
§ Values shown are number of mice with $>50\%$ cells positive for membrane immunofluorescence with goat anti-MLV serum/total mice tested.
‖ Values shown are number of mice with an anti-FV antibody titer of ≥ 4 in the antibody plus complement cytotoxicity assay per total mice tested.
¶ 14 A.BY and 9 A mice were used.

nearly equally between high and low levels. 22 mice had titers of <4, and 28 had titers ≥4 (Table I). Therefore, recovery from viremia, loss of FV-induced cell-surface antigens, and generation of cytotoxic anti-FV antibody were each influenced by a single genetic locus.

By comparing these three parameters in individual backcross mice, we determined that all three were influenced by the same gene, Rfv-3. A strong association was found between recovery from viremia, loss of FV-induced cell-surface antigens, and presence of anti-FV antibody (Table II). Mice which had high plasma F-MuLV ($>10^3$ PFU/ml) were usually found to have high virus-specific immunofluorescence. Mice which had low plasma F-MuLV ($<10^3$ PFU/ml) were typically found to have low virus-specific membrane immunofluorescence. Similarly, the presence of cytotoxic anti-FV antibodies in plasma from leukemic mice was associated with low plasma F-MuLV levels and with low incidences of virus-specific immunofluorescent-positive cells in spleens. Low cytotoxic anti-FV antibody titers in plasma from leukemic backcross mice were associated with high plasma F-MuLV levels and with high percent virus-specific membrane fluorescence on spleen cells. Thus, approximately one-half of the backcross mice were similar to the (B10.A × A)F₁ parents and appeared to have the dominant B10.A allele of the Rfv-3 gene associated with recovery from viremia, loss of FV-induced cell-surface antigens, and generation of cytotoxic anti-FV antibody. The other half of the backcross mice were similar to the A strain and appeared to lack the B10.A allele of Rfv-3.

Kinetics of Anti-FV Antibody Development Vs. Loss of FV-Specific Cell-Surface Antigens. The simplest explanation for the association of a single gene with clearance of viremia, decreased antigen expression, and appearance of antibody would be that one gene controls a single parameter which affects all three variables. For example, Rfv-3 may regulate the production of anti-FV antibodies which might then act to clear viremia and decrease FV antigen expression on the surface of spleen cells. If anti-FV antibody does decrease FV-induced cell-surface antigens, it should be possible to detect a rise in anti-FV antibody titer concurrently with or slightly before the time at which cell-surface antigens decrease. This was observed when (B10.A × A)F₁ mice
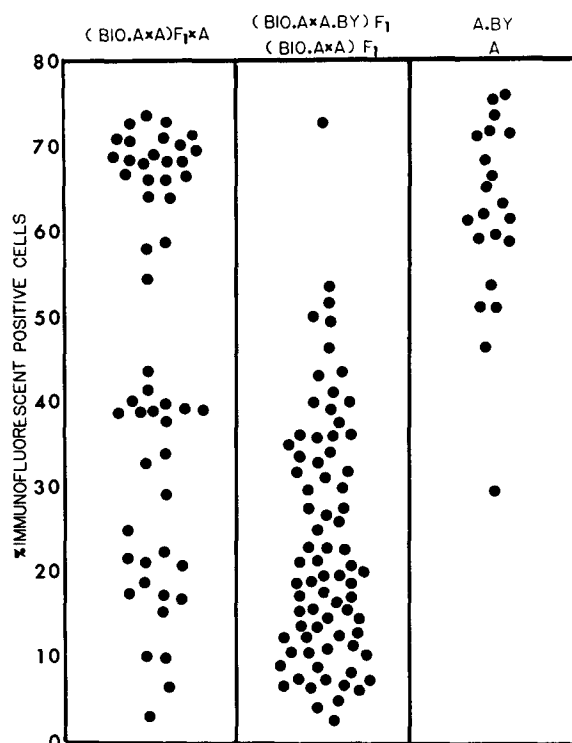
FIG. 1. Percentage of leukemic spleen cells from (B10.A X A)F₁ X A backcross, (B10.A X A)F₁, (B10.A X A.BY)F₁, A.BY and A mice with virus-specific membrane immunofluorescence 35-37 days following FV inoculation. The two strains of late F₁ mice were pooled, as were the A and A.BY mice, because they were indistinguishable with respect to membrane immunofluorescence (9). The cells were reacted with fluorescein-conjugated goat anti-MLV serum. Each point represents one mouse spleen.

were examined for membrane immunofluorescence and anti-FV antibody at various times after FV inoculation. The percent of spleen cells with FV-induced cell-surface antigens increased rapidly to a plateau (60-70% positive in the fluorescent antibody assay) which persisted from about day 8 to around day 20 after FV injection. Thereafter, the percentage of positive cells decreased to levels characteristic of late spleens (Fig. 2). Starting on about day 21, anti-FV antibody was first detected in plasma of leukemic mice. Some mice with demonstrable antibody still had a high percent of cells with FV cell membrane antigen. However, the close association between antibody production and antigen loss supports the hypothesis that anti-FV antibody is involved in the induction of antigen loss.

Loss of FV Antigens after Passive Antibody Administration. In an attempt to directly demonstrate that anti-FV antibodies were capable of inducing FV antigen loss, we passively administered anti-FV antibody to mice. 25 million viable early (8-9 d) (B10.A X A)F₁ spleen cells were given i.v. to lethally irradiated (900 rads) nonimmune (B10.A X A.BY)F₁ recipients. 5 and 6 d after cell transfer 0.5 ml of either anti-FV antiserum or normal mouse serum was administered i.v.; on the 7th d spleens were harvested. Spleens from mice which had received immune serum showed low percentages of cells which were positive for virus-specific membrane immunofluorescence

TABLE II

Correlation of Viremia, FV-Induced Cell-Surface Antigen Expression, and Anti-FV Antibody in
(B10.A × A)F₁ × A Backcross Mice 36 D After FV Inoculation (1,500 FFU)

		Viremia*					FV antigen‡					Anti-FV antibody§	
		Low	High				Low	High				Low	High
FV Antigen‡	High	4	20	Anti-FV Antibody§	High	21	7	Viremia*	High	17	6
	Low	23	4		Low	6	16		Low	5	22

* Based on the analysis of the A and (B10.A × A)F₁ parents of this backcross, high F-MuLV viremia was defined as >10³ PFU/ml plasma (8). Values shown are the number of mice in each category.

‡ FV-induced cell-surface antigens were detected by direct membrane immunofluorescence using fluorescein-conjugated goat anti-MLV serum. High was defined as >50% of cells positive.

§ High antibody was defined as a titer ≥4 in the antibody plus complement cytotoxicity assay using AA41 leukemia cells as targets. One mouse was not tested for anti-FV antibody.
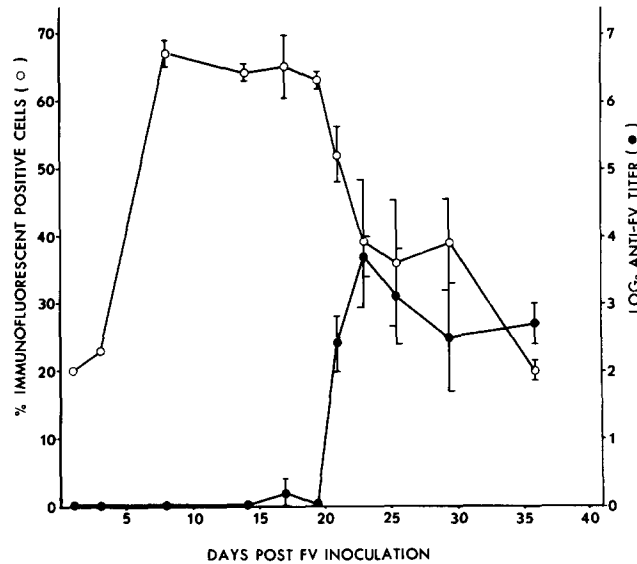

DAYS POST FV INOCULATION

FIG. 2.   Comparison of virus-specific membrane immunofluorescence of (B10.A × A)F₁ spleen cells and circulating cytotoxic anti-FV antibody from mice at various times after inoculation. The cells were reacted with fluorescein-conjugated goat anti-MLV serum. The F₁ serum was tested for anti-FV cytotoxic activity using the AA41 tumor cell line as a target in the antibody plus complement cytotoxicity assay. Points are averages from several mice ± SEM.

(30–32%), whereas spleens from mice which had received normal mouse serum or no serum at all, had high percentages of positive cells (57–69%) (Table III). All spleens examined had high levels of virus-specific cytoplasmic immunofluorescence (60–74%), indicating that the immune serum had not specifically eliminated virus-infected cells. These results strongly suggest that immune serum induces virus-specific cell-surface antigen loss in vivo.

FV Antigen Loss on A.BY Leukemic Cells.   A.BY leukemic spleen cells do not normally lose FV antigens during the course of FV leukemia (Table I, Fig. 1). We wanted to know whether the failure of A.BY mice to produce anti-FV antibody could be responsible for FV-antigen retention, or whether A.BY leukemic spleen cells were intrinsically resistant to modulation of FV antigens. Therefore, high antigen A.BY

TABLE III

Loss of FV-Induced Cell-Membrane Antigens after Passive Transfer of Immune
Serum

Mouse no.	Serum received	Virus-specific membrane immunofluorescence	Virus-specific cytoplasmic immunofluorescence
		%	%
1	Anti-FV	32	74
2	Anti-FV	31	60
3	Anti-FV	30	64
4	NMS	68	69
5	NMS	64	65
6	NMS	60	63
7	NMS	69	72
8	No serum	57	60
9	No serum	58	70

2.5×10^7 viable early (8–9 d) (B10.A × A/WySn)F_1 spleen cells were transferred i.v. to lethally irradiated (900 rads) (B10.A × A.BY)F_1 mice. These cells were 68% positive in the membrane immunofluorescence assay. 0.5 ml of either anti-FV serum or normal B10.A mouse serum (NMS) was administered on days 5 and 6 after cell transfer, and on day 7 spleens were harvested. Spleen cells were tested for cell-surface and intracellular (cytoplasmic) virus-specific immunofluorescence.

TABLE IV

FV-Induced Cell-Surface Antigen Loss on Early (8–9 D) A.BY Leukemic
Spleen Cells Transferred to Lethally Irradiated Immune Recipients

Experiment	Membrane immunofluorescence*		
	Donor	900-rads immune recipient	900-rads nonimmune recipient
	%	%	%
1	66	11, 18	67, 60
2	65	11, 23	51, 70

* 8–9 d (early) A.BY leukemic spleen cells were transferred into lethally irradiated (900 rads) (B10.A × A.BY)F_1 hybrids which were previously immunized against FV-induced cell-surface antigens or into nonimmune lethally irradiated recipients. Recipient spleen cells were examined for viral antigens by immunofluorescence 7 d after cell transfer. Percent of cells showing direct membrane immune fluorescence with fluoresceinated goat anti-Moloney LV serum is shown.

spleen cells were exposed to anti-FV antibody by transfer to lethally irradiated hyperimmune (B10.A × A.BY)F_1 recipients. It was observed that early A.BY leukemic spleen cells lost FV-specific cell-surface antigens 6 d after transfer (Table IV). Because these cells were capable of FV antigen loss, this data suggested that lack of production of anti-FV leukemia cell antibody was responsible for the high FV cell-surface antigen levels seen in A.BY mice in late stages of leukemia.

Discussion

The present results indicate that A.BY and A mice differ from (B10.A × A.BY)F_1 and (B10.A × A)F_1 mice in recovery from FV viremia, loss of FV-induced cell-surface

antigens, and production of cytotoxic anti-FV leukemia cell antibodies. Furthermore, in (B10.A × A)F_1 × A backcross mice, these three parameters appeared to be controlled by the same segregating genetic locus, Rfv-3. Thus, in the F_1 and backcross mice the Rfv-3$^{r/s}$ genotype was associated with recovery from viremia, loss of FV cell-surface antigens, and anti-FV leukemia cell antibody production. Conversely, in the A, A.BY and backcross mice the Rfv-3$^{s/s}$ genotype was associated with lack of recovery from viremia, retention of FV antigen, and failure to generate antibodies. It seemed likely that these three phenomena were linked by a common mechanism. A number of lines of evidence suggested that anti-FV antibody might be responsible for the observed antigen loss and clearance of FV viremia. (a) Loss of FV-induced cell-surface antigen coincided with the appearance of cytotoxic anti-FV antibody in the plasma of leukemic mice. (b) High antigen early (8–9 d) F_1 (9) and A.BY leukemic spleen cells could be induced to lose FV antigen upon transfer to lethally irradiated hyperimmune mice which had demonstrable cytotoxic anti-FV plasma antibody. (c) Adoptive transfer of early (B10.A × A)F_1 (high antigen) spleen cells to lethally irradiated recipients, followed by passive transfer of anti-FV immune serum, provided direct evidence that anti-FV antiserum was capable of inducing FV-antigen loss from leukemic spleen cell surfaces. It appeared that antibody was acting to modulate FV-induced cell-surface antigens (14–18) and was not simply killing virus-infected cells, because the spleen cells retained intracellular virus-specific antigens. Furthermore, because immune serum was transferred 1 and 2 d before spleen cells were examined, it would appear highly unlikely that a population of low FV cell-surface antigen cells could have overgrown the spleen in that time.

If the mechanism of Rfv-3$^{r/s}$-associated antigen loss operates via control of the production of anti-FV antibodies, then one would expect that if high antigen leukemic spleen cells from A or A.BY mice (Rfv-3$^{s/s}$) were introduced into an environment with anti-FV antibodies, they would lose FV cell-surface antigens. This was observed upon the transfer of high antigen A.BY spleen cells to lethally irradiated hyperimmune recipients. These results suggested that the failure of A.BY leukemic spleen cells to lose FV antigen during the normal disease course was not a result of an intrinsic inability of A.BY cells to lose (or modulate) antigen. It seemed likely that FV antigen was not lost after 30 d postinoculation because A.BY mice were not capable of generating anti-FV leukemia cell antibodies.[2]

Interestingly, although the A.BY and A mice usually do not produce cytotoxic anti-FV leukemia cell antibodies late in the disease, they do develop significant FV-neutralizing antibody titers (B. Chesebro. Manuscript in preparation). It may be that clearance of virus from plasma by neutralizing antibody is not sufficient to effect recovery from viremia. Apparently, anti-leukemia cell antibodies are required to decrease virus production. Because cell-surface FV gp70 may be essential for recognition of viral cores before virus budding, antibody-mediated modulation or dispersion of cell-surface viral proteins could interfere with virus budding and release (19). This possibility was supported by our observation of a substantial decrease in the number of F-MuLV producing cells (scored as infectious centers) in late leukemic (B10.A ×

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[2] Preliminary experiments using an indirect membrane fluorescence assay indicated that late leukemic A and A.BY mice had no noncytotoxic leukemia cell binding antibodies in their sera (data not shown).

A)F_1 spleens (B. Chesebro, K. Wehrly, D. Doig, and J. Nishio. Manuscript in preparation).

Although we think it most likely that Rfv-3 acts as an immunoregulatory gene (20), that influences production of cytotoxic anti-FV antibodies, other possibilities exist. Rfv-$3^{r/s}$ could act to increase interferon production (21, 22), which would decrease virus production, and in turn, FV-antigen expression. Alternately, Rfv-$3^{r/s}$ could increase production of SFFV defective interfering particles, which could contribute to recovery from F-MuLV viremia (23). Kumar et al. (24) have demonstrated the existence of a gene (FV-3) which confers resistance to nonspecific immunosuppression induced by FV. Rfv-3 may similarly regulate susceptibility to immunosuppression (25) but is not likely to be identical to FV-3 because Rfv-3^r is dominant for antibody production, and FV-3^s is dominant for susceptibility to immunosuppression.

Several mouse genes were recently shown to influence MLV viremia and leukemia (26). Because FV and MLV are immunologically cross-reactive, it would seem likely that any mouse genes which influence immunological recognition of FV or MLV antigens might be of importance to both systems. However, at present it appears that Rfv-3 has no influence on MLV viremia as B10 and A.BY mice, which differ at Rfv-3, have similar responses to MLV (26).

Wheelock et al. (27) have suggested that statolon-induced anti-FV antibody may induce both regression of FV splenomegaly and antigenic modulation of FV antigens which could lead to the persistence of leukemia cells. Our results confirmed that FV leukemia cells could persist in the presence of cytotoxic antibody, and FV cell-surface antigens were quantitatively decreased on these leukemia cells (9). However, antibody alone appeared unable to induce regression of massive splenomegaly. Furthermore, our preliminary experiments indicate that low antigen late leukemic F_1 spleen cells grow in hyperimmune recipient mice (data to be published). It seems likely that other mechanisms such as cell-mediated cytotoxicity (CMC) may be necessary for recovery from splenomegaly (28). It remains unclear whether modulation of cell-surface antigens has any bearing on growth of tumor cells in the face of a tumor-specific immune response. Antibody-induced antigenic modulation may interfere with CMC or other host defense mechanisms essential for elimination of tumor. If so, the kinetics of the CMC response compared with the antibody response would be of critical importance to whether or not recovery from leukemia ultimately occurs.

Summary

A single genetic locus, Rfv-3, influenced Friend virus (FV) viremia, loss of FV-induced cell-surface antigens from leukemia cells, and generation of anti-FV antibodies. 30–90 d after FV infection leukemic spleen cells from (B10.A $\times$ A)F_1 and (B10.A $\times$ A.BY)F_1 mice (Rfv-$3^{r/s}$) were found to have low FV-induced cell-surface antigen expression compared to leukemic spleen cells from A and A.BY mice (Rfv-$3^{s/s}$). In addition, these F_1 mice recovered from viremia and generated cytotoxic anti-FV antibodies. A and A.BY mice did not recover from viremia and failed to generate anti-FV antibodies. Anti-FV leukemia cell antibody appeared to mediate FV-antigen loss because decrease of FV cell-surface antigens occurred at the same time as anti-FV antibody appeared in the plasma of F_1 mice, and passive transfer of anti-FV antisera induced modulation of FV cell-surface antigens. Rfv-3 did not influence an intrinsic

ability of FV antigens to be modulated from Rfv-3$^{s/s}$ leukemia cells because FV antigen loss from Rfv-3$^{s/s}$ spleen cells occurred after transfer of cells to an immune environment.

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