Immunological mechanisms involving both humoral and cellular immunity may be important in host defense against viral-induced neoplastic diseases. Humoral antibodies can neutralize many viruses (1), and some antibodies can kill virus-infected or transformed cells in the presence of complement, normal lymphoid cells, or peritoneal exudate cells (2-5). In some systems cytotoxic antibodies correlate with tumor rejection (6-8) and passively transferred antibodies can protect against subsequent tumor cell challenge (9, 10). However, humoral factors, presumably specific antibodies or antigen-antibody complexes, can also exert an inhibitory effect on the host cell-mediated immune response to tumor cells in vitro (11, 12) and can enhance tumor progression in vivo (13, 14).

Spontaneous recovery from Friend virus (FV) mouse leukemia is often seen in certain mouse strains (15). The host immune responses to both the virus and the neoplastic cells probably play a critical role in this recovery. Furthermore, recovery has been shown to be markedly influenced by a single gene (Rfv-1) located in the D-region of the major histocompatibility complex (H-2) (16). Since this gene primarily affects recovery from leukemia rather than susceptibility to virus infection or cell transformation, it seems likely that some aspects of the host specific immune response might be influenced by this gene. The present experiments were undertaken to investigate the roles of host humoral antiviral and antileukemia cell immune responses in congenic mouse strains differing only at the H-2 complex. The data suggest that neutralizing anti-FV antibody may play a key role in control of viral infection. However, in spite of the fact that neutralizing or cytotoxic antibodies can inhibit focal leukemia cell growth in vivo, these antibodies are not sufficient to induce recovery from leukemia. In addition, other non-H-2-linked genes were shown to affect host control of viral infection and Rfv-1-mediated recovery from leukemia.

Materials and Methods

Buffer: pH 7.3, phosphate-buffered balanced salt solution (PBBS) contained NaCl, 7,200 mg/liter; KCl, 320 mg/liter; Na₂HPO₄, 1,150 mg/liter; KH₂PO₄, 200 mg/liter; CaCl₂, 140 mg/liter;

\[1\] Abbreviations used in this paper: B/T-L, BALB Tennant leukemia virus; FFU, focus-forming units; FV, Friend leukemia virus; PBBS, phosphate-buffered balanced salt solution; RML, Rocky Mountain Laboratory.
Humoral Immunity and Recovery from Leukemia

MgCl₂·6H₂O, 200 mg/liter; MgSO₄·7H₂O, 200 mg/liter; glucose, 1,000 mg/liter; phenol red, 10 mg/liter. PBBS with 5% fetal calf serum was used as diluent in neutralization and cytotoxic antibody tests.

**Animals.** Mouse strains used were previously described (16). In addition, F₁ hybrid mice (B10.A × A/WySn)F₁ and (B10.D2 × BALB/c)F₁ and backcross mice (C57BL/10 × BALB.B)F₁ and BALB.B were bred at the Rocky Mountain Laboratory (RML). For neutralization tests, RML random bred white mice were used.

**Virus.** The B-tropic strain of Friend virus was grown, stored, inoculated, and assayed for focus-forming units (FFU) as before (16). FV stocks were also made in (C57BL/10 × A.BY)F₁ and (B10.A × A/WySn)F₁ mice from spleen homogenates 8 days after i.v. inoculation of 15,000 FFU of virus. NB-tropic Friend virus was used in neutralization tests only. This strain was obtained from Dr. Frank Lilly, Department of Genetics, Albert Einstein College of Medicine, Bronx, N.Y. and was propagated in BALB/c mice.

**Recovery Experiments.** 11-16-wk-old mice were injected i.v. with FV and were palpated weekly for splenomegaly while under ether anesthesia, as previously described (16).

**Virus Growth.** Kinetics of FV growth in the spleens of infected mice was followed by i.v. inoculation of dilutions of freshly prepared 10% spleen homogenates into (BALB/c × A/J)F₁ mice. The spleens of the recipient mice fixed in Bouin's solution 9 days later, and foci were counted (17). Results were expressed as FFU/donor spleen.

**Viremia.** Mice were tail bled into heparinized capillary tubes. Plasma was separated by centrifugation. Presence of viremia was determined by i.v. inoculation of 4 µl fresh plasma in 0.5 ml PBBS into (BALB/c × A/J)F₁ mice. These mice were palpated weekly for 8 wk for splenomegaly. Those without splenomegaly were considered to have received nonviremic plasma.

**Virus Neutralization.** 10 µl of serial threefold dilutions of plasma or serum was mixed with 50 µl diluent containing 200 FFU NB-tropic FV. The mixtures were incubated 3 h at 37°C, diluted with 1.5 ml diluent at 0°C, and 0.5 ml was injected i.v. into RML mice. Mice were killed 9 days later, and spleens were fixed and foci counted as usual. The titer was expressed as the highest dilution giving >50% reduction in the number of foci observed.

**Antibody Plus Complement Mediated Cytotoxicity.** Targets were either spleen cells from mice that had received 15,000 FFU FV i.v. 9 days earlier or BC-3A lymphoma cells derived from a primary lymphoma occurring 2 mo after inoculation of newborn BALB/c mice with B/T-L (Tennant) leukemia virus (18). BC-3A cells were propagated in vivo as an ascites tumor line or in vitro in stationary suspension cultures in RPMI 1640 with 10% fetal calf serum and 10 µM 2-mercaptoethanol. These cells have the Friend-Moloney-Rauscher antigen (2) on their surface, demonstrated by cross-absorption experiments, and all sera tested simultaneously against both BC-3A and 9-day FV spleen targets gave identical cytotoxic antibody titers.

10–50 × 10⁶ target cells were labeled with 150 µCi ⁵¹Cr (Na₂CrO₄) in 0.6 ml PBBS at 37°C for 30 min. Cells were washed three times with diluent and resuspended at a concentration of 4 × 10⁶ cells/ml for use. 25 µl serial twofold dilutions of sera or plasmas to be tested were made in Linbro round bottomed well microtiter trays (Linbro Chemical Co., New Haven, Conn.). 25 µl containing 1 × 10⁶ labeled targets was added, and the mixture incubated 30 min at 37°C. Due to the frequent occurrence of anticomplementary activity in the sera, targets were washed twice with diluent before addition of complement (C'). After the second wash, 25 µl diluent was added to each well, and 50 µl of a one:four or one:eight dilution of absorbed rabbit serum was added as C' source. The mixture was incubated 45 min at 37°C and the trays centrifuged 1,000 rpm for 5 min. 50 µl of supernate was removed from each well and counted in an automatic gamma counter. Control wells contained targets plus C' alone and targets plus diluent alone. Percent lysis was calculated as:

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\frac{\text{cpm with antibody} - \text{cpm with diluent only}}{\text{total releasable cpm targets} - \text{cpm with diluent only}} \times 100
\]

Rabbit serum for C' was absorbed at 0°C for 30 min in 0.01 M EDTA with washed ascites leukemia cells (BC-3A or LSTRA) in a ratio of 1 ml packed cells per 5 ml rabbit serum. After absorption the cells were sedimented, serum removed, recalcified with CaCl₂, and frozen at –70°C in small aliquots. After absorption percent lysis of target cells by C' alone varied from 2–12%. The titer of cytotoxic antibodies in sera was expressed as the highest dilution giving >15% lysis above C' alone.
Table I
Effect of Passive Antibody Transfer on FV Spleen Foci

| Treatment* | Day | Foci | P$ |
|------------|-----|------|----|
| None       | --  | 13.6 ± 2.1 | -- |
| NMS§       | 1   | 12.0 ± 3.7  | <0.05 |
| Anti-FV serum | 1   | 2.0 ± 1.1   | <0.02 |
| NMS        | 3   | 27.5 ± 6.0  | >0.1  |
| Anti-FV serum | 3   | 5.5 ± 3.2   | 0.02  |
| NMS        | 6   | 21.7 ± 6.1  | >0.1  |
| Anti-FV serum | 6   | 21.7 ± 2.4  | >0.1  |

* (B10.A x A.BY)F~ mice received FV (20 FFU, i.v.) on day 0 and 0.2 ml serum i.p. on the subsequent day as indicated.
† P values comparing treatment with anti-FV serum and normal mouse serum given on the same day.
§ Normal mouse serum.

Passive Antibody Transfer. Hyperimmune anti-FV serum was produced in (B10.A x A.BY)F~ mice by i.v. inoculation of 300 FFU of FV. 30 days later 15,000 FFU was inoculated i.v., and this dose was repeated at 14-day intervals. Mice were bled 7 days after the third, fourth, and sixth inoculations. Pooled sera from each strain had an anti-FMR cytotoxic antibody titer of 128–256 and a FV neutralizing titer of 258–900.

Passive antibody transfer experiments were done in (B10.A x A.BY)F~ mice given 25 FFU of FV i.v. Groups of 4–5 mice were then given 0.3 ml syngeneic anti-FV serum or normal syngeneic serum i.p. 1, 3, or 6 days later. One group received no serum. All mice were sacrificed on the 9th day after virus and spleen foci were counted.

Results

Effect of Passive Antibody Transfer on Spleen Foci. To assess the in vivo effects of humoral antibodies on FV infection and development of leukemia in mice with a low incidence of spontaneous recovery, passive antibody transfer experiments were carried out. Anti-FV serum, containing FV neutralizing antibodies as well as cytotoxic antibodies for FV-induced cell surface antigens, or normal serum was given to (B10.A x A.BY)F~ mice at 1, 3, or 6 days after FV inoculation, and mice were examined for spleen foci on the 9th day. The results (Table I) indicate that anti-FV serum given 1 or 3 days after virus reduced the number of virus-induced spleen foci seen. Antiserum given on day 6 was not effective. Since cell transformation in the spleen can occur as early as several hours after FV inoculation (19), antibody transferred 1 or 3 days after virus probably does not act solely by interfering with primary infection and transformation. It is likely that, analogous to the situation in vitro, antibodies to virus-induced cell surface antigens together with complement or normal lymphoid cells can mediate lysis or inhibit growth of virus-transformed cells and thus could theoretically form an effective host defense mechanism against leukemia cells.

Friend Virus Growth and Neutralizing Antibodies. To investigate possible correlations between humoral antibody response and recovery from FV-induced leukemia, a kinetic experiment was set up to examine splenomegaly, virus growth, and appearance of antibody in two congenic mouse strains differing only
HUMORAL IMMUNITY AND RECOVERY FROM LEUKEMIA

FIG. 1. Comparison of virus growth, spleen weight, incidence of viremia, and neutralizing antibody titer after the inoculation of congenic F₁ mice with 15,000 FFU FV ([C57BL/10 × A.BY]F₁ stock). Each point represents the geometric mean of the values obtained from 3–8 mice.

for genes within the H-2 complex. Female (B10.A × A.BY)F₁ (H-2<sup>ab</sup>) and C57BL/10 × A.BY)F₁(H-2<sup>bb</sup>) mice were inoculated i.v. with 15,000 FFU of B-tropic FV ([C57BL/10 × A.BY]F₁ stock), and on subsequent days groups of 3–8 mice were examined for viremia, FV neutralizing and cytotoxic antibodies, spleen weight, and total amount of FV per spleen. Results are shown in Fig. 1. In agreement with previous data concerning the effect of the Rfu-1 gene on recovery from FV leukemia (16), both strains of mice developed splenomegaly by the 9th day. However, during the following weeks the spleens of H-2<sup>bb</sup> mice returned to near normal size while those of the H-2<sup>ab</sup> mice remained grossly enlarged. In spite of the strain differences in recovery from splenomegaly, there appeared to be very little difference in the kinetics of viral growth and elimina-
tion. Both strains had peak spleen FV titers and 100% incidence of viremia on day 9, and both strains showed a progressive drop in spleen FV titer and incidence of viremia over the following 1–3 wk. By day 22 no mice had viremia, and by day 34 no spleen FV was detectable. Coincident with the fall in spleen and plasma virus levels all mice developed significant titers of FV neutralizing antibody (Fig. 1) and cytotoxic antibody (data not shown). There were some differences between the strains. H-2^a^ mice had slightly higher spleen FV levels on days 3 and 9 and eliminated the virus at a slower rate than H-2^b^ mice. The time of reduction of virus levels varied from day 13 to 32 among individual H-2^a^ mice, however, there was always a direct correlation in individuals between presence of neutralizing and cytotoxic antibody and reduction in spleen FV level. On days 9, 13, and 17 several individuals had both viremia and neutralizing antibody at the same time. This could probably be explained by the existence of some infectious virus-antibody immune complexes in these plasmas.

The excellent correlation between humoral antiviral immune response and control of virus infection indicates that neutralizing antibodies could play a crucial role in restricting FV growth. However, there was a striking contrast in H-2^a^ mice between control of infection and lack of control of the leukemic process in the presence of good neutralizing and cytotoxic antibody responses.

**Cytotoxic Antibodies.** The role of cytotoxic antibodies in recovery from FV leukemia was investigated by examining hybrid mice of the Rfv-1 high (b/b) and low (d/b or a/b) recovery genotypes 30–35 days after FV inoculation. BALB hybrids, (C57BL/10 × BALB/c)F1 (H-2^d^) and (C57BL/10 × BALB.B)F1 (H-2^b^), were given 150 FFU of FV raised in BALB.B mice. A.BY hybrids, (C57BL/10 × A.BY)F1 (H-2^b^) and (B10.A × A.BY)F1 (H-2^a^), were given 15,000 FFU of FV raised in (C57BL/10 × A.BY)F1 mice. All BALB hybrids developed cytotoxic antibody titers of eight or greater. Although the incidence of recovery from splenomegaly was markedly different between strains (H-2^d^, 1/9; H-2^b^, 9/11), there was no difference in the range of cytotoxic antibody titers observed, which suggests that cytotoxic antibodies are not the critical factor in determining the different recovery incidences seen in these strains. Examination of A.BY hybrids revealed similar results with significant cytotoxic antibody titers present in both strains regardless of the status of recovery from splenomegaly (H-2^b^, 30/30; H-2^a^, 11/33). Neutralizing antibody titers in all the mice were uniformly high (72–258). There were 5 H-2^a^ individuals with splenomegaly that had no detectable cytotoxic antibody titer at 30 days, however, on subsequent bleeding 4 wk later they all had titers of eight or greater and still had gross splenomegaly.

Because of the unexpected occurrence of cytotoxic antibodies in splenomegalic mice 30 days or more after virus inoculation, spleen cells of some of these mice were tested for FV-induced cell membrane antigens by direct cytototoxicity. Of a total of 17 A.BY hybrid mice examined 30–105 days after FV, spleen cells of only 2 (11.8%) were lysed by anti-FV serum and C’', whereas all mice examined 8–11 days after virus had spleen cells which were lysed. These results could explain the observed coexistence of cytotoxic antibodies and leukemic splenomegaly.

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2 BALB hybrids are much more susceptible to high FV doses than are A.BY hybrids (16), and the doses used here were found optimal for expression of the Rfv-1 genotype.
Role of Non-H-2 C57BL Genes. The present observations of recovery from FV infection and the detection of neutralizing and cytotoxic antibodies in C57BL hybrid mice independent of recovery from splenomegaly are in contrast to data obtained with other mouse strains (20) where neutralizing antibodies are only found in association with regression of splenomegaly. These findings suggest that non-H-2 C57BL genes play a role in control of FV infection, and it is possible that this control could be a necessary prerequisite for ultimate recovery from splenomegaly. To assess whether the control of FV infection seen in (C57BL/10 × A.BY)F1 and (B10.A × A.BY)F1 mice was found only in C57BL hybrid mice, we examined several C57BL hybrid mouse strains as well as their non-C57BL parents for presence of viremia 30 days after FV inoculation. The data, shown in Table II, concern only mice with splenomegaly and indicate that in each group the C57BL hybrid mice alone have a high incidence of recovery from viremia in the presence of splenomegaly. Mice lacking C57BL genes only rarely recover from viremia in the presence of splenomegaly. These results are not influenced by genes of the H-2 complex.

It has previously been noted that H-2 (Rfv-1) gene-mediated regression of splenomegaly can be clearly demonstrated only in C57BL hybrid mice (16). One or more non-H-2 C57BL genes appears to be necessary for expression of this H-2-
TABLE II

Incidence of Viremia 30 Days after FV Inoculation in Splenomegalic Mice

| Mouse strain               | Number with viremia/total with splenomegaly | Virus dose |
|----------------------------|---------------------------------------------|------------|
| BALB/c                     | 2/2                                         | FFU        |
| BALB.B                     | 7/7                                         | 300        |
| (BALB/c × A/J)F₁           | 19/20                                       | 300        |
| (B10 × BALB)F₁*            | 2/18                                        | 300        |
| A.BY                       | 7/8                                         | 3,000      |
| (B10 × A)F₁‡               | 4/43                                        | 3,000 or 30,000 |
| DBA/2                      | 5/7                                         | 3,000      |
| (C57BL/6 × DBA/2)F₁        | 0/11                                        | 3,000      |

* Includes (C57BL/10 × BALB.B)F₁, (B10.A × BALB.B)F₁, (C57BL/10 × BALB/c)F₁, and (B10.D2 × BALB/c)F₁.
‡ Includes (C57BL/10 × A.BY)F₁, (B10.A × A.BY)F₁, and (B10.A × A/WySn)F₁.

associated effect. BALB.B mice (H-2<sup>b</sup>) were never observed to regress at doses studied, while (C57BL/10 × BALB.B)F₁ mice (H-2<sup>b</sup>) had a high incidence of regression. Since the effect of C57BL genes is dominant in the F₁, we studied the incidence of regression of splenomegaly in the backcross, (C57BL/10 × BALB.B)F₁ × BALB.B. If a single, dominant, non-H-2 C57BL gene were involved, one would expect near 50% incidence of regression; if two genes were involved, 25% regression; if three genes were involved, 12.5% regression, etc.

Table III gives the results of studying 63 backcross mice. Only three regressors were found (4.8% incidence), which implies that three or more non-H-2 C57BL genes are necessary for expression of the H-2 (Rfu-1)-mediated effect on regression. Unfortunately these backcross mice were not examined for viremia, and so it is not possible to say whether the effect of C57BL genes on recovery from FV infection is also multigenic involving the same genes as the effect on regression of splenomegaly.

Discussion

The present work has attempted to clarify some of the roles of humoral immunity in host defense against FV leukemia in mice. FV-induced antibody, together with complement, can lyse FV leukemia cells in vitro. As demonstrated in the present experiments, similar antisera can inhibit FV-induced neoplastic spleen foci even when administered as late as 3 days after virus inoculation.

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<sup>3</sup> It is theoretically possible that a maternal effect could explain this difference since the mothers of the F₁ mice used were C57BL/10, not BALB.B. The reciprocal F₁ cross will have to be tested to rigorously exclude this possibility. In the backcross experiment a maternal effect is unlikely since the regressor F₁ mice were the mothers of the backcross progeny, which in turn were over 95% progressors.
Bearing in mind that humoral antibodies can kill neoplastic cells in vitro and inhibit formation of neoplastic cell foci in vivo (21), we looked for evidence of their effects in the spontaneous recovery from FV leukemia controlled by the $R{v}_0$-1 gene. Kinetic studies of FV growth and viremia in congenic mice differing at this gene suggest that neutralizing antibody plays a major role in control of FV infection. However, no such direct correlation between recovery from the leukemia and presence or titer of antibodies was demonstrated. Both progressor and regressor C57BL hybrid mice were able to restrict virus proliferation and develop high titers of neutralizing and cytotoxic antibodies. Antibody may be necessary for recovery to occur, but its presence alone is not sufficient to induce recovery. It should be noted that to get a countable number of spleen foci in the passive antibody transfer experiment the virus dose used was very low compared to the dose used in most recovery experiments. Initial virus dose has a profound effect on recovery incidence (15, 16), and it is possible that cytotoxic antibody may be an effective defense mechanism only against low virus or leukemia cell burdens.

The occurrence of neutralizing and cytotoxic antibodies in the face of concurrent splenomegaly raises questions about the expression of virus-induced cell surface antigens on the leukemic spleen cells in the later stages of the disease. Presence of the antibodies in plasma implies that they are in excess over the amount of antigen exposed on cells in the spleen and other organs. The spleen cells should be coated with immunoglobulin if they possess the appropriate membrane antigens. Examination of these spleen cells with fluorescent antimalouse Ig serum has provided no evidence that this is the case. Furthermore, most late (>30 day) leukemic spleen cells used directly for targets in cytotoxicity tests with antibody and complement were negative for virus-induced antigens. Together these observations imply that tumor antigens on the leukemic spleen cells are decreased or altered at this time, and this may explain the persistence of leukemic splenomegaly in the face of high titers of cytotoxic antibody in these mice. The mechanism of this antigenic change is unknown. At the virus doses used many separate clones of neoplastic cells are transformed initially. Little is known about the pressures of selection on these clones during the course of disease. Antibody or other host defenses could selectively eliminate clones with a high antigen density leaving only those few clones with low antigen density to

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4 Chesebro, B., and K. Wehrly. 1975. Unpublished observations.
proliferate. It is also possible that antibody itself could play a role in inducing in vivo antigenic modulation (22).

The loss of virus-induced cell membrane antigens seen late in FV leukemia has been noted previously to be influenced by the H-2 locus and could provide an explanation for the association of H-2^b^b with recovery from FV-induced splenomegaly in certain strains. On the BALB genetic background, H-2^a^a mice lose cell membrane antigens and H-2^a^b mice do not (23). Theoretically host immune defenses might more easily recognize and eliminate the neoplastic cells in the H-2^b^b mice. However, we have not seen a similar H-2 association of this effect using C57BL hybrid mice. The 5–10% of Y10 (H-2^b^b) mice which do not recover from splenomegaly do have decreased or altered virus-induced spleen cell membrane antigens similar to congenic H-2^a^b or H-2^a^a mice. It is possible that the progres-sor individuals which we are examining have not recovered because their spleen cells have decreased amounts of virus-induced antigen. Evidence against this possibility is the observation that recoveries do occur later than 30 days after FV inoculation, i.e., at a time when virus-induced cell surface antigen has already decreased. Thus it is unclear at this time whether or not the frequently observed reduction in virus-induced cell surface antigens has any relationship to the phenomenon of recovery from splenomegaly.

It is clear that our results on viral growth kinetics, which were obtained in C57BL hybrid mice, are not identical to what has been observed in other mouse strains which lack C57BL genes (24). The Rfv-1 gene effect can be demonstrated dramatically only C57BL hybrids (16). Experiments using (C57BL/10 × BALB.B)F_1 × BALB.B backcross mice showed that at least three other C57BL genes are involved in the expression of the Rfv-1 recovery phenomenon. Fv-2 (25, 26) may be one of these genes. Data on control of viremia in various strains suggested a similar effect of C57BL genes independent of Rfv-1, although the number of genes involved was not determined. These results on recovery and viremia could be due to similar mechanisms possibly involving antibody. Mice that are not C57BL hybrids are able to make FV neutralizing antibody and cytotoxic antibody after appropriate immunization. Thus there is no absolute inability to synthesize these antibodies, but these mice could be more susceptible to immunosuppression induced by FV than C57BL hybrids (27). Differences in interferon production could also be important in control of virus proliferation (28), or enhancement (29), or suppression (30, 31) of the immune response. However, since both groups of mice can support virus replication to equal peak levels in the spleen, there doesn’t appear to be any critical target cell deficiency or initial impediment to virus proliferation in C57BL hybrids.

In summary, our data suggest that neutralizing anti-FV antibody may play a major role in elimination of viral infection, and that this may be a necessary, but not alone sufficient, step in the process of recovery from FV-induced splenomegaly. In several systems such antibodies have been shown by passive transfer to be effective in host defense against tumor cells (10, 32, 33). However, similar to our results, in the Moloney sarcoma virus system there is a very poor correlation of presence of these antibodies with tumor regression, and mice with progressing tumors have been found to have antibody titers in the same range as many regressors (34–37). Antibody to virus-induced cell surface antigens can
function in vivo against FV-transformed cells, but its occurrence in the presence of persistent splenomegaly suggests that it is not the major effector of the Rfv-1 gene-controlled recovery phenomenon. In addition the observation of similar titers of neutralizing and cytotoxic antibody titers in mice of differing Rfv-1 genotypes suggests that this gene does not act by controlling these aspects of the host immune response. The role of the specific cellular immune response in recovery will be examined in the following paper (38).

Summary

The humoral immune response to Friend virus leukemia was studied in congenic F1 mice differing in their incidence of recovery from leukemia. Anti-viral neutralizing antibodies rose in titer in vivo concurrently with disappearance of viremia and fall in spleen virus levels. Cytotoxic antileukemia cell antibodies also appeared at this time. Passive transfer of these antibodies could inactivate low numbers of leukemia cells in vivo; however, mice of both high and low recovery genotypes produced antibodies in equal titer and recovered from viral infection in spite of striking differences in recovery from leukemic splenomegaly. Mice lacking C57BL genes did not produce antibodies or recover from viremia except in rare instances. Recovery from splenomegaly was found to be influenced by three or more C57BL genes independent of the H-2 complex.

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