H-2D CONTROL OF RECOVERY FROM FRIEND VIRUS LEUKEMIA: H-2D REGION INFLUENCES THE KINETICS OF THE T LYMPHOCYTE RESPONSE TO FRIEND VIRUS

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Specific immune T lymphocytes play an important role in the resistance to a variety of viral diseases (1). In the case of spontaneous recovery from Friend virus (FV)[1]-induced leukemia, the appearance of FV-specific cytotoxic T lymphocytes closely correlates with regression of leukemic splenomegaly (2). Furthermore, recovery from FV leukemia has been shown to be strongly influenced by an H-2D-associated gene, Rfv-1 (3). After high FV doses, mice of the H-2D$^{b/b}$ genotype have a high incidence of recovery from FV leukemia, whereas mice of the H-2D$^{d/b}$ and H-2D$^{d/d}$ genotypes have a low incidence of recovery (3, 4). The mechanism of this genetic influence on recovery is unknown, however, previous findings suggested that the H-2D region might control the T lymphocyte response to FV (5). We recently showed that shortly after inoculation with a low dose of FV, mice of both the H-2D$^{b/b}$ and H-2D$^{d/b}$ genotypes developed cytotoxic T lymphocytes (CTL) that recognized FV in association with their respective H-2D gene products.[2] These findings indicated that both genotypes could develop an appropriate CTL response and suggested that a more subtle, perhaps quantitative difference in this response to FV might account for the differences in recovery observed between mice of the H-2D$^{b/b}$ and H-2D$^{d/b}$ genotypes after high FV doses.

To study quantitative aspects of the T lymphocyte response to FV in these mice, we developed an FV-specific T lymphocyte proliferative assay (6). In contrast to the assay for FV-specific CTL, this assay allowed us to detect a T lymphocyte response even in the presence of FV leukemia cells. Our results indicated that H-2D$^{d/b}$ mice had a significantly delayed development of the FV-specific T lymphocyte proliferative response as compared with congenic H-2D$^{b/b}$ mice. This delay was mapped to the D region of H-2, and could explain the differences in recovery incidence observed in these mice after high virus doses. Furthermore, congenic H-2$^{a/a}$ mice never developed

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[1] *Abbreviations used in this paper:* C′, complement; Con A, concanavalin A; CTL, cytotoxic T lymphocytes; FCS, fetal calf serum; FV, Friend virus; i.p., intraperitoneal; i.v., intravenous; SFFU, spleen focus-forming units.

[2] Britt, W. J., K. Wehrly, and B. Chesebro. 1983. Anti-Friend virus cytotoxic T lymphocytes from both low and high recovery mouse strains are restricted to the H-2D region. Manuscript submitted for publication.

a significant FV-specific T lymphocyte response. This lack of responsiveness was virus-specific and mapped to the left of the D region of H-2.

Materials and Methods

Animals. The following mice were purchased from The Jackson Laboratory, Bar Harbor, ME: C57BL/10Sn, H-2^b = K^bD^b; B10.A, H-2^a = K^kD^d; B10.A(2R), H-2^{h2} = K^kD^b; A.BY, H-2^b = K^bD^b; and A/WySn, H-2^a = K^kD^d. F_1 hybrids were bred at the Rocky Mountain Laboratories, Hamilton, MT. Mice used in all experiments were between 2 and 4 mo of age.

Cells and Virus. The B-tropic strain of Friend virus complex (FV) was obtained from Dr. F. Lilly, Albert Einstein School of Medicine, Bronx, NY. The virus stocks used for inoculation were prepared in (C57BL/10Sn × A.BY)F_1 mice and assayed as previously described (3). The origins and properties of the FV leukemia cell lines AA41 and clone 2C of Y57 (Y57-2C) have been described (2, 7). Cell lines were maintained in RPMI 1640 supplemented with 4% fetal calf serum (FCS) and 2-mercaptoethanol at a final concentration of 2×10^{-5} M.

Elimination of Cells Expressing Thy-1 Antigen. Depletion of Thy-1 antigen-positive cells using monoclonal rat anti-Thy-1 antibody and rabbit serum as a source of complement (C′) has been described previously (8).

Concanavalin A Stimulation of Spleen Cells. Nylon wool nonadherent spleen cells were added to 96-well flat bottom microtiter plates in 0.2 ml of Click's media (9), supplemented with 5% FCS. 0.025 ml of Click's media plus 1.0 μg of Concanavalin A (Con A) (Miles-Yeda Laboratories, Rehovot, Israel) was added. The cultures were incubated for 48 h at 37°C in a humidified atmosphere containing 5% CO_2, pulsed with 1 μCi [^{3}H]thymidine (New England Nuclear, Boston, MA) for 12 h and harvested on a MASH II (Microbiological Associates, Bethesda, MD). The cpm incorporated was determined. Wells containing no Con A were considered to be background and were consistently between 1,000–2,000 cpm ± 10%. The response to Con A was usually in the range of 200,000–250,000 cpm.

FV-Specific T Lymphocyte Proliferation Assay. This assay has been described previously (6). Briefly, 100–200 × 10^6 spleen cells were passaged over nylon wool columns. Nonadherent cells were washed twice with phosphate-buffered balanced salt solution (PBBS), resuspended in Click's media supplemented with 5% FCS (screened for low background stimulation). 5×10^5 cells were added to flat bottom microtiter wells containing 1×10^5 mitomycin C (Sigma Chemical Co., St. Louis, MO)-treated Y57-C2 or AA41 leukemia cells. The total volume of the culture was 0.2 ml. The cultures were incubated for 4 d at 37°C in a humidified atmosphere containing 5% CO_2. The cultures were then pulsed with 1 μCi [^{3}H]thymidine for 12 h and harvested. Control cultures containing only nylon wool nonadherent cells and no mitomycin C-treated leukemia cells (media only) were considered background cpm incorporated. The background counts consistently ranged between 1,000 and 3,000 cpm. If the background counts were outside of these limits, individual mice were excluded from the study. Results were expressed as Δcpm, calculated by subtracting cpm of unstimulated control cultures from cpm of cultures stimulated with mitomycin C-treated leukemia cells.

Greater than 90% of the nylon wool nonadherent cells expressed cell surface Thy-1, and depletion of the nylon wool nonadherent cells with anti-Thy-1 and rabbit C′ completely abrogated the FV-specific proliferative response. Therefore, the cells proliferating in the cultures appeared to be T lymphocytes. In addition, membrane immunofluorescence studies of the proliferating cells 4 d after mixing with mitomycin C-treated FV leukemia cells revealed that ~80–90% expressed Lyt-1 and <10% expressed Lyt-2 (data not shown).

Statistical Methods. To determine a significant response above background incorporation, normal (C57BL/10Sn × A.BY)F_1 and (B10.A × A.BY)F_1 mice were stimulated with mitomycin C-treated Y57-2C leukemia cells. The Δcpm from 32 normal (C57BL/10Sn × A.BY)F_1 and 28 normal (B10.A × A.BY)F_1 mice were determined. The results from both strains of mice were pooled, as no significant differences were observed. The mean and standard deviation were calculated. The result of this calculation was 2,000 ± 300, thus giving a 99% confidence interval (3 standard deviations) of ~1,000–3,000. Mice whose cells incorporated >3,000 cpm were thus considered significantly different from normal mice. Similar methods were used to determine a confidence interval for the proliferative response of (B10.A × A/WySn)F_1 mice when the mitomycin C-treated AA41 leukemia cell line was used.

Results

Lack of Suppression of the T Lymphocyte Response to Con A in F_1 Congenic Mice Inoculated With Friend Virus. Earlier reports suggested that mice with FV-induced leukemic splenomegaly showed suppression of various T lymphocyte functions such as the in vitro responses to Con A, phytohemagglutinin, and allogeneic cells, and in vivo skin allograft and tumor homograft rejection (10–12). However, using nylon wool-purified splenic T lymphocytes, Kumar et al. (13) found no suppression of Con A responsiveness in the presence of FV or FV leukemia cells. Although we showed previously that anti-FV antibody production was unimpaired even in the presence of massive leukemic splenomegaly in F_1 mice of the Rfv-$3^{r/s}$ genotype, we did not directly test T lymphocyte function in these mice (14). Therefore, to determine the effect of FV on T lymphocyte reactivity, we measured the proliferative response of nylon wool nonadherent spleen cells to Con A at various times after intravenous inoculation of mice with 1,000 spleen focus-forming units (SFFU) of FV-B. The majority of mice tested had responses comparable to normal, uninoculated, syngeneic mice (Fig. 1), in spite of the fact that mice of the H-$2D^{d/b}$ and H-$2D^{d/d}$ genotypes were grossly leukemic (spleen weight >1.0 g) by day 18 postvirus inoculation.

Mice of the H-$2D^{d/b}$ Genotype Generate a T Lymphocyte Proliferative Response to FV During Progressive Leukemia. Once we found that splenic T lymphocytes of FV-infected

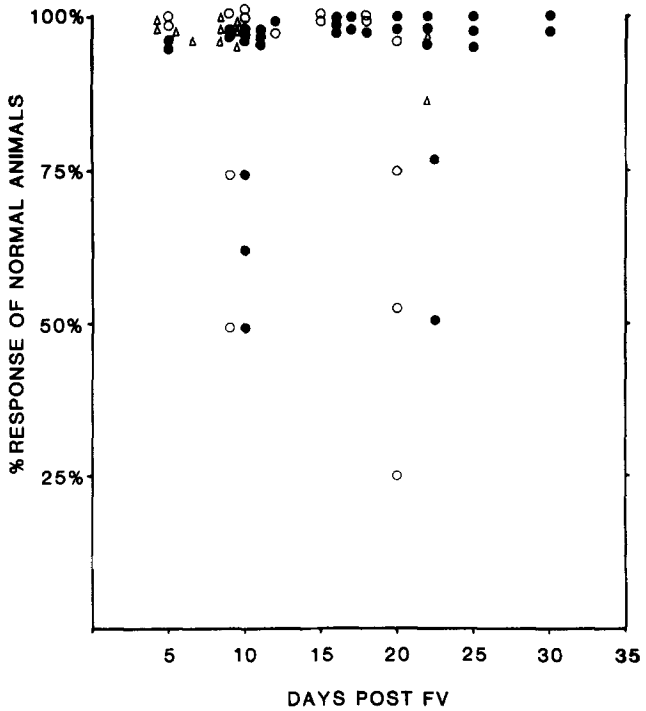

FIG. 1. (○), (C57BL/10Sn × A.BY)F_1, H-$2D^{b/b}$; (●), (B10.A × A.BY)F_1, H-$2D^{d/b}$; and (△), (B10.A × A/WySn)F_1, H-$2D^{d/d}$; mice were given i.v. 1,000 FFU of FV-B. On the days indicated after inoculation, nylon wool nonadherent spleen cells were cultured with 1 μg of Con A as described in Materials and Methods. The results are expressed as the percent response of nylon wool enriched spleen cells from a normal, uninoculated syngeneic animal cultured under the same conditions on the same day.

leukemic H-2D$^{d/b}$ mice could respond to Con A, we then tested whether these mice could generate a T lymphocyte response specific for FV antigens. Mice with the H-2D$^{d/b}$ genotype had leukemic splenomegaly at the time of analysis, but most generated a significant FV-specific T lymphocyte response equal in magnitude to H-2D$^{b/b}$ mice that had recovered from leukemia (Fig. 2). Therefore, the low incidence of recovery in mice of the H-2D$^{d/b}$ genotype could not simply be ascribed to a failure to mount a T lymphocyte response to FV.

H-2D$^{d/b}$ Mice Exhibit a Delayed Development of the T Lymphocyte Response to FV as Compared with H-2D$^{b/b}$ Mice. Our previous findings demonstrated that mice of H-2D$^{d/b}$ genotype had a high incidence of recovery if inoculated with a low dose of FV (10–20 FFU), but failed to recover if inoculated with a high virus dose (1,000–2,000 FFU) (4). These results suggested that H-2D$^{d/b}$ mice could develop the effector mechanisms necessary for recovery from FV leukemia, and that the effect of FV dose on recovery might be explained by quantitative or kinetic aspects of the FV-specific immune response rather than the presence or absence of such responses. Therefore, we investigated the kinetics of the development of the T lymphocyte response to FV in H-2D$^{b/b}$ and H-2D$^{d/b}$ mice after a high FV dose. The majority of H-2D$^{b/b}$ mice exhibited a T lymphocyte proliferative response to FV as early as 6 d after virus inoculation and by day 10 >90% of mice examined had a significant T lymphocyte response (Fig. 3 and Table I). In contrast, only a small percentage of mice of the H-2D$^{d/b}$ genotype developed a T lymphocyte response before day 16 after virus

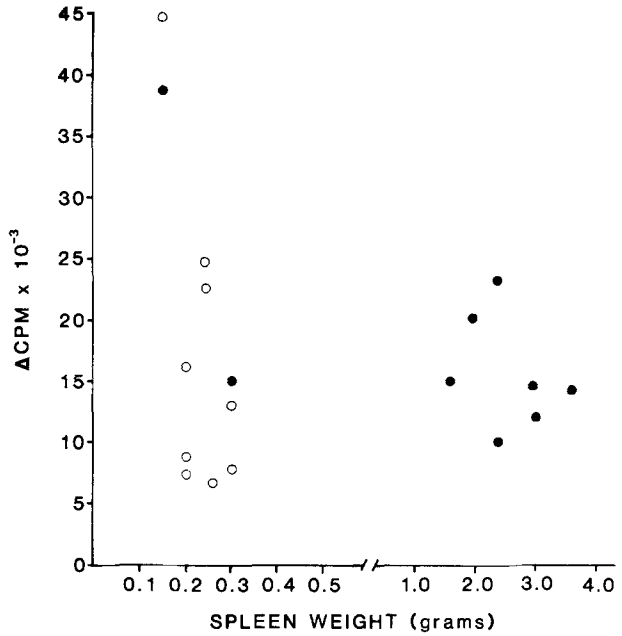

FIG. 2. (○), (C57BL/10Sn × A.BY)F$_1$, H-2D$^{b/b}$; and (●), (B10.A × A.BY)F$_1$, H-2D$^{d/b}$; mice were given i.v. 1,000 FFU of FV-B. 18 d after virus inoculation, spleens were removed and weighed, and nylon wool nonadherent spleen cells were cultured with mitomycin C-treated Y57-clone 2C FV leukemia cells for 4 d. The cultures were then pulsed with 1 μCi [^{3}H]thymidine and harvested 12 h later. The Δcpm incorporated was determined as described in Materials and Methods. Values greater than 3,000 Δcpm were considered significant responses. Mice with spleen weights >1.0 g were considered leukemic animals.

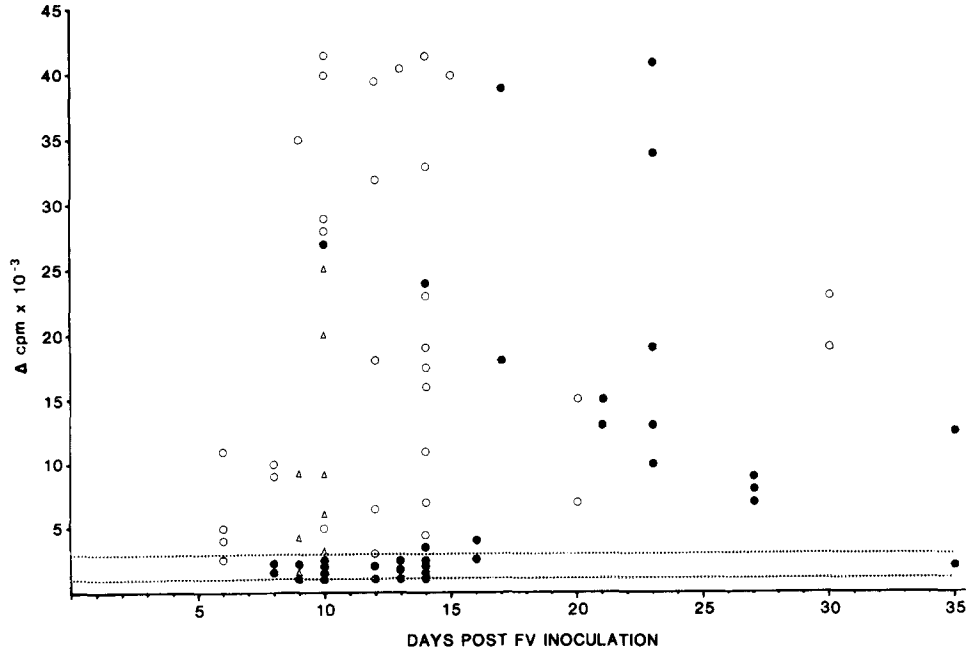

FIG. 3. (○), (C57BL/10Sn × A.BY)F_1, H-2$D^{b/b}$; (●), (B10.A × A.BY)F_1, H-2$D^{d/b}$; and (△), (B10.A(2R) × A.BY)F_1, H-2$D^{b/b}$; were given i.v. 1,000 FFU of FV-B. At various days after virus inoculation, nylon wool nonadherent spleen cells were cultured with mitomycin C-treated Y57-clone 2C FV leukemia cells for 4 d. Cultures were pulse labeled with [^{3}H]thymidine, harvested, and Δcpm incorporated was determined as described in Materials and Methods. Values >3,000 Δcpm were considered significant responses.

TABLE I

Accelerated Development of an FV-Specific T Lymphocyte Proliferative Response Maps to the D Region of H-2

Strain	H-2 Genotype				Responders/total number tested*		
	K	I	S	D	5–10d‡	11–16d‡	17–40d‡
(B10 × A.BY)F_1	b/b	b/b	b/b	b/b	11/12 (90%)	16/16 (100%)	4/4 (100%)
(B10.A(2R) × A.BY)F_1	k/b	k/b	d/b	b/b	7/8 (88%)	NT	NT
(B10.A × A.BY)F_1	k/b	k/b	d/b	d/b	1/9 (13%)	3/13 (23%)	11/12 (92%)

* Nylon wool-passed splenic lymphocytes were cultured with mitomycin C-treated Y57-clone 2C FV leukemia cells for 4 d. After a 12-h pulse with 1 μCi [^{3}H]thymidine, cells were collected and cpm incorporated into DNA was measured. A response above 3,000 Δcpm was considered significant.

‡ Number of days between FV inoculation (1,000 FFU FV-B, i.v.) and assay of T lymphocyte response.

NT, not tested.

inoculation (Fig. 3 and Table I); however, almost all of these mice developed a significant T lymphocyte response to FV when tested later than 16 d after virus inoculation (Fig. 3). The lack of an FV-specific T lymphocyte response in H-2$D^{d/b}$ mice before day 16 could not be attributed to general suppression of all T lymphocyte reactivity, as the lymphocytes of these same mice had a normal response when stimulated with Con A (Fig. 1).

To determine which region of the H-2 complex influenced the rate of development

of the T lymphocyte response to FV, we investigated the FV-specific T lymphocyte response in (B10.A(2R) × A.BY)F_1 mice (H-2$D^{b/b}$). These mice differed from the (B10.A × A.BY)F_1 (H-2$D^{d/b}$) only in the H-2D subregion. Almost all of (B10.A(2R) × A.BY)F_1 mice developed a significant T lymphocyte response to FV by day 10 after virus inoculation (Fig. 3 and Table I). This result indicated that the accelerated T lymphocyte response to FV mapped to the H-2D subregion and appeared to be a recessive trait, as it was seen in H-2$D^{b/b}$ but not in H-2$D^{d/b}$ mice. These findings were analogous to the known recessive influence of these H-2D genotypes on recovery from high doses of FV in these same mouse strains (3, 4).

Absence of a T Lymphocyte Response to FV in (B10.A × A)F_1 Mice. In order to study the FV-specific T lymphocyte response of H-2$D^{d/d}$ mice syngeneic AA41 FV leukemia cells were used as stimulator cells. (B10.A × A.BY)F_1 (H-2$D^{d/b}$) mice were positive, but surprisingly all (B10.A × A)F_1 H-2$D^{d/d}$ mice were negative (Table II). This failure to respond to FV could be demonstrated in animals that exhibited normal responses to Con A (Fig. 1) and allogeneic cells in a mixed lymphocyte culture (data not shown). The region of H-2 controlling this response was analyzed by using H-2 recombinant mice. (B10.A(18R) × A.BY)F_1 mice, which were identical to the (B10.A × A)F_1 mice in the H-2D region but differed at all regions to the left of H-2D, were found to be good responders in the FV-specific T lymphocyte proliferation assay (Table II). Thus, a region to the left of H-2D, perhaps the H-2I region, controlled this response, and in H-2 heterozygous mice (B10.A × A.BY)F_1 responsiveness appeared to be dominant.

Passively Transferred Immune T Lymphocytes Induce Recovery from FV Leukemia Only If Transferred Early After Virus Inoculation. To correlate the in vitro findings with previous in vivo results (3), we transferred FV-immune spleen cells into H-2$D^{d/b}$ mice inoculated with a high dose of FV (2,000 FFU) that caused progressive leukemia in the majority of mice of this genotype (4). Based on our findings in vitro, we predicted that transfer of syngenic, immune spleen T lymphocytes early after FV inoculation (day 6) would result in a high incidence of recovery as compared with transfer later after FV inoculation (day 16). Our results confirmed this prediction in that passive transfer of immune spleen cells into animals inoculated with FV 6 d earlier resulted in high incidence of recovery (78%) (Table III). However, the transfer of this same population of immune spleen cells into animals that received FV 16 d before cell transfer did not result in an increased incidence of recovery as compared to control

TABLE II

Absence of an FV-Specific T Lymphocyte Proliferative Response Maps to the K, I, or S Subregion of H-2‡*

Strain	H-2 genotype				Responders/total tested
	K	I	S	D	
(B10.A × A.BY)F_1	k/b	k/b	d/b	d/b	6/8 (75%)
(B10.A(18R) × A/WySn)F_1	b/k	b/k	b/d	d/d	5/6 (83%)
(B10.A × A/WySn)F_1	k/k	k/k	d/d	d/d	0/10 (0%)

* Nylon wool passed splenic lymphocytes were cultured with and without mitomycin C-treated AA41 leukemia cells of (B10.A × A/WySn)F_1 origin (H-2$^{a/a}$) for 4 d. After a 12-h pulse with 1 μCi [^{3}H]thymidine, cpm incorporated into DNA was measured. Mice with a Δcpm above 3,000 cpm were considered responders.

‡ Mice were tested 17–30 d after intravenous inoculation of 1,000 FFU of FV-B.

TABLE III

Early Transfer of Immune Spleen T Lymphocytes Induces Recovery From FV Leukemia

Day of cell transfer*	Treatment of donor cells	Recovered/total‡
6	None	14/18 (78%)
6	C′ only	20/31 (65%)
6	a-Thy-1 + C′	10/31 (32%)
16	None	4/16 (25%)
Control§	No cells transferred	5/19 (26%)

* Recipient (B10.A × A.BY)F_1 mice were given 2,000 FFU of FV i.v. on day 0. 6 or 16 d after virus inoculation, recipient animals were given 35 × 10^6 viable spleen cells intravenously from (B10.A × A.BY)F_1 mice given 100 FFU i.p. 16 d earlier ("immune spleen cell donors"). Spleen cells were either untreated, treated with C′ alone or treated with monoclonal anti-Thy-1 plus C′.

† Mice recovering from FV-induced splenomegaly over total number tested.

§ Control (B10.A × A.BY)F_1 animals were given 2,000 FFU of FV only.

animals. Furthermore, the cells responsible for inducing recovery appeared to be T lymphocytes since recovery was reduced in 6-d recipients when the donor spleen cells were depleted of T lymphocytes by treatment with anti-Thy-1 plus complement (Table III).

Discussion

The data in the present report indicated that Friend virus-specific immune spleen T lymphocytes could induce recovery from FV leukemia if given at 6 d, but not at 16 d following FV inoculation. This finding emphasized the importance of immune T lymphocytes in the recovery process (2, 8), and also demonstrated that the timing of the immune response relative to the virus infection was of critical importance. Furthermore, it was found that a significant difference existed between H-2D$^{b/b}$ and H-2D$^{d/b}$ mice in the kinetics of the development of the FV-specific in vitro T lymphocyte proliferative response. This influence on the response kinetics was mapped to the H-2D region, and correlated exactly with the known influence of an H-2D gene, Rfv-1, on spontaneous recovery from FV leukemia (3). This suggested that the mechanism by which Rfv-1 influenced recovery might operate by controlling the kinetics of development of the T lymphocyte response to FV. An early T lymphocyte response might lead to recovery by restricting virus spread and eliminating leukemia cells early in the course of the disease when the leukemia cell burden was low (15).

Lack of an early response to FV in H-2D$^{d/b}$ might also could explain the previously observed influence of virus dose on recovery in mice with this genotype (4). These mice would appear to have the mechanisms necessary for recovery since after low FV doses they produce anti-FV CTL (2),[2] can recover from leukemia (4), and their spleen T lymphocytes can adoptively transfer ability to recover (Table III). However, after use of high FV inocula, many more cells would be infected and transformed initially leading to a large virus and leukemia cell burden earlier after inoculation. In this situation, the observed delay in the anti-FV T-lymphocyte response might be a critical disadvantage that could not be overcome in H-2D$^{d/b}$ mice. In contrast, the more rapid response of H-2D$^{b/b}$ mice appeared capable of eliminating this increased leukemia

cell burden.

The mechanism by which H-2D might influence the kinetics of a T lymphocyte response is unknown. In several systems, the H-2K or H-2D regions appear to control the generation of CTL (16, 17). In these instances, nonresponsiveness was dominant, similar to our results. However, in no case previously has the difference been found to be a kinetic one. It is possible that, based on their increased quantity of H-2D^b cell surface antigen, H-2D$^{b/b}$ FV leukemia cells might induce a more rapid FV-specific T lymphocyte response than H-2D$^{d/b}$ cells. However, our previous attempts to detect such increased immunogenicity in vivo failed to detect any differences (8). Therefore, we feel this is an unlikely explanation for our present findings. Alternatively, these results might be explained by a quantitative difference in the number of precursor cells involved at a critical point in the immune network leading to generation of FV-specific CTL. Future studies will attempt to evaluate quantitatively some of these precursor cell populations.

Use of the in vitro T lymphocyte proliferative assay has allowed us to identify a second H-2-associated effect on the FV immune response. (B10.A × A)F_1 mice were nonresponders at both early and late times after FV inoculation. Responsiveness in this case was dominant, and mapped to the left of the H-2D region (Table II). The action of this gene appeared consistent with the effects of a typical I-region immune response gene (18). Such a genetic effect had been predicted previously, based on studies of FV tumor rejection (19) and recovery from FV leukemia (4), where it was referred to as the Rfv-2 gene. Lack of in vitro responsiveness in the (B10.A × A)F_1 mice went along with the absence of spontaneous recovery from FV leukemia. However, our preliminary studies indicate that nonresponsiveness to FV in these mice may be highly virus-specific because we have observed a high incidence of recovery after inoculation of Moloney leukemia virus pseudotypes of the defective spleen focus-forming virus component of the Friend virus complex (data not shown).

The role of immunosuppression in the effects reported here is not clear. FV inoculation of certain mouse strains (BALB/c and DBA/2) leads to profound immunosuppression (10, 12). However, in the F_1 mice used throughout this report, we found no evidence for suppression of the T-lymphocyte response to Con A, even in mice that did not respond to FV at any time after FV inoculation. We used nylon wool-filtered splenic T lymphocytes in all our experiments, and it is possible that some suppressive cell populations were removed by this step (13). If suppressor cells were responsible for either the late response or absence of a response to FV in certain F_1 mice, these cells might function in vivo before the time of nylon wool separation of spleen cells for analysis. However, our previous in vivo adoptive cell transfer experiments (8), as well as the present data (Table III), do not support this interpretation. Suppressor cells might also remain in the nylon wool-purified populations. But if so, they must either be FV-specific in their effects and/or too weak in potency to suppress responsiveness to Con A. Appropriate cell mixing experiments will have to be carried out to evaluate these possibilities.

Summary

A Friend virus (FV)-specific T lymphocyte proliferation assay was used to compare the T lymphocyte responses of H-2 congenic mice that differed in their ability to recover from FV leukemia after inoculation of high virus doses. Gene(s) of the H-2D

region influenced the kinetics of this response such that H-2D$^{b/b}$ homozygous mice were positive 6–8 d earlier than H-2D$^{d/b}$ mice. This correlated with the Rfv-1, H-2D-linked influence on recovery from FV by these mice, and also appeared to explain the prominent effect of virus dose on recovery incidence. These findings were supported by the ability of passively transferred immune splenic T lymphocytes to induce recovery from leukemia at 6 d after FV inoculation, but not at 16 d. H-2$^{a/a}$ mice were found to be unresponsive in the FV-specific T lymphocyte proliferation assay. This effect mapped to the left of H-2D, possibly in the H-2I region, and may be an in vitro manifestation of the Rfv-2 gene. No evidence for nonspecific immunosuppression of the T lymphocyte response to concanavalin A was observed in any of the H-2 congenic F_1 mice studied.

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